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DISSERTATION

The impact of the interplay between nonionics and the cell membrane on the nanoparticle-cell association and stability of Caco-2 cells

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The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!" (I found it!) but 'That's funny ...'

Isaac Asimov (1920 - 1992)

Content

1. Aims of the present thesis

Colloidal drug delivery represents a new area of research with exciting and promising perspectives especially for drugs from the biotech pipeline with delicate molecular structure e.g. peptides, proteins, and DNA. Whereas in case of conventional formulations the biodistribution is guided by the drug substance alone, nanoscaled formulations are distributed in the body in response to the characteristics of the nanoparticle. At present, all kind of cell interaction experiments require labelling of nanoparticles, however, associated with the risk of dye leakage and probably falsified results. Thus, new characterization methods are required. Among others, flow cytometry offers a reasonable solution since the granularity of the cell surface granularity of Caco-2 cells upon binding of nanoparticles is altered and is monitored by the side scatter signal.

The second, major part of this work is dedicated to elucidation and improvement of the nanoparticle-cell interaction. Currently non-ionic surfactants such as Poloxamer 188, Polysorbate 20 and Polysorbate 80 are required for preparation of nanoparticles, for stabilization of nanoparticles during storage and for coating to escape the reticuloendothelial system. Since the Caco-2 cell line of human origin represents a model for intestinal cells and is recommended by the FDA for absorption studies, this cells were chosen to elucidate the nanoparticle-cell interaction. The analytical method of choice was flow cytometry, enabling observation of few thousands sigle cells in one run.

Another aspect of the present work includes the estimation of the potential cytotoxicity of the surfactants. Since one surfactant exerted no negative effects on cell viability, elucidation of the potential to improve everydays cell

cultivation procedures i.e. splitting and cryopreservation, represents the third part of the present thesis. Ongoing from evaluation of surfactants' influence on the nanoparticle-cell interaction, as well as the impact of tensides on cell proliferation and viability, finally the work was focused on identification of the underlying mechanism of the surfactant-cell interplay by applying the micropipette aspiration technique.

All in all, the studies reveal new aspects for improvement of colloidal drug delivery and cultivation of pharmaceutically relevant preclinical cell culture models.

2. Introduction

As a result of the nanohype, colloidal drug carriers are nowadays being thoroughly investigated. They offer exciting new perspectives for drug delivery, especially for diseases that attack selectively a tissue or organ but with reduced systemic side effects. Nonetheless, huge efforts are necessary in order to guide the drug-loaded delivery systems to the target tissue, to facilitate overcoming membrane barriers, and to deliver the drug at therapeutically relevant concentrations. All of these required issues have to be fulfilled without or minimal toxic effects.

Preclinical evaluation of new drugs and drug delivery systems requires isolated growing cells and the broad cell cultivation procedures including numerous splitting cycles followed by seeding to proliferate and form confluent tissues in order to perform and repeat the tests with high and reliable reproducibility.

2.1 Nanoparticles

Representing colloidal carriers nanoparticles have been extensively investigated in medical and biotechnological sciences as drug delivery systems because their variable diameter in the range from 10 nm to 1000 nm makes them acceptable for many routs of administration. Additionally, in line with the literature particle size represents a decisive parameter for the interaction with cells. Among many advantages of nanoparticles, one of the most important is the ability to cross physiological epithelial barriers, e.g. 100 nm particles have been detected intracellularly in Caco-2 cells [1]. To enhance the nanoparticle-cell association many approaches have been followed, just one of them is modification of the particle surface with wheat germ agglutinin

(WGA), which possesses and mediates cytoadhesive and cytoinvasive characteristics [2].

Biodegradable colloidal carriers attracted increased attention as promising drug delivery systems for new chemical entities and also already marketed drugs, peptides, proteins, and DNA [3, 4]. To date, the most extensively investigated polymer is poly(D,L-lactide-co-glycolide) (PLGA), which is also approved by the FDA for medicinal and pharmaceutical purposes. This biodegradable polyester, well known as a resorbable suture material in surgery, is randomly hydrolyzed in the organism to yield the fully biocompatible metabolites lactic and glycolic acid, which enter the citric cycle to be finally transformed to carbon dioxide and water. The rate of biodegradation depends on lactic/glycolic acid ratio so that 50:50 PLGA, which is used in this work, is fully degraded within one month.

Among the high number of different preparation procedures for nanoparticles, the solvent evaporation technique was used to produce PLGA nanoparticles. In order to be able to incorporate not only hydrophobic but also water soluble drugs, a modified technique, the so-called double emulsion or (W/O)/W–technique was employed. Briefly, a small volume of distilled water finally containing the hydrophilic drug was emulsified with the organic solution of PLGA by sonication to yield a W/O-emulsion. The addition of a large volume of aqueous stabilizer solution and sonication resulted in a (W/O)/W-emulsion. The nanodroplets were hardened under stirring by addition of aqueous stabilizer solution in excess and evaporation of the organic phase, first at atmospheric pressure then under vacuum. The nanoparticle suspension was stored frozen at –80°C.

2.2 Cell culture and Caco-2 cells

The identification of new drugs, characterisation of their biopharmaceutical properties and their clinical safety as well as efficacy in animal and/or human

studies requires huge experimental efforts, is time consuming, expensive and ethically restricted. Additionally, species-related differences in biopharmaceutical characteristics limit the utility of animal trials at early stages of development. Therefore alternative models for prediction of drug uptake, bioavailibility and metabolism which additionally allow reliable in-vitro – in-vivo correlation are in the focus of preclinical research. Not only in medicine but also in pharmaceutics human cell culture models gained increased interest as valuable alternatives to animal trials especially for prediction of drug uptake. The term "cell culture" covers cell populations which have been established from one cell and are cultivated for longer time through multiple generations [5]. The Caco-2 cell line was recognized by both regulatory authorities, the FDA and EMEA, as a standard cell line for screening permeability and consequently classification of new active pharmaceutical ingredients according to the Biopharmaceutics Classification System (BCS) [6].

The Caco-2 cell line was established 35 years ago from the colon adenocarcinoma of a 72-years old male Caucasian. The Caco-2 cells spontaneously differentiate morphologically and functionally to yield monolayers that mimick the human intestinal epithelium despite of originating from the colon. Meanwhile they are used in daily routine for prediction of drug transport as monolayers grown on permeable filters, the so-called "transwell system". Preferably active transport systems are investigated [7]. Furthermore, Caco-2 single cells and monolayers are established to determine the cytotoxicity of drugs, drug delivery systems and even pharmaceutical excipients [8]. In spite of its broad application in research, this cell does not fully mimick the intestinal barrier towards absorption in humans. Caco-2 cells produce only negligible amounts of mucus. In humans, however, mucus forms a thick gel layer which represents another barrier towards absorption. In addition, it is difficult to study drugs with poor permeability [9].

According to textbook knowledge tumor cells are characterized by unlimited proliferation. Nevertheless, in daily practice, seeded Caco-2 cells proliferate to reach confluency. If they are not subcultivated in this status, they will stop proliferation. Hence, in order to cultivate the cells continuously, the cells have to be splitted and seeded again. Many factors can influence the rate of proliferation, among them the composition of the cell culture medium [10]. Continuous cell culture as necessary in research also requires stocks of cells, since several cellular functions can change with time in culture. In order to guarantee unaltered cellular functions that are dependent from passage number cryo-preservation of cells is obligatory. Cryo-preservation reveals advantages and limitations: while maintaining supplying with cells, it partially damages the cells during the processing. During freezing and thawing, ice crystals are formed inside and outside the cells, which can damage the cell mechanically or osmotically. Finally this results in low proliferation rates after thawing so that certain freezing rates and rapid thawing is recommended [11]. For long time storage of frozen cells, addition of cryo-protective agents (CPAs) is inevitable such as DMSO (dimethyl sulfoxide), or glycerin. Nevertheless CPAs improve cell stability and proliferation only by part, because of the cytotoxity of DMSO and in addition they can cause dramatic osmotic changes in cells upon addition and/or removal.

2.3 Surfactants

Surfactants are applied in many and different branches of industry: pharmaceutics, cosmetics, food, paints, etc. In pharmaceutical field they are most frequently applied to improve dissolution, stabilization and absorption of drug substances. In this work the influence of several commonly used non-ionic surfactants on the nanoparticle-cell interaction, the cyto-toxicity, and proliferation of cells was examined in a dose dependent manner.

Poloxamer 188 (Pluronic® F-68, PF-68), is a non-ionic surfactant and belongs to block copolymers, which are arranged in the basic triblock structure $EO_{n/2}$-PO_m-$EO_{n/2}$. The hydrophilic ethylene oxide blocks (EO, n= 152) form the ends of the polymer chain, whereas the hydrophobic core consists of propylene oxide blocks (PO, m= 30). The average molecular weight is 8400 Da. Poloxamer 188 is approved by the FDA for various application routes ranging from 0.01% in emulsions for topical application up to 2.50% in suspensions for oral administration [12]. Poloxamer is also widely applied for preparation, stabilization and coating of nanoparticles in pharmaceutics [2]. As it is impossible to fully remove the surfactant from the nanosuspension the influence of residual surfactant on the nanoparticle-cell interaction was assessed in this work but using commercially available surfactant free latex nanoparticles.

Furthermore, Poloxamer 188 possesses some other interesting characteristics that can influence to the cells' physiology: (i) As a supplement in cell culture medium it saved cells from starvation death and protected them against high ion concentrations or trace metal ions [13]. (ii) Poloxamer 188 inhibits the P-gp – function and thereby increases the intestinal absorption of various drugs or peptides that are P-gp substrates [14]. (iii) The surfactant also minimized cell death from shear stress in flow cytometric chambers [15]. It was suggested that these effects are due to interaction of Poloxamer 188 with the cell membrane resulting in a decreased fluidity of the plasma membrane [16].

The other two nonionics under investigation are **Polysorbate 20** (Tween® 20) and **Polysorbate 80** (Tween® 80). Polysorbate 20 and 80 are polyoxyethylene derivatives of sorbitan monolaurate and sorbitan monooleate, respectively. The hydrophilic moieties of these surfactants are polyethers of 30 molecules ethylene oxide. These surfactants are used for preparation of solid-lipid nanoparticles [17], proved to essentially increase the absorption across Caco-2 monolayers, considerably reduced the apical efflux of drugs from Caco-2

monolayers and inhibited intestinal P-glycoprotein (P-gp) activity [18]. According to the literature the Polysorbates also increase cell viability and protect cells from stress damage [19].

2.4 Methods used in this thesis

Only two fundamental methods used in this work, flow cytometry and the micropipette aspiration technique, will be described, whereas all other methods are either considered as routine techniques, such as nanoparticle preparation and characterization or standardized tests, such as the BrdU – test for determination of cell proliferation which were performed according to the manufacturers instructions with slight modifications.

As **Flow cytometry** allows screening and analysis of several parameters of a couple of thousand single cells in one run, lasting for only a few seconds, this technique became a method of choice in cell biology for last three decades. There are only a few basic requirements: a suspension of single cells, a fluorescence label emitting at a detectable wavelength, and finally a binding event linking these two features. In the flow system, the cells are stringed one by one in a sheath fluid stream and then they separately enter the detection system. According to the arrangement of the fluorescence collection optics the detection system comprises the forward scatter (FS), the side scatter (SS) and the fluorescence collection system [20]. The FS collects the light scattered by the cells opposite to the illuminating light beam and its intensity corresponds to the volume and thus the size of the cell. The SS collects fluorescence light emitted rectangular to the incident beam and offers information about roughness of the cell surface. Together with the SS the fluorescence collection optics detects the cell associated fluorescence intensity. All in all, flow cytometry represents a high throughput technique which allows a multiparametric analysis of thousands of cells within less than one minute.

The **Micropipette aspiration technique** is applied to collect information about the mechanical properties of the cell, cell membrane and the underlying cytoskeleton under stress conditions and also in response to added substances [21].

According to the set-up, three experimental approaches can be followed:

i) The adherend cell is deformed in a way that it is partially sucked into the pipette with a smaller diameter than the cell.

ii) The non-adherend cell attached to the orifice is being sucked into a pipette with a smaller diameter than the cell.

iii) The entire cell is sucked into a pipette with a nearly same aperture as the cell diameter.

The second mode was used in present work. The micropipettes were pulled from borosilicate glass capillaries. The micropipette was mounted on a syringe and connected via an U-tube to a reservoir filled with PBS (phosphate buffered saline). According to the adjustment of the reservoir along a vertical axis a positive or negative pressure was applied to the cell. The micropipette was filled with PBS and under microscopic inspection the pipette was approached to the cell until the tip touched the cell membrane. The pressure necessary to attach the cell was at the orifice and the corresponding apex of the cell was set as "zero tension state". Then a stepwise increasing negative pressure was applied and the cell membrane deformed due to suction into the aperture of the micropipette. According to images acquired at each step, the relationship between the shift in length of the cell's apex and the applied negative pressure was used to assess the stiffness of the cell membrane.

2.5 Abbreviations

BSC	Biopharmaceutics Classification System
CPA	cryo-protective agents
DMSO	dimethyl sulfoxide
EMEA	European Medicines Agency
EO	ethylene oxide
FDA	Food and Drug Administration
FS	forward scatter
NP	Nanoparticle
PBS	phosphate buffered saline
Pg-p	P-glycoprotein
PLGA	poly(D,L-lactide-co-glycolide)
PO	propylene oxide
SS	side scatter
WGA	wheat germ agglutinin
(W/O)/W	(Water-in-Oil)-in-Water

2.6 References

[1] M. Gaumet, R. Gurny, F. Delie. **Localization and quantification of biodegradable particles in an intestinal cell model: The influence of particle size.** *European Journal of Pharmaceutical Sciences* (2009) 36: 465 – 473

[2] A. Weissenböck, M. Wirth, F. Gabor. **WGA-grafted PLGA-nanospheres: preparation and association with Caco-2 single cells.** *Jr of Controlled Release* (2004) 99: 383 – 392

[3] J. Panyam, V. Labhasetwar. **Biodegradible nanoparticles for drug and gene delivery to cells and tissues.** *Advanced Drug Delivery reviews* (2003) 3: 329 – 347

[4] M. N. V. Ravi Kumar, U. Bakowsky, C. M. Lehr. **Preparation and characterization of cationic PLGA nanospheres as DNA carriers.** *Biomaterials* (2004) 25: 1771 – 1777

[5] G. Gstraunthaler. **Standardisation in cell and tissue culture – the need for specific GLP guidelines in the cell culture laboratory (Good Cell Culture Practice – GCCP).** ALTEX (2006) 23: 274 – 277

[6] J. S. Kim, S. Mitchell P. Kijek, Y. Tsume, J. Hilfinger, G. L. Amidon. **The suitability of an in-situ perfusion model for permeability determinations: Utility for BCS Class I Biowaiwer requests.** *Molecular Pharmaceutics* (2006) 3: 686 – 694

[7] P. Artursson, K. Palm, K. Luthman. **Caco-2 monolayers in experimental and theoretical predictions of drug transport.** *Advanced Drug Delivery Reviews* (2001) 46: 27 – 43

[8] P. Shah, V. Jogani, T. Bagchi, A. Misra. **Role of Caco-2 cell monolayers in prediction of intestinal drug absorption.** *Biotechnol. Prog* (2006) 22: 186 – 198

[9] P. Artursson, R. T. Borchard. **Intestinal drug absorption and metabolism in cell cultures: Caco-2 and beyond.** *Pharm Research* (1997) 14: 1655 – 1658

[10] Y. Sambuy, I. De Angelis, G. Ranaldi, M. L. Scarino, A. Stammati, F. Zucco. **The Caco-2 cell line as a model of the intestinal barrier: influence of cell and culture-related factors on caco-2 cell functional characteristics.** *Cell Biology and Toxicology* (2005) 21: 1 – 26

[11] E. J. Woods, J. D. Benson, Y. Agca, J. K. Critser. **Fundamental cryobiology of reproductive cells and tissues.** *Cryobiology* (2004) 48: 146–156

[12] www.accessdata.fda.gov/scripts/cder/iig/getiigWEB.cfm (23.04.2009)

[13] P. Hellung-Larsen, F. Assaad, S. Pankratova, B. L. Saietz, L. T. Skovgaard. **Effects of Pluronic F-68 on Tetrahymena cells: protection against chemical and physical stress and prolongation of survival under toxic conditions.** *Journal of Biotechnology* (2000) 76: 185 – 195

[14] E. V. Batrakova, H. Y. Han, V. Yu. Alakhov, D. W. Miller, A. V. Kabanov. **Effect of Pluronic block copolymers on drug absorption in Caco-2 cell monolayers.** *Pharmaceutical Research* (1998) 15: 850 - 855

[15] M. Al-Rubeai, A. N. Emery, S. Chalder, M. H. Goldman. **A flow cytometric study of hydrodynamic damage to mammalian cells.** *Journal of Biotechnology* (1993) 31: 161 – 177

[16] O. T. Ramirez, R. Mutharasan. **The role of the plasma membrane fluidity on the shear sensitivity of hybridomas grown under hydrodynamic stress.** *Biotechnology and Bioengineering* (1990) 36: 911 – 920

[17] A. del Pozo-Rodriguez, D. Delgado, M. A. Solinis, A. R. Gascon, J. L. Pedraz. **Solid lipid nanoparticles: Formulation factors affecting cell transfection capacity.** *International Journal of Pharmaceutics* (2007) 339: 261 – 268

[18] L. Yu-li: **Relationship between the hydrophilic-lipophilic balance values of pharmaceutical excipients and their multidrug resistance modulating effect in Caco-2 cells and rat intestines.** *Journal of Controlled Release* (2003) 90: 37 – 48.

[19] S. H. Choi, M. B. Gu: **A portable toxicity biosensor using freeze-dried recombinant bioluminescent bacteria.** *Biosensors & Bioelectronics* (2002) 17: 433 – 440

[20] T. G. M. Schalkhammer. Analytical Biotechnology: Immunoanalytical Methods (F. Gabor, O. Hoffmann, F. Pittner, M. Wirth) Birkhäuser (2002)

[21] R. M. Hochmuth. **Micropipette aspiration of living cells.** *Journal of Biomechanics* (2000) 33: 15 – 22

3. Specific Topics

Author's contribution

I hereby declare to have significantly contributed to the realization of the studies included in the present thesis.

The review in the **first part** (*The role of surface functionalization in the design of PLGA micro- and nanoparticles*) was prepared together with the co-authors. Concerning next article in this part (*Characterization of binding and uptake of biomimetic nanoparticles by flow cytometry*) I contributed to the study design and carried out some experiments. I was involved in data analysis and interpretation of the results.

Regarding the **second part** (manuscripts: *Nonionic surfactants increase cell-binding of nanoparticles by modulation of membrane stiffness* and *The interaction of Poloxamer 188 with the cell membrane increases the cell-association of nanoparticles*) I participated in the study design, contributed to the cell culture maintenance and performed all micropipette aspiration experiments. Additionally, I did the data analysis, interpretation of the results and I wrote the manuscripts.

In the **third part** (manuscript: *Poloxamer 188 supplemented culture medium increases the vitality of Caco-2 cells after subcultivation and freeze/thaw cycles*) I designed the study, carried out the cell culture and did some experiments, performed analysis and interpretation of data, and furthermore, I wrote the manuscript.

Vienna, March 2010

Mag. Vera Kerleta

3.1 First Part

THE ROLE OF SURFACE FUNCTIONALIZATION IN THE DESIGN OF PLGA MICRO- AND NANOPARTICLES

G. Ratzinger, C. Fillafer, **V. Kerleta**, M. Wirth, F. Gabor

Critical Reviews 2010

CHARACTERIZATION OF BINDING AND UPTAKE OF BIOMIMETIC NANOPARTICLES BY FLOW CYTOMETRY

K. Trimmel, G. Ratzinger, **V. Kerleta**, C. Fillafer, M. Wirth, F. Gabor

J. Drug Del. Sci. Tech 2008

THE ROLE OF SURFACE FUNCTIONALIZATION IN THE DESIGN OF PLGA MICRO- AND NANOPARTICLES

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Abstract

Nano- and microcarriers prepared from the biocompatible and biodegradable polymer poly(D,L-lactide-co-glycolide) (PLGA) are being extensively studied for drug delivery purposes. Apart from size, their fate in the body is mainly determined by surface characteristics, which govern the interaction of the particles with their environment.

The present review provides an overview of the currently established concepts for the surface functionalization of particles made from PLGA. In the first part, a concise description of the material-borne surface features and the related functionalization strategies are given followed by current methods for the physical and chemical characterization of the particle surface. The second part highlights the aims of functionalization, which include improved drug delivery, vaccination and imaging. Targeting approaches for site-specific delivery of drug-loaded particles to certain tissues or even to intracellular targets are presented as well as stealth coatings for a prolonged blood circulation, labeling methods for imaging purposes, and strategies for the immobilization of macromolecular drugs on the particle surface. Finally, present limitations as well as future challenges will be discussed with a focus on the surface modification procedure and essential demands on functional particulate systems posed by the dynamic and complex *in vivo* environment.

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IV. FUTURE CHALLENGES

V. OUTLOOK

I. INTRODUCTION

As indicated by 3869 published research papers, 3351 issued patents and 393 disclosed theses by the end of July 2009 poly(d,l-lactide-co-glycolide) (PLGA) is one of the most extensively investigated polymers for drug delivery and tissue engineering.[1,2] Although only a few pharmaceuticals are available on the market to date (see Table I), the high number of patents vs. research papers points to a powerful and promising excipient together with high commercial interest. PLGA offers unique properties for drug delivery purposes like world-wide approval for medical use, biodegradability, biocompatibility, and controlled release. However, some issues are not manageable by a single polymer e.g. targeting the diseased tissue, cellular uptake together with pre-programmed intracellular trafficking, and escaping the reticuloendothelial system (RES). As the contact with the body and the consequences thereof are mediated via the surface of the device, surface modification of sub-mm PLGA-particles by grafting with selected biomimetic ligands can meet some of these ambitious challenges to pave the way towards a more efficacious medication with reduced side effects and improved patient's compliance.

Table I. Currently approved drug formulations based on PLGA microparticles (U.S. and European Union; modified from Ref. 22)

Active pharmaceutical ingredient	Product	License holder
Peptides and proteins		
buserelin acetate	Suprecur Depot® [b]	Hoechst
lanreotide acetate	Somatuline Depot® [a]	Beaufour Ipsen
	Somatuline LA® [b], Somatuline retard® [b]	Ipsen
leuprolide acetate	Lupron Depot®, -3, -4, -PED [a]	Abbott Labs
	Prostap SR® [b], Enantone® [b]	Takeda
octreotide acetate	Sandostatin LAR® [a,b]	Novartis
somatropin recombinant	Nutropin Depot® [a,c]	Genentech
triptorelin acetate	Gonapeptyl Depot® [b]	Ferring
	Decapeptyl SR® [b]	Ipsen
triptorelin embonate	Pamorelin® [b], Pamorelin LA® [b]	Debioclinic, Ipsen
triptorelin pamoate	Trelstar Depot® [a], Trelstar LA® [a]	Watson Labs
Small molecules		
minocycline hydrochloride	Arestin® [a,b]	OraPharma
naltrexone	Vivitrol® [a]	Alkermes
risperidone	Risperdal Consta® [a,b]	Ortho McNeil Janssen, Janssen-Cilag

[a] approved by FDA (according to http://www.accessdata.fda.gov/Scripts/cder/DrugsatFDA; February 3rd 2010)
[b] approved in ≥1 countries of the European Union (according to http://www.hma.eu/mri.html (Mutual Recognition Index); http://emc.medicines.org.uk/default.aspx (Great Britain); http://pharmaweb.ages.at/pharma_web/index.jsf (Austria); February 3rd 2010); actual name of the marketed products may differ between countries
[c] commercialisation discontinued in 2004

At present, GMP-grade PLGA is marketed as Lactel® (Polymers International, Pelham, AL, U.S.A.), Medisorb® (Alkermes, Cambridge, MA, U.S.A.), Purasorb® (Purac resp. CSM, Amsterdam, Netherlands) and Resomer® (Boehringer Ingelheim, Germany). Usually, the polymer is prepared by ring opening polymerization of the cyclic dimeric anhydrids, D,L-lactide and glycolide in presence of Sn(II)-2-ethyl-hexanoate, Zn or Zn-lactate as a catalyst.[3] Due to toxicological concerns regarding Sn and esterification of

the hydroxyl-group at one end of the polymer chain, which yields a more hydrophobic polymer, the latter two catalysts are preferred. In general, increasing the amount of catalyst generates more polymerization nuclei so that the molecular weight of the polymer decreases. Moreover, the higher reactivity of glycolide facilitates formation of glycolide microblocks rather than lactide ones.[4] Most important for selection of the underlying mechanism of surface modification is the choice of the chain length controller. Employing lactic acid to stop polymerisation yields a polymer with a free carboxylic end-group termed as uncapped, whereas lactic acid ethyl ester yields end-capped PLGA. Particles made from the carboxylate polymers or so-called "H-type" products open two basic pathways for rather stable surface modification, the covalent binding of bioactive moieties preferably via carbodiimide and the grafting via ionic interactions due to the negative surface charge of particles made thereof (see Figure 1). The third approach towards surface modification relies on the overall hydrophobicity of both "H-type" and "non-H-type" PLGA which allows adsorptive coating, however, with the risk of rapid desorption of the coat in biological fluids due to swelling and erosion of the particles.[5-7]

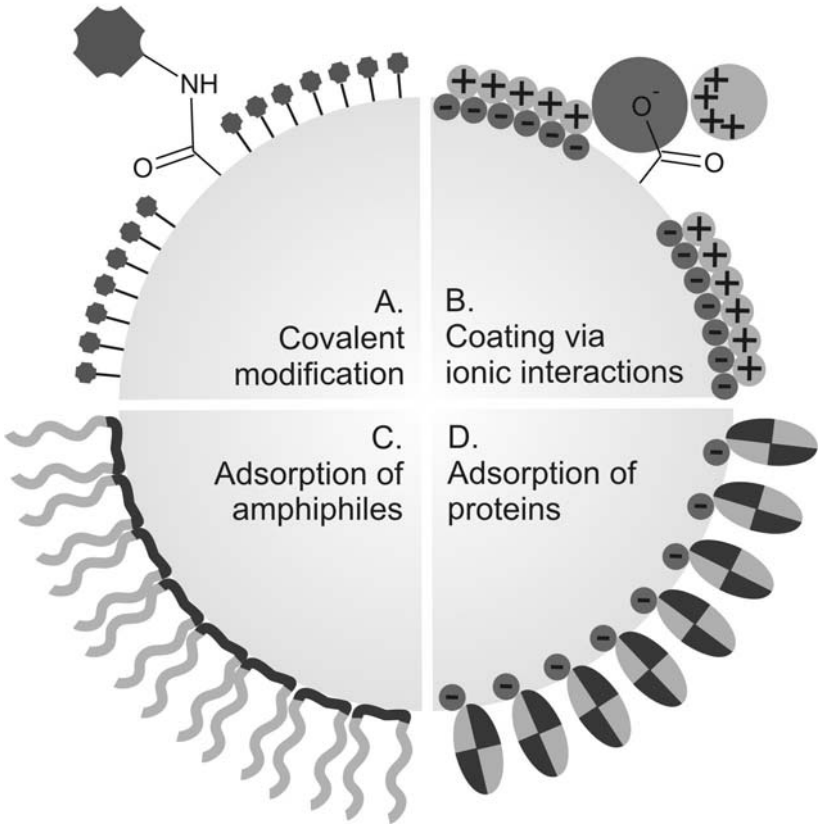

Figure 1: Strategies for surface modification of PLGA-particles.

Generally, PLGA particles are prepared by so-called top-down processes starting with the polymer. For preparation of microparticles from PLGA various techniques are available at the lab-scale basically relying on solvent extraction/evaporation, phase separation and spray drying. According to the type of emulsion applied, several modifications of solvent extraction/evaporation are distinguished: (i) The o/w technique relies on an emulsion prepared from a solution containing PLGA and the hydrophobic drug in a volatile organic solvent and an aqueous stabilizer solution. The organic solvent is removed by evaporation or extraction into the continuous cohesive phase causing hardening of the droplets. (ii) In case of the s/o/w method, instead of a solution solid small-sized drug material is processed as

before. (iii) The incorporation of a hydrophilic drug by the w/o/w method comprises emulsification of a small volume of an aqueous drug solution in a PLGA-rich organic phase followed by dispersion in a second aqueous phase containing a stabilizer. Diffusion of the organic solvent through the second aqueous phase and evaporation yields solid particles. (iv) In case of the o/o technique the first oily solution, usually acetonitrile, contains PLGA and a hydrophobic drug with a certain water solubility. Then the first oily solvent is extracted by the second oily solvent, e.g. cotton seed oil, leading to hardening of the droplets.[8] The second basic technique is phase separation or coacervation comprising dispersion of a solid or emulsified drug in the solution of PLGA followed by addition of a non-solvent so that a coacervate is formed at the interface. In case of PLGA, salting out is a modification of this process in that the polymer is precipitated by addition of water to an emulsion of PLGA dissolved in organic, water-miscible solvent and viscous PVA/salt solution.[9] Finally, spray-drying is particularly suited for hydrophobic drugs but problematic for hydrophilic drugs especially proteins and in case of small batches.[10,11] Alkermes Inc. and Genentech Inc. reported about a cryogenic spray technique, meanwhile known as Alkermes' ProLease® technology, at the developmental scale for preparation of Zn-rhGH (recombinant human growth hormone)-loaded PLGA microspheres. At this, homogenized Zn-rhGH lyophilisate was dispersed in PLGA/dichloromethane and sprayed into liquid nitrogen. The organic solvent was extracted from the

frozen droplets step-wise with liquid ethanol, first at – 105°C and then at – 40°C.[12]

The basic mechanisms for the formation of PLGA-nanoparticles are similar to those for microparticles but require smaller droplet-diameters to enter the nano-scale. In case of solvent extraction/evaporation higher energy input is usually provided by sonication, high pressure homogenization or vigorous mechanical stirring.[13,14] The nanoprecipitation technique relying on the interfacial deposition of PLGA by a non-solvent following displacement of a semi-polar solvent miscible with water from a lipophilic solution yields nanoparticles in the range of 100-300 nm with narrow size distribution in a one step procedure.[15-17] Table II presents some basic parameters for nanoparticle preparation; for more detailed information, the reader is referred to some excellent reviews covering preparation of PLGA micro- as well as nanoparticles.[18-22]

As confirmed by the long and successful history of the absorbable sutures "Vicryl®" (Polyglactin® 910 by Ethicon Inc., now subsidiary of Johnson & Johnson; 8% L-lactic acid and 92% glycolic acid), PLGA matrices are biodegradable.[23] In aqueous biological environment the polymer is first hydrated by adsorption of water and swelling of the matrix, a process lasting for days to months. Both hydrophilicity and crystallinity are key issues in this initial process. Among the different types of PLGA, the polymer composed of equal amounts of lactic and glycolic acid exhibits highest hydrophilicity and lowest crystallinity leading to fastest degradation. As free carboxylate groups

are more easily hydrated than ester moieties, the uncapped PLGA degrades faster than the end-capped polymer. This water-uptake mediates random hydrolytic scission of ester bonds yielding water-insoluble oligomers. Consequently, the mean molecular weight of the polymer decreases but the mass of the particle remains rather constant. Since the PLGA-oligomers contain a free carboxylate on one end and ester hydrolysis is acid-catalysed, carboxylated oligomers promote further degradation and a drop in pH. This so-called "acidic microclimate" is observed in microparticles which in turn catalyzes scission of the polymer backbone. As soon as the molecular weight of the oligomers drops below about 5.2 kDa, the oligomers become water-soluble, diffuse out of the matrix, and erosion of the particle is indicated by mass loss.[24] As opposed to surface erosion, homogenous or bulk erosion is the mechanism generally accepted for degradation of PLGA particles less than 300 µm in diameter.[25] According to the degradation mechanism, a triphasic release profile is most commonly observed in PLGA-microparticles: The so-called first burst effect, mainly due to release of surface-associated drug but also to pore formation upon water-entry,[26] is followed by a lag-phase with diffusion-controlled slow release until erosion accelerates the release again.[14,27] As compared to *in vitro*, *in vivo* biodegradation of PLGA, however, was shown to be faster because of a plasticizing effect of lipids, the release of radicals in consequence of a local immune response, autocatalytic cleavage of the polymer due to particle-aggregation or contribution of enzymes to degradation.[22,28] Due to bulk erosion upon biodegradation it is expected that

surface-modified PLGA-particles retain their biorecognitive coating and thus their functionality even during release of the API as opposed to particles made from surface eroding polymers.

Regarding *in vivo* administration, biocompatibility is another issue for drug delivery purposes. The tissue response after subcutaneous injection of PLGA microparticles occurs in three phases:[29] (i) Within the first two weeks a minimal acute or finally chronic inflammatory response is observed at the site of administration associated predominantly with monocytes. (ii) The second phase comprises the foreign body reaction, which is associated with macrophages in case of smaller microspheres or foreign body giant cells in case of large microspheres and development of a fibrous capsule as well as granulation tissue. (iii) Upon erosion, the particles of microspheres are phagocytosed by either macrophages or foreign body giant cells according to their size within weeks. In addition, some long-term studies reported inflammatory responses sometimes causing tissue necrosis but diminishing with time. This was observed in case of 15 µm PLGA particles and also attributed to degraded oligomers.[30,31] After intraperitoneal administration of PLGA particles a similar response comprising chronic inflammation and phagocytosis was reported in case of microparticles, however, nanoparticles caused minimal phagocytic activity most likely due to clearance from the peritoneum within two days.[32] As the open diameter of the smallest capillaries is 5-6 µm, particles smaller than 3 µm can be administered intravenously. Haemocompatibility studies with alendronate-loaded PLGA

nanoparticles revealed no significant effect on haemolysis, leukocyte number, platelet activation, activated partial thromboplastin time, and complement consumption as well as no cytotoxic effects on endothelial cells of blood vessels.[33] Upon use of simulated blood fluid, however, formation of 750 nm aggregates from 100 nm particles was observed and attributed to decreased electrostatic repulsion due to adsorption of cations.[34] Finally, the end-products of hydrolytic degradation of PLGA contribute to the biocompatibility of PLGA. Lactate is converted to pyruvate, which enters the Krebs cycle via acetylation of coenzyme A, and carbon dioxide, which is mainly eliminated by respiration. Part of the glycolate is excreted directly via the urine, another part is oxidized to glyoxylate, which is converted to glycine, serin and pyruvate. Pyruvate again enters the Krebs cycle to yield finally carbon dioxide and water.[24,35]

As to the biocompatibility of PLGA, the utility of micro- and nanospheres as adjuvants for vaccination seems to be contradictory at the first sight (see also section IIB, IIIB and IIIE for details). The immune response, however, is modified by design: Particles less than 5 µm are taken up by antigen presenting cells like macrophages or dendritic cells. Additionally, the large surface area presents multiple copies of the adsorbed antigen. Finally, the matrix traps and retains the antigen in local lymph nodes and protects it from degradation resulting in prolonged stimulation of the immune system.[36] Considering these issues, nanoparticles seem to be best suited for vaccination since they offer an increased surface area for antigen

adsorption, possibly enhanced immunogenicity due to higher uptake rates and they are sterilizable by simple filtration.[37]

Whereas simply the size of the PLGA particles is the key issue for successful vaccination, application of these powerful potential carriers for a certain therapeutic indication requires some further modifications to meet the given specific demands. Considering the physicochemical characteristics of a pre-formed PLGA particle, the surface carboxylate groups of uncapped PLGA allow for covalent and electrostatic conjugation of ligands whereas the hydrophobicity of the PLGA matrix can be exploited for adsorption of hydrophobic or even amphiphilic ligands. This review is intended to give an overview about the current knowledge of surface modification techniques followed by a short description of the methods available for characterization. Further chapters deal with the different aims of functionalization including non-specific and specific bioadhesion, improved internalization, pre-programmed intracellular trafficking, imaging, prolonged circulation time and stabilization of biomacromolecules. After discussing future challenges an outlook tries to give a vision of this emerging field of research.

	Emulsion evaporation	Emulsion diffusion	Salting out	Solvent diffusion or displacement Nanoprecipitation
Solvent	non-highly toxic	non-highly toxic	non-highly toxic but explosive	non-highly toxic
Drug	hydrophilic (double emulsion) and hydrophobic (single emulsion)	hydrophobic	hydrophobic	poorly water-soluble, highly soluble in polar solvent

Energy consumption	high (emulsification and evaporation)	low except for high speed homogenization	low	low
Time requirement	moderate	high	low, but additional purification step required	high

Table II: Comparison of some basic decisive parameters for nanoparticle preparation summarized from Astete and Sabliov 2006.[20]

II. SURFACE CHARACTERISTICS AND RELATED FUNCTIONALIZATION STRATEGIES

II.A. Carboxylate groups - covalent modification

As already outlined above PLGA is available in two forms: uncapped, i.e. containing terminal carboxylate groups, and end-capped, i.e. terminated by an alkyl ester. The terminal carboxylates are often used for covalent conjugation of ligands either to the dissolved polymer prior to particle formation or to surface-exposed carboxylic groups of preformed particles. Owing to the high stability of covalent linkages, these approaches are generally preferable to other immobilization strategies in order to guarantee efficient functionalization. Many chemically sensitive ligands, e.g. proteins or peptides, should not be coupled to PLGA prior to particle preparation as they are prone to denaturation by organic solvents or shear stress during the emulsification process. Moreover, for a number of applications the immobilized ligands have to be displayed at the particle surface. To meet

these requirements, covalent coupling to the surface of preformed PLGA particles may be advantageous.

There are only few chemical groups that specifically react with carboxylates. As carboxylic acids are rather weak nucleophiles in aqueous solutions, they do not easily couple via nucleophilic addition.[38] The most important chemical reaction for the covalent modification of PLGA carboxylates is the carbodiimide mediated cross-linking with amine-containing molecules. Thereby, the carboxylic group reacts with a carbodiimide to yield an O-acylisourea intermediate, which is highly reactive and forms amide bonds with amine nucleophiles. Most importantly, the reaction works in aqueous buffers under mild conditions including neutral pH, which makes it applicable to proteins, peptides and other easily degradable molecules. At this, the water-soluble derivative 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) can either be used alone or together with N-hydroxysulfosuccinimide (sulfo-NHS) or N-hydroxysuccinimide (NHS). While the O-acylisourea intermediate is prone to rapid hydrolysis, sulfo-NHS and NHS give more stable active ester intermediates, which finally react with the amine. The increased stability may result in a higher coupling efficiency. Moreover, the application of the succinimide enables a two-step procedure for conjugation, which may be advantageous for ligands that bear not only amine but also carboxylic groups in order to avoid cross-linking. To saturate unreacted binding sites, an excess of small amine-containing ligands such as glycine or ethanolamine may be

used. Until now, the carbodiimide method has been successfully used for conjugating a broad range of different amine-containing molecules, especially targeters, e.g. peptides, lectins, and antibody fragments, but also polycations.[39-42]

To enable the coupling of ligands that cannot directly react with carboxylate groups, various spacers, such as diamines, polyamines, or dihydrazides may be applied.[43-45] Depending on their length and flexibility spacers may enable or enhance the conjugation of certain ligands with hardly accessible reactive groups. Moreover, they can influence the orientation of coupled ligands, which might have an impact on their bioactivity.

Although the number of carboxylic groups available for coupling to the particle surface has been criticized for being limited, it has proven sufficient to obtain a targeting effect as already confirmed by enhanced particle-cell interactions.[40] However, for efficient coupling to PLGA carboxylates the presence of steric stabilizers such as PVA or poloxamer should be considered, as the adsorbed stabilizer may compromise the reaction via steric hindrance.[46,47]

II.B. Surface charge – coating via ionic interactions

Nano- and microparticles made from uncapped PLGA are characterized by a negative surface charge at physiological pH. Primarily, this charge is due to carboxyl groups which arrange at the liquid/particle interface in course of the

preparation procedure. Since the p*K*as of the carboxyl groups of lactic and glycolic acid are 3.86 and 3.83 respectively, these groups increasingly exist in their dissociated form with increasing the suspension's pH above 4. Making use of these ionized groups, the particle surface can be coated with cationic polyelectrolytes via ionic interactions. The adsorption of polyelectrolytes onto the surface of particles in suspension is complex and influenced by several parameters. The packing and structure of the adsorbed layer is widely determined by the particle surface charge density, polyelectrolyte charge density, as well as the pH and ionic strength of the suspension. The degree of dissociation of ionizable groups on particle surface and polymer can be varied by adjusting pH. Thereby, the affinity between surface and polyelectrolyte is regulated. Besides pH, ionic strength is decisive for the structure of adsorbed polymer.[48] Generally, in aqueous solutions of low ionic strength, polyelectrolytes have an expanded and rather rigid conformation due to intrachain repulsive forces. The adsorption of such stretched polymer molecules onto particles can lead to compensation of the surface charge but does not necessarily introduce a surplus of cationic groups.[49] However, for most applications an inversal of the negative surface charge by the cationic polyelectrolytes is desired. This can be achieved by adsorption from solutions containing appropriate amounts of salt. Increasing the solution's ionic strength leads to screening of repulsive intra- and interchain interactions and consequently the polymer can adopt coiled and more flexible conformations. Due to this structural flexibility and reduced interpolymer repulsion, the

adsorption of coiled polyelectrolytes can lead to higher deposition densities. Since not all of the ionized groups participate in binding to the particle, an overcompensation of surface charge and thus inversal of the zeta potential occurs.[50] However, if the ionic strength of the adsorption medium exceeds a specific threshold, the charges on the polymer and on the particle surface might be screened to such an extent that adsorption is drastically reduced.[48]

Coating of negatively charged particles has been found to be advantageous for several applications. The positive charges imparted at the particle surface, for example, efficiently complex anionic macromolecules such as plasmid DNA (pDNA). This has received considerable interest for the formulation of vaccines (see Section III.E). Moreover, decoration with polycations is considered to be a rather unspecific but simple approach to enhance the adhesion of particles to mucus[51,52] and cells.[53-56] The mechanisms involved and possible influences of coating with cationic polyelectrolytes on internalization[53] and endosomal processing[57] will be discussed in Section III.A.1. To make use of these potential benefits, natural, processed natural and synthetic polyelectrolytes have been employed for the coating of negatively charged PLGA nano- and microparticles. These include protamine,[56] chitosan,[51,55,58-66] gelatine,[67] diethylaminoethyl dextran,[68] Eudragit® RL/RS,[52] poly(ethylene imine) (PEI),[69-73] and poly(L-lysine) (PLL).[7,54,70,74-76]

Protamine sulfate is an arginine rich protein (Mw~4 kDa) which shares structural similarities with the HIV tat peptide and is characterized by

membrane-translocating as well as nuclear-localizing activity.[77] Possibly due to these membrane-penetrating properties, microparticles coated with cationic protamin possessed transfection activity in HEK cells and increased immunogenicity as compared to plain particles.[56]

A considerable number of studies have addressed the usability of chitosan for the surface modification of nano- and microparticles made from PLGA. Chitosan, which is a partially deacetylated derivative of the polysaccharide chitin, is commercially available in a variety of molecular weights (Mw) and deacetylation degrees (DD). The considerable variety of these materials is reflected in the studies dealing with coating, which report use of chitosans with Mw~48 kDa (DD: 75-80%),[65,66] Mw~50 kDa (DD: 80%),[60] Mw>50 kDa (DD: 85%),[51] Mw~88 kDa (DD: 85%)[63] as well as Mw~150 and 150-400 kDa.[62] Primarily, surface modification of PLGA particles has been achieved by using chitosan/poly(vinyl alcohol) (PVA) blends as stabilizers in course of the particle preparation procedure.[51,58,59,61,63,64] The incorporation of chitosan in the particle surface can be confirmed qualitatively by derivatization of the introduced amino groups with NHS-PEG-biotin, subsequent addition of Oregon Green®-streptavidin and flow cytometric analysis of the particle-associated fluorescence intensity.[61] Similarly, an assay using fluorescamine can be used to gain information on the qualitative and quantitative degree of polyamine adsorption.[50,61,63,72] This assay is based on the reaction of the non-fluorescent compound fluorescamine with primary amino groups yielding fluorescent pyrrolinones (excitation/emission: 390/475-

490 nm). As an alternative for the quantification of chitosan, complexation of the anionic dye Orange IIC with ammonium groups in acidic solution has been reported.[50] According to a systematic study by Guo et al. the amount of adsorbed chitosan increases with the polyelectrolyte concentration employed. As illustrated by fitting experimental data to adsorption isotherm models, the coating mechanism involved complies with a multilayer adsorption behaviour on a heterogeneous surface.[63] Although adsorption in course of the preparation procedure is a facile and supposedly effective approach, it only offers limited control over the conformation and quantity of adsorbed polyelectrolyte. In this regard, adsorption of chitosan onto preformed PLGA particles from solution seems preferable and has been achieved.[55,60,65,66] Chitosan adsorption from solution is a spontaneous process governed by the electrostatic interactions of the polyamine with the negatively charged particle surface. However, additional anchoring of adsorbed polymer molecules can occur via hydrophobic interactions.[50] In a detailed study, the coating of poly(D,L-lactic acid) (PLA) nanoparticles, which are expected to bear similar surface characteristics as PLGA particles, with various chitosans has been investigated. It was found that the amount of adsorbed polymer increased with rising Mw with a deposition maximum for chitosan of Mw~150 kDa. Adsorption was also increased for chitosans with lower DD. Supposedly, less deacetylated polymer chains exhibit reduced interactions with the aqueous solvent and consequently tend to adopt condensed conformations. In

conjunction with decreased interpolymer repulsion at the surface this leads to higher mass deposition rates.[50]

In contrast to chitosan which bears a rather rigid carbohydrate backbone, synthetic polyamines like PEI and PLL are characterized by enhanced polymer flexibility and higher charge densities. In the case of PEI which is commercially available in linear and branched forms ranging from Mw <1 kDa to 1.3 x 10^3 kDa, every third atom is a nitrogen atom that can be ionized by protonation. While linear PEIs mainly consist of secondary amino groups, branched PEIs are characterized by a theoretical primary to secondary to tertiary amine ratio of 1:2:1. These polymers have found widespread use for gene delivery *in vitro* and *in vivo* owing to an enhanced interaction with the negative cell membrane, the "proton sponge" effect and the high potential for complexing anionic macromolecules (pDNA, antisense oligonucleotides).[78-80] To confer these features on PLGA nano- and microparticles, coating with PEI in course of the preparation procedure[69-71,81,82] or onto preformed particles[72,73] has been investigated. Yang et al. also showed that a layer-by-layer approach is feasible by using dextran sulfate as an anionic counter-polyelectrolyte.[73] While in most studies PEIs with a Mw of 25 - 70 kDa have been used, high Mw compounds (600 - 1000 kDa) have also been employed. From a toxicological point of view, the latter seems questionable since systemic administration of 800 kDa PEI has caused considerable toxicity possibly due to a high potential for erythrocyte agglomeration.[79] Coating with branched PEI (Mw~25 kDa) has been shown to

generate PLGA particles with a distinctly increased surface binding capacity for pDNA.[71,72] Furthermore, successful polyamine adsorption was indicated by a clear increase of the buffering capacity towards HCl as compared with plain particles.[72] Trimaille et al. studied the coating of PLA nanoparticles with branched PEI (Mw~10 kDa and 25 kDa) and found that the optimum pH for ionization of surface carboxyl groups as well as polyelectrolyte amino groups was 5.8. The amount of non-adsorbed PEI was determined by a Coomassie Blue assay. Moreover, visual proof of surface-deposited polymer was given by SEM. Consistent with theory, coating from solutions with low ionic strength resulted in the adsorption of flat molecules and a zeta potential of ~-1 mV while coating in the presence of higher salt concentrations led to the deposition of coiled polymers and charge inversion.[49]

The synthetic polycation PLL has also been used for coating of negatively charged PLGA particles via ionic interactions in several studies. As with PEI, low molecular weight polymers should be applied to avoid toxic side effects.[83] According to Cui et al., ionization of the ε-amino groups of PLL (Mw~150-300 kDa) by titration can be used to regulate the hydrophile-lipophile balance and secondary structure of the polyamine.[74] By using a reaction assay for the quantification of PLL via o-phthaldialdehyde, it was found that a dissociation degree of ≥68% and an α-helix content of ≥50% is needed for sufficient surface entrapment of the polymer and stable microparticle formation. An alternative and quite promising approach has relied on the coating of preformed PLGA particles with multifunctional PLL-

g-PEG polymers.[7,75,76] Two syntheses of PLL-*g*-PEG have been described in the literature. Spencer et al. conjugated a PLL backbone (Mw~20 kDa) with N-hydroxysuccinimidyl esters of methoxypoly(ethylene glycol) propionic acid (Mw~2 kDa) and attained a grafting ratio of lysine units to PEG chain of 3.5:1.[6,7,75] Thereby, sufficient ε-amino groups were still available for electrostatic interactions with surface carboxyl groups of the particles.[7,75] By using PLL backbones grafted with methoxy-capped PEGs, protein repellent coatings can be introduced to PLGA microparticles.[75] Moreover, it has been shown that PLL can be conjugated with RGD-capped PEGs and that subsequent adsorption of the PLL-*g*-PEG-RGD onto preformed microparticles yields target specific carriers.[7] An alternative approach to the synthesis of PLL-*g*-PEG has been reported by Kim et al.[76] The terminal primary amino group of a PLL backbone (Mw~ 2 kDa) with carbobenzoxy-protected ε-amino groups (ε-CBZ-PLL) was conjugated to carbodiimide-activated COOH-PEG-folate (Mw~ 3.4 kDa). Upon removal of CBZ with hydrogen bromide in acetic acid and coating of PLGA nanoparticles with polycationic PLL-*g*-PEG-folate, an enhanced binding of the colloids to folate receptor overexpressing cells was observed.[76] The direct electrostatic immobilization of a proteinaceous targeting agent at the particle surface has been achieved by Kou et al. who expressed a single chain antibody with a polylysine tag in *E. coli* and characterized its adsorption onto PLGA nanospheres.[84]

As reported, the coating of nano- and microparticles made from PLGA by adsorption of polycations can be realized by two techniques. The

hydrophile-lipophile balance and concentration of polyamine govern the incorporation of polyelectrolyte chains into the particle surface and matrix in course of the preparation procedure. In contrast, adsorption of polycations onto preformed particles is dominated by electrostatic interactions with negatively charged surface groups. However, the advantages and limits for pharmaceutical applications of either of the coating approaches have rarely been discussed. In general, systematic studies dealing with the effects of polyelectrolyte Mw and charge density, solution pH and ionic strength on the adsorption process are scarce for PLGA particles. Moreover, most reports in the literature lack a clear description of the adsorption protocol and specifications regarding the pH and ionic strength of the medium used for zeta potential measurements. Without these parameters, the contribution of free carboxyl groups to the measured zeta potential can not be estimated correctly. The necessity of a critical interpretation of the zeta potential is further illustrated by the fact that even particles prepared from end-capped PLGA exhibit a negative zeta potential[85] probably due to the adsorption of anions to the particle surface. In this context, the impact of stabilizers or surfactants on the surface carboxyl density of PLGA particles has not been addressed sufficiently. Although the formation of a non-removable corona on the particle surface has been reported in the case of PVA,[81,86,87] it is not clear to which extent the coating via ionic interactions is affected. Finally, since the anchoring stability of an adsorbed polyelectrolyte layer can be drastically compromised by electrolytes, tensides and proteins,[88] investigations carried

out in physiological media are needed to identify potential limits of polyelectrolyte coatings for pharmaceutical applications.

II.C. Hydrophobicity – adsorption of amphiphiles

Due to the rather hydrophobic nature of PLGA, hydrophobic or amphiphilic molecules, polymers, and other substances may be adsorbed via hydrophobic interactions. Adsorption is defined as the accumulation or concentration of materials of one phase at the interfacial surface of the other phase.[89] The extent of adsorption increases with a decreasing solubility of an adsorbate in a solvent. The strong attraction between hydrophobic molecules and surfaces in water is a mainly entropic phenomenon.[90] In this section, adsorption that is predominantly based on hydrophobic interactions is described. As protein adsorption is also strongly determined by electrostatic interactions, it is discussed separately (see Section II.D).

During particle preparation via the solvent evaporation technique or similar procedures, an organic solution of PLGA is emulsified in an aqueous medium. In order to avoid coalescence, the dispersed organic phase droplets must be prevented from contacting each other. This can be achieved by adding amphiphilic substances such as surfactants, polymers, or proteins that arrange themselves at the polar-apolar interface forming mono- or multibilayers.[91] Stabilizing polymers adsorb at the interface and may extend into both phases, preferably into the continuous phase. The polymer fractions

located at the outside of the droplets repel each other and therefore stabilize the emulsion. After solvent removal and particle solidification, amphiphiles are still necessary to complement the electrostatic stabilization of the suspension. While some stabilizers are adsorbed at the particle surface in a reversible manner, others may be physically entrapped in the particle matrix and build a residual layer at the surface that resists washing. Therefore, the choice of a certain stabilizer governs the surface characteristics of PLGA particles and can be exploited for designing carriers with tailored features. Moreover, even preformed particles may be exposed to hydrophobic molecules in order to achieve a surface-modification via adsorption.

Poly(vinyl alcohol) (PVA) is one of the most frequently used emulsifiers for the preparation of micro- and nanoparticles from PLGA and related polymers. PVA is prepared by partial hydrolysis of poly(vinyl acetate) and therefore consists of rather hydrophobic vinyl acetate moieties and rather hydrophilic vinyl alcohol moieties. Upon particle preparation via a solvent evaporation technique, PVA and PLGA form an interconnected network at the interface with PVA anchored via its hydrophobic vinyl acetate moieties.[87] While PVA has been suspected to be carcinogenic,[92-94] the International Agency for Research on Cancer regards it as "not classifiable as to carcinogenicity to humans".[95] Another commonly used group of emulsifiers are the poloxamers (Pluronics®), which are amphiphilic ABA triblock copolymers consisting of a hydrophobic poly(propylene glycol) (PPG) middle block and two hydrophilic poly(ethylene glycol) (PEG) outer blocks. They are

approved by the FDA for topical, oral, and parenteral application[96] and listed in the European Pharmacopoeia. Concerning the thickness of the adsorption layer, which depends on the hydrophobicity of the particle surface and the HLB of the respective Pluronic type, the values in the literature range from 3 nm to even 20 nm.[97,98] At high concentrations Pluronic hemimicelles are adsorbed. Further emulsifiers include semisynthetic derivatives of cellulose, like methylcellulose or hydroxypropylmethyl cellulose, and polysorbates (Tween®).[22] However, for Tween 20 a strong toxicity was observed by a dramatically increased paracellular transport of [^{14}C]sucrose in a blood-brain barrier endothelial cell culture.[99] Besides non-ionic stabilizers, also ionic surfactants have been assessed. Cationic emulsifiers like cetyltrimethylammonium bromide (CTAB), dimethyl dioctadecyl ammonium bromide (DDA), and 1,2-dioleoyl-1,3-trimethylammoniopropane (DOTAP) were used during particle preparation to stabilize the emulsion owing to their amphiphilic properties. At the same time they furnish the resulting particles with a positive surface charge that allows the adsorption of DNA.[100,101] Accordingly, anionic emulsifiers like sodium dodecyl sulfate (SDS) and dioctyl sodium sulfosuccinate (DSS) were employed for the preparation of PLGA particles with negative surface charge in order to enable the adsorption of antigens for immunization purposes.[102,103] Nevertheless, for toxicity reasons the repertory of possible surfactants for parenteral administration is limited and some of the mentioned emulsifiers such as SDS will not be applicable.

Recently, there have been some approaches to substitute traditional non-biodegradable surfactants by fully degradable alternatives. Phospholipids, especially those with saturated chains such as 1,2-didecanoylphosphatidylcholine (DDPC) or 1,2-dipalmitoylphosphatidylcholine (DPPC), were proposed as efficient emulsifiers for the preparation of PLGA nanospheres.[104] As compared to PVA, a higher emulsifying efficiency was observed for DPPC, which was attributed to a more complete surface coating. In another approach, PLGA nanoparticles were coated with a pegylated-lipid envelope composed of poly(ethylene glycol) distearoylphosphatidyl-ethanolamine (PEG-DSPE), phosphatidylcholin, and cholesterol resulting in the formation of a so-called nanocell.[105] Upon encapsulation of a drug within the PLGA matrix and incorporation of a second lipophilic agent within the envelope, a temporal release of the two drugs was reported. Hydroxyethyl starch (HES) is a well-established plasma volume expander that could be an interesting alternative to the non-biodegradable PEG.[106] HES can be hydrophobically modified by the formation of fatty acid esters. Using HES laurate for stabilization, PLGA nanoparticles with a narrow size distribution and a mean particle size of 110 nm were prepared and further characterized in protein adsorption assays using HSA and fibrinogen.[98] Thereby, HES laurate provided a stealth character comparable to Pluronic® F127 and even superior to Pluronic® F68, which was also confirmed by *in vitro* phagocytosis assays with murine macrophages. Another promising approach is the use of

alkyl polyglucosides, which are non-ionic surfactants consisting of glucose units and a fatty alcohol.[94]

The adsorption characteristics of amphiphiles also play an important role for the covalent surface modification of PLGA particles. In presence of PVA, the conjugation of polyclonal antibodies to PLGA nanoparticles was reduced by 48%.[46] In presence of high concentrations of Pluronic® F68, ligand coupling decreased by up to 65%.[47] Thus, easily removable emulsifiers may be preferred in order to enable access to the PLGA carboxylate groups. However, there are an increasing number of studies that exploit the irreversible adsorption of amphiphilic molecules for the functionalization of PLGA particles. This might be achieved either by using high-affinity emulsifiers that remain stably associated with the particle surface and confer an inherent additional functionality to the particles in order to alter their interaction with cells, or by covalent coupling of targeters and other substances to surface-anchored emulsifiers.

Currently, there are attempts to prepare particles with a specific surface functionality in a one-step procedure by enhancing the surface activity of functional molecules. The vitamin-PEG conjugate D-α-tocopheryl polyethylene glycol 1000 succinate (TPGS) is an amphiphilic and water-soluble derivative formed by conjugation of vitamin E succinate with PEG.[107] TPGS inhibits P-glycoprotein mediated drug transport and might thus improve the bioavailability of P-gp substrates.[108] A similar approach was assessed for vitamin D using cholecalciferol polyethylene glycol succinate

(CPGS).[109] Amphiphilic derivatives of hyaluronic acid were used as surfactants for the preparation of PLA nanoparticles.[110] The resulting particles bound preferentially to chondrocytes owing to hyaluronate-targeting of the CD44 receptor. As a versatile modification platform, avidin-fatty acid conjugates were prepared and added to the PVA solution upon microparticle preparation via solvent evaporation.[111] This should allow for a quick immobilization of various biotinylated ligands to surface-exposed avidin, which resulted in a maximum of 2.5 µg biotin-phycoerythrin (240 kDa) per mg polymer. For a new approach in vaccination, lipopolysaccharides have been added to the stabilizer solution during particle preparation to act as inflammasome-activating adjuvant.[112] By contrast, aiming at an enhanced blood and tissue compatibility certain salts or amino acid complexes of heparin have been used for the preparation of microparticles.[113] Nevertheless, it has to be considered that a major fraction of surface associated heparin and TPGS can be removed from the particle surface by repeated washing.[114] In general, the adsorption of amphiphilic molecules for a specific surface modification is a rapid and convenient method, provided that the interaction is strong enough to resist premature desorption.

Several biorecognitive molecules are not amphiphilic and cannot be physically entrapped during particle preparation. Thus, they need to be immobilized to preformed particles. The classical approach to couple ligands to terminal PLGA carboxylate groups exposed at the particle surface may often not be feasible due to steric hindrance or inappropriate coupling

chemistry. As an alternative, covalent conjugation to certain functional groups that are present at the particle surface due to physically entrapped stabilizers has been reported. At this, glutaraldehyde was used to couple lectins to PVA- or BSA-stabilized PLA microspheres.[115] When two adjacent hydroxyl groups of PVA formed an acetal with glutaraldehyde, the remaining aldehyde group could then either react with the lectin or with another PVA molecule resulting in PVA cross-linking. Alternatively, a multifunctional epoxy linker that reacts with PVA hydroxyl groups and with amine groups of a ligand was used for the conjugation of transferrin and trans-activating transcriptor (tat) peptide to surface-anchored PVA.[116,117] However, it should be considered that PVA is not biodegradable and only low Mw PVA is quickly eliminated from the body. In animal studies, medium and high Mw PVA have been found deposited in various organs.[93] Thus, cross-linked PVA might not be eliminated from the body and thus accumulate in certain organs causing inflammatory reactions. For another approach, antibodies were coupled to surface-anchored PVA via cyanogen bromide, which creates a link between PVA hydroxyl groups and primary amines.[118] Although the reagent is well established for the preparation of matrices for affinity chromatography, its applicability in drug delivery systems might be limited due to its acute toxicity. To overcome the PVA-shielding effect, PVA was replaced by poly(ethylene-alt-maleic acid) (PEMA), which contains carboxylic acid side chains and thus allows for carbodiimide-mediated coupling of amine-containing ligands to the surface-anchored stabilizer.[119] Similarly, when the

hydroxyl groups of Pluronic® F127 were succinylated, the resulting carboxylated poloxamer could be used as an emulsifier for the preparation of PLGA nanoparticles followed by covalent coupling of a peptide via a modified carbodiimide method.[120] Besides neutral or carboxylated stabilizers, cationic amphiphiles were used to introduce reactive groups. Polylysine that is usually immobilized at negatively charged surfaces via electrostatic interactions (see Section II.B) is unable to stabilize emulsions at neutral pH due to its high charge density. Upon addition of sodium hydroxide, however, the polypeptide becomes amphiphilic and the secondary structure is shifted from random-coil to α-helix. Under these conditions the polypeptide was applied as a surfactant for the preparation of PLGA microparticles by a solvent evaporation technique.[74] The surface-anchored polylysine was then coupled via its ε-amino groups to thiol groups of a synthetic peptide using the bifunctional crosslinker sulfo-GMBS.[121]

To conclude, surface adsorption of different substances via hydrophobic interactions has an impact on the stability of the suspension, the access to PLGA carboxylate groups for covalent surface modification, and it can even be exploited for imposing certain characteristics to the surface itself. The main prerequisite for the latter approach is a strong hydrophobic interaction, which resists desorption during repeated washings. For the future, only biocompatible stabilizers should be applied, either being biodegradable or at least easily eliminated via urinary or biliary excretion.

II.D. Protein adsorption

The adsorption of proteins to the surface of PLGA particles is discussed separately, because it relies on a combination of hydrophobic and electrostatic interactions. On the one hand, protein adsorption is being exploited for the preparation of carriers for therapeutic proteins or protein vaccines as well as for targeting purposes. On the other hand, the adsorption of blood proteins upon parenteral administration is most often unwanted as it leads to a rapid clearance of administered colloids via the RES. Protein adsorption to the particle-liquid interface has also an impact on the stability of a suspension. Moreover, adsorption phenomena may even affect the release of encapsulated protein drugs.

To elucidate the mechanisms that govern protein adsorption, model proteins containing different amounts of charged amino acids were used. Amphoteric molecules often have their maximum adsorption capacity at the isoelectric point (IEP), where the net charge of the adsorbate becomes zero.[89] Nevertheless, a strong dependence on electrostatic interactions has also been described. An examination of the surface-affinity of proteins with different isoelectric points from 4.6 to 10.7 under varying pH conditions revealed that protein adsorption was significantly enhanced by attractive electrostatic interactions, while a certain extent of binding even took place under electrostatically repulsive conditions via non-Coulomb forces.[122] In another study, the positively charged model protein lysozyme was adsorbed onto

negatively charged nanoparticles prepared from either PLGA alone or from blends with the strongly negative poly(styrene-co-4-styrene-sulfonate) (PSS). Thereby, increasing lysozyme loading was observed in case of the enhanced negative surface charge.[123] When the negative charge of PLGA particles was increased by using anionic surfactants like sodium dodecyl sulfate (SDS) or dioctyl sodium sulfosuccinate (DSS) for particle preparation, recombinant p55 gag protein from HIV-1 or antigens from *Neisseria meningitidis* type B could be adsorbed to the charged particles. A potent immune response was elicited upon immunization of mice, which was superior to that achieved by co-administration of antigen and PVA-stabilized PLGA microparticles.[102,103]

Upon adsorption of positively charged protein, the initially negative zeta potential of the particle surface shifts towards zero and the particles start to aggregate.[123] Since electrostatic repulsion is probably the most important factor for maintaining stable suspensions, the impact of protein adsorption must not be neglected.

Another interesting aspect is the influence of non-specific protein adsorption on the release of encapsulated protein drugs.[124,125] When the inner surface of the particles expands in the course of polymer degradation increasing amounts of the therapeutic protein may be adsorbed, which results in a slower release. Generally, the type of interaction with the surface seems to affect the release kinetics of the adsorbed protein. A stronger contribution of electrostatic interactions was associated with a quicker release as compared to predominantly hydrophobic interactions.[123] In contrast to

hydrophobic interactions, electrostatic forces are affected by changes in pH or salt concentration.

Sensitive proteins that would be degraded during encapsulation due to shear forces or organic solvents or during covalent coupling might thus be immobilized via simple adsorption to the particle surface. The functionality of adsorbed proteins was assessed for different applications. For targeting purposes, transferrin-coated PLGA nanoparticles were prepared by incubation of blank nanoparticles with the protein.[99] The uptake of the transferrin-grafted particles into blood brain barrier cells was increased 20-fold as compared to blank nanoparticles and 2-fold as compared to BSA-coated particles. This effect could be inhibited by an excess of free transferrin, which points to selective endocytosis. Upon comparing covalently immobilized and surface-adsorbed monoclonal antibodies, it was reported that only the nanoparticles with adsorbed antibody were specifically taken up into MCF-10A neoT cells.[126] This observation was attributed to an inactivation of the antibody during the carbodiimide-mediated coupling procedure.

To conclude, the adsorption of various proteins to PLGA particle surfaces has been described as a rapid and simple alternative for the delivery of sensitive proteins and for the immobilization of targeters. The main problems, however, have not been addressed adequately so far. The stability of the adsorptive protein-particle interaction under physiological conditions remains questionable as adsorbed molecules may be displaced by competitive adsorption of other substances, for example by plasma proteins. Moreover, pH

and ionic strength influence the affinity of the adsorbed protein to the surface. Last but not least, particles that easily adsorb proteins at their surface are expected to be rapidly opsonized and eliminated by the RES, which might limit their applicability *in vivo*. Thus, the most important aspect concerning protein adsorption is generally not how to enforce it, but how to reduce it. Strategies to reduce opsonization via the so-called stealth effect are discussed in section III.C.

II. E. Methods for surface characterization

Several sensitive and sophisticated techniques are available for the physicochemical characterization of PLGA-particles including their surface properties. It is obvious that a single method cannot cover the whole spectrum of analytical questions and thus the available techniques listed in Table II need to be combined to fully elucidate the surface modification of particles.

Size and polydispersity represent key parameters not only for biodistribution and clearance but they are also useful as a rough estimate of the particle surface available for coupling. Laser diffractometry (LD), dynamic light scattering (DLS) also known as photon correlation spectroscopy (PCS) and less frequently multi angle laser light scattering (MALLS) after flow field flow fractionation (FFFF) represent light scattering techniques for particle size determination. Due to multiple pitfalls associated with these

techniques, it is highly recommended to confirm particle size by an imaging technique.[135] These microscopic methods such as scanning electron microscopy (SEM), transmission electron microscopy (TEM), Cryo-TEM and recently atomic force microscopy (AFM) provide additional information about particle morphology (see also Section IV).

For determination of the specific surface area (SSA) the Brunauer-Emmett-Teller method (BET) is applied which relies on adsorption-desorption isotherms of N_2 and Kr gases at the particle surface. Although this method is preferably used to characterize porous microparticles and the associated initial burst release,[127,128] it might also be a versatile tool for the determination of the surface area available for coupling of ligands considering porosity, size, and polymer composition of PLGA micro- and nanoparticles.

Table III: Analytical methods for characterization of surface-modified PLGA-particles

Technique	Abbr.	Parameter investigated	Reference
Dynamic light scattering	DLS	Hydrodynamic diameter (5 - 5000nm)	129,130
or Photon correlation spectroscopy	PCS		
or Quasi elastic light scattering	QELS		
Laser diffractometry	LD	Volume-based particle size distribution (50nm – 1000µm)	130
Flow field-flow fractionation with Multi angle light scattering (MALS)	FFFF	Hydrodynamic size Radius of gyration (up to 500nm)	131-133
Scanning electron microscopy	SEM	Number-based particle size distribution (50 nm – 100 µm), particle morphology	134,135 136
Transmission electron microscopy	TEM	Number-based particle size distribution (50 nm – 100 µm), particle morphology	137,138
Cryogenic transmission electron microscopy	Cryo-TEM	Number-based particle size distribution (50 nm – 100 µm), particle	139

		morphology	
Atomic force microscopy	AFM	Number-based particle size	140
or Scanning probe microscopy	SPM	distribution, particle morphology, binding forces	
Small angle X-ray scattering	SAXS	Radius of gyration, shape and surface structure	131,141
Electrophoretic techniques	---	Electrophoretic mobility, zeta potential	142-145
Hydrophobic interaction chromatography	HIC	Hydrophobicity / hydrophilicity	146
Contact angle measurement	---	Hydrophobicity / hydrophilicity	147
Underwater contact angle measurement	---	Hydrophobicity / hydrophilicity	148
Rose Bengal adsorption assay	RB	Hydrophobicity / hydrophilicity	137,149
Resonant mirror system	RMS	Ligand-ligate interaction	150
Two dimensional gel electrophoresis	2D-PAGE	Protein adsorption	147; 151
X-ray photoelectron spectroscopy	XPS	Surface chemistry (penetration depth:	81,86,138,148
or Electron spectroscopy for chemical analysis	ESCA	2 – 10 nm)	
Secondary ion mass spectrometry	SIMS	Surface chemistry (penetration depth 1 nm)	152
Time-of-flight Secondary ion mass spectrum	TOF-SIMS	Surface chemistry	153
Static Secondary ion mass spectrometry	SSIMS	Surface chemistry (thermally labile compounds)	86
Fourier transform infrared spectroscopy – attenuated total reflection	FTIR ATR	Chemical composition	59
Flow cytometry	FCM	Mean fluorescence per particle	47
MicroRaman spectroscopy	---	Molecular composition, crystal localisation (penetration depth in µm-range)	136
Brunnauer-Emmett-Teller method	BET	Specific surface area	127,128

Besides the surface morphology and size, two additional parameters describing the characteristics of particles are their surface charge and hydrophobicity. The surface charge strongly influences the stability of aqueous nanosuspensions as well as particle cell interactions.[144,145] Usually, the surface charge is assessed via the mobility of the particles in an electrical field and expressed as electrophoretic mobility (μ) or converted to zeta (ζ) potential, which represents the potential at the hydrodynamic shear plane of

the particle. For the theoretical background as well as calculation of mobility and ζ-potential the reader is referred to the literature.[142] The surface charge is dependent on the degree of ionization of particle surface groups and on ion adsorption. In practice, zeta potential measurements are highly sensitive to the conductivity of the dispersant medium and its pH. Generally, values > 30 mV indicate physically stable PLGA-particle suspensions which are widely stabilized via electrostatic repulsion.[143]

Surface hydrophobicity can influence the adsorption of excipients and proteins. Whereas the contact angle measurements of water and octane drops as well as air bubbles are only applicable to PLGA films,[147] the hydrophilicity and vice versa the hydrophobicity of particles can be determined by a Rose Bengal adsorption assay.[137,149] At this, nanoparticles are incubated with an aqueous solution of the hydrophilic dye, spun down and subsequently the dye content in the supernatant is determined by spectrometry at 564 nm. From the difference between blank and sample the amount of dye adsorbed by the nanoparticles is calculated considering the density and diameter of the particles. In addition, hydrophobic interaction chromatography (HIC) proved useful to monitor changes in particle surface hydrophobicity upon modification with hydrophilic ligands[146] (see III.C.).

X-ray photoelectron spectroscopy (XPS), also called electron spectroscopy for chemical analysis (ESCA), is routinely applied for particle surface analysis. The samples are prepared on substrates such as glass slides or aluminum foil by drop casting of aqueous particle suspensions followed by

drying. X-ray irradiation causes emission of photoelectrons with specific binding energies according to the electron core level at the site of ejection. The peak intensity is proportional to the atomic concentration within the sample. XPS provides qualitative information about the surface chemistry as well as quantitative information about the element composition and functional groups at the particle surface with high sensitivity, usually 1 atom in 1000.[86] Unfortunately, interpretation of the spectra is complex since varying ligand coverage of the particle surface and/or the penetration depth in the range of 2 – 10 nm might lead to interference between strong signals from the underlying PLGA matrix and only weak signals from the modified surface. In addition, precise quantification of data may be difficult.[148] Nevertheless, XPS analysis at a penetration depth of 10 nm revealed that rhodamine was not present at the surface of PLGA nanoparticles but embedded in the core.[81] Additionally, XPS has proven as a powerful tool to detect the presence of a chitosan- or PVA-shell on PLGA nanoparticles.[138,148]

A surface analytical technique complementary to XPS is secondary ion mass spectrometry (SIMS). At this, secondary ions emitted from the particles under high vacuum are analyzed according to their mass/charge (m/z) ratio. As compared to XPS, SIMS offers the advantage of detecting all elements including their isotopic distribution, has low detection limits and a low penetration depth of only 1 nm of the particle surface,[152] but the drawback of sample damage. Time-of-flight (TOF-SIMS) is preferentially applied for mass analysis of high molecular weight samples and static SIMS (SSIMS) is used

to collect the mass spectra of thermally labile organic compounds. Both complementary surface analytical techniques, XPS and SSIMS, have been employed to determine residual surfactant at the surface of PLGA nanoparticles after purification by centrifugation or gel permeation chromatography.[86] Moreover, XPS and TOF-SIMS have been applied for surface characterization of PLGA microparticles after vaccine antigen adsorption. Although some amino acid residues known to be specific for certain proteins were identified by TOF-SIMS, the spectra were too complex to provide specific information about protein identity and quantity. However, the combination of the two techniques allowed for the identification and quantification of both the protein and the surfactant adsorbed at the outermost surface and revealed that the antigen concentration decreased with increasing surfactant concentration.[153]

Whereas the chemical composition of polymers can be elucidated by Fourier transform infrared (FT-IR) spectroscopy, the ATR-mode (attenuated total reflectance) is applied for surface characterization. After spreading the nanoparticle suspension onto the ATR crystal and drying with nitrogen, it was possible to detect the presence of positively charged chitosan as well as surfactant at the surface of PLGA-nanoparticles.[59]

In addition, the adhesive capacity of surface modified particles as well as their binding specificity was assessed by the resonant mirror system (RMS). The RMS is an optical biosensor based on the phase shift of an incident beam that occurs in reflected light, which passes through a prism

underlying the resonant mirror. This label-free technique allows monitoring of the interaction between a dissolved molecule or a dispersed nanoparticle of interest (ligate) and its biospecific partner (ligand) immobilized at the waveguide sensing surface. Depending on the extent of binding a change in the resonance angle is observed. Using this technique, the interaction between biotinylated nanoparticles and Neutr-Avidin-coated surfaces was assessed. At this, a strong and specific binding was observed, but no equilibrium presumably due to rearrangement of nanoparticles.[150]

Among the fluorimetric methods, fluorescence microscopy and fluorimetry allow for detection and quantification of fluorescence labeled ligands coupled onto the particle surface. Flow cytometry, relying on forward versus side scatter analysis of a few thousand single microparticles in a sheath flow one by one, offers a multiparametric analysis in terms of size, granularity, and fluorescence intensity after surface modification with fluorescent labeled ligands.[47]

MicroRaman spectroscopy allows for studying molecule vibrations in micron-sized materials due to their interaction with photons. Accomplishing a penetration depth in the µm-range, this technique comprises the surface as well as the core of particles. Applying this technique, the distribution of drug crystals in PLGA-microparticles was found to be close to the surface in high molecular weight PLGA matrices but rather in the core of low molecular weight PLGA.[136]

Perhaps the most important aspect of surface characterization is the interaction of the functionalized nano- and microparticles with the biological environment, which requires either *ex vivo* studies with human cell culture models[40] or *in vivo* studies in animals.

III. AIMS OF FUNCTIONALIZATION

III.A. Modified bioadhesion and potential internalization

III.A.1 Non-specific mechanisms

A rather non-specific approach to enhance the binding of polymer particles to human cells makes use of the net negative charge of the extracellular face of the plasma membrane. Although the quantitative and qualitative compositions of the charged groups contributing to the overall negative zeta potential on different cell types have not been fully resolved yet, it seems clear that negative charges generally prevail.[154] Theoretically, these charges bear a considerable potential for attractive electrostatic interactions with positively charged drug carriers and consequently might be exploited to increase the cell-binding[53,155] and transfection efficiency of particulate carrier systems.[156] Nano- and microparticles prepared from uncapped PLGA and stabilized by PVA or poloxamers, however, are usually negatively charged due to free surface carboxyl groups. To induce a positive zeta potential on these particles, several coating techniques with cationic excipients have been

developed (see Sections II.B, II.C, III.E). As expected, PLGA nanoparticles coated with didodecyl dimethyl ammonium bromide (DMAB) preferentially associated with the negative endothelial layer in a femoral artery *ex vivo* model.[157] This led to a seven- to tenfold enhanced delivery of an antiproliferative agent as compared to plain particles. Interestingly, the density of negative charges in the vasculature was found to be further increased in malignantly transformed tissue.[158] Supposedly, this is due to an induced exposure of anionic phospholipids, most likely phosphatidylserine, on the cell membrane. This surplus of negative charges as compared to the normal vasculature in combination with the reportedly low pH in tumours could be exploited for the accumulation of cationic drug carriers or imaging agents.[65] Besides increasing cytoadhesion, coating with cationic excipients also affects the internalization rate and intracellular processing of particles. While negatively charged polystyrene beads were only taken up to a low extent by macrophages and dendritic cells (DCs), positively charged beads were readily internalized and induced maturation of DCs.[53] These positively charged particles were characterized by a similarly high cell binding affinity to antigen presenting cells (APCs) as IgG-modified beads. This led to phagocytosis and subsequent engulfment in tightly apposed phagosomes which do not undergo maturation. In contrast, albumin-modified particles were taken up in loosely apposed phagosomes which rapidly fuse with lysosomes.[57] Thus, coating of drug and vaccine carriers with cationic polyelectrolytes could not only result in enhanced uptake into APCs but also

in intraphagosomal protection of the payload-to-be-delivered. However, besides these promising reports with *in vitro* and *ex vivo* setups, studies performed under physiological conditions are rare. In particular, it is not clear to which extent the adsorption of serum proteins will alter the cationic charge density of the particle surface and consequently effectiveness of the electrostatic bioadhesion approach. This issue deserves attention, especially since a recent report indicated that the adsorption of serum onto PLL-coated PS microspheres leads to reduced uptake into DCs as compared to untreated cationic beads.[155] The probably limited influence of particle surface charge in presence of serum was also indicated by Roser et al., who observed no differences in the blood circulation times and biodistribution of cationic, anionic and neutral albumin nanospheres in rats.[159]

If not administered via the parenteral or topical route, drug carrier systems will encounter the sticky, viscous and elastic mucus layer which lines all mucosal tissues.[160,161] Due to its constant secretion, transport and excretion, mucus functions as a dynamic protective barrier efficiently removing foreign material from the body. This severely limits the residence time and thus efficiency of sustained release drug carrier systems. Therefore, mucoadhesion and mucopenetration have been investigated as approaches to counteract premature removal[161,162] and to enhance transport of particulate drug carriers through the mucin mesh to underlying tissues.[160] Polymers utilized to formulate mucoadhesive drug delivery systems have comprised polyanions, polycations, and thiolated polymers. Polyanions such as

poly(acrylic acid) (PAA) as well as sodium carboxymethylcellulose (CMC) and their derivatives supposedly bind to mucus via hydrogen bonding. In contrast, polycations like chitosan are anchored by a combination of hydrogen bonds and electrostatic interactions with sulfonic - or sialic acid groups of glycosylated moieties of mucus.[161] To transfer mucoaffinity to PLGA nano- and microparticles, coating with mucoadhesive polymers like PAA,[163,164] chitosan and thiolated derivatives,[51,60,64,163,165] PEI[81] as well as Eudragit® RL/RS[52] has been investigated. As opposed to particles prepared in the presence of poloxamer 188 and PVA, PLGA nanoparticles produced with PAA as a stabilizer were indeed characterized by mucoadhesive properties.[164] According to Yamamoto et al., surface modification of particles with chitosan led to higher retention rates in the lung of guinea pigs after administration by nebulization as compared to plain colloids.[60] The prolonged pharmacological action of calcitonin co-administered in these particles was attributed to the mucoadhesive properties and probably an opening of tight junctions. Furthermore, it was shown that chitosan-coated PLGA nanoparticles exhibit higher binding to a rat everted intestinal sac model than PAA- and alginate-modified controls.[163] These proof-of-principle studies illustrate that mucoadhesive properties can be integrated into a priori non-mucoadhesive particles made from PLGA by surface coating. Whether PLGA is suited as a sustained release polymer for mucoadhesive systems, however, is questionable. Even if mucoadhesion is established, the particles are expected to be removed from the body at a rate similar to that of mucus

turnover. However, these turnover rates range from ~20 min in the nasal tract to ~10-20 min for the luminal layer in the respiratory tract and ~4-6 h in the gastrointestinal tract[160] and thus lie well below the degradation half life of PLGA.

Very recently, it has been found, that mucopenetrating nanoparticles can be engineered with potential advantages over traditional, mucoadhesive formulations. While mucoadhesive particles adhere to the outer luminal mucus layer, which is rapidly cleared, mucopenetrating nanoparticles are supposed to diffuse into low-viscosity aqueous pores of the mucin mesh and to thereby reach deeper adherent mucus layers.[160,166] The surface functionalization rationales that are used to generate mucopenetrating properties have been deduced from studying nature. Using fluorescence recovery after photobleaching and multiple image photography, Olmsted et al. investigated the diffusion of viruses and polystyrene particles (59-1000 nm) in human cervical mucus.[167] While viruses with a net neutral surface charge like human papilloma virus (~55 nm) and Norwalk virus (~38 nm) diffused as rapidly in mucus as in saline, negatively charged polystyrene particles were trapped and adhered to mucin via hydrophobic interactions. Ongoing from these and similar findings it was proposed that sufficiently small particles with neutral surface charge would exhibit minimal interactions with mucus and thus be able to diffuse relatively unhindered.[160,166] A well established strategy to render the particle surface inert and neutral is based on the modification with poly(ethylene glycol)

(PEG). Upon conjugation of polystyrene nanoparticles with high surface densities of low molecular weight PEGs of 2 kDa and 5 kDa, particles with enhanced diffusivity in mucus were generated.[168] In contrast, conjugation with higher molecular weight PEG (>10 kDa) might induce mucoadhesive properties due to the increased penetration of longer polymer chains into the mucin network. After surface modification with PEG, nanoparticles with mean diameters of 200 nm and 500 nm, which are clearly larger than the reported mucin mesh pore size (10-200 nm), diffused in mucus at rates only four- to sixfold slower than in water.[169] Consequently, surface modification with PEGs of appropriate Mw might drastically improve the transport of particles towards the underlying epithelium. Making use of the same principle, but via a different approach to surface modification, PLGA nanoparticles have been rendered mucopenetrating.[170] Avidin-decorated particles (170±57 nm) were prepared by using palmitate-avidin/PVA blends as stabilizer in course of the preparation procedure. By adding different amounts of biotinylated PEG to these particles, varying degrees of grafting were achieved. With increasing the surface grafting density of PEG, an increase in the diffusivity of the PLGA nanoparticles in human cervical mucus was observed.[170] Increased diffusion coefficients in mucus upon alteration of the surface charge of particles have also been monitored without PEGylation.[171] By modification of PLGA nanoparticles with dimethyl dioctadecyl ammonium bromide (DDAB) and subsequent loading with plasmid DNA, 10-fold higher diffusion rates were achieved as compared to

negatively charged polystyrene nanoparticles of similar size. These reports seem promising for the delivery of drugs, genes or antigens through the mucus barrier to the underlying epithelium. However, the therapeutic advantages of mucopenetrating particles over mucoadhesive systems have yet to be proven *in vivo*. Furthermore, PEG-decorated nanoparticles will be expected to have a low uptake rate into epithelial cells due to the hydrophilic surface coating. In this regard, grafting of mucopenetrating nanoparticles with low molecular weight targeting moieties, which enhance uptake into epithelial cells, might be advantageous.

III.A.2 Biorecognitive mechanisms

More specifically, bioadhesion can be enhanced by a biorecognitive interaction between particle surface-immobilized active targeters and tissue-characteristic structures at the cell surface. These targets comprise a broad range of receptors including cell adhesion molecules, carriers and other membrane proteins or glycoproteins. The fundamental problem is the identification of appropriate tissue-specific targets. However, few of them are uniquely present in only one single tissue and it is thus important to consider the relative selectivity of the targeted moiety in relation to the potency of the delivered drug. Moreover, these tissue markers have to be accessible to allow for an interaction with the targeted particles. The route of administration is also crucial for successful targeting. If a drug delivery system attaches to the target cells via non-internalizing epitopes, high local drug concentrations at

the outer surface of the target cell may result in a higher therapeutic efficacy than free drug released in the circulation. However, even upon localized release only part of the drug will enter the target cells. Therefore, most strategies focus on internalizing epitopes. As ligands for active targeting, proteins such as antibodies or lectins, as well as peptides and peptide-analogues, aptamers, vitamins, and other natural-derived, semisynthetic or synthetic molecules that bind to certain target structures with high affinity and specificity are applicable.

The application of antibodies for clinically applied immunoconjugates is a well-established strategy in cancer treatment. Similarly, drug delivery devices might be guided to their target cells via surface-immobilized immunoglobulins. At this, antibodies against the human epidermal growth factor receptor (EGFR; HER) for targeted breast cancer therapy,[172,173] against the siglec-7 (CD33-like) receptor that is expressed on most acute myeloid leukaemias,[174] and against the Fas (CD95/Apo-1) death receptor[175] mediated enhanced PLGA nanoparticle internalization. Antibodies against vascular endothelial growth factor receptor-2 (VEGFR-2) for systemic targeting to angiogenic sites in prostate tumors resulted in enhanced cytoadhesion of microparticles.[118] To mimic leukocyte adhesion to inflamed endothelium, PLGA microspheres were decorated with two different ligands, i.e. an antibody against intercellular cell adhesion molecule-1 (ICAM-1) and the selectin ligand sialyl Lewis^X for glycotargeting.[176]

Glycotargeting is another extensively studied strategy, which relies on the specific interaction between non-enzymatic sugar-binding proteins, so-called lectins, and certain carbohydrate moities.[177] Either endogenous lectins or, inversely, endogenous sugars serve as targets. A prominent group of mammalian lectins are the selectins, a family of adhesion receptors, which can be addressed by the above-mentioned sialyl Lewis^X. Another example is the galactose-specific asialoglycoprotein receptor, which is expressed at the surface of hepatocytes and overexpressed in hepatocarcinoma. Recently, pDNA-loaded cationic nanoparticles were prepared from a blend of PLGA and 1,2-dioleoyl-3-(trimethylammonium) propane (DOTAP) followed by adsorption of the asialoglycoprotein receptor ligand asialofetuin for targeted delivery to liver tumor cells.[178] In a mouse xenograft tumor model, complete tumor regression was reported for 75% of mice treated with these targeted gene carriers. For targeting cartilage, PLA nanoparticles were coated with the glycosaminoglycan hyaluronate, which has a high affinity to the CD44 receptor that is present at the surface of chondrocytes and other articular cells.[110] Nevertheless, the majority of glycotargeting approaches relies on lectin-mediated targeting to specific carbohydrates. Each mammalian cell bears a sugar coat, the so-called glycocalyx, which is composed of the oligosaccharide moieties of membrane-anchored proteoglycans, glycolipids and glycoproteins. The glycosylation pattern is not only tissue-specific, but it may also change upon malignant transformation.[179] Screenings of the binding pattern of a range of plant lectins with different carbohydrate binding

specificities to different cell types pave the way for lectin-mediated targeting.[180-184] Moreover, sugars are also present in the mucus and the extracellular matrix. Wheat germ agglutinin (WGA), the lectin derived from *Triticum vulgare*, specifically interacts with N-actetyl-D-glucosamine and sialic acid residues. These sugars form part of the intestinal mucus and the glycocalyx of human intestinal epithelium.[185] Moreover, they are present in the glycosylated extracellular domain of the epidermal growth factor (EGF) receptor.[186] WGA and WGA-decorated PLGA particles are not only cytoadhesive, but they may even be taken up into enterocytes via receptor-mediated endocytosis,[187] which might open the way to improved peroral drug delivery. Not only enterocytes may be targeted in the intestine, but also M cells being addressed in peroral immunization. Thus, allergen-loaded PLGA microparticles were functionalized with the α-L-fucose-specific *Aleuria aurantia* lectin (AAL) for binding to the glycocalyx of murine M cells.[188] In mice, peroral gavage of the targeted formulation resulted in a favorable shift from a Th2-type allergic to a Th1-type immune response. Similar results were obtained with the α-L-fucose-specific *Ulex europaeus* 1 agglutinin (UEA-1).[189] However, the glycosylation pattern of M cells exhibits species-related variations and for human M cells other targets than α-L-fucose will be necessary.[189] Besides peroral application, there are also possible targets in other tissues demanding other routes of application. For example, human macrophage-like THP-1 cells preferably internalized WGA-modified PLGA nanoparticles,[184] which might enable a new approach for the treatment of

infectious diseases provoked by intracellular pathogens. Furthermore, lectin-binding to bladder cancer cells might be exploited for designing targeted drug carriers with a prolonged residence time in the bladder upon instillation.[183]

Another strategy is the targeting of the transferrin receptor, which plays an essential role in the iron metabolism of cells and is overexpressed in certain tissues such as in the liver, epidermis, intestinal epithelium, vascular endothelium of the brain capillary, certain blood cells and in malignant tissues. Functionalization of paclitaxel-loaded PLGA nanoparticles with transferrin enhanced the antiproliferative activity of the encapsulated drug in MCF-7 and drug-resistent MCF-7/Adr breast cancer cells, which was attributed to a sustained intracellular drug retention.[116] Transferrin has also been proposed for brain delivery of nanoparticles. In an attempt to study the endocytosis of targeted particulates by blood-brain barrier (BBB) cells, an *in vitro* model of the BBB made of co-cultured endothelial cells and astrocytes was incubated with transferrin-coated PLGA nanoparticles.[99] The interaction was found to be specific and caveolae-mediated endocytosis was proposed.

Other approaches are aimed at mimicking the interaction of different bacteria with their hosts. Various bacteria such as *Yersinia*, *Shigella*, *Salmonella* and *Listeria* species enter the body through the intestine aided by enteroinvasive proteins. The *Yersinia* adhesin invasin binds to a subset of ß1 integrin receptors at the apical membrane of M cells leading to host cell invasion.[190] Although ß1 integrin expression is generally limited to the basolateral membranes of polarized epithelia, there is evidence that they also

occur at the apical side of M cells.[191] A large fusion protein containing the carboxyl terminal 479 amino acids of invasin (MBP-Inv479) was covalently coupled to the surface of PLGA nanoparticles.[192] For these modified particles an enhanced interaction with invasin binding cells was reported that could be inhibited by free MBP-Inv479 or the competing ligand RGD, which points to a specific interaction with integrins. Another approach is based on the neuronal transport of tetanus toxin. The non-toxic C fragment of tetanus toxin (TTC) binds with high affinity to the neuronal ganglioside $GT1_b$, which leads to efficient endocytosis and retrograde transport from the distal axonal terminus to the neuronal cell body thus allowing to bypass the blood-brain barrier and to enter the central nervous system (CNS). This transport mechanism seems promising for new treatment opportunities in neurodegenerative diseases. To study a possible strategy for targeted drug delivery to neurons, PLGA nanoparticles were surface-modified with TTC.[193] Flow cytometric studies with neuroblastoma, liver and endothelial cells revealed selective targeting to the neuroblastoma cells.

However, application of proteinaceous targeters bears some possible disadvantages. They may elicit undesirable immune responses; they are degraded by proteolytic enzymes; they change their conformation under unfavorable conditions, which may impair their binding properties; their biotechnological production is often subject to a significant batch-to-batch variability; it is often difficult to immobilize these large molecules in an adequate orientation being favorable for cell interactions; due to their size

they provide a large area for possible non-specific binding and may thus counteract a stealth effect regardless of an existing PEG coat; and last but not least, they are generally rather expensive. Most of these problems might be avoided by using peptides or peptidomimetics instead. Respective binding peptides may be identified by affinity selection such as phage display, yeast surface display, messenger RNA display or peptide-on-bead display.[194] The most prominent peptide for targeting purposes is the cell binding motif arginine-glycine-aspartic acid (RGD), which efficiently triggers integrin-stimulated cell adhesion.[195] The affinity of RGD-containing peptides to different integrins is influenced by the conformation of the RGD-containing loop and by the neighboring amino acids. Cells differ as to their typical integrin pattern, which may be exploited with an appropriate RGD-containing peptide. Surface-grafted RGD was employed as a model ligand to demonstrate the specific uptake of targeted stealth PLGA microparticles by phagocytes *in vitro*.[7] In another example, enhanced delivery of drug to integrin-overexpressing cancer cells upon RGD-functionalization was reported.[196] Upon oral immunization in a mouse model, RGD grafting was reported to provoke a slightly enhanced immune response, which was attributed to M cell targeting.[197] The targeting effect observed in the *in vitro* co-culture model was however more pronounced than the *in vivo* results. Thus, a partial degradation of the RGD peptide in the gastrointestinal tract was suspected and alternative peptide analogues were tested.[198] Peroral immunization of mice with PLGA nanoparticles that were grafted either with

an RGD peptidomimetic or a leucine-aspartic acid-valine (LDV) tripeptide derivative revealed an increased IgG production. However, there were differences observed depending on the relative affinity of the targeters to M cells and APCs that probably induced different induction pathways.

For delivery to the pulmonary epithelium, the above-mentioned ICAM-1 has been proposed, which is expressed on inflammatory and immune effector cells, fibroblasts, endothelial and epithelial cells and which is up-regulated in some types of carcinomas such as lung carcinoma.[120] ICAM-1 may not only be targeted by antibodies, but also by the cyclic peptide cyclo-(1,12)-PenITDGEATDSGC (cLABL). Conjugated to PLGA nanoparticles, cLABL enhanced the endocytosis into human umbilical cord vascular endothelial cells (HUVECs) with upregulated ICAM-1 and into A549 lung epithelial cells.[120,199] In an attempt to enable PLGA nanoparticles to cross the BBB, PLGA was derivatized with different short peptides that were similar to synthetic opioid peptides.[200] Functionalized fluorescent-labeled particles were tested in an *in vivo* experiment with rats. In contrast to plain PLGA particles, confocal microscopic analyses of tissue cuts revealed some penetration into the cerebral tissue with some of the modified particle preparations. Recently, PLGA was conjugated with the glycosylated heptapeptide H_2N-Gly-L-Phe-D-Thr-Gly-L-Phe-L-Leu-L-Ser(O-ß-D-Glucose)-$CONH_2$ (g7) and loperamide-loaded nanoparticles were prepared.[201] *In vivo* nociceptive testing in rats using the hot plate test revealed an extraordinary analgesic effect of these

functionalized drug-loaded carriers, which was attributed to a successful transport of loperamide across the BBB.

Aptamers are DNA or RNA oligonucleotides with unique tertiary conformations, which allow for antigen binding with high affinity and specificity. They are non-immunogenic, highly stable in a wide pH and temperature range and in presence of organic solvents. Docetaxel-loaded PLGA nanoparticles were surface modified with the A10 2'-fluoropyrimidine RNA aptamer, which binds to prostate specific membrane antigen (PSMA) that is overexpressed in prostate cancer.[202] In a mouse xenograft tumor model, the targeted particles gave promising results inducing complete tumor regression in five of seven mice at a 109 day survivability of 100%.

Interestingly, also vitamins can be exploited for targeting purposes. The most prominent representative of this group of targeters is folate (vitamin B9), a small, innocuous, and non-immunogenic molecule, which is accessible for conjugation via its γ-carboxylate group without losing its binding properties. Moreover, it is highly stable in presence of organic solvents and under different pH and temperature conditions. Folate is taken up into cells either by the ubiquitous low-affinity folate carriers or by high-affinity folate receptors (FR), which occur only in a limited range of tissues such as certain tumor cells and activated macrophages.[203] In contrast to the carrier, the folate receptor is also able to endocytose folate-linked cargo, which makes it an interesting target in cancer and inflammation therapy. Folate-decorated doxorubicin-loaded PLGA particles were not only taken up

into FR-overexpressing KB cells to a higher extent than non-targeted carriers in an *in vitro* experiment, but they also reduced tumor growth *in vivo* in a xenograft mouse model.[204] An enhanced particle uptake into KB cells was also observed with poly(L-lysine)-poly(ethylene glycol)-folate coated PLGA nanoparticles.[76] Doxorubicin-loaded nanoparticles prepared with vitamin E TPGS-folate exhibited significantly higher cytotoxicity towards C6 glioma cells than free drug or the non-targeted formulation.[205] Besides folate, other vitamins such as thiamine (vitamin B1) or cobalamine (vitamin B12) might also provide interesting targeting opportunities.[206,207]

Furthermore, low-molecular weight synthetic molecules may be applied for active targeting. In an approach to selectively deliver drug carriers to bone, PLGA was modified with the bisphosphonate alendronate, which has a high affinity to hydroxyapatite, the major inorganic component of bone and teeth.[208]

However, even the highest affinity and selectivity of a ligand-target interaction will sometimes not suffice for successful nanoparticle delivery as various barriers may limit the access of a particulate carrier. Upon peroral administration, particles need to overcome the mucus barrier in order to interact with the underlying cells.[189] In solid tumors the diffusion of targeted nanoparticles is impaired and in dense avascular tissues such as cartilage the extracellular matrix limits the entry of drug carriers.[194] Moreover, functionalized carriers are only efficient if they are not prematurely

eliminated. Thus, systemically administered particulates will require a combination of optimum targeting and optimum stealth characteristics.

III.B. Directed intracellular trafficking

For some applications it may be sufficient to deliver a drug to a certain tissue, while others must be delivered to a specific intracellular target. These targets can be located in the cytoplasm, e.g. for proteins or siRNA, in the nucleus, e.g. for DNA, antisense oligonucleotides or DNA intercalators, in mitochondria, e.g. for antiapoptotic drugs, or in other compartments.[209,210] Especially for successful gene delivery, directed intracellular delivery remains the main challenge. First of all, the mechanism of particle uptake into the cell seems to affect their intracellular sorting and thus the extent of possible exocytosis.[116] The fraction that remains inside the cell has to evade lysosomal degradation. Subsequently, the carrier or its respective payload has to migrate through the cytoplasm to the specific target. In the case of gene delivery, the vehicle or its respective payload finally has to enter the nucleus.

One possible mechanism for endosomal escape of polymeric vectors via an intrinsic endosomolytic activity is described by the proton sponge hypothesis.[211] Due to their high buffer capacity, branched polyamines like polyethyleneimine (PEI) or polyamidoamine (PAMAM) dendrimers that contain numerous secondary and tertiary amines counteract the acidification during endocytic trafficking.[212] Moreover, the resulting influx of ions leads to

osmotic swelling and finally to the rupture of the endosomes and the release of their content into the cytoplasm. This mechanism has been exploited with PEI-PLGA nanoparticles with surface-immobilized DNA for gene delivery to pulmonary epithelium.[69] Upon incubation of Calu-3 human airway submucosal epithelial cells with DNA-loaded nanocarriers, a rhodamine-labeled plasmid DNA that drives the expression of a green fluorescent protein was primarily found in lysosomes. However, also a low amount of green fluorescent protein was detected, which pointed to endosomal escape and nuclear delivery of a certain percentage of the administered DNA. Recently, PEI-PLGA nanoparticles have been loaded with a DNA vaccine encoding a *Mycobacterium tuberculosis* latency antigen.[213] The particles stimulated human monocyte-derived dendritic cells and induced their maturation, which was concluded from an increase in the expression of surface markers and the secretion of cytokines that was comparable to the positive control. In mice, pulmonary application resulted in more efficient immunization than intramuscular application, which was attributed to a more efficient uptake. In another study, PEI-PLGA particles were prepared either by PEI adsorption or by covalent coupling via the carbodiimide chemistry.[72] The zeta potential of the PEI-conjugated PLGA particles amounted to ~ +35 mV, which was significantly higher than for PEI-adsorption (+10 to 24 mV). Uptake studies in RAW murine macrophages and subsequent determination of the gene and protein expression revealed a high amount of PEI-modified particles in the cytoplasm and a successful transfection, whereas unmodified particles

were accumulated in phagolysosomes. For PEI and other polycations, cytotoxicity issues should however be considered.[80] Surface-conjugated PEI seems to exhibit a lower cytotoxicity than free PEI.[72] Besides the proton sponge hypothesis for polycations, a mechanism for endosomal escape has been proposed also for PVA-stabilized PLGA nanoparticles.[214] Upon uptake into human arterial smooth muscle cells (HASMCs), their intracellular localization pointed to an endosomal escape of the particles, which was attributed to a cationization of the particle surface in acidic pH followed by a localized destabilization of the endo-lysosomal membrane.

A recent approach for direct delivery to the cytoplasm relies on the conjugation with "cell-penetrating peptides" (CPPs) or "protein transduction domains" (PTDs) that mediate membrane-transport.[210,215,216] One prominent representative of this group is the viral protein tat, which might be responsible for cell penetration by lipid raft-dependent macropinocytosis[217] and for the delivery of genetic material to the nucleus.[215] Fluorescence-labeled tat-PLGA nanoparticles were incubated with human keratinocytes HaCaT, which resulted in higher fluorescence intensities at the cell membrane and in the cytoplasm upon confocal laser scanning microscopic imaging than observed for non-targeted particles.[218] Another example of a CPP is the arginine peptide (RRRRRRRRCK-FITC).[219]

Another barrier for intracellularly migrating drug delivery systems is the cytoplasm itself. The cytoplasm is crowded with proteins, cytoskeletal filaments and other organelles. Thus, particles greater than 500 kDa or 20

nm are largely immobile unless there is some kind of assisted transport.[220] Among others, intracellular transport of organelles relies on active transport mechanisms that are mediated by cytoskeleton-dependent motor proteins such as myosin, kinesin, and dynein. Particle tracking experiments revealed that some PEI/DNA complexes are actively transported by motor proteins along microtubules similar to endogenous organelles or invading pathogens.[221] An interesting approach for the active intracellular transport of PLGA particles relies on a mechanism that is used by several bacteria such as *Listeria monocytogenes*.[222] The protein ActA, which is expressed at the bacterial surface, initiates actin polymerization by interacting with host cell proteins and thus promotes actin-based motility. PLGA particles with surface-adsorbed ActA also were found to polymerize actin, which resulted in comet-tail propulsion. Interestingly, this effect was only observed for anionic carriers, whereas cationic PLGA-PEI particles did not form comet tails. Although the direction of this kind of transport was rather random, it enabled the particles to overcome restricted diffusion and thus the carriers might rather reach the perinuclear region. Interestingly, actin-based motility was about 100-fold faster than passive diffusion and larger particles moved quicker than smaller ones.

Last but not least, therapeutic genes have to be delivered to the nucleus. All types of transport, active as well as passive, into and out of the nucleus have to pass through nuclear pore complexes (NPCs).[220] While small molecules of up to 40 kDa or 10 nm may overcome this barrier by passive

diffusion, molecules >45 kDa must contain a nuclear localization signal (NLS) to be recognized by importins, which mediate the nuclear transport. The upper size limit for active transport through the NPC is reported to be about 40-60 nm. However, it may not always be necessary to deliver the whole carrier into the nucleus. Instead, the payload may be released in the perinuclear region facilitating transport through the NPC. Recently, the delivery of NLS peptide-functionalized PLGA nanoparticles to the nucleus was reported.[223] Briefly, FITC-loaded PLGA nanoparticles were prepared and covalently grafted with aminoPEG-coated quantum dots (QDs). The remaining QD-amine groups were coupled with thiol-terminated NLS peptides. Upon modification, the diameter of the particles increased from 72 nm to 168 nm. The modified particles were tested for their uptake into human cervical cancer cells (HeLa) and their intracellular localization was analyzed by confocal laser scanning microscopy revealing that targeted nanocarriers not only attained the cytoplasm, but also the nucleus. However, the modified carriers widely surpassed the reported size limit of the NPC. Thus the authors hypothesized that the carriers were able to pass due to size losses upon degradation, but PLGA is usually not degraded that quickly (see Section I) and more importantly, it is generally difficult to distinguish between labeled carriers and released marker. Encapsulated fluorescent dye is quickly released from the hydrophobic PLGA matrix (see Section III.D.), and some of the QDs might be cleaved from the PLGA surface. Thus, the observed fluorescence might not necessarily represent the localization of the

PLGA carriers. This was highlighted by a comparison between PLGA nanoparticles that physically encapsulated Nile red and PLGA nanoparticles prepared from fluoresceinamine-coupled polymer.[224] Xu et al. reported that the increase in intracellular fluorescence intensity observed with physically entrapped markers was rather a result of dye transfer than particle uptake.

To sum up, there are still several crucial open questions about the fate of endocytosed PLGA particles and potential intracellular targeting strategies. Targeting to other organelles than the nucleus such as mitochondria is still in its beginnings.[210] For a further elucidation labeling procedures should be applied that guarantee a stable association of the marker with the carrier throughout the whole study. Despite the mentioned challenges, the increasing knowledge about the mechanisms of intracellular pathogen trafficking might enable new strategies for organelle-specific delivery in the future.[225,226]

III.C. Prolonged circulation time

When "foreign" nano- or microparticles are injected into the bloodstream, these materials are rapidly cleared from systemic circulation. This removal is the consequence of a coordinated interplay between the adsorption of serum proteins at the particle surface and a subsequent uptake of the colloids by cells of the reticuloendothelial system (RES). Phagocytosis of particles is predominantly achieved by Kupffer cells in the liver but also by macrophages

in the spleen and, to a lower extent, by macrophages in the bone marrow. It has been known since 1903 that specific serum proteins are involved in the labeling of particles as "foreign".[227] These opsonins can be categorized into immune opsonins which interact with receptors on macrophages to stimulate endocytosis (IgG, complement proteins (C3, C3b)) and non-immune opsonins (fibronectin, C-reactive protein, tuftsin, mannose-binding protein, lipopolysaccharide-binding protein) which alter the particle's surface characteristics and thus render it more adhesive to phagocytes.[228] In contrast to opsonins, dysopsonins like IgA and α_1-acid glycoprotein have been suggested to function as adsorptive serum components which play a regulatory role in inhibiting phagocytosis.[228-230] For a more detailed discussion of the mechanisms underlying the sequestration of particles from the bloodstream, the interested reader is directed to excellent reviews from recent years.[228,230-235] As a consequence of the efficient removal by cells of the RES it arises that if the spleen or liver are not the primary targets of nanoparticulate delivery systems, the particles will have to be disguised in order to evade the body's defense mechanisms and to avoid inflammatory responses. Appropriate engineering of stealth particles will consequently be necessary for the successful application of nano- and microparticles as circulating drug reservoirs with controlled release properties, as artificial oxygen carriers, vasculature imaging agents, and passive as well as active targeting devices.[236] In order to reduce the interaction of particles with macrophages and to limit protein adsorption, adaption of the particle size and

surface coating techniques have been proposed. Generally, keeping the size of the particles <100nm seems to be advantageous for prolonging blood half-life. Supposedly, this is due to the low surface area per particle in combination with the high curvature which does not promote adsorption of the proteins needed for complement activation in a proper geometric configuration.[230,235,236] The complement system consists of more than 20 plasmatic proteins with enzymatic or binding capabilities and some receptors on cells. Adsorption of serum complement proteins and subsequent activation of the complement cascade can be determined with the complement activation assay[235,237,238] and should always be validated by using zymosan particles as a positive control.[233] Besides particle size, high charge density and hydrophobicity of the surface have been identified as characteristics which promote serum protein adsorption.[230,235] This is in line with theoretical predictions which indicate that ionic and hydrophobic interactions, along with an entropy gain caused by changes in protein conformation, represent the driving forces for protein adsorption.[239] Consequently, in order to render biodegradable PLGA nanoparticles long-circulating, the hydrophobic and negatively charged particle surface has to be shielded. This is expected not only to decrease opsonization but also to minimize interactions with macrophages. To hydrophilize colloids, coating techniques with hydrophilic macromolecules have been investigated. While polysaccharides such as dextran and heparin have been employed for this purpose and might be advantageous due to their biodegradability,[240] most studies have used

poly(ethylene glycol) (PEG) and its copolymers for surface modification. Since PEG contains a high number of ether groups which can bind water molecules via hydrogen bonding, their anchoring at the particle surface introduces a highly hydrated hydrophilic coating layer. This flexible layer sterically stabilizes the particles, screens underlying surface charges and reduces the interfacial free energy, thus minimizing attractive forces for protein adsorption.[235] In practice, the introduction of a PEG layer is accompanied by a decrease of the particle's zeta potential due to a shift of the shear plane away from the particle surface.[146,237] The efficiency of protein repulsion is dependent on the Mw of the PEG, the distance between the surface grafting points and the conformational flexibility of the PEG chains.[235] For a detailed review on the interconnections between these parameters see Vonarbourg et al.[235] According to a theoretical model,[241] the optimal distance between two terminally attached PEG chains should be in the range of ~1 nm to repulse small proteins (~2 nm) and ~1.5 nm to repulse large proteins (6-8 nm).[242] These theoretical predictions imply that the particle surface has to be entirely covered by the PEG coating to achieve sufficient repellence and have been confirmed in studies with PLA nanoparticles.[151,243] Due to the wide variety of particle matrix materials and grafting techniques described in the literature, it is difficult to identify an optimal PEG chain length. However, coating with PEGs in the Mw size range between 1.5-3.5 kDa at appropriate grafting densities seems to generate a high degree of protein repulsion in most systems.[235] Generally, techniques for the PEGylation of PLGA nanospheres

have comprised the production of particles from blends of PLGA/PEG-PLGA,[244-249] the use of PEG-copolymers as surfactants in course of the particle preparation procedure,[85,146,250] as well as the adsorptive coating of preformed particles with PEG-copolymers.[5,7,75,76,85,251]

By introducing hydrophilic PEG-blocks to the hydrophobic PLGA backbone, polymers with amphiphile characteristics can be prepared.[252] Despite higher water uptake into the particle matrix, particles prepared from PEG-[PLGA oligomers] are expected to be characterized by similar degradation characteristics as particles prepared from PLGA.[253] Upon cleavage of the ester bonds, the PEG molecules will be liberated from the carrier and excreted mainly via the kidneys if the Mw is in the range of 1-20 kDa.[252] The preparation of nano- and microparticles from PEG-PLGA can be achieved with or without additional stabilizer by emulsification solvent evaporation procedures, solvent displacement, salting out,[254] and hydrodynamic flow focusing,[255] respectively. At this, the surface density of PEG chains can be controlled by varying the ratio of the PLGA/PEG-PLGA blends used for particle preparation. In combination with the Mw of the PEG, surface density determines whether the coating layer will mainly exist in a brush- or mushroom-like conformation.[235,252] As illustrated by studies with PLA/PEG-PLA blends, the majority of PEG chains orientate themselves towards the outer aqueous phase in course of particle formation.[151] When incubated with serum and subsequently analyzed regarding the particles' protein adsorption pattern by 2D-PAGE, protein repellent properties were

already observed at blending ratios of 0.5:99.5 (PEG-PLA:PLA). However, the highest protein repellence as compared to nanoparticles prepared from plain PLA was observed with blending ratios of at least 5:95 and a conjugate of PLA (Mw~ 45 kDa) with a PEG chain of at least 5 kDa Mw.[151] The calculated distance between two terminally attached PEGs in this system corresponded to ~1.4 nm, which is in the theoretically predicted range for optimal protein repulsion.[242] In relation to these findings, a recent study has indicated that nanoparticles made from PEG-PLGA might be characterized by an even higher resistance to protein adsorption.[246] When comparing the protein adsorption capacity of particles made from copolymers of PEG with the polyesters poly(ε-caprolactone) (PCL), PLA and PLGA, the highest degree of polymer core protection from opsonization was observed for PLGA-PEG. Similarly prepared PEGylated PLGA nanoparticles have not only exhibited low protein adsorption, but also altered biodistribution.[245,247,248] In order to be able to track the nanoparticles *in vivo* and to determine their uptake into different tissues, ^{125}I-cholesterylaniline was encapsulated as radiolabel. Upon intravenous injection in rats, a clearly prolonged blood circulation half life was observed for the PEGylated nanoparticles ($t_{1/2}$~ 7 h) as compared to colloids produced from plain PLGA ($t_{1/2}$~ 15-35 s). Furthermore, uptake of the particles into spleen and liver was drastically reduced.[245] These results were confirmed by the same authors in mice, however, it should be highlighted that the nanoparticles used in all three studies exhibited a broad size distribution as indicated by polydispersity indices of ≥0.3.[248] Using a similar

approach to radiolabeling, Li et al. encapsulated ^{125}I-BSA as a model protein drug into nanoparticles made from PLGA or PEG-PLGA.[244] After encapsulation into PEGylated particles, the plasma half life of BSA was increased from 13.6 min for plain PLGA carriers to ~4.5 h. Moreover, the biodistribution profile of PEG-PLGA particles was altered, indicating increased localization of BSA-loaded particles in the spleen and lung instead of the liver.[244]

Besides the production of colloids from PEG-PLGA, PEGylation of preformed PLGA nano- and microparticles has been achieved by surface modification with a variety of PEG-copolymers via hydrophobic or electrostatic interactions. Since a very profound body of knowledge had been generated on the successful coating of PS nanoparticles with poloxamers and poloxamines in the 1980's and 1990's,[233,236] surface modification of PLGA with these block copolymers of PEG and PPG suggested itself. Indeed, addition of poloxamer 407, poloxamine 904 or 908 during the preparation procedure or adsorption onto preformed PLGA nanospheres, generated long-circulating colloids with altered biodistribution in rats and rabbits.[85] However, while 39% and 28% of the administered dose of poloxamer 407- and poloxamine 908-coated particles respectively were detected 3 hours post injection, only ~5% of plain and poloxamine 904-modified particles remained circulating in the bloodstream. The rather high sequestration of poloxamine 904-modified nanospheres was attributed to the comparably short PEG blocks (4x Mw~ 0.6 kDa) as compared to poloxamer 407 (2x Mw~ 4 kDa) and poloxamine 908 (4x

Mw~ 5kDa). Interestingly, coating in course of the preparation procedure or onto preformed particles led to similar alterations of the biodistribution profiles.[85] This is remarkable, since it has been shown that poloxamer 407 and poloxamine 908 adsorbed onto preformed PLGA nanoparticles are displaced by serum proteins.[5] To monitor the displacement rate, the two surfactants were radiolabeled with ^{125}I Bolton-Hunter reagent and adsorbed onto particles made from PLGA and PS. Upon incubation in phosphate buffered saline (PBS) for 24 hours, a removal of ~5% of surfactant was monitored. However, upon incubation in serum ~20% of surfactant was removed from PS nanospheres. In the case of PLGA nanoparticles, displacement was even more pronounced as illustrated by 71% removal of poloxamer 407 and 78% of poloxamine 908.[5] Although the studied PEG-copolymers are obviously rather weakly linked to the PLGA matrix, these carriers were characterized by prolonged plasma half-lives and a biodistribution shifted away from the liver.[251] When PEG-PLA was used as the coating polymer, varying biodistribution profiles were observed.[146] By coating PS- as well as PLGA-nanoparticles with PEG-PLA copolymers or poloxamine 908, hydrophilic colloids were generated as monitored by hydrophobic interaction chromatography (HIC). However, while all coated PLGA nanospheres were characterized by prolonged plasma half-lives upon injection in rats, only PS particles coated with poloxamine 908 exhibited stealth properties three hours post injection. Although the PS nanospheres coated with PLA-PEG were characterized by an altered biodistribution

shortly after administration, no differences were observed after three hours as compared to plain particles. This was attributed to potential differences in the affinity of the PLA-block to PS and PLGA as particle matrix materials.[146]

The modification of preformed PLGA nano- and microparticles has also been achieved using an electrostatic coating approach with PLL-PEG.[7,75,76] At this, the PEG segments are anchored to the particle surface via ionic interactions between the polycationic PLL-backbone and carboxyl groups at the particle interface. In addition, coating of PLGA nanoparticles with protein repellent carbohydrate derivatives has been investigated.[98] Hydroxyethylstarch (HES), which is fully biodegradable, was conjugated to lauric acid and the resulting amphiphilic HES-laurates were used as a stabilizer in course of the production procedure. These PLGA nanoparticles adsorbed similarly low levels of BSA and fibrinogen as poloxamer 407-coated colloids. Moreover, reduced phagocytosis of the HES-laurate-modified particles by a monocyte macrophage cell line was observed.[98]

In conclusion, the coating of hydrophobic and negatively charged PLGA nanoparticles with PEG or polysaccharide derivatives results in hydrophilization of the particle surface. Even with theoretically optimal PEG surface densities, no complete inhibition but only a reduction of serum protein adsorption was observed.[151] Nevertheless, several reports have described a drastically increased plasma half life and altered biodistribution upon coating of PLGA nanoparticles with PEG indicating success of this approach for engineering of long-circulating nanoparticles. However, it

remains to be addressed whether PEGylated PLGA nanoparticles retain their long-circulating properties upon repeated administrations. In the case of poloxamer- and poloxamine-modified PS nanospheres, it has been shown that PEGylated particles administered in a second injection 3-13 days after the first injection are rapidly removed by the RES.[256] This was attributed to an acquired ability of liver and spleen macrophages to recognize the injected colloids. Furthermore, it is not clear whether the coating techniques generate homogenous PEG layers at the particle surface. Using HIC, a study has indicated that heterogeneities in surface coating might be responsible for the premature removal of fractions of PEGylated carriers.[237] Finally, the introduction of cell-specific homing moieties at the protein repellent surface layer has been achieved recently and represents a crucial step forward to fully explore the potential of long-circulating PLGA nanoparticles as targeted drug carrier devices.[7,76,202]

III.D. Tracking

Sensitive analytical detectability is a crucial prerequisite for *in vivo* and *in vitro* studies dealing not only with the biodistribution and elimination kinetics, but also with cytoadhesion, cytoinvasion and intracellular trafficking of nano- and microparticles made from PLGA. At this, the most commonly employed labeling techniques are based on the tagging of particles with fluorophores or radioisotopes.

III.D.1 Fluorescence labeling

Fluorescence-based labeling techniques have been the methods of choice to render polymer particles trackable for *in vitro* cell interaction studies. The most frequently used concept is based on the encapsulation of a fluorescent dye in the polymer matrix in course of the preparation procedure of the particles. Thereby, PLGA nano- and microparticles mainly have been labeled with hydrophobic fluorophores such as 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI),[98,257] 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO),[137] BODIPY® 493/503,[258] BODIPY® FL,[259] coumarin 6,[7,55,170,214] pyren[60] or nile red.[224] However, hydrophilic dye molecules like rhodamine 123,[201] rhodamine 6G,[81,260] rhodamine B,[261] dextran-rhodamine[72] indocyanine green[262] as well as quantum dots[263] have also been employed. Additionally, Panyam et al. developed a dual labeling technique with coumarin 6 and osmium tetroxide yielding colloids that can be detected by fluorescence- and electron microscopy.[264] Dye-labeled PLGA conjugates primarily have been synthesized by carbodiimide-mediated activation of the uncapped polymer backbone and subsequent reaction either directly with the fluorescein derivatives fluoresceinamine[224,265] or fluorescein cadaverine[40] or via a diamine spacer with carboxyfluorescein[266] or fluorescein isothiocyanate.[76] Moreover, Tosi et al. have described a method for synthesizing a versatilely applicable biotin-capped derivative of PLGA. Particles made from this polymer were tracked in

tissue sections by electron microscopy after reaction with a streptavidin-peroxidase conjugate and subsequent incubation with diaminobenzidine.[266] From experience in our lab, the binding of avidin to biotin-modified nanoparticles, however, can be clearly limited in the presence of proteins. This is probably due to protein adsorption at the particle surface which leads to steric obscuration of the small biotin binding site (unpublished results). Considering this, a versatile two-step approach for the decoration of particles with biotin as reported by Müller et al. seems more promising.[75] A cationic PLL-g-PEG-biotin conjugate, which adsorbs to the negatively charged PLGA surface via the PLL-block, was used to introduce sterically flexible biotin for subsequent labeling with Oregon Green®-streptavidin.

Furthermore, the covalent fluorescence labeling of preformed particles has been investigated. Following activation of surface carboxyl groups and amination with ethylene diamine, the introduced amino groups on PLGA microspheres were subsequently conjugated with fluorescein isothiocyanate (FITC).[267] Besides this rather complicated two-step procedure, direct surface modification of PLGA nano- and microparticles with fluorescent entities also has been reported. At this, the well established carbodiimide chemistry was used to covalently immobilize fluorescein-tagged proteins[268,269] or amine-functionalized quantum dots.[223]

III.D.2 Radioactive labeling

The techniques for the modification of PLGA particles with radioactive isotopes rely on similar rationales as in the case of fluorescence labeling. Primarily, radioactive labeled particles have been used analytically to investigate the effect of surface modifications with PEG or PEG-containing surfactants on the biodistribution of PLGA particles in rodents.[244,270-273] In addition, the possibility to use radioactive particles for the delivery of a radiation dose to tumor tissue was discussed.[274] Several studies report the labeling of PLGA particles by encapsulation of small molecules or proteins conjugated to radioactive isotopes. These include ^{188}Re-dimercaptosuccinic acid,[274] ^{111}In-oxine,[85,146,270] ^{3}H-paclitaxel,[275] ^{125}I-cholesterylaniline,[245] ^{125}I-tetanus toxoid[51] and ^{125}I-tagged bovine serum albumin (BSA).[244] Direct labeling of PLGA has been achieved by reaction of ^{14}C-acetic acid anhydride with terminal hydroxyl groups of the polymer chains.[276] Furthermore, the widely used approach for the labeling of proteins and proteinaceous colloids with metastable ^{99m}Tc by stannous reduction has been applied to preformed PLGA particles.[271-273] While the reaction mechanism underlying the conjugation of ^{99m}Tc to proteins is rather clear,[277] detailed information about the labeling of polymeric particles is scarce. A lowering of the valency state of ^{99m}Tc by the reducing agent stannous chloride and subsequent complexation with amine groups has been proposed as the labeling mechanism for chitosan nanoparticles.[278] Probably, the adsorption of ^{99m}Tc onto PLGA particles is driven by the high affinity of multivalent cations for negatively charged interfaces.

Radiolabeling of PLGA nanoparticles has also been achieved by coating of plain colloids with radioactively tagged poloxamers and poloxamines.[5,272] At this, the hydroxyl end group of the PEG-containing surfactants is aminated and subsequently conjugated with ^{125}I-hydroxyphenylpropionic acid. The interested reader is directed to Neal et al. for a comprehensive discussion of reaction schemes and for alternative approaches to the radioactive labeling of PEGs.[5]

In conclusion, it has to be highlighted that adequate labeling of particles made from PLGA, especially using fluorescent dyes, is not trivial. Although the degradation half life of PLGA lies well beyond the time scale of most *in vitro* cell interaction studies, marker is already expected to be released from the particles in course of short experiments.[224,279] As a rule of thumb, small hydrophilic dye molecules cannot be efficiently loaded and are poorly retained in the particle matrix. However, low molecular weight hydrophobic compounds also suffer from premature release. As illustrated by recent reports, the extent of this leakage probably has been underestimated so far, due to the use of protein- and lipid-free buffer systems for the liberation tests.[224,280] The consequences thereof have not necessarily hampered the interpretation of studies using microparticles since their relation to free label in physical size is unambiguous. However, in case of nanoparticles optical microscopy hits on its resolution limits and discrimination between free marker molecules and particles becomes a

tremendous challenge. The resulting susceptibility of nanoparticle-cell interaction studies to misinterpretations has been discussed recently.[224,280] Considering this, it becomes clear that results solely based on fluorescence detection should be interpreted with particular care. To improve the value of studies in this field, limitations of existing protocols have to be addressed and the development of more effective labeling approaches is strongly needed. From the current perspective, covalent modification of PLGA seems promising due to the stable linkage between marker molecules and the polymer. The encapsulation of hydrophobic high molecular weight species, such as quantum dots, might prove to be a valuable alternative since their diffusion coefficients in the particle matrix are expected to be clearly lower than those of low molecular weight compounds. On the long run however, an integration of ultrastructure-resolving techniques will be needed to fully resolve the cytoadhesive and cytoinvasive properties as well as the subcellular trafficking of polymer nanoparticles.

III.E. Stabilization of biomacromolecules

Since it has been shown in the early 1990's, that biomacromolecules can be encapsulated into PLGA microspheres and might thereby be protected from degradation,[281] numerous studies have investigated the delivery of peptides, proteins, oligonucleotides and DNA using carriers made from PLGA.[21,282,283] However, several difficulties have been found to be associated

with this concept. Firstly, although the encapsulation of hydrophilic molecules is feasible using water-in-oil-in-water solvent evaporation techniques, the loading efficiency is often limited. Secondly, the dispersion steps involved in particle preparation are associated with contact to organic solvents and shear stress due to sonication or homogenization. These processes might result in denaturation of the biomacromolecules during encapsulation.[284,285] In addition, molecules incorporated in a PLGA matrix are increasingly exposed to an acidic microclimate by time. This has been visualized recently by confocal laser scanning microscopy and is a consequence of polymer hydrolysis as well as the accumulation of degradation products in aqueous pores.[286,287] While the stability of drug released during the "first burst" phase is not necessarily compromised thereby, pronounced degradation might occur in course of later stages of release.[288] Several strategies have been proposed for the stabilization of labile biomacromolecules under these circumstances including complexation of proteins with zinc, addition of PEG as well as co-encapsulation of antacid excipients.[285,289] An alternative approach to circumvent these limitations is based on the adsorption of the biomacromolecules to be delivered onto the surface of preformed nano- and microparticles made from PLGA.[71,290] Since the coating is applied to preformed particles, exposure of the biomacromolecules to potentially deleterious solvent and shear conditions is avoided. Furthermore, the release of the payload from the carrier proceeds relatively quickly and is not strictly dependant on the slow bulk erosion

release kinetics of PLGA.[72] This might prove especially advantageous in the case of vaccination, as a typical phagocytic cell only has a lifespan of several days.[156] Since it has been reported by several groups that loading of biomacromolecules onto the particle surface can provide sufficient protection against enzymatic degradation,[71,100] the delivery of proteins,[70,102,103,122,267,291,292] plasma membrane preparations,[293] tumor cell lysates,[293] oligonucleotides[62,294] and pDNA[39,59,69,71,72,82,100,295-299] has been investigated. While direct coating of plain PLGA particles has been achieved,[291,300] precedent surface functionalization with excipients has proven advantageous for enhancing the efficiency of the subsequent coating. Heparin, for example, is characterized by a high binding affinity to growth factors such as fibroblast growth factor (FGF), vascular endothelial growth factor (VEGF), heparin binding epidermal growth factor (HBEGF) and transforming growth factor-ß (TGF-ß). Making use of this affinity, Chung et al. showed that conjugation of heparin to the surface of highly porous uncapped PLGA particles via carbodiimide chemistry generates microspheres with fourfold higher FGF-loading capacity as compared to plain particles.[267]

Coating of PLGA particles with cationic polyelectrolytes (see Section II.B) or amphiphilic molecules (see Section II.C) can also enhance the binding capacity for biomacromolecules. PLGA nano- and microparticles have been regarded as promising biodegradable antigen carriers and adjuvants for the formulation of vaccines.[156,301-303] Since vaccines made from purified preparations from pathogenic organisms or recombinant proteins are often

not sufficiently immunogenic, site specific delivery systems are needed to optimally present the antigen to the innate and adaptive immune systems.[302] Nanoparticles might prove beneficial for this purpose since they diffusively spread upon injection and can reach lymphe nodes.[156] Up to date, however, microparticles have been studied more extensively. Due to their relatively large size, microparticles are hardly taken up by non-phagocytic cells but can be internalized by antigen presenting cells (APCs) such as macrophages or DCs. This passive targeting effect supposedly leads to preferential delivery to APCs and has been reported to be most pronounced for particles in the size range of 1-3 µm.[302]

Microparticles produced from PLGA have been investigated as potential carriers for protein antigens. One approach is based on the formation of particles from blends of end-capped PLGA and the anionic amphiphile dioctyl sulfosuccinate. The resulting microparticles are characterized by a negative zeta potential and have been shown to adsorb proteins such as ovalbumin, carbonic anhydrase, lysozyme, lactic acid dehydrogenase, bovine serum albumin,[122] the recombinant proteins MB1/MB2 from *Neisseria meningitides*,[103,122] recombinant p55 gag from HIV-1[102] and the recombinant HIV envelope glycoprotein gp120dV2.[122,292] In case of the latter, adsorptive coating of the particles retained the antigenic structure of the glycoprotein, while encapsulation into PLGA microparticles did not. According to protein quantification by size exclusion chromatography and the bicinchonic acid (BCA) assay, the highest loading rate with gp120dV2

(IEP ~8.5) was achieved at pH 5. This indicates a preferential adsorption of positively charged protein molecules onto the negatively charged carriers via ionic interactions.[292] The importance of the IEP in protein adsorption was confirmed in the case of MB1/MB2, where highest loading rates on negatively charged PLGA microparticles were observed for the positively and non-charged form of the protein respectively.[103] In addition, positively charged microparticles have been investigated as protein carriers. Mandal et al. used PEI- and PLL-coated PLGA microparticles for delivery of granulocyte-macrophage colony-stimulating factor (GM-CSF) which is a differentiation factor for hematopoietic progenitor cells and may act as an adjuvant.[70] Similar amounts of GM-CSF adsorption were monitored on plain and polyamine-modified particles, hinting at a dominant role of the hydrophobic PLGA matrix for this protein.

In recent years, gene-based vaccination has evolved as an alternative to traditional vaccine strategies.[156,283] At this, the transfection of cells with pDNA results in sustained intracellular antigen production which in turn can lead to a coordinated activation of humoral and cell-mediated immune responses. Since delivery of naked pDNA is severely limited due to premature enzymatic degradation,[304] alternative approaches have been investigated using polymeric particles as carriers.[156,302] For example, particles were prepared containing encapsulated naked or polyamine-complexed pDNA.[283,305] Since naked pDNA is prone to degradation in course of the particle preparation process,[284] approaches based on the encapsulation of

pDNA complexed with PLL[304] or PEI[306,307] have proven to be more promising. At this, the polyamines form stable complexes with pDNA due to electrostatic interactions between amine and phosphate groups. This has been shown to stabilize pDNA during particle formation and limits its susceptibility to enzymatic degradation by DNAse I.[304] Furthermore, free amino groups of the polyelectrolyte are expected to buffer the intraparticular pH drop during polymer erosion which will additionally contribute to the stability of the formulation.[306] As an alternative approach, pDNA has been loaded onto the surface of PLGA particles modified with cationic surfactants or polyelectrolytes. Several works, especially by Singh and coworkers, have dealt with the applicability of the cationic amphiphiles cetyl trimethyl ammonium bromide (CTAB), dimethyl dioctadecyl ammonium bromide (DDAB) and 1,2-dioleoyl-1,3-trimethylammoniopropane (DOTAP) for the introduction of positive charges at the surface of end-capped PLGA microparticles during particle preparation.[100,171,295,296,298,308] Positively charged carriers, primarily modified with CTAB, have been loaded with plasmids encoding antigens from *Mycobacterium tuberculosis*,[309] avian metapneumovirus,[310] foot and mouth disease virus,[311] hepatitis B virus,[312] hepatitis C virus,[308] HIV,[100,295,296,298] and measles virus.[313] By varying the amount of CTAB used for particle coating, the loading efficiency and release rate of pDNA can be regulated.[296,298] The amount of pDNA associated with the microparticles was determined by agarose gel electrophoresis or after ultracentrifugation of the loaded particles into an OptiPrep® density gradient, complexation with ethidium bromide and

fluorimetric analysis of the gradient fractions. When compared to naked pDNA, CTAB-modified particles loaded with pDNA were found to elicit higher immune responses, possibly due to an adjuvant effect of the cationic particles.[100,295,296] The sequential loading of two plasmids encoding the antigens p55 gag and gp-140 of HIV-1 has also been reported.[298] While only low loading levels were achieved for unmodified particles, the plasmids were efficiently adsorbed to cationic PLGA particles. Furthermore, protection of surface-adsorbed pDNA from degradation by DNAse I was observed *in vitro*.[298] In contrast to these observations, Oster et al. have reported that coating of negatively charged particles with CTAB does not provide protection of pDNA against degradation by DNAse I.[71] However, surface modification with branched PEI (Mw~ 25 kDa) led to highly positive carriers which protect surface-adsorbed plasmids from enzymatic cleavage. While naked pDNA was degraded by DNAse I within 5 min, pDNA adsorbed onto PEI-coated particles was stable for ~12 hours. PEI/PLGA blends also have been frequently used for the preparation of nanoparticulate transfection vectors which bear a positive surface charge and have been successfully loaded with plasmids.[69,82] Moreover, covalent conjugation of PEI onto the particle surface has been investigated.[39,72,297] At this, polyamine anchoring at the surface of preformed microparticles is probably a result of a combination of electrostatic interactions and the formation of covalent bonds via active esters. According to Pai Kasturi et al., covalently modified PEI-PLGA particles are characterized by 5-fold enhanced pDNA adsorption efficiency as compared to

plain particles.[39] Furthermore, it was observed that surface modification with linear PEI (Mw~25 kDa) led to a quicker release of plasmid and decreased buffering capacity towards acid titration as compared with branched PEI (Mw~25 kDa and ~70 kDa). Whether these observations owe to varying degrees of complexation of pDNA by linear and branched PEI could be investigated with an ethidium bromide replacement assay.[49] By mixing plasmids with ethidium bromide, pDNA with intercalated fluorophores was obtained. Upon adsorption of pDNA prepared in such a manner onto PLA particles coated with branched PEI, different amounts of free "squeezed out" ethidium bromide were detected. Coating with "coiled" PEI resulted in particles with a high potency for DNA condensation and thus high replacement of ethidium bromide. In comparison, particles coated with PEI that had been adsorbed in a "stretched" conformation were characterized by less free surface charges and a consequently decreased potency for complexation.[49] Chitosan has also been used for the preparation of positively charged PLGA nanoparticles which were subsequently coated with plasmids[59] or antisense oligonucleotides.[62] According to AFM-studies, pDNA-chitosan complexes appeared to form clusters at the particle surface.[59] Another interesting approach for the introduction of a surplus of positive charges on uncapped PLGA particles is the conjugation with poly(amidoamine) (PAMAM) dendrimers.[299] Microparticles were conjugated with third to sixth generation PAMAM dendrimers by carbodiimide coupling chemistry. As determined by UV-spectrophotometric analysis (absorption: 260nm) of the

coating solution's residual pDNA content, fivefold higher pDNA loading efficiencies were obtained on PAMAM-modified as compared to plain particles. While the zeta potential and buffering capacity towards acid titration increased with increasing dendrimer generation, the transfection efficiency was constantly higher than that of pDNA-loaded plain particles but not influenced by dendrimer generation.[299]

The development of particle-based vaccination strategies has also included the loading of microparticles with immunostimulatory substances. Non-methylated nucleotide sequences containing cytosine linked to guanine by a phosphodiester (CpG DNA) belong to this group of molecules. Sequences containing the CpG motif are frequently found in prokaryotic DNA but in vertebrate DNA they do not occur as abundantly in their non-methylated form.[294] Since CpG DNA induces the conversion of immature DCs to mature APCs, they are considered to be a promising class of vaccine adjuvants. By adsorbing phosphorothioate oligonucleotides containing CpG on cationic PLGA microparticles, a potent immunostimulatory effect was observed in mice as compared to free CpG.[294] Similarly, Poly(inosine)-poly(cytidylic acid) (poly(I:C)), which is a synthetic analogue of viral double-stranded RNA characterized by affinity for toll-like receptor 3, also serves as a maturation signal for DCs.[314] Poly(I:C) electrostatically adsorbed onto PLGA microparticles coated with cationic diethylaminoethyl dextran was observed to be a more potent inductor of DC maturation as compared to the free soluble substance.[68]

As illustrated, the rationale of adsorbing plasmids, proteins or antisense oligonucleotides onto the surface of preformed particles represents a promising approach for gene- and antisense-delivery as well as vaccination. Moreover, surface-based techniques offer the possibility to co-deliver substances encapsulated in the particles. Feasibility of this approach has been shown by adsorption of a plasmid encoding luciferase onto the surface of PLGA microspheres with encapsulated FITC-BSA.[72] These first proofs of principle could stimulate the development of bifunctional vaccine formulations with pDNA/protein antigen at the particle surface for a "first burst" and encapsulated pDNA/protein for sustained delivery to enhance the immune response.[282] Moreover, biodegradable delivery systems carrying the antigen and immunomodulatory substances could be prepared and decorated with targeting moieties. However, for these approaches to be successful, the surface adsorption protocols yielding high loading efficiencies as well as protection of the biomacromolecules from degradation have to be identified. It also remains to be addressed whether adsorptive coating techniques generate sufficiently stable linkages, since relatively quick displacement of proteins from the carrier system can occur due to competitive adsorption of serum proteins.[70]

IV. FUTURE CHALLENGES

Despite the mentioned large number of successful proof-of-concept studies, several challenges still have to be overcome on the way to successful clinical application of surface-modified PLGA-based carriers. Until now, most of the reported methods for the preparation and surface modification of PLGA particles involve rather small batches. A scale-up to large production volumes will certainly implicate additional challenges. Moreover, reasonable production costs will remain an important prerequisite for successful application.

In order to prevent unwanted effects that might be caused by residual reagents, efficient purification methods are needed. The removal of reagents applied for surface modification has rather been neglected so far. Currently, particle suspensions are most often purified by methods that exploit the difference in size between the particles and the employed reagents, such as centrifugation,[41,42,47,315] ultracentrifugation,[173,178] diafiltration,[316] size exclusion chromatography,[192,200] or dialysis.[116,208] However, these methods are generally intricate and time-consuming and they do not allow for a quantitative elimination of unwanted reagents. Future functionalization strategies will have to consider these limitations.

As a basis for further advancements also a more detailed understanding of PLGA particle morphology is required. Although electron microscopy enables a very high magnification, the resolution that may be

achieved upon imaging of PLGA particles is strictly limited. Due to the low glass transition temperature of PLGA of about 40°C the particles quickly start to "melt" upon irradiation with the electron beam, which may result in artifacts. So far, scanning electron microscopy (SEM) enabled valuable insights concerning the size and porosity of PLGA microparticles.[317] With cryogenic transmission electron microscopy (cryoTEM) or via freeze fracture replica even PLGA particles in the submicron range have been visualized.[131] However, the only unquestionable information is that about the size and the overall shape of the PLGA nanoparticles. At this, electron microscopy is a valuable complement for other frequently used sizing techniques such as photon correlation spectrometry (PCS), which can only determine the hydrodynamic diameter of suspended particles and is influenced by numerous parameters.[316] As the molecules typically used for surface modification of PLGA particles have a diameter of only a few nanometers at the largest, their presence and conformation cannot be evaluated by currently available electron microscopic techniques. At the best, it is possible to distinguish between smooth, rough, and porous surfaces.[81]

Alternatively, atomic force microscopy (AFM, scanning probe microscopy)[318] has been explored to characterize the morphology of PLGA particles. In AFM, the sample surface is scanned with a mechanical probe to generate a topographic map of the sample. Additionally, this technique can be used to gain information about the rigidity of the sample or even about the affinity of ligand-receptor interactions. In contrast to SEM, not only dry but

also liquid samples can be imaged, and it is not necessary to work under vacuum. Nevertheless, AFM is most useful for the characterization of rather flat surfaces, and it is not possible to examine steep walls or overhangs. Moreover, the particles need to be immobilized prior to imaging, which is often a tedious task. Concerning PLGA particles, the method is still in its infancy. So far, it is possible to reliably determine the particle size and shape, and to distinguish between smoother or rougher surfaces.[59]

Aside from the characterization of the nano- and microparticles themselves, their distribution in a complex organism and their interaction with specific cells needs to be investigated using appropriate models. Typically, *in vitro* experiments are performed in stationary setups with particles dispersed in buffer. However, the stability of plain and surface-modified particles might be compromised substantially in physiological media with high protein content.[66] It has been observed that charged particles preferentially accumulate in certain tissues,[319,320] but it is not clear whether this is due to ionic interactions between particles and endothelial cells or whether plasma protein adsorption or microaggregate formation also play a decisive role. To accurately study the interaction of particles with tissues that are exposed to flow *in vivo* (endothelium, urinary tract epithelium, GI tract), alternatives to currently used stationary assays have to be developed.[269,321] Using a microfluidic flow chip, it has been shown recently that plain PLGA microparticles are characterized by negligible bioadhesion in the presence of hydrodynamic drag.[269] Consequently, in order to engineer drug carriers which

efficiently adhere to a target tissue in the presence of shear forces, sophisticated surface functionalisation strategies might have to be developed.

V. OUTLOOK

In 2004, Nutropin Depot, the first and only marketed protein-loaded PLGA microparticle formulation, was withdrawn from sale because of high costs. This fact does not encourage further research in this area, but only at the first sight. Apart from profit and demand, PLGA micro- and nanoparticles including covalently functionalized ones will gain ground in three fields of application in future.

In the broad and sometimes sophisticated area of targeted therapy, covalent conjugation of targeting ligands offers the advantage of stable attachment as compared to possible detachment or even loss of the ligand in case of adsorptive immobilization. Additionally, the biorecognitive ligand is antennary exposed towards the biological environment allowing optimal biointeraction as compared to electrostatic or adsorptive coating. The latter approaches sometimes suffer from unknown folding of amphiphilic ligands probably shielding the target moiety, especially when cross-linking is required to stabilize the coat. Nevertheless, two issues are to be met: Firstly, the biocompatibility and biodegradability of surfactants used for preparation of PLGA particles has rarely been considered. Secondly, the covalent surface

modification requires processing of the drug loaded particle which remains time consuming in spite of optimization. On the one hand, there is the risk of premature drug loss due to the first burst effect, on the other hand this drawback might turn to an advantage when the release rate is supposed to be controlled by diffusion and/or erosion alone.

Targeted diagnostics will be another field of application for surface modified PLGA-nanoparticles. Especially multi-labeling by simultaneous covalent immobilization at the surface of targeted particles with contrast labels for MRI, PET, CT and ultrasound[322] is a promising approach and beneficial for the patient. In clinical practice, however, some combinations might be pointless. Whereas fluorescent imaging is very useful in research, its utility in man is highly questionable. With respect to patient's comfort, imaging should be feasible in one step e.g. by a combination of PET and MRI.

Finally, the most frequently stressed combination of therapy and diagnosis (theranostics) by one particulate formulation might hit a snag. Apart from the high costs for the health care system, the benefit for a patient with unknown disease remains questionable since the particle will release the drug irrespective of sickness or healthiness.

All in all, the increasing knowledge in surface modification of PLGA-particles will considerably contribute to realize Paul Ehrlich's dream of magic bullets for targeted therapy, although the bullets will be invisible owing to their small size in the micro- or nanometer range.

References:

1. Scopus.com [database on the Internet]. Amsterdam: Elsevier B.V.; 2009 [cited 2009 Aug 4]. Available from: http://www.scopus.com/home.url.
2. Okada H, Toguchi H. Biodegradable microspheres in drug delivery. Crit Rev Ther Drug Carrier Systems. 1995;12:1-99.
3. Gilding DK, Reed AM. Biodegradable polymers for use in surgery: polyglycolic/poly(lactic acid) homo- and copolymers. 1. Polymer. 1979;20:137-143.
4. Dorta MJ, Manguia O, Llabres M. Effects of polymerization variables on PLGA properties: molecular weight, composition and chain structure. Int J Pharm. 1993;100:9-14.
5. Neal JC, Stolnik S, Schacht E, Kenawy ER, Garnett MC, Davis SS, Illum L. In vitro displacement by rat serum of adsorbed radiolabeled poloxamer and poloxamine copolymers from model and biodegradable nanospheres. J Pharm Sci. 1998;87(10):1242-1248.
6. Huang N-P, Michel R, Voros J, Textor M, Hofer R, Rossi A, Elbert DL, Hubbell JA, Spencer ND. Poly(l-lysine)-g-poly(ethylene glycol) layers on metal oxide surfaces: Surface-analytical characterization and resistance to serum and fibrinogen adsorption. Langmuir. 2001;17(2):489-498.
7. Faraasen S, Voros J, Csucs G, Textor M, Merkle HP, Walter E. Ligand-specific targeting of microspheres to phagocytes by surface modification with poly(L-lysine)-grafted poly(ethylene glycol) conjugate. Pharm Res. 2003;20(2):237-246.
8. Hermann J, Bodmeier R. Biodegradable, somatostatin acetate containing microspheres prepared by various aqueous and non-aqueous solvent evaporation methods. Eur J Pharm Biopharm. 1998;45:72-85.

9. Rafler G, Jobmann M. Controlled release systems of biodegradable polymers. 5th communication: Microparticle preparation by a salting out process. Pharm Ind. 1997;59:620-627.
10. Pavanetto F, Genta I, Giunchedi P, Conti P. Evaluation of spray-drying as a method for polylactide and polylactide-co-glycolide microsphere preparation. J Microencaps. 1993;10:487-497.
11. Johansen P, Merkle HP, Gander B. Technological considerations related to up-scaling of protein microencapsulation by spray-drying. Eur J Pharm Biopharm. 2000;50:413-417.
12. Herbert P, Murphy K, Johnson O, Dong N, Jaworowicz W, Tracy MA, Cleland JL, Putney SD. A large-scale process to produce microencapsulated proteins. Pharm Res. 1998;15:357-362.
13. Lecaroz C, Gamazo C, Renedo MJ, Blanco-Prieto MJ. Biodegradable micro- and nanoparticles as long-term delivery vehicles for gentamycin. J Microencaps. 2006;23:782-792.
14. Corrigan OI, Li X. Quantifying drug release from PLGA nanoparticulates, Eur J Pharm Sci. 2009;37:477-485.
15. Govender T, Stolnik S, Garnett MC, Illum L, Davis SS. PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug. J Control Release. 1999;57:171-185.
16. Bilati U, Allemann E, Doelker E. Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles. Eur J Pharm Sci. 2005;24:67-75.
17. Mehta AK, Yadav KS, Sawant KK. Nimodipine loaded PLGA nanoparticles: Formulation optimisation using factorial design, characterization and in vitro evaluation. Curr Drug Deliv. 2007;4:185-193.
18. Bala I, Hariharan S, Kumar R. PLGA-nanoparticles in drug delivery: The state of the art. Crit Rev Ther Drug Carrier Syst. 2004;21:387-422.

19. Freitas S, Merkle HP, Gander B. Microencapsulation by solvent extraction/evaporation: reviewing the state of the art of microsphere preparation process technology. J Control Release. 2005;102:313-332.
20. Astete CE, Sabliov CM. Synthesis and characterisation of PLGA nanoparticles. J Biomater Sci Polym Ed. 2006;17:247-289.
21. Mundargi RC, Babu VR, Rangaswamy V, Patel P, Aminabhavi TM. Nano/micro technologies for delivering macromolecular therapeutics using poly(d,l-lactide-co-glycolide) and its derivatives. J Control Release. 2008;125(3):193-209.
22. Wischke C, Schwendeman SP. Principles of encapsulating hydrophobic drugs in PLA/PLGA microparticles. Int J Pharm. 2008;364:298-327.
23. Brannon-Peppas L, Vert M. Polylactic and Polyglycolic acid as drug delivery carriers. Wise, DL, editor. Marcel Dekker (New York): Handbook of pharmaceutical controlled release technology; 2000.
24. Perrin DA, English JP. Polyglycolide and Polylactide. Domb AJ, Kost J, Wiseman DM, editors. Harwood academic publishers (Amsterdam): Handbook of biodegradable polymers; 1997.
25. Spenlehauer G, Vert M, Benoit JP, Boddaert A. In vitro and in vivo degradation of poly(D,L-lactide/glycolide) type microspheres made by solvent evaporation method. Biomaterials. 1989;10:557-563.
26. Kang J, Schwendeman SP. Pore closing and opening in biodegradable polymers and their effect on the controlled release of proteins. Mol Pharm. 2007;4:104-118.
27. Makino K, Mogi T, Ohtake N, Yoshida M, Ando S, Nakajima T, Ohshima H. Pulsatile drug release from poly (lactide-co-glycolide) microspheres: How does the composition of the polymer matrices affect the time interval between the initial burst and the pulsatile release of drugs? Colloids Surf B Biointerfaces. 2000;19:173-179.
28. Cai Q, Shi GX, Bei JZ, Wang SG. Enzymatic degradation behaviour and mechanism of poly(lactide-co-glycolide) foams by trypsin. Biomaterials. 2003;24:629-638.

29. Anderson JM, Shive MS. Biodegradation and biocompatibility of PLA and PLGA microspheres. Adv Drug Deliv Rev. 1997;28:5-24.
30. Avgoustakis K, Nixon JR. Biodegradable controlled release tablets: III. Effect of polymer characteristics on drug release from heterogeneous poly(lactide-co-glycolide) matrices. Int J Pharm. 1993;99:247-252.
31. Hua N, Sun J. Body distribution of poly(D,L-lactide-co-glycolide) copolymer degradation products in rats. J Mater Sci Mater Med. 2008;19:3243-3248.
32. Kohane DS, Tse JY, Yeo Y, Padera R, Shubina M, Langer R. Biodegradable polymeric microspheres and nanospheres for drug delivery in the peritoneum. J Biomed Mat Res. 2006;77:351-361.
33. Cenni E, Granchi D, Avnet S, Fotia C, Salerno M, Micieli D, Sarpietro MG, Pignatello R, Castelli F, Baldini N. Biocompatibility of poly(D,L-lactide-co-glycolide) nanoparticles conjugated with alendronate. Biomaterials. 2008;29:1400-1411.
34. Kim D, El-Shall H, Dennis D, Morey T. Interaction of PLGA nanoparticles with human blood constituents. Colloids Surf B Biointerfaces. 2005;40:83-91.
35. Ignatius AA, Claes LE, In vitro biocompatibility of bioresorbable polymers: Poly(L,DL-lactide) and poly(L-lactide-co-glycolide). Biomaterials. 1996;17:831-839.
36. Malyala P, O'Hagan DT, Singh M. Enhancing the therapeutic efficacy of CpG oligonucleotides using biodegradable particles. Adv Drug Deliv Rev. 2009;61:218-225.
37. Wendorf J, Singh M, Chesko J, Kazzaz J, Soewanan E, Ugozzoli M, O'Hagan D. A practical approach to the use of nanoparticles for vaccine delivery. J Pharm Sci. 2006;95:2738-2750.
38. Hermanson GT. Bioconjugate techniques. San Diego: Academic Press; 1996.
39. Kasturi PS, Qin H, Thomson KS, El-Bereir S, Cha S-C, Neelapu S, Kwak LW, Roy K. Prophylactic anti-tumor effects in a B cell lymphoma

model with DNA vaccines delivered on polyethyleneimine (PEI) functionalized PLGA microparticles. J Control Release. 2006;113(3):261-270.

40. Gabor F, Trimmel K, Ratzinger G, Kerleta V, Fillafer C, Wirth M. Characterization of binding and uptake of biomimetic nanoparticles by flow cytometry. J Drug Delivery Sci Technol. 2008;18(1):51-57.
41. Chen H, Gao J, Lu Y, Kou G, Zhang H, Fan L, Sun Z, Guo Y, Zhong Y. Preparation and characterization of PE38KDEL-loaded anti-HER2 nanoparticles for targeted cancer therapy. J Control Release. 2008;128:209-216.
42. Brandhonneur N, Chevanne F, Vie V, Frisch B, Primault R, Le Poitier M-F, Le Corre P. Specific and non-specific phagocytosis of ligand-grafted PLGA microspheres by macrophages. Eur J Pharm Sci. 2009;36:474-485.
43. Sharon JL, Puleo DA. The use of N-terminal immobilization of PTH(1-34) on PLGA to enhance bioactivity. Biomaterials. 2008;29:3137-3142.
44. Ratzinger G, Agrawal P, Körner W, Lonkai J, Sanders HMHF, Terreno E, Wirth M, Strijkers GJ, Nicolay K, Gabor F. High-relaxivity contrast agents for MRI: Surface modification of PLGA nanospheres with Gd-DTPA and Gd-DOTA. Submitted to Small.
45. Ratzinger G, Wang XY, Wirth M, Gabor F. Targeted PLGA-microparticles as a novel and convenient strategy to treat lactose intolerance. Submitted to J Control Release.
46. McCarron PA, Marouf WM, Donnelly RF, Scott C. Enhanced surface attachment of protein-type targeting ligands to poly(lactide-co-glycolide) nanoparticles using variable expression of polymeric acid functionality. J Biomed Mater Res. 2008;87:873-884.
47. Ratzinger G, Länger U, Neutsch L, Pittner F, Wirth M, Gabor F. Surface modification of PLGA particles: The interplay between stabilizer, ligand size, and hydrophobic interactions. Langmuir. 2009;In press. DOI: 10.1021/la902602z.

48. Dahlgren MAG. Effect of counterion valency and ionic strength on polyelectrolyte adsorption. Langmuir. 1994;10(5):1580-1583.
49. Trimaille T, Pichot C, Delair T. Surface functionalization of poly(D,L-lactic acid) nanoparticles with poly(ethylenimine) and plasmid DNA by the layer-by-layer approach. Colloids Surf A Physicochem Eng Asp. 2003;221:39-48.
50. Messai I, Delair T. Adsorption of chitosan onto poly(D,L-lactic acid) particles: A physico-chemical investigation. Macromol Chem Phys. 2005;206(16):1665-1674.
51. Vila A, Sánchez A, Tobío M, Calvo P, Alonso MJ. Design of biodegradable particles for protein delivery. J Control Release. 2002;78:15-24.
52. Dillen K, Vandervoort J, Van den Mooter G, Ludwig A. Evaluation of ciprofloxacin-loaded Eudragit® RS100 or RL100/PLGA nanoparticles. Int J Pharm. 2006;314(1):72-82.
53. Thiele L, Rothen-Rutishauser B, Jilek S, Wunderli-Allenspach H, Merkle HP, Walter E. Evaluation of particle uptake in human blood monocyte-derived cells in vitro. Does phagocytosis activity of dendritic cells measure up with macrophages? J Control Release. 2001;76:59-71.
54. Vasir JK, Labhasetwar V. Quantification of the force of nanoparticle-cell membrane interactions and its influence on intracellular trafficking of nanoparticles. Biomaterials. 2008;29(31):4244-4252.
55. Kim BS, Kim CS, Lee KM. The intracellular uptake ability of chitosan-coated poly (D,L-lactide-co-glycolide) nanoparticles. Arch Pharm Res. 2008;31(8):1050-1054.
56. Martínez Gómez JM, Csaba N, Fischer S, Sichelstiel A, Kündig TM, Gander B, Johansen P. Surface coating of PLGA microparticles with protamine enhances their immunological performance through facilitated phagocytosis. J Control Release. 2008;130(2):161-167.

57. Thiele L, Merkle HP, Walter E. Phagocytosis and phagosomal fate of surface-modified microparticles in dendritic cells and macrophages. Pharm Res. 2003;20(2):221-228.
58. Kumar MNVR, Mohapatra SS, Kong X, Jena PK, Bakowsky U, Lehr CM. Cationic poly(lactide-co-glycolide) nanoparticles as efficient in vivo gene transfection agents. J Nanosci Nanotechnol. 2004;4(8):990-994.
59. Kumar MNRV, Bakowsky U, Lehr CM. Preparation and characterization of cationic PLGA nanospheres as DNA carriers. Biomaterials. 2004;25:1771-1777.
60. Yamamoto H, Kuno Y, Sugimoto S, Takeuchi H, Kawashima Y. Surface-modified PLGA nanosphere with chitosan improved pulmonary delivery of calcitonin by mucoadhesion and opening of the intercellular tight junctions. J Control Release. 2005;102(2):373-381.
61. Fischer S, Foerg C, Ellenberger S, Merkle HP, Gander B. One-step preparation of polyelectrolyte-coated PLGA microparticles and their functionalization with model ligands. J Control Release. 2006;111:135-144.
62. Nafee N, Taetz S, Schneider M, Schaefer UF, Lehr CM. Chitosan-coated PLGA nanoparticles for DNA/RNA delivery: Effect of the formulation parameters on complexation and transfection of antisense oligonucleotides. Nanomedicine. 2007;3(3):173-183.
63. Guo C, Gemeinhart RA. Understanding the adsorption mechanism of chitosan onto poly(lactide-co-glycolide) particles. Eur J Pharm Biopharm. 2008;70(2):597-604.
64. Manca M-L, Mourtas S, Dracopoulos V, Fadda AM, Antimisiaris SG. PLGA, chitosan or chitosan-coated PLGA microparticles for alveolar delivery?: A comparative study of particle stability during nebulization. Colloids Surf B Biointerfaces. 2008;62(2):220-231.
65. Yang R, Shim W-S, Cui F-D, Cheng G, Han X, Jin Q-R, Kim D-D, Chung S-J, Shim C-K. Enhanced electrostatic interaction between

chitosan-modified PLGA nanoparticle and tumor. Int J Pharm. 2009;371(1-2):142-147.

66. Yang R, Yang SG, Shim WS, Cui F, Cheng G, Kim IW, Kim DD, Chung SJ, Shim CK. Lung-specific delivery of paclitaxel by chitosan-modified PLGA nanoparticles via transient formation of microaggregates. J Pharm Sci. 2009;98(3):970-984.
67. Tsung MJ, Burgess DJ. Preparation and characterization of gelatin surface modified PLGA microspheres. AAPS PharmSci. 2001;3(2):E11.
68. Wischke C, Zimmermann J, Wessinger B, Schendler A, Borchert HH, Peters JH, Nesselhut T, Lorenzen DR. Poly(I:C) coated PLGA microparticles induce dendritic cell maturation. Int J Pharm. 2009;365(1-2):61-68.
69. Bivas-Benita M, Romeijn S, Junginger HE, Borchard G. PLGA-PEI nanoparticles for gene delivery to pulmonary epithelium. Eur J Pharm Biopharm. 2004;58(1):1-6.
70. Mandal B, Kempf M, Merkle HP, Walter E. Immobilisation of GM-CSF onto particulate vaccine carrier systems. Int J Pharm. 2004;269(1):259-265.
71. Oster CG, Kim N, Grode L, Barbu-Tudoran L, Schaper AK, Kaufmann SHE, Kissel T. Cationic microparticles consisting of poly(lactide-co-glycolide) and polyethylenimine as carriers systems for parental DNA vaccination. J Control Release. 2005;104(2):359-377.
72. Kasturi SP, Sachaphibulkij K, Roy K. Covalent conjugation of polyethyleneimine on biodegradable microparticles for delivery of plasmid DNA vaccines. Biomaterials. 2005;26: 6375-6385.
73. Yang YW, Hsu PYJ. The effect of poly(d,l-lactide-co-glycolide) microparticles with polyelectrolyte self-assembled multilayer surfaces on the cross-presentation of exogenous antigens. Biomaterials. 2008;29(16):2516-2526.

74. Cui C, Schwendeman SP. Surface entrapment of polylysine in biodegradable poly(DL-lactide-co-glycolide) microparticles. Macromolecules. 2001;34:8426-8433.
75. Müller M, Voros J, Csucs G, Walter E, Danuser G, Merkle HP, Spencer ND, Textor M. Surface modification of PLGA microspheres. J Biomed Mater Res A. 2003;66(1):55-61.
76. Kim SH, Jeong JH, Chun KW, Park TG. Target-specific cellular uptake of PLGA nanoparticles coated with poly(L-lysine)-poly(ethylene glycol)-folate conjugate. Langmuir. 2005;21:8852-8857.
77. Reynolds F, Weissleder R, Josephson L. Protamine as an efficient membrane-translocating peptide. Bioconjug Chem. 2005;16(5):1240-1245.
78. Boussif O, Lezoualc'h F, Zanta MA, Mergny MD, Scherman D, Demeneix B, Behr JP. A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: Polyethylenimine. Proc Natl Acad Sci USA. 1995;92(16):7297-7301.
79. Kircheis R, Wightman L, Wagner E. Design and gene delivery activity of modified polyethylenimines. Adv Drug Deliv Rev. 2001;53(3):341-358.
80. Neu M, Fischer D, Kissel T. Recent advances in rational gene transfer vector design based on poly(ethylene imine) and its derivatives. J Gene Med. 2005;7(8):992-1009.
81. Shakweh M, Besnard M, Nicolas V, Fattal E. Poly (lactide-co-glycolide) particles of different physicochemical properties and their uptake by peyer's patches in mice. Eur J Pharm Biopharm. 2005;61(1-2):1-13.
82. Kim I-S, Lee S-K, Park Y-M, Lee Y-B, Shin S-C, Lee KC, Oh I-J. Physicochemical characterization of poly(l-lactic acid) and poly(d,l-lactide-co-glycolide) nanoparticles with polyethylenimine as gene delivery carrier. Int J Pharm. 2005;298(1):255-262.

83. Arnold LJ, Dagan A, Gutheil J, Kaplan NO. Antineoplastic activity of poly(L-lysine) with some ascites tumor cells. Proc Natl Acad Sci U S A. 1979;76(7):3246-50.
84. Kou G, Gao J, Wang H, Chen H, Li B, Zhang D, Wang S, Hou S, Qian W, Dai J, Zhong Y, Guo Y. Preparation and characterization of paclitaxel-loaded PLGA nanoparticles coated with cationic SM5-1 single-chain antibody. J Biochem Mol Biol. 2007;40(5):731-739.
85. Dunn SE, Coombes AGA, Garnett MC, Davis SS, Davies MC, Illum M. In vitro cell interaction and in vivo biodistribution of poly(lactide-co-glycolide) nanospheres surface modified by poloxamer and poloxamine copolymers. J Control Release. 1997;44:65-76.
86. Scholes PD, Coombes AG, Illum L, Davis SS, Watts JF, Ustariz C, Vert M, Davies MC. Detection and determination of surface levels of poloxamer and PVA surfactant on biodegradable nanospheres using SSIMS and XPS. J Control Release. 1999;59(3):261-278.
87. Sahoo SK, Panyam J, Prabha S, Labhasetwar V. Residual polyvinyl alcohol associated with poly (D,L.lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. J Control Release. 2002;82:105-114.
88. Rojas OJ, Ernstsson M, Neuman RD, Claesson PM. Effect of polyelectrolyte charge density on the adsorption and desorption behavior on mica. Langmuir. 2002;18(5):1604-1612.
89. Kim C-J. Advanced pharmaceutics: Physicochemical principles. 1st ed. Boca Raton (FL): CRC; 2004.
90. Israelachvili J. Intramolecular and surface forces. 2nd ed. London: Academic Press; 1991.
91. Evans DF, Wennerström H. The colloidal domain: Where physics, chemistry, biology, and technology meet. 2nd ed. Hoboken (NJ): Wiley-VCH; 1999.
92. Lacasse FX, Filion MC, Phillips NC, Escher E, McMullen JN, Hildgen P. Influence of surface properties at biodegradable microsphere

surfaces: Effects on plasma proteins adsorption and phagocytosis. Pharm Res. 1998;15:312-317.

93. DeMerlis CC, Schoneker DR. Review of the oral toxicity of polyvinyl alcohol (PVA). Food Chem Toxicol. 2003;41:319-326.
94. Patel AR, Kulkarni S, Nandekar TD, Vavia PR. Evaluation of alkyl polyglucoside as an alternative surfactant in the preparation of peptide-loaded nanoparticles. J Microencaps. 2008;25(8):531-540.
95. Monographs.IARC.fr [homepage on the Internet]. Lyon Cedex: World Health Organization, International Agency for Research on Cancer; 2009 [updated 2009 Apr 2; cited 2009 Oct 2]. Available from: http://monographs.iarc.fr/ENG/Classification/Listagentsalphorder.pdf.
96. Accessdata.fda.gov [database on the Internet]. Silver Spring: U.S. Food and Drug Administration; 2009 [cited 2009 Oct 2]. Available from: http://www.accessdata.fda.gov/scripts/cder/iig/index.cfm.
97. Santander-Ortega MJ, Jodar-Reyes AB, Csaba N, Bastos-Gonzalez D, Ortega-Vinuesa JL. Colloidal stability of Pluronic F68-coated PLGA nanoparticles: A variety of stabilization mechanisms. J Colloid Interface Sci. 2006;302:522-529.
98. Besheer A, Vogel J, Glanz D, Kressler J, Groth T, Mäder K. Characterization of PLGA nanospheres stabilized with amphiphilic polymers: hydrophobically modified hydroxyethyl starch vs Pluronics. Mol Pharm. 2009;6(2):407-415.
99. Chang J, Jallouli Y, Kroubi M, Yuan X-B, Feng W, Kang C-S, Pu P-Y, Betbeder D. Characterization of endocytosis of transferrin-coated PLGA nanoparticles by the blood-brain barrier. Int J Pharm. 2009;379(2):285-292.
100. Singh M, Briones M, Ott G, O'Hagan D. Cationic microparticles: A potent delivery system for DNA vaccines. Proc Natl Acad Sci U S A. 2000;97(2):811-816.
101. Otten GR, Schaefer M, Doe B, Liu H, Srivastava I, Megede J, Kazzaz J, Lian Y, Singh M, Ugozzoli M, Montefiori D, Lewis M, Driver

DA, Dubensky T, Polo JM, Donnelly J, O'Hagan DT, Barnett S, Ulmer JB. Enhanced potency of plasmid DNA microparticle human immunodeficiency virus vaccines in rhesus macaques by using a priming-boosting regimen with recombinant proteins. J Virol. 2005;79(13):8189-8200.

102. Kazzaz J, Neidleman J, Singh M, Ott G, O'Hagan DT. Novel anionic microparticles are a potent adjuvant for the induction of cytotoxic T lymphocytes against recombinant p55 gag from HIV-1. J Control Release. 2000;67:347-356.

103. Singh M, Kazzaz J, Chesko J, Soenawan E, Ugozzoli M, Giuliani M, Pizza M, Rappouli R, O'Hagan DT. Anionic microparticles are a potent delivery system for recombinant antigens from Neisseria meningitidis serotype B. J Pharm Sci. 2004;93(2):273-282.

104. Feng S-S, Huang G. Effects of emulsifiers on the controlled release of paclitaxel (Taxol®) from nanospheres of biodegradable polymers. J Control Release. 2001;71:53-69.

105. Sengupta S, Eavarone D, Capila I, Zhao G, Watson N, Kiziltepe T, Sasisekharan R. Temporal targeting of tumor cells and neovasculature with a nanoscale delivery system. Nature 2005; 436:568-572.

106. Besheer A, Hause G, Kressler J, Mäder K. Hydrophobically modified hydroxyethyl starch: Synthesis, characterization, and aqueous self-assembly into nano-sized polymeric micelles and vesicles. Biomacromolecules. 2007;8:359-367.

107. Mu L, Feng SS. Vitamin E TPGS used as emulsifier in the solvent evaporation/extraction technique for fabrication of polymeric nanospheres for controlled release of paclitaxel (Taxol®). J Control Release. 2002;80:129-144.

108. Win KY, Feng SS. Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs. Biomaterials. 2005;26:2713-2722.

109. Zhao HZ, Tan EC, Yung LYL. Potential use of cholecalciferol polyethylene glycol succinate as a novel pharmaceutical additive. J Biomed Mater Res. 2008;84A:954-964.

110. Laroui H, Grossin L, Leonard M, Stoltz J-F, Gillet P, Netter P, Dellacherie E. Hyaluronate-covered nanoparticles for the therapeutic targeting of cartilage. Biomacromolecules. 2007;8:3879-3885.

111. Fahmy TM, Samstein RM, Harness CC, Saltzman WM. Surface modification of biodegradable polyesters with fatty acid conjugates for improved drug targeting. Biomaterials. 2005;26:5727-5736.

112. Demento SL, Eisenbarth SC, Foellmer HG, Platt C, Caplan MJ, Saltzman WM, Mellman I, Ledizet M, Fikrig E, Flavell RA, Fahmy TM. Inflammasome-activating nanoparticles as modular systems for optimizing vaccine efficacy. Vaccine. 2009;27:3013-3021.

113. Cui C, Schwendeman SP. One-step surface modification of poly(lactide-co-glycolide) microparticles with heparin. Pharm Res. 2007;24(12):2381-1393.

114. Mu L, Feng SS. A novel controlled release formulation for the anticancer drug paclitaxel (Taxol®): PLGA nanoparticles containing vitamin E TPGS. J Control Release. 2003;86:33-48.

115. Montisci M.-J., Giovannuci G, Duchene D, Ponchel G. Covalent coupling of asparagus pea and tomato lectins to poly(lactide) microspheres. Int J Pharm. 2001;215:153-161.

116. Sahoo SK, Labhasetwar V. Enhanced antiproliferative activity of transferrin-conjugated paclitaxel-loaded nanoparticles is mediated via sustained intracellular drug retention. Mol Pharm. 2005;2(5):373-383.

117. Rao KS, Reddy MK, Horning JL, Labhasetwar V. TAT-conjugated nanoparticles for the CNS delivery of anti-HIV drugs. Biomaterials. 2008;29:4429-4438.

118. Lu J, Jackson JK, Gleave ME, Burt HM. The preparation and characterization of anti-VEGFR2 conjugated, paclitaxel-loaded PLLA

or PLGA microspheres for the systemic targeting of human prostate tumors. Cancer Chemother Pharmacol. 2008;61(6):997-1005.

119. Keegan ME, Falcone JL, Leung TC, Saltzman WM. Biodegradable microspheres with enhanced capacity for covalently bound surface ligands. Macromolecules. 2004;37:9779-9784.

120. Chittasupho C, Xie S-X, Baoum A, Yakovleva T, Siahaan TJ, Berkland CJ. ICAM-1 targeting of doxorubicin-loaded PLGA nanoparticles to lung epithelial cells. Eur J Pharm Sci. 2009;37(2):141-150.

121. Cui C, Stevens VC, Schwendeman SP. Injectable polymer microsperes enhance immunogenicity of a contraceptive peptide vaccine. Vaccine. 2007;25:500-509.

122. Chesko J, Kazzaz J, Ugozzoli M, O'Hagan DT, Singh M. An investigation of the factors controlling the adsorption of protein antigens to anionic PLG microparticles. J Pharm Sci. 2005;94(11):2510-2519.

123. Cai C, Bakowsky U, Rytting E, Schaper AK, Kissel T. Charged nanoparticles as protein delivery systems: A feasibility study using lysozyme as model protein. Eur J Pharm Biopharm. 2008;69:31-42.

124. Crotts G, Park TG. Stability and release of bovine serum albumin encapsulated within poly(D,L-lactide-co-glycolide) microparticles. J Control Release. 1997;44:123-134.

125. Crotts G, Sah H, Park TG. Adsorption determines in-vitro protein release rate from biodegradable microspheres: quantitative analysis of surface area during degradation. J Control Release. 1997;47:101-111.

126. Kocbek P, Obermajer N, Gegnar M, Kos J, Kristl J. Targeting cancer cells using PLGA nanoparticles surface modified with monoclonal antibody. J Control Release. 2007;120:18-26.

127. Wang J, Wang BM, Schwendeman P. Mechanistic evaluation of the glucose-induced reduction in initial burst release of octreotide

acetate from poly(D,L-lactide-co-glycolide) microspheres. Biomaterials. 2004;25:1919-1927.

128. Xu Q, Crossley A, Czernuszka J. Preparation and characterization of negatively charged poly(lactic-co-glycolic acid) microspheres. J Pharm Sci. 2009;98:2377-2389.

129. Villari V, Micali N. Light scattering as spectroscopic tool for the study of disperse systems useful in pharmaceutical sciences. J Pharm Sci. 2008;97:1703-1730.

130. Müller RH, Jacobs C, Kayser O. Nanosuspensions as particulate drug formulations in therapy: Rationale for development and what we can expect for the future. Adv Drug Deliv Rev 2001;47:4-12.

131. Augsten C, Kiselev MA, Gehrke R, Hause G, Mäder K. A detailed analysis of biodegradable nanospheres by different techniques - A combined approach to detect particle sizes and size distributions. J Pharm Biomed Anal. 2008;47:95-102.

132. Fraunhofer W, Winter G. The use of asymmetrical flow field-flow fractionation in pharmaceutics and biopharmaceutics. Eur J Pharm Biopharm. 2004;58:369-383.

133. Schimpf M, Caldwell K, Giddings JC. Field-flow fractionation handbook. Wiley-Intersceince 2000

134. Musyanovych A, Schmitz-Wienke J, Mailänder V, Walther P, Landsfester K. Preparation of biodegradable polymer nanoparticles by miniemulsion technique and their cell interactions. Macromol Biosci. 2008;8:127-139.

135. Gaumet M, Vargas A, Gurny R, Delie F. Nanoparticles for drug delivery: The need for precision in reporting particle size parameters. Eur J Pharm Biopharm. 2008;69:1-9.

136. Giovagnoli S, Blasi P, Ricci M, Schoubben A, Perioli L, Rossi C. Phsysicochemical characterization and release mechanism of a novel prednisone biodegradable microsphere formulation. J Pharm Sci. 2008;97:303-317.

137. Gaumet M, Gurny R, Delie F. Fluorescent biodegradable PLGA particles with narrow size distributions: Preparation by means of selective centrifugation. Int J Pharm. 2007;342:222-230.

138. Guan XP, Quan DP, Liao KR, Wang T, Xiang P, Mai KC. Preparation and characterization of cationic chitosan-modified poly(D,L-lactide-co-glycolide) copolymer nanospheres as DNA carriers. J Biomater Appl. 2008;22:353-371.

139. Gvili K, Benny O, Danino D, Machluf M. Poly(D,L-lactide-co-glycolide acid) nanoparticles for DNA delivery: Waiving preparation complexity and increasing efficiency. Biopolymers. 2007;85:379-391.

140. Dorati R, Patrini M, Perugini P, Pavanetto F, Stella A, Modena T, Genta I, Cont B. Surface characterization by atomic force microscopy of sterilized PLGA microspheres. J Microencapsul. 2006;23:123-133.

141. Bunjes H, Unruh T. Characterization of lipid nanoparticles by differential scanning calorimetry, X-ray and neutron scattering. Adv Drug Deliv Rev. 2007;59:379 – 402.

142. Agnihotri SM, Ohshima H, Terada H, Tomoda K, Makino K. Electrophoretic mobility of colloidal gold particles in electrolyte solutions. Langmuir. 2009;25:4804 – 4807.

143. Kesisoglou F, Panmai S, Wu Y. Nanosizing - Oral formulation development and biopharmaceutical evaluation. Adv Drug Deliv Rev. 2007;59:631-644.

144. Shinkai M, Yanase M, Honda H, Wakabayashi T, Yoshida J, Kobayashi T. Intracellular hyperthermia for cancer using cationic liposomes: in vitro study. Jpn J Cancer Res. 1996;87:1179-1183.

145. Ito A, Shinkai M, Honda H, Kobayashi T. Medical application of functionalized magnetic nanoparticles. J Biosci Bioeng. 2005; 100:1-11.

146. Stolnik S, Dunn SE, Garnett MC, Davies MC, Coombes AG, Taylor DC, Irving MP, Purkiss SC, Tadros TF, Davis SS, Illum L. Surface modification of poly(lactide-co-glycolide) nanospheres by

biodegradable poly(lactide)-poly(ethylene glycol) copolymers. Pharm Res. 1994;11(12):1800-1808.

147. Lück M, Pistel KF, Li YX, Blunk T, Müller RH, Kissel T. Plasma protein adsorption on biodegradable microspheres consisting of poly(D,L-lactide-co-glycolide), poly(L-lactide) or ABA triblock copolymers containing poly(oxyethylene); Influence of production method and polymer composition. J Control Release. 1998;55:107-120.

148. Shakesheff KM, Evora C, Soriano I, Langer R. The adsorption of poly(vinyl alcohol) to biodegradable microparticles studied by X-ray photoelectron spectroscopy (XPS). J Colloid Interface Sci. 1996;185:538-547.

149. Gaumet M, Gurny R, Delie F. Localization and quantification of biodegradable particles in an intestinal cell model: The influence of particle size. Eur J Pharm Sci. 2009;36:465-473.

150. Weiss B, Schneider M, Muys L, Taetz S, Neumann D, Schaefer UF, Lehr CM. Coupling of biotin-(poly(ethylene glycol))amine to poly(D,L-lactide-co-glycolide) nanoparticles for versatile surface modification. Bioconjug Chem. 2007;18:1087-1094.

151. Gref R, Luck M, Quellec P, Marchand M, Dellacherie E, Harnisch S, Blunk T, Muller RH. 'Stealth' corona-core nanoparticles surface modified by polyethylene glycol (PEG): influences of the corona (PEG chain length and surface density) and of the core composition on phagocytic uptake and plasma protein adsorption. Colloids Surf B Biointerfaces. 2000;18(3-4):301-313.

152. Ha CS, Gardella JA. Surface chemistry of biodegradable polymers for drug delivery systems. Chem Rev. 2005;105:4205-4232.

153. Chesko J, Kazzaz J, Ugozzoli M, Singh M, O'Hagan DT, Madden C, Perkins M, Patel N. Characterization of antigens adsorbed to anionic PLG microparticles by XPS and TOF-SIMS. J Pharm Sci. 2008;97:1443-1453.

154. Mehrishi JN, Bauer J. Electrophoresis of cells and the biological relevance of surface charge. Electrophoresis. 2002;23(13):1984-1994.

155. Thiele L, Diederichs JE, Reszka R, Merkle HP, Walter E. Competitive adsorption of serum proteins at microparticles affects phagocytosis by dendritic cells. Biomaterials. 2003;24(8):1409-1418.

156. Nguyen DN, Green JJ, Chan JM, Langer R, Anderson DG. Polymeric materials for gene delivery and DNA vaccination. Adv Mater. 2009;21(8):847-867.

157. Labhasetwar V, Song C, Humphrey W, Shebuski R, Levy RJ. Arterial uptake of biodegradable nanoparticles: Effect of surface modifications. J Pharm Sci. 1998;87(10):1229-1234.

158. Ran S, Downes A, Thorpe PE. Increased exposure of anionic phospholipids on the surface of tumor blood vessels. Cancer Res. 2002;62(21):6132-6140.

159. Roser M, Fischer D, Kissel T. Surface-modified biodegradable albumin nano- and microspheres. II: effect of surface charges on in vitro phagocytosis and biodistribution in rats. Eur J Pharm Biopharm. 1998;46(3):255-263.

160. Lai SK, Wang YY, Hanes J. Mucus-penetrating nanoparticles for drug and gene delivery to mucosal tissues. Adv Drug Deliv Rev. 2009;61(2):158-171.

161. Andrews GP, Laverty TP, Jones DS. Mucoadhesive polymeric platforms for controlled drug delivery. Eur J Pharm Biopharm. 2009;71(3):505-18.

162. Ponchel G, Irache J-M. Specific and non-specific bioadhesive particulate systems for oral delivery to the gastrointestinal tract. Adv Drug Deliv Rev. 1998;34(2-3):191-219.

163. Kawashima Y, Yamamoto H, Takeuchi H, Kuno Y. Mucoadhesive DL-lactide/glycolide copolymer nanospheres coated with chitosan to improve oral delivery of elcatonin. Pharm Dev Technol. 2000;5(1):77-85.

164. Vandervoort J, Yoncheva K, Ludwig A. Influence of the homogenisation procedure on the physicochemical properties of PLGA nanoparticles. Chem Pharm Bull. 2004;52(11):1273-1279.

165. Grabovac V, Bernkop-Schnurch A. Development and in vitro evaluation of surface modified poly(lactide-co-glycolide) nanoparticles with chitosan-4-thiobutylamidine. Drug Dev Ind Pharm. 2007;33(7):767-774.

166. Cu Y, Saltzman WM. Drug delivery: Stealth particles give mucus the slip. Nat Mater. 2009;8(1):11-3.

167. Olmsted SS, Padgett JL, Yudin AI, Whaley KJ, Moench TR, Cone RA. Diffusion of macromolecules and virus-like particles in human cervical mucus. Biophys J. 2001;81(4):1930-1937.

168. Wang YY, Lai SK, Suk JS, Pace A, Cone R, Hanes J. Addressing the PEG mucoadhesivity paradox to engineer nanoparticles that "slip" through the human mucus barrier. Angew Chem Int Ed Engl. 2008;47(50):9726-9729.

169. Lai SK, O'Hanlon DE, Harrold S, Man ST, Wang Y-Y, Cone R, Hanes J. Rapid transport of large polymeric nanoparticles in fresh undiluted human mucus. Proc Natl Acad Sci U S A. 2007;104(5):1482-1487.

170. Cu Y, Saltzman WM. Controlled surface modification with poly(ethylene)glycol enhances diffusion of PLGA nanoparticles in human cervical mucus. Mol Pharm. 2008;6(1):173-181.

171. Dawson M, Krauland E, Wirtz D, Hanes J. Transport of polymeric nanoparticle gene carriers in gastric mucus. Biotechnol Prog. 2004;20(3):851-857.

172. Sun B, Ranganathan B, Feng S-S. Multifunctional poly(D,L-lactide-co-glycolide)/montmorillonite (PLGA/MMT) nanoparticles decorated by trastuzumab for targeted chemotherapy of breast cancer. Biomaterials. 2008;29(4):475-486.

173. Acharya S, Dilnawaz F, Sahoo SK. Targeted epidermal growth factor receptor nanoparticle bioconjugates for breast cancer therapy. Biomaterials. 2009;30:5737-5750.

174. Scott CJ, Marouf WM, Quinn DJ, Buick RJ, Orr SJ, Donnelly RF, McCarron PA. Immunocolloidal targeting of the endocytotic siglec-7 receptor using peripheral attachment of siglec-7 antibodies to poly(lactide-co-glycolide) nanoparticles. Pharm Res. 2008;25(1):135-146.

175. McCarron PA, Marouf WM, Quinn DJ, Fay F, Burden RE, Olwill SA, Scott CJ. Antibody targeting of camptothecin-loaded PLGA nanoparticles to tumor cells. Bioconjug Chem. 2008;19(8):1561-1569.

176. Eniola AO, Hammer DA. In vitro characterization of leukocyte mimetic for targeting therapeutics to the endothelium using two receptors. Biomaterials. 2005;26:7136-7144.

177. Sharon N, Lis H. History of lectins: from hemagglutinins to biological recognition molecules. Glycobiology. 2004;14(11):53-62.

178. Diez S, Navarro G, de Ilarduya CT. In vivo targeted gene delivery by cationic nanoparticles for treatment of hepatocellular carcinoma. J Gene Med. 2009;11(1):38-45.

179. Gabor F, Wirth M. Lectin-mediated drug delivery: Fundamentals and perspectives. STP Pharma Sciences. 2003;13:3-16.

180. Gabor F, Stangl M, Wirth M. Lectin-mediated bioadhesion: Binding characteristics of plant lectins on the enterocyte-like cell lines Caco-2, Ht-29 and HCT-8. J Control Release. 1998;55:131-142.

181. Gabor F, Klausegger U, Wirth M. The interaction between wheat germ agglutinin and other plant lectins with prostate cancer cells Du-145. Int J Pharm. 2001;221:35-47.

182. Toegel S, Harrer N, Plattner VE, Unger FM, Viernstein H, Goldring MB, Gabor F, Wirth M. Lectin binding studies on C-28/I2 and T/C-28a2 chondrocytes provide a basis for new tissue engineering and

drug delivery perspectives in cartilage research. J Control Release. 2007;117(1):121-129.

183. Plattner VE, Wagner M, Ratzinger G, Gabor F, Wirth M. Targeted drug delivery: Binding and uptake of plant lectins using human 5637 bladder cancer cells. Eur J Pharm Biopharm. 2008;70(2):572-576.

184. Plattner VE, Ratzinger G, Engleder ET, Gallauner S, Gabor F, Wirth M. Alteration of the glycosylation pattern of monocytic THP-1 cells and its impact on lectin-mediated drug delivery. Eur J Pharm Biopharm. 2009; DOI: 10.1016/j.ejpb.2009.07.004.

185. Gabor F, Wirth M, Jurkovich B, Haberl I, Theyer G, Walcher G, Hamilton G. Lectin-mediated bioadhesion: Proteolytic stability and binding-characteristics of Wheat germ agglutinin and Solanum tuberosum lectin on Caco-2, HT-29 and human colonocytes. J Control Release. 1997;49:27-37.

186. Lochner N, Pittner F, Wirth M, Gabor F. Wheat germ agglutinin binds to the epidermal growth factor receptor of artificial Caco-2 membranes as detected by silver nanoparticle enhanced fluorescence. Pharm Res. 2003;20(5):833-839.

187. Gabor F, Bogner E, Weissenboeck A, Wirth M. The lectin-cell interaction and its implications to intestinal lectin-mediated drug delivery. Adv Drug Deliv Rev. 2004;56:459-480.

188. Roth-Walter F, Schöll I, Untersmayr E, Ellinger A, Boltz-Nitulescu G, Scheiner O, Gabor F, Jensen-Jarolim E. Mucosal targeting of allergen-loaded microspheres by Aleuria aurantia lectin. Vaccine. 2005;23(21):2703-2710.

189. Jepson MA, Clark MA, Hirst BH. M cell targeting by lectins: A strategy for mucosal vaccination and drug delivery. Adv Drug Deliv Rev. 2004;56:511-525.

190. Palumbo RN, Wang C. Bacterial invasin: Structure, function, and implication for targeted oral gene delivery. Curr Drug Deliv. 2006;3(1):47-53.

191. Des Rieux A, Fievez V, Garinot M, Schneider Y-J, Preat V. Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach. J Control Release. 2006;116(1):1-27.

192. Dawson GF, Halbert GW. The in vitro cell association of invasin coated polylactide-co-glycolide nanoparticles. Pharm Res. 2000;17(11):1420-1425.

193. Townsend SA, Evrony GD, Gu FX, Schulz MP, Brown RH, Langer R. Tetanus toxin C fragment-conjugated nanoparticles for targeted drug delivery to neurons. Biomaterials. 2007;28:5176-5184.

194. Rothenfluh DA, Bermudez H, O'Neil CP, Hubbell JA. Biofunctional polymer nanoparticles for intra-articular targeting and retention in cartilage. Nat Mat. 2008;7:248-254.

195. Hersel U, Dahmen C, Kessler H. RGD modified polymers: biomaterials for stimulated cell adhesion and beyond. Biomaterials. 2003;24:4385-4415.

196. Wang Z, Chui W-K, Ho PC. Design of a multifunctional PLGA nanoparticulate drug delivery system: Evaluation of its physicochemical properties and anticancer activity to malignant cancer cells. Pharm Res. 2009;26(5):1162-1171.

197. Garinot M, Fievez V, Pourcelle V, Stoffelbach F, des Rieux A, Plapied L, Theate I, Freichels H, Jerome C, Marchand-Brynaert J, Schneider Y-J, Préat V. PEGylated PLGA-based nanoparticles targeting M cells for oral vaccination. J Control Release 2007;120:195-204.

198. Fievez V, Plapied L, Des Rieux A, Pourcelle V, Freichels H, Wascotte V, Vanderhaeghen M-L, Jerome C, Vanderplasschen A, Marchand-Brynaert J, Schneider Y-J, Preat V. Targeting nanoparticles

to M cells with non-peptidic ligands for oral vaccination. Eur J Pharm Biopharm. 2009;73:16-24.

199. Zhang N, Chittasupho C, Duangrat C, Siahaan TJ, Berkland C. PLGA nanoparticle-peptide conjugate effectively targets intercellular cell-adhesion molecule-1. Bioconjug Chem. 2008;19(1):145-152.

200. Costantino L, Gandolfi F, Tosi G, Rivasi F, Vandelli MA, Forni F. Peptide-derivatized biodegradable nanoparticles able to cross the blood-brain barrier. J Control Release. 2005;108:84-96.

201. Tosi G, Costantino L, Rivasi F, Ruozi B, Leo E, Vergoni AV, Tacchi R, Bertolini A, Vandelli MA, Forni F. Targeting the central nervous system: in vivo experiments with peptide-derivatized nanoparticles loaded with loperamide and rhodamine-123. J Control Release. 2007;122(1):1-9.

202. Farokhzad OC, Cheng J, Teply BA, Sherifi I, Jon S, Kantoff PW, Richie JP, Langer R. Targeted nanoparticle-aptamer bioconjugates for cancer therapy in vivo. Proc Natl Acad Sci U S A. 2006;103(16):6315-6320.

203. Hilgenbrink AR, Low PS. Folate receptor-mediated drug targeting: From therapeutics to diagnostics. J Pharm Sci. 2005;94(10):2135-2146.

204. Yoo HS, Park TG. Folate receptor targeted biodegradable polymeric doxorubicin micelles. J Control Release. 2004;96(2):273-283.

205. Zhang Z, Lee SH, Feng S-S. Folate-decorated poly(lactide-co-glycolide)-vitamin E TPGS nanoparticles for targeted drug delivery. Biomaterials. 2007;28:1889-1899.

206. Lockman PR, Oyewumi MO, Koziara JM, Roder KE, Mumper RJ, Allen DD. Brain uptake of thiamine-coated nanoparticles. J Control Release. 2003;93:271-282.

207. Salman HH, Gamazo C, de Smidt PC, Russell-Jones G, Irache JM. Evaluation of bioadhesive capacity and immunoadjuvant

properties of vitamin B(12)-Gantrez Nanoparticles. Pharm Res. 2008;25(12):2859-2868.

208. Choi S-W, Kim J-H. Design of surface-modified poly(D,L-lactide-co-glycolide) nanoparticles for targeted drug delivery to bone. J Control Release. 2007;122:24-30.

209. Vasir JK, Labhasetwar V. Biodegradable nanoparticles for cytosolic delivery of therapeutics. Adv Drug Deliv Rev. 2007;59:718-728.

210. Breunig M, Bauer S, Goepferich A. Polymers and nanoparticles: Intelligent tools for intracellular targeting? Eur J Pharm Biopharm. 2008;68:112-128.

211. Behr J-P. The proton sponge: A trick to enter cells the viruses did not exploit. Chimia. 1997;51:34-36.

212. Pack DW, Hoffmann AS, Pun S, Stayton PS. Design and development of polymers for gene delivery. Nat Rev Drug Discov. 2005;4:581-593.

213. Bivas-Benita M, Lin MY, Bal SM, Meijgaarden KE, Franken KLMC, Friggen AH, Junginger HE, Borchard G, Klein MR, Ottenhoff THM. Pulmonary delivery of DNA encoding Mycobacterium tuberculosis latency antigen Rv1733c associated to PLGA-PEI nanoparticles enhances T cell responses in a DNA prime/protein boost vaccination regimen in mice. Vaccine. 2009;27:4010-4017.

214. Panyam J, Zhou W-Z, Prabha S, Sahoo SK, Labhasetwar V. Rapid endo-lysosomal escape of poly(DL-lactide-co-glycolide) nanoparticles: Implications for drug and gene delivery. FASEB J. 2002;16:1217-1226.

215. Gupta B, Levchenko TS, Torchilin VP. Intracellular delivery of large molecules and small particles by cell-penetrating proteins and peptides. Adv Drug Deliv Rev 2005;57:637-651.

216. Foerg C, Merkle HP. On the biomedical promise of cell penetrating peptides: Limits versus prospects. J Pharm Sci 2008;97(1):144-162.

217. Wadia JS, Stan RV, Dowdy SF. Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat Med. 2004;10(3):310-315.

218. Nam YS, Park JY, Han S-H, Chang I-S. Intracellular drug delivery using poly(D,L-lactide-co-glycolide) nanoparticles derivatized with a peptide from a transcriptional activator protein of HIV-1. Biotechnol Lett. 2002;24:2093-2098.

219. Lee S-J, Jeong J-R, Shin S-C, Huh Y-M, Song H-T, Suh J-S, Chang Y-H, Jeon B-S, Kim J-D. Intracellular translocation of superparamagnetic iron oxide nanoparticles with peptide-conjugated poly(D,L-lactide-co-glycolide). J Appl Phys. 2005;97(10):1-3.

220. Pouton CW, Wagstaff KM, Roth DM, Moseley GW, Jans DA. Targeted delivery to the nucleus. Adv Drug Deliv Rev. 2007;59:698-717.

221. Suh J, Dawson M, Hanes J. Real-time multiple-particle tracking: applications to drug and gene delivery. Adv Drug Deliv Rev. 2005;57:63-78.

222. Ng CP, Goodman TT, Park I-K, Pun SH. Bio-mimetic surface engineering of plasmid-loaded nanoparticles for active intracellular trafficking by actin comet-tail motility. Biomaterials. 2009;30:951-958.

223. Cheng F-Y, Wang SP-H, Su C-H, Tsai T-L, Wu P-C, Shieh D-B, Chen J-H, Hsieh PC-H, Yeh C-S. Stabilizer-free poly(lactide-co-glycolide) nanoparticles for multimodal biomedical probes. Biomaterials. 2008;29(13):2104-2112.

224. Xu P, Gullotti E, Tong L, Highley CB, Errabelli DR, Hasan T, Cheng J-X, Kohane DS, Yeo Y. Intracellular drug delivery by poly(lactic-co-glycolic acid) nanoparticles, revisited. Mol Pharm. 2008;6(1):190-201.

225. Gruenberg J, Van der Goot FG. Mechanisms of pathogen entry through the endosomal compartments. Nat Rev Mol Cell Biol. 2006;7:495-504.

226. Medina-Kauwe LK. "Alternative" endocytic mechanisms exploited by pathogens: New avenues for therapeutic delivery? Adv Drug Deliv Rev. 2007;59: 798-809.

227. Wright AE, Douglas SR. An experimental investigation of the role of the blood fluids in connection with phagocytosis. Proc R Soc Lond. 1903;72:357-370.

228. Patel HM. Serum opsonins and liposomes: Their interaction and opsonophagocytosis. Crit Rev Ther Drug Carrier Syst. 1992;9(1):39-90.

229. Moghimi SM, Muir IS, Illum L, Davis SS, Kolb-Bachofen V. Coating particles with a block co-polymer (poloxamine-908) suppresses opsonization but permits the activity of dysopsonins in the serum. Biochim Biophys Acta. 1993;1179:157-165.

230. Harashima H, Matsuo H, Kiwada H. Identification of proteins mediating clearance of liposomes using a liver perfusion system. Adv Drug Deliv Rev. 1998;32(1-2):61-79.

231. Gref R, Domb A, Quellec P, Blunk T, Müller RH, Verbavatz JM, Langer R. The controlled intravenous delivery of drugs using PEG-coated sterically stabilized nanospheres. Adv Drug Deliv Rev. 1995;16(2-3):215-233.

232. Ogawara KI, Higaki K, Kimura T. Major determinants in hepatic disposition of polystyrene nanospheres: Implication for rational design of particulate drug carriers. Crit Rev Ther Drug Carrier Syst. 2002;19(4-5):277-306.

233. Moghimi SM, Szebeni J. Stealth liposomes and long circulating nanoparticles: critical issues in pharmacokinetics, opsonization and protein-binding properties. Prog Lipid Res. 2003;42(6):463-478.

234. Owens DE, Peppas NA. Opsonization, biodistribution, and pharmacokinetics of polymeric nanoparticles. Int J Pharm. 2006;307(1):93-102.

235. Vonarbourg A, Passirani C, Saulnier P, Benoit JP. Parameters influencing the stealthiness of colloidal drug delivery systems. Biomaterials. 2006;27(24):4356-4373.

236. Moghimi SM, Hunter AC, Murray JC. Long-circulating and target-specific nanoparticles: Theory to practice. Pharmacol Rev. 2001;53(2):283-318.

237. Gbadamosi JK, Hunter AC, Moghimi SM. PEGylation of microspheres generates a heterogeneous population of particles with differential surface characteristics and biological performance. FEBS Lett. 2002;532(3):338-344.

238. Meng F, Engbers GHM, Gessner A, Müller RH, Feijen J. Pegylated polystyrene particles as a model system for artificial cells. J Biomed Mater Res A. 2004;70(1):97-106.

239. Claesson PM, Blomberg E, Fröberg JC, Nylander T, Arnebrant T. Protein interactions at solid surfaces. Adv Colloid Interface Sci. 1995;57:161-227.

240. Lemarchand C, Gref R, Couvreur P. Polysaccharide-decorated nanoparticles. Eur J Pharm Biopharm. 2004;58(2):327-341.

241. Jeon SI, Lee JH, Andrade JD, De Gennes PG. Protein-surface interactions in the presence of polyethylene oxide: I. Simplified theory. J Colloid Interface Sci. 1991;142(1):149-158.

242. Jeon SI, Andrade JD. Protein-surface interactions in the presence of polyethylene oxide: II. Effect of protein size. J Colloid Interface Sci. 1991;142(1):159-166.

243. Vittaz M, Bazile D, Spenlehauer G, Verrecchia T, Veillard M, Puisieux F, Labarre D. Effect of PEO surface density on long-circulating PLA-PEO nanoparticles which are very low complement activators. Biomaterials. 1996;17(16):1575-1581.

244. Li Y, Pei Y, Zhang X, Gu Z, Zhou Z, Yuan W, Zhou J, Zhu J, Gao X. PEGylated PLGA nanoparticles as protein carriers: synthesis, preparation and biodistribution in rats. J Control Release. 2001;71(2):203-211.

245. Panagi Z, Beletsi A, Evangelatos G, Livaniou E, Ithakissios DS, Avgoustakis K. Effect of dose on the biodistribution and pharmacokinetics of PLGA and PLGA-mPEG nanoparticles. Int J Pharm. 2001;221(1-2):143-152.

246. Ameller T, Marsaud R, Legrand P, Gref R, Barratt G, Renoir JM. Polyester-poly(ethylene glycol) nanoparticles loaded with the pure antiestrogen RU 58668: Physicochemical and opsonization properties. Pharm Res. 2003;20(7):1063-70.

247. Avgoustakis K, Beletsi A, Panagi Z, Klepetsanis P, Livaniou E, Evangelatos G, Ithakissios DS. Effect of copolymer composition on the physicochemical characteristics, in vitro stability, and biodistribution of PLGA-mPEG nanoparticles. Int J Pharm. 2003;259(1-2):115-27.

248. Beletsi A, Panagi Z, Avgoustakis K. Biodistribution properties of nanoparticles based on mixtures of PLGA with PLGA-PEG diblock copolymers. Int J Pharm. 2005;298(1):233-241.

249. Duan Y, Sun X, Gong T, Wang Q, Zhang Z. Preparation of DHAQ-loaded mPEG-PLGA-mPEG nanoparticles and evaluation of drug release behaviors in vitro/in vivo. J Mater Sci: Mater Med. 2006;17(6):509-516.

250. Taek KJ, Yu MO, Shin BC. Preparation of poly(DL-lactide-co-glycolide) nanoparticles by PEG-PPG diblock copolymer. Polymer (Korea). 2003;27(4):370-376.

251. Redhead HM, Davis SS, Illum L. Drug delivery in poly(lactide-co-glycolide) nanoparticles surface modified with poloxamer 407 and poloxamine 908: In vitro characterisation and in vivo evaluation. J Control Release. 2001;70(3):353-363.

252. Olivier J-C. Drug transport to brain with targeted nanoparticles. NeuroRx. 2005;2(1):108-119.

253. Burkersroda F, Gref R, Göpferich A. Erosion of biodegradable block copolymers made of poly(lactic acid) and poly(ethylene glycol). Biomaterials. 1997;18(24):1599-1607.

254. Avgoustakis K. Pegylated poly(lactide) and poly(lactide-co-glycolide) nanoparticles: preparation, properties and possible applications in drug delivery. Curr Drug Deliv. 2004;1(4):321-33.

255. Karnik R, Gu F, Basto P, Cannizzaro C, Dean L, Kyei-Manu W, Langer R, Farokhzad OC. Microfluidic platform for controlled synthesis of polymeric nanoparticles. Nano Lett. 2008;8(9):2906-2912.

256. Moghimi SM. Prolonging the circulation time and modifying the body distribution of intravenously injected polystyrene nanospheres by prior intravenous administration of poloxamine-908. A 'hepatic-blockade' event or manipulation of nanosphere surface in vivo? Biochim Biophys Acta. 1997;1336(1):1-6.

257. Denis-Mize KS, Dupuis M, Singh M, Woo C, Ugozzoli M, O'Hagan DT, Donnelly JJ, Ott G, McDonald DM. Mechanisms of increased immunogenicity for DNA-based vaccines adsorbed onto cationic microparticles. Cell Immunol. 2003;225(1):12-20.

258. Fillafer C, Friedl DS, Wirth M, Gabor F. Fluorescent bionanoprobes to characterize cytoadhesion and cytoinvasion. Small. 2008;4(5):627-633.

259. Kang J, Schwendeman SP. Determination of diffusion coefficient of a small hydrophobic probe in poly(lactide-co-glycolide) microparticles by laser scanning confocal microscopy. Macromolecules. 2003;36(4):1324-1330.

260. Eniola AO, Hammer DA. Artificial polymeric cells for targeted drug delivery. J. Control Release. 2003;87(1-3):15-22.

261. Jalón EG, Blanco-Príeto MJ, Ygartua P, Santoyo S. PLGA microparticles: Possible vehicles for topical drug delivery. Int J Pharm. 2001;226(1-2):181-184.

262. Saxena V, Sadoqi M, Shao J. Indocyanine green-loaded biodegradable nanoparticles: preparation, physicochemical characterization and in vitro release. Int J Pharm. 2004;278(2):293-301.

263. Nehilla BJ, Allen PG, Desai TA. Surfactant-free, drug-quantum-dot coloaded poly(lactide-co-glycolide) nanoparticles: Towards multifunctional nanoparticles. ACS Nano. 2008;2(3):538-544.

264. Panyam J, Sahoo SK, Prabha S, Bargar T, Labhasetwar V. Fluorescence and electron microscopy probes for cellular and tissue uptake of poly(D,L-lactide-co-glycolide) nanoparticles. Int J Pharm. 2003;262(1-2):1-11.

265. Weiss B, Schaefer UF, Zapp J, Lamprecht A, Stallmach A, Lehr CM. Nanoparticles made of fluorescence-labelled poly(L-lactide-co-glycolide): preparation, stability, and biocompatibility. J Nanosci Nanotechnol. 2006;6(9-10):3048-3056.

266. Tosi G, Rivasi F, Gandolfi F, Costantino L, Vandelli MA, Forni F. Conjugated poly(D,L-lactide-co-glycolide) for the preparation of in vivo detectable nanoparticles. Biomaterials. 2005;26(19):4189-4195.

267. Chung HJ, Kim HK, Yoon JJ, Park TG. Heparin immobilized porous PLGA microspheres for angiogenic growth factor delivery. Pharm Res. 2006;23(8):1835-1841.

268. Mo Y, Lim LY. Mechanistic study of the uptake of wheat germ agglutinin-conjugated PLGA nanoparticles by A549 cells. J Pharm Sci. 2004;93(1):20-28.

269. Fillafer C, Ratzinger G, Neumann J, Guttenberg Z, Dissauer S, Lichtscheidl IK, Wirth M, Gabor F, Schneider MF. An acoustically-driven biochip - Impact of flow on the cell-association of targeted drug carriers. Lab Chip. 2009; 9(19):2782-2788.

270. Hawley AE, Illum L, Davis SS. Lymph node localisation of biodegradable nanospheres surface modified with poloxamer and poloxamine block co-polymers. FEBS Lett. 1997;400(3):319-323.

271. Delgado A, Soriano I, Sánchez E, Oliva M, Évora C. Radiolabelled biodegradable microspheres for lung imaging. Eur J Pharm Biopharm. 2000;50(2):227-236.

272. Park YJ, Nah SH, Lee JY, Jeong JM, Chung JK, Lee MC, Yang VC, Lee SJ. Surface-modified poly(lactide-co-glycolide) nanospheres for targeted bone imaging with enhanced labeling and delivery of radioisotope. J Biomed Mater Res A. 2003;67(3):751-760.

273. Halder KK, Mandal B, Debnath MC, Bera H, Ghosh LK, Gupta BK. Chloramphenicol-incorporated poly lactide-co-glycolide (PLGA) nanoparticles: Formulation, characterization, technetium-99m labeling and biodistribution studies. J Drug Target. 2008;16(4):311-320.

274. Shukla J, Bandopadhyaya GP, Varma IK. 188Rhenium(V)-dimercaptosuccinic acid loaded poly(lactic-co-glycolic)acid microspheres for targeted radiotherapy: Production and effectivity. Pharmazie. 2005;60(8):583-587.

275. Mo Y, Lim LY. Paclitaxel-loaded PLGA nanoparticles: Potentiation of anticancer activity by surface conjugation with wheat germ agglutinin. J Control Release. 2005;108(2-3):244-262.

276. Le Ray AM, Vert M, Gautier JC, Benoit JP. End-chain radiolabeling and in vitro stability studies of radiolabeled poly(hydroxy acid) nanoparticles. J Pharm Sci. 1994;83(6):845-851.

277. Dadachova E, Mirzadeh S. The role of tin in the direct labelling of proteins with rhenium-188. Nucl Med Biol. 1997;24(6):605-608.

278. Banerjee T, Singh AK, Sharma RK, Maitra AN. Labeling efficiency and biodistribution of Technetium-99m labeled nanoparticles: Interference by colloidal tin oxide particles. Int J Pharm. 2005;289(1-2):189-195.

279. Fillafer C, Friedl DS, Ilyes AK, Wirth M, Gabor F. Bionanoprobes to study particle-cell interactions. J Nanosci Nanotechnol. 2009;9(5):3239-3245.

280. Pietzonka P, Rothen-Rutishauser B, Langguth P, Wunderli-Allenspach H, Walter E, Merkle HP. Transfer of lipophilic markers from PLGA and polystyrene nanoparticles to Caco-2 monolayers mimics particle uptake. Pharm Res. 2002;19(5):595-601.

281. Cohen S, Yoshioka T, Lucarelli M, Hwang LH, Langer R. Controlled delivery systems for proteins based on poly(lactic/glycolic acid) microspheres. Pharm Res. 1991;8(6):713-20.

282. Jiang W, Gupta RK, Deshpande MC, Schwendeman SP. Biodegradable poly(lactic-co-glycolic acid) microparticles for injectable delivery of vaccine antigens. Adv Drug Deliv Rev. 2005;57(3):391-410.

283. Abbas AO, Donovan MD, Salem AK. Formulating poly(lactide-co-glycolide) particles for plasmid DNA delivery. J Pharm Sci. 2008;97(7):2448-61.

284. Ando S, Putnam D, Pack DW, Langer R. PLGA microspheres containing plasmid DNA: Preservation of supercoiled DNA via cryopreparation and carbohydrate stabilization. J Pharm Sci. 1999;88(1):126-30.

285. Wolf M, Wirth M, Pittner F, Gabor F. Stabilisation and determination of the biological activity of L-asparaginase in poly(D,L-lactide-co-glycolide) nanospheres. Int J Pharm. 2003;256(1-2):141-152.

286. Fu K, Pack DW, Klibanov AM, Langer R. Visual evidence of acidic environment within degrading poly(lactic-co- glycolic acid) (PLGA) microspheres. Pharm Res. 2000;17:100-106.

287. Ding AG, Schwendeman SP. Acidic microclimate pH distribution in PLGA microspheres monitored by confocal laser scanning microscopy. Pharm Res. 2008;25(9):2041-2052.

288. Walter E, Moelling K, Pavlovic J, Merkle HP. Microencapsulation of DNA using poly(DL-lactide-co-glycolide):

stability issues and release characteristics. J Control Release. 1999;61(3):361-374.

289. Schwendeman SP. Recent advances in the stabilization of proteins encapsulated in injectable PLGA delivery systems. Crit Rev Ther Drug Carrier Syst. 2002;19(1):73-98.

290. Singh M, Kazzaz J, Ugozzoli M, Malyala P, Chesko J, O'Hagan DT. Polylactide-co-glycolide microparticles with surface adsorbed antigens as vaccine delivery systems. Curr Drug Deliv. 2006;3(1):115-120.

291. Chung YI, Tae G, Hong Yuk S. A facile method to prepare heparin-functionalized nanoparticles for controlled release of growth factors. Biomaterials. 2006;27(12):2621-2626.

292. Singh M, Chesko J, Kazzaz J, Ugozzoli M, Kan E, Srivastava I, O'Hagan DT. Adsorption of a novel recombinant glycoprotein from HIV (env gp120dV2 SF162) to anionic PLG microparticles retains the structural integrity of the protein, whereas encapsulation in PLG microparticles does not. Pharm Res. 2004;21(12):2148-2152.

293. Sapin A, Clavreul A, Garcion E, Benoit JP, Menei P. Evaluation of particulate systems supporting tumor cell fractions in a preventive vaccination against intracranial rat glioma. J Neurosurg. 2006;105(5):745-752.

294. Singh M, Ott G, Kazzaz J, Ugozzoli M, Briones M, Donnelly J, O'Hagan DT. Cationic microparticles are an effective delivery system for immune stimulatory CpG DNA. Pharm Res. 2001;18(10):1476-1479.

295. O'Hagan D, Singh M, Ugozzoli M, Wild C, Barnett S, Chen M, Schaefer M, Doe B, Otten GR, Ulmer JB. Induction of potent immune responses by cationic microparticles with adsorbed human immunodeficiency virus DNA vaccines. J Virol. 2001;75(19):9037-9043.

296. Singh M, Ugozzoli M, Briones M, Kazzaz J, Soenawan E, O'Hagan DT. The effect of CTAB concentration in cationic PLG

microparticles on DNA adsorption and in vivo performance. Pharm Res. 2003;20(2):247-251.

297. Zhang XQ, Intra J, Salem AK. Comparative study of poly (lactic-co-glycolic acid)-poly ethyleneimine-plasmid DNA microparticles prepared using double emulsion methods. J Microencaps. 2008;25:1-12.

298. Briones M, Singh M, Ugozzoli M, Kazzaz J, Klakamp S, Ott G, O'Hagan D. The preparation, characterization, and evaluation of cationic microparticles for DNA vaccine delivery. Pharm Res. 2001;18(5):709-712.

299. Zhang XQ, Intra J, Salem AK. Conjugation of polyamidoamine dendrimers on biodegradable microparticles for nonviral gene delivery. Bioconjug Chem. 2007;18(6):2068-2076.

300. Stivaktakis N, Nikou K, Panagi Z, Beletsi A, Leondiadis L, Avgoustakis K. Immune responses in mice of β-galactosidase adsorbed or encapsulated in poly(lactic acid) and poly(lactic-co-glycolic acid) microspheres. J Biomed Mater Res A. 2005;73(3):332-338.

301. Johansen P, Men Y, Merkle HP, Gander B. Revisiting PLA/PLGA microspheres: An analysis of their potential in parenteral vaccination. Eur J Pharm Biopharm. 2000;50(1):129-146.

302. O'Hagan DT, Singh M, Ulmer JB. Microparticle-based technologies for vaccines. Methods. 2006;40(1):10-19.

303. Johansen P, Martínez Gomez JM, Gander B. Development of synthetic biodegradable microparticulate vaccines: A roller coaster story. Expert Rev Vaccines. 2007;6(4):471-474.

304. Capan Y, Woo BH, Gebrekidan S, Ahmed S, DeLuca PP. Preparation and characterization of poly (D,L-lactide-co-glycolide) microspheres for controlled release of poly(L-lysine) complexed plasmid DNA. Pharm Res. 1999;16(4):509-513.

305. Howard KA, Alpar HO. The development of polyplex-based DNA vaccines. J Drug Target. 2002;10(2):143-151.

306. Zhou X, Liu B, Yu X, Zha X, Zhang X, Wang X, Jin Y, Wu Y, Chen Y, Shan Y, Chen Y, Liu J, Kong W, Shen J. Controlled release of PEI/DNA complexes from PLGA microspheres as a potent delivery system to enhance immune response to HIV vaccine DNA prime/MVA boost regime. Eur J Pharm Biopharm. 2008;68(3):589-595.

307. Howard KA, Li XW, Somavarapu S, Singh J, Green N, Atuah KN, Ozsoy Y, Seymour LW, Alpar HO. Formulation of a microparticle carrier for oral polyplex-based DNA vaccines. Biochim Biophys Acta. 2004;1674(2):149-157.

308. O'Hagan DT, Singh M, Dong C, Ugozzoli M, Berger K, Glazer E, Selby M, Wininger M, Ng P, Crawford K, Paliard X, Coates S, Houghton M. Cationic microparticles are a potent delivery system for a HCV DNA vaccine. Vaccine. 2004;23(5):672-680.

309. Mollenkopf HJ, Dietrich G, Fensterle J, Grode L, Diehl KD, Knapp B, Singh M, O'Hagan DT, Ulmer JB, Kaufmann SHE. Enhanced protective efficacy of a tuberculosis DNA vaccine by adsorption onto cationic PLG microparticles. Vaccine. 2004;22(21-22):2690-2695.

310. Liman M, Peiser L, Zimmer G, Pröpsting M, Naim HY, Rautenschlein S. A genetically engineered prime-boost vaccination strategy for oculonasal delivery with poly(d,l-lactic-co-glycolic acid) microparticles against infection of turkeys with avian Metapneumovirus. Vaccine. 2007;25(46):7914-7926.

311. Choudary S, Ravikumar P, Ashok Kumar C, Suryanarayana V, Reddy G. Enhanced immune response of DNA vaccine (VP1-pCDNA) adsorbed on cationic PLG for foot and mouth disease in guinea pigs. Virus Genes. 2008;37(1):81-87.

312. He X, Jiang L, Wang F, Xiao Z, Li J, Liu SL, Li D, Ren D, Jin X, Li K, He Y, Shi K, Guo Y, Zhang Y, Sun S. Augmented humoral and cellular immune responses to hepatitis B DNA vaccine adsorbed onto cationic microparticles. J Control Release. 2005;107(2):357-372.

313. Pan C-H, Nair N, Adams RJ, Zink MC, Lee E-Y, Polack FP, Singh M, O'Hagan DT, Griffin DE. Dose-dependent protection against or exacerbation of disease by a polylactide glycolide microparticle-adsorbed, alphavirus-based measles virus DNA vaccine in Rhesus macaques. Clin Vaccine Immunol. 2008;15(4):697-706.

314. Verdijk RM, Mutis T, Esendam B, Kamp J, Melief CJM, Brand A, Goulmy E. Polyriboinosinic polyribocytidylic acid (poly(I:C)) induces stable maturation of functionally active human dendritic cells. J Immunol. 1999;163(1):57-61.

315. Cartiera MS, Johnson KM, Rajendran V, Caplan MJ, Saltzman WM. The uptake and intracellular fate of PLGA nanoparticles in epithelial cells. Biomaterials. 2009;30:2790–2798.

316. Fillafer C, Wirth M, Gabor F. Stabilizer-induced viscosity alteration biases nanoparticle sizing via dynamic light scattering. Langmuir. 2007; 23(17):8699-8702.

317. Pean J-M, Venier-Julienne M-C, Boury F, Menei P, Denizot B, Benoit J-P. NGF release from poly(D,L-lactide-co-glycolide) microspheres. Effect of some formulation parameters on encapsulated NGF stability. J Control Release. 1998;56:175-187.

318. Binnig G, Quate CF, Gerber C. Atomic force microscope. Phys Rev Lett. 1986;56(9):930-933.

319. Wilkins DJ, Myers PA. Studies on the relationship between the electrophoretic properties of colloids and their blood clearance and organ distribution in the rat. Br J Exp Pathol. 1966;47(6):568-576.

320. Thurston G, McLean JW, Rizen M, Baluk P, Haskell A, Murphy TJ, Hanahan D, McDonald DM. Cationic liposomes target angiogenic endothelial cells in tumors and chronic inflammation in mice. J Clin Invest. 1998;101:1401-13.

321. Ainslie KM, Lowe RD, Beaudette TT, Petty L, Bachelder EM, Desai TA. Microfabricated Devices for Enhanced Bioadhesive Drug Delivery: Attachment to and Small-Molecule Release Through a Cell

Monolayer Under Flow. Small. 2009; DOI: 10.1002/smll.200901254 (final page number not assigned).

322. Wheatley MA, Forsberg F, Oum K, Ro R, El-Sherif D. Comparison of in-vitro and in-vivo acoustic response of a novel 50 : 50 PLGA contrast agent. Ultrasonics. 2006;44:360-367.

CHARACTERIZATION OF BINDING AND UPTAKE OF BIOMIMETIC NANOPARTICLES BY FLOW CYTOMETRY

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Characterization of binding and uptake of biomimetic nanoparticles by flow cytometry

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To establish a method for characterization of the nanoparticle-cell interaction without labeling, the changes in granularity of Caco-2 cells due to association of biomimetic nanoparticles, consisting of PLGA and wheat germ agglutinin (WGA), were monitored by side scattering using flow cytometry. The reliability was confirmed by a temperature-dependent linear correlation between side scatter data and cell-associated fluorescence intensities of cells loaded with fluorescent biomimetic nanoparticles. Moreover, not only the binding to but also the uptake of the nanoparticles into the cells was detected. Whereas the cell-binding of nanoparticles with lowest WGA-densities was controlled by temperature-dependent diffusional processes, the cytoinvasive properties of the WGA-corona prevailed at higher lectin densities. Due to the multiparametric character of the flow cytometric analysis useful additional information on the viability and agglomeration of cells is provided at the same time.

Key words: Caco-2 – Cell-association – Flow cytometry – Nanoparticles – Poly (d,l-lactide-co-glycolide) – Wheat germ agglutinin.

A recent advance in nanotechnology is the functionalization of polymeric nanoparticles with biorecognitive molecules that give a command to the cellular machinery to induce or suppress processes similar to biological structures [1-3]. According to the subclassification of these biomimetic systems, biohybrid structures combine synthetic structures and natural biological molecules [4]. For drug delivery purposes, biohybrid systems are represented by nanoparticles that were grafted with vitamin B12, antibodies, folate, transferrin, and lectins. The challenge is to target cells or improve cellular binding and uptake by opening different pathways for adhesion and/or internalization [5-7]. To achieve deeper understanding of the functionality and the mechanisms involved, techniques are necessary to investigate the interaction at the cellular level.

The methods most commonly used to investigate the nanoparticle-cell interaction comprise scanning electron microscopy, laser scanning microscopy, fluorimetry, and flow cytometry. Whereas scanning electron microscopy and laser scanning microscopy offer semiquantitative results at best, the other methods provide quantitative information. Both of the techniques that rely on sensitive fluorimetric detection require, however, preparation of fluorescent nanoparticles or staining with labeled antibodies prior to analysis. According to the literature, fluorescent nanoparticles are prepared by simple entrapment of Nile red [8], coumarin-derivatives, texas red [9] or doxorubicin [10] with the risk of dye leakage during the experiment probably falsifying the results. Alternatively, labeling the biomolecules of the biohybrid nanoparticles can modify or inhibit bioactivity. Hence, a method that avoids the need for labeling would appear preferable.

Flow cytometry represents a well established method for characterization of the interaction between cells and fluorescent particles since the forward and side scatter as well as fluorescence detectors enable a multiparametric analysis of changes upon association of particles with the cells [11]. The forward scatter collection optics enable changes in diameter of the cells to be determined and the fluorescence collection optics provide information on cell-associated fluorescence intensities. Whereas these former parameters are frequently used for analysis, the side scatter collection optics, which monitor the granularity of cells independent of any labeling, has not been used to characterize the interaction between colloidal carriers and cells.

To detect possible changes in the granularity of cells upon association with nanoparticles without any label, PLGA-nanoparticles were chosen. This biocompatible and biodegradable polymer is not only used in medicine but also for the controlled release of entrapped drugs [12, 13]. In order to be able to correlate the side scattering of the cells with the mean cell-associated fluorescence intensity of the nanoparticles and to prove the potential of the side scatter for label-free analysis, nanoparticles made from fluorescent PLGA were prepared. For that purpose the PLGA was covalently conjugated with a fluorescein-derivative to prevent leakage of the dye and to allow correct correlations [14].

To check the utility of the side scatter for the analysis of biohybrid systems, the surface of the nanoparticles was grafted with the lectin from *Triticum vulgare* to mediate adhesion of the nanoparticles to the cells. Wheat germ agglutinin specifically binds to sialic acid and N-acetyl-D-glucosamine moieties present at the surface of the glycocalyx of enterocytes of the human intestine. In addition to cytoadhesion, it has been shown that the lectin also mediates uptake of nanoparticles into the cells [7, 15, 16]. Since the Caco-2 model is well established in pharmaceutical technology to study the binding, uptake, and transport of drugs [17], Caco-2 single cells were chosen for flow cytometric analysis of the nanoparticle-cell interaction.

The aim of this contribution is to estimate whether the side scatter of cells is a valuable parameter for characterization of the interaction between non-labeled nanoparticles and cells.

I. MATERIALS AND METHODS

1. Chemicals

Resomer RG 503H (PLGA, lactide/glycolide ratio 50:50, inherent viscosity 0.32-0.44 dL/g, acid number > 3 mg KOH/g) was obtained from Boehringer Ingelheim (Ingelheim, Germany). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), N-hydroxysuccinimide (NHS), sulfo-NHS, and Pluronic F-68 were purchased from Sigma (Vienna, Austria). Wheat germ agglutinin (WGA) and its fluorescein-labeled derivative (F-WGA) with a molar fluorescein/protein ratio of 3.2 were obtained from Vector Laboratories (Burlingham, CA, USA). 2-(4-(2-Hydroxyethyl)-1-piperazinyl)-ethanesulfonic acid (HEPES) and N,N´-dicyclohexylcarbodiimide (DCC) were bought from Merck

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(Darmstadt, Germany). Fluorescein cadaverine (5-((5-aminopentyl) thioureidyl)fluorescein, dihydrobromide salt) was from Molecular Probes (Eugene, OR, USA). All other chemicals in use were of analytical grade.

2. Preparation of fluorescein-labeled PLGA

For the preparation of fluorescent nanospheres, the fluorescein cadaverine was covalently coupled to the polymer. The carboxylic groups of the polymer were activated by mixing 500 µL of each, a 0.4% NHS, a 0.51% DCC, and a 5.0% PLGA solution in methylene chloride. After end-over-end incubation for 2h at room temperature, 0.4mg (1.5%) or 0.8mg (3.1%) F-cadaverine dissolved in a mixture of 500 µL dichloromethane and 22 µL pyridine were added and incubated overnight. The labeled polymer was purified by repeated washings with 5 mmol HCl until the aqueous layer remained colorless followed by precipitation with methanol for 2 h at 4°C. The fluorescein cadaverine-labeled PLGA (F-PLGA) was lyophilized and stored at 4°C until use.

3. Preparation of PLGA nanospheres

The nanospheres were prepared by a modified water-in-oil-in-water solvent evaporation technique. Briefly, 200 µL distilled water were emulsified with 1 mL ethyl acetate containing 200mg PLGA by sonication for 30 sec (Sonifier: Bandelin electronic UW 70/HD 70, tip: MS72/D, Berlin, Germany). After addition of 3 mL 10% (w/v) aqueous solution of Pluronic the emulsion was sonified again for 25 s. The resulting double emulsion was poured into 50 mL 1% (w/v) aqueous Pluronic solution containing 2% isopropanol and maintained under mechanical stirring at 600 rpm for 1 h under a hood. The residual ethyl acetate was evaporated under vacuum. Aliquots (1.5 mL) of the nanosphere suspension were washed twice with 20 mmol HEPES/NaOH buffer pH 7.0 containing 1% Pluronic by centrifugation (7000 rpm, 10 min, 4°C) and resuspension. The nanospheres were finally stored at -80°C until use.

4. Preparation of F-PLGA nanospheres

The F-PLGA nanospheres were prepared as described above but 10 or 20% of the PLGA were replaced by F-PLGA. In brief, the nanospheres were prepared from a mixture of either 20 mg F-PLGA and 180 mg PLGA or 40 mg F-PLGA and 160 mg PLGA using both 1.5% F-cadaverine PLGA and 3.1% F-cadaverine PLGA.

5. Particle size analysis

The mean diameter and particle size distribution were examined by laser diffraction (Shimadzu Laser Diffraction Particle Size Analyzer SALD-1100). The nanospheres were suspended in 10 mL water containing 0.2% Tween 20 as a wetting agent to minimize agglomeration, sonicated for 1min, and analyzed under continuous stirring in duplicate. Sizes were expressed as volume diameters, which are indicated by the 90 and 50% value, meaning that 90 or 50% of the particles are smaller than the value given.

6. Surface modification of the nanospheres

Lectin-grafted nanospheres were prepared from both unlabeled PLGA and a mixture of 90% unlabeled PLGA and 10% F-PLGA containing 3.1% F-cadaverine.

To modify the surface of the nanoparticles, 500 µL 2, 3.5, or 7% EDAC solution in 20 mmol HEPES/NaOH buffer pH 7.0 alone or these solutions containing additionally either 0.086, 0.15, 0.3% NHS or 2.49, 4.36, 8.72% sulfo-NHS were added to 500 µL nanosphere suspension (about 2.0 mg dry nanospheres) in the same buffer and incubated end-over-end for 2 h. The suspensions were centrifuged, the supernatant was discarded to remove excessive cross-linking agent, and the pellet was resuspended in 500 µL HEPES/NaOH buffer pH 7.0. To modify the surface of the nanospheres 100 µL WGA solution (0, 0.0001, 0.001, 0.002, 0.005, 0.025, 0.05, 0.075, 0.1 or 0.2% lectin in buffer, w/v) were added and the suspension was incubated end-over-end for 18 h. Excessive coupling sites were blocked by the addition of 100 µL 20% glycine solution in buffer and end-over-end incubation for 1h. The particles were washed by centrifugation and resuspension in 20 mmol HEPES/NaOH buffer pH 7.0 containing 1% Pluronic followed by isotonic HEPES/NaOH buffer pH 7.4 containing 1% Pluronic. Finally, the pellet was dispersed in 500 µL isotonic HEPES/NaOH buffer pH 7.4 and stored at -80°C until use.

7. Cell culture

Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells (passage number 60-80) were grown in RPMI-1640 cell culture medium containing 10% fetal calf serum, 4 mmol L-glutamine, and 150 µg/mL gentamycine in a humidified 5% CO_2/95% air atmosphere at 37°C and subcultured by trypsination.

8. Photometry and fluorimetry

To determine the amount of surface immobilized F-WGA or the fluorescein content of F-PLGA nanoparticles, the nanospheres were repeatedly washed with water, lyophilized, and dissolved in 0.1N NaOH/5% SDS. The fluorescein label of the polymer or the lectin was quantified either by the absorption at 490 nm (U-2000 UV/Vis Spectrophotometer, Hitachi, Austria) or by fluorimetry at 485/535nm (Spectrafluor-reader, Tecan, Grödig, Austria) using serial dilutions of F-WGA or fluorescein sodium in the same solvent for calibration.

9. Flow cytometry

The amount of nanoparticles associated with single cells was determined by mixing 50 µL cell suspension (5×10^6 cells/mL culture medium) with 50 µL nanosphere suspension (1.0 mg/mL in isotonic HEPES/NaOH-buffer pH 7.4) and incubation for 20 min at 4 or 37°C. To remove free and loosely associated particles, the cells were centrifuged (5 min, 1000 rpm, 4°C) and washed twice with 150 µL isotonic HEPES/NaOH-buffer pH 7.4 each. The cell suspension was resuspended in 1000 µL cell pack and analyzed by flow cytometry (Epics XL-MCL Flow cytometer, Coulter, Miami, USA) using a forward versus side scatter gate for inclusion of the single-cell population and exclusion of debris and cell aggregates. Intact single cells were detected in gate G, single cells with membrane bound nanospheres in gate A, and agglomerated cells were accumulated in gate D. Debris and fragments deriving from dead cells form a new population appearing in the histograms below gate A. The fluorescence labeling of the cells was detected at 525 nm (10 nm bandwidth) and the mean channel number of the logarithmic intensities of individual peaks was used for further calculations. Amplification of the fluorescence signals was adjusted to put the autofluorescence signal of unlabeled cells in the first decade of the 4-decade log range. For each analysis data of 3000 cells was accumulated. All the tests were performed at least in triplicate.

II. RESULTS

1. Elaboration of appropriate bioadhesive fluorescent nanoparticles

1.1. Preparation of F-WGA-grafted PLGA nanospheres

As a prerequisite for elucidation of the cell-nanoparticle interaction by flow cytometry, nanoparticles grafted with sufficient amounts of cytoadhesive ligands are required. Thus, different immobilization strategies for WGA relying on carbodiimide mediated cross-linking were investigated (*Table I*). Although the same amount of F-WGA (100 µg) was added to each batch, the lectin density at the surface of the nanospheres differed considerably. When EDAC/Sulfo-NHS were used as cross-linking agents, the amount of nanoparticle-bound F-WGA was below the limit of fluorimetric detection after dissolving

Table I - Influence of the type and concentration of the crosslinker on the F-WGA-density at the surface of the nanoparticles. The P90 and the P50 values indicate that 90% or 50% of the particles are smaller than the given value.

Coupling agent	F-WGA-density of NS (µg F-WGA/mg nanospheres)	Nanosphere diameter (nm)		Nanosphere recovery yield (mg/500 µl suspension)	F-WGA coupling efficiency (%)
		P90	P50		
2.0% EDAC	1.99	590	320	0.66 ± 0.04	1.31
3.5% EDAC	55.73	11070	350	0.78 ± 0.07	43.75
7.0 % EDAC	62.59	3210	390	0.91 ± 0.05	56.95
2.0% EDAC/0.08% NHS	1.89	590	420	0.65 ± 0.11	1.13
3.5% EDAC/0.15% NHS	44.89	810	360	0.67 ± 0.04	30.07
7.0 % EDAC/0.3% NHS	62.58	5860	460	0.96 ± 0.08	60.38
2.0% EDAC/2.49% sNHS	n.d.	540	360	0.58 ± 0.06	n.d.
3.5% EDAC/4.36% sNHS	n.d.	570	360	0.64 ± 0.09	n.d.
7.0 % EDAC/8.72% sNHS	n.d.	540	370	0.64 ± 0.02	n.d.

n.d.: not detectable.

the particles in NaOH/SDS. In accordance, the diameter of the nanospheres remained unaltered indicating a lack of surface modification. Upon use of EDAC or EDAC/NHS for covalent immobilization of F-WGA, the lectin density at the surface concurrently increased with the concentration of the cross-linking agent. On average, 1.22 ± 0.12% (2.0% EDAC), 36.91 ± 9.67(3.5% EDAC), and 58.66 ± 2.42% (7.0% EDAC) of the amount of F-WGA added were covalently coupled to the nanospheres. Concurrent with the lectin-density at the surface the nanosphere recovery yield as well as the mean diameter of the nanoparticles increased exceeding that of EDAC/sulfo-NHS treated nanoparticles. The homogeneity of the particle size distribution, however, seemed to be dependent on the presence or absence of NHS. Whereas the particle diameter was comparable upon use of 2% EDAC, partial agglomeration of the nanoparticles was observed at higher EDAC concentrations. This was indicated by the relatively high 90%-values in the micrometer range. Interestingly, half of the particles were not prone to aggregation as indicated by the unaltered 50% values. In contrast, when using NHS, aggregates only appeared upon coupling with the highest EDAC concentration. The mean nanosphere recovery yield amounted to 1.44 ± 0.26 mg/mL suspension. Since the use of 3.5% EDAC/0.15% NHS for immobilization of F-WGA at the surface of the nanoparticles resulted in a high lectin density as well as low tendency for agglomeration, this cross-linking procedure was retained for all further nanoparticle preparations.

1.2. Adjustment of the fluorescein-content of WGA-F-PLGA nanospheres

In an attempt to estimate whether the granularity of nanoparticle-loaded Caco-2 cells can be correlated with the mean cell-associated fluorescence intensity, different batches of WGA-grafted fluorescent nanoparticles were prepared. Using F-PLGA made from 1.5 or 3.1% F-cadaverine, nanoparticles were prepared from blends containing either 10 or 20% of the fluorescent polymer (*Table II*). Upon flow cytometric analysis of Caco-2 bound WGA-grafted nanoparticles it became apparent that nanoparticles prepared with 1.5% F-cadaverine-PLGA provoked similar granularity of the cells independent of the weight ratio of PLGA/F-PLGA. On the contrary, doubling the 1.5% F-cadaverine-PLGA content of the nanospheres increased the mean cell-associated fluorescence intensity 1.5-fold as a result of the twofold fluorescein-content. Additionally, the mean diameter of the nanoparticles prepared from 1.5% F-cadaverine-PLGA was in the same range.

In the case of nanoparticles made from 3.1% F-cadaverine-PLGA, a doubling of the fluorescein-content provoked no increase of the mean cell-associated fluorescence intensity but a 50% reduction of the read out. This decreased cell-binding of the nanospheres prepared from 20% 3.1% F-cadaverine-PLGA was also indicated by the roughness of the cell surface amounting to only half of that of nanospheres made from 10% 3.1% F-cadaverine-PLGA (*Table II*). Particularly in the case of the 3.1% F-cadaverine-PLGA nanoparticles it is likely that doubling the F-PLGA content reduces the number of carboxylate groups available for coupling of WGA. This results in considerably lower cell-association as indicated by the strong decline of the mean granularity of the cells. Additionally, this effect is reflected by a 50% decrease in the mean cell-associated fluorescence intensity.

Due to the high cell-association, which is reflected by the highest read outs of cell-associated mean fluorescence intensity as well as granularity of the Caco-2 cells, WGA-grafted nanospheres prepared from a mixture of PLGA and 3.1% F-cadaverine PLGA at a weight ratio of 9 + 1 were used for all further experiments.

2. Interaction between labeled as well as non-labeled nanoparticles with varied WGA content and Caco-2 single cells

2.1. Volume diameter of the nanoparticles

Based on the assumption that the volume diameter of the nanoparticles attached to the surface of Caco-2 cells strongly influences the side scattering of the cells, the particle size distribution of both the lectin-grafted nanoparticles and the plain nanospheres was determined (*Table III*). Interestingly, the grafting procedure even in the absence of lectin increased the diameter of the nanospheres by 70 (PLGA) or 50 nm (F-PLGA) as compared to the parent nanospheres. The coupling in the presence of lectin, however, provoked no considerable additional increase in the size of the nanospheres. On average the volume diameter

Table II - Characteristics of WGA-F-PLGA nanospheres prepared from different batches of F-PLGA at different weight ratios of F-PLGA and PLGA.

F-PLGA				
F-cadaverine added to PLGA (%)	1.5		3.1	
Fluorescein content of F-PLGA (µg/g)	748.8 ± 56.6		1804.7 ± 382.4	
WGA-F-PLGA-nanospheres				
F-PLGA added for nanosphere preparation (%)	10	20	10	20
Mean diameter (nm)	520	550	670	580
Fluorescein content of nanospheres (µg/g)	80.6 ± 12.0	163.3 ± 57.9	169.3 ± 40.6	390.2 ± 32.4
Mean cell-associated fluorescence intensity	4.9 ± 0.2	7.6 ± 0.2	11.5 ± 0.7	6.1 ± 2.8
Mean granularity of cells	444.2 ± 15.0	420.1 ± 6.4	530.7 ± 24.0	268.9 ± 1.4

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Table III - Volume diameter of WGA-PLGA and WGA-F-PLGA nanoparticles used for cell-association assays (mean, SEM ± 10 nm).

	WGA-PLGA nanospheres		WGA-F-PLGA nanospheres	
	90% value (nm)	50% value (nm)	90% value (nm)	50% value (nm)
Plain nanospheres	490	360	540	370
Glycine-saturated NS	560	380	590	430
WGA-grafted NS	563	386	621	455

of the nanoparticles was comparable and amounted to 563 ± 11 nm (WGA-PLGA-nanospheres) or 621 ± 36 nm (WGA-F-PLGA nanospheres). Thus, the slight differences in size of the nanoparticles used are assumed to exert no considerable influence on the read outs.

2.2. Influence of Pluronic F68 on the granularity of the cells

Since some detergents are known to exert toxic effects on cells, the influence of Pluronic F68, a constituent of the HEPES-buffer, on the granularity of the cell membrane was examined. Flow cytometry of Caco-2 cells incubated in buffer with and without 1% Pluronic F68 revealed a mean granularity of 131.3 ± 2.2 AU (with Pluronic) or 131.2 ± 0.9 AU (without Pluronic). Furthermore, the percentage of cells located within gate G was 93.5 ± 0.7% (with Pluronic) and 94.9 ± 0.2% (without Pluronic), respectively, indicating non-toxicity of this detergent at the concentrations used.

2.3. Experiments at 4°C

To assess the influence of cell-bound nanoparticles on the granularity of Caco-2 cells, the surface of non-labeled as well as labeled PLGA-nanoparticles was modified with increasing amounts of WGA presumably resulting in a stepwise increasing lectin-density. Upon incubation of the cells with unlabeled particles lacking a lectin-corona, the granularity of the cells increased by 39.6 ± 1.7 AU (arbitrary units) as compared to cells without nanoparticles pointing to non-specific binding of PLGA-nanoparticles (*Figure 1*). Upon addition of 0.1 μg WGA for grafting of the PLGA-nanoparticles, no considerable difference in roughness of the cells was observed which might be attributed to a lack of or relatively low density of the bioadhesive lectin at the surface of the nanoparticles. In contrast, over the range from 1 to 75 μg WGA a steep increase in granularity up to 344.5 ± 5.5 AU concurrent with increasing amounts of lectin for surface modification of the nanospheres was observed. Further increase of the lectin density revealed potential saturation of the lectin binding sites at the cell-surface since the roughness remained unaltered. Although the plateau of granularity was reached at 75 μg WGA, the percentage of viable single cells in gate A decreased with a further increase in lectin density. Whereas 92.8 ± 1.57% of the cells were single cells up to 50 μg WGA, the proportion decreased to 82.8 ± 0.8% (75 μg WGA), 77.7 ± 4.4% (100 μg WGA), and 68.8 ± 3.1% (200 μg WGA). This might be attributed to aggregation of the cells by the multivalent nanoparticles followed by accumulation in gate D (*Figure 2*).

As compared to non-labeled bioadhesive nanoparticles, the onset of increasing granularity of labeled WGA-F-PLGA nanoparticles was delayed to higher WGA concentrations starting at 25 μg WGA. Additionally, the slope in granularity was relatively smooth exhibiting no plateau even upon surface modification of the particles with 200 μg WGA. Interestingly, as compared to WGA-PLGA nanospheres, this smooth increase in granularity in the case of WGA-F-PLGA nanospheres correlated well with the percentage of viable single cells. Up to 100 μg WGA 90.1 ± 0.8% of the cell population was detected in gate A referring to single cells, whereas this percentage decreased to 85.8 ± 0.8% at 200 μg WGA.

Determination of the mean cell-associated fluorescence intensity

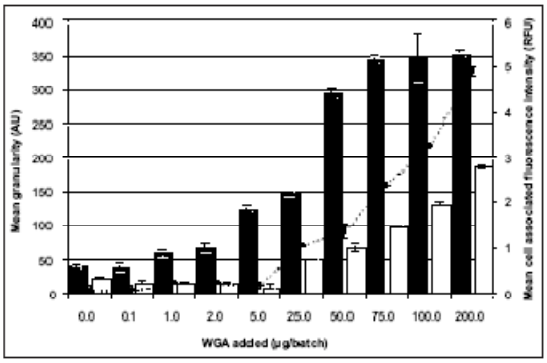

Figure 1 - Mean granularity (WGA-PLGA nanospheres, black columns; WGA-F-PLGA nanospheres, white columns) and mean cell-associated fluorescence intensity (WGA-F-PLGA nanospheres, line) of Caco-2 single cells after incubation with WGA-grafted nanospheres at 4°C. The WGA-values represent the amount of lectin applied for surface modification of the nanospheres (mean ± SD, n = 4).

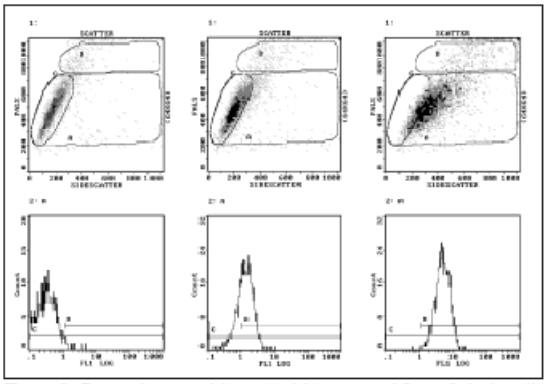

Figure 2 - Forward versus side scatter histograms of Caco-2 single cells incubated with F-PLGA nanospheres prepared without WGA (top row, left), with 50 µg WGA (top row, mean) or 200 µg WGA (top row, right) and mean cell-associated fluorescence intensity of viable single cells in gate A after incubation with F-PLGA nanospheres prepared without WGA (bottom row, left), with 50 µg WGA (bottom row, mean) or 200 µg WGA (bottom row, right).

revealed a similar tendency as that monitored by the side scattering. Whereas the mean cell-associated fluorescence intensity amounted to 0.17 ± 0.06 RFU (relative fluorescence units) up to 25 μg WGA, higher amounts of WGA for surface modification resulted in an increasing cell-associated fluorescence intensity up to 4.91 ± 0.10 RFU at 200 μg WGA.

2.4. Experiments at 37°C

When the same assays as described above were performed at 37°C the surface modification of PLGA nanoparticles with no or the lowest amounts of WGA elicited a side scatter of 70.2 ± 3.9 AU, which is clearly due to non-specific association of the nanoparticles with Caco-2 single cells (*Figure 3*). Similar to the side scattering at 4°C, the roughness of the cell surface increased from 85.7 ± 3.4 AU to 296.8 ± 8.0 AU concurrent with increasing amounts of lectin applied for surface modification from 1 to 75 μg WGA. The plateau of side scattering was reached at 75 μg WGA but exhibited a lower total roughness of 296.8 ± 8.0 AU on average and was accompanied by a decreasing number of single cells. Whereas the percentage of single cells associated with WGA-PLGA nanoparticles was relatively constant

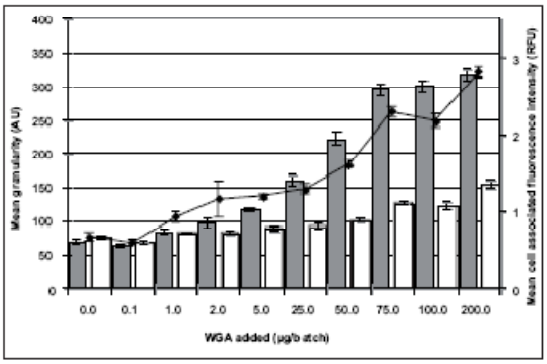

Figure 3 - Mean granularity (WGA-PLGA nanospheres, grey columns; WGA-F-PLGA nanospheres, white columns) and mean cell-associated fluorescence intensity (WGA-F-PLGA nanospheres, line) of Caco-2 single cells after incubation with WGA-grafted nanospheres at 37°C. The WGA-values represent the amount of lectin applied for surface modification of the nanospheres (mean ± SD, n = 4).

amounting to 96.0 ± 1.4% at the mean up to 50 µg WGA, upon higher WGA concentrations for surface modification it was reduced to 82.0 ± 1.4% (75 µg WGA), 79.5 ± 1.1% (100 µg WGA), and 68.9 ± 2.0% (200 µg WGA), respectively.

The association of WGA-F-PLGA nanospheres with Caco-2 cells at 37°C was characterized by a lag of side scattering with slightly fluctuating individual read outs amounting to 84.2 ± 11.5 up to 25 µg WGA. Ongoing from 25 µg WGA the granularity of the cells slightly increased from 93.1 ± 4.09AU (25 µg WGA) to 154.6 ± 6.2AU (200 µg WGA) without any hints of saturation. Monitoring the mean cell-associated fluorescence intensity of Caco-2-cells after incubation with WGA-F-PLGA nanospheres revealed a similar trend as already observed from the side scatter values. Up to 25 µg WGA the mean cell-associated relative fluorescence intensity increased only slightly but over the range from 25 µg WGA (1.3 ± 0.1 RFU) to 200 µg WGA (2.8 ± 0.1 RFU) a correlation between the amount of WGA added and the readout was observed. Upon use of more than 100 µg WGA for coupling, cross-linking of the cells by the increasingly dense lectin corona was observed. Whereas 96.5 ± 0.2% of the cells were located in gate G up to 75 µg WGA, the proportion of viable single cells was reduced to 89.7 ± 1.5% (100 µg WGA) and 77.8 ± 4.1% (200 µg WGA).

3. Comparison of the mean cell-associated granularity and the mean cell-associated fluorescence intensity acquired at different temperature levels

When comparing the side scatter values of cells acquired at 4 and 37°C, the run of the curves revealed that the interaction between the WGA-PLGA nanoparticles and the cells was characterized by three different sections which were strongly dependent on the amount of WGA added (*Figure 4*). Up to 2 µg WGA the granularity of the cells incubated at 37°C was higher than that incubated at 4°C as indicated by a difference of 28.6 ± 3.2 AU. Over the range from 5 to 25 µg WGA it is assumed that the granularity of the cells was independent of the temperature level of incubation. Finally, upon addition of more than 50 µg WGA for surface modification an inverted tendency was observed. The granularity of the cells incubated at 4°C exceeded that of cells incubated at 37°C by 48.6 ± 15.0 AU.

In the case of WGA-F-PLGA nanospheres the overall changes in granularity were less pronounced but followed the same tendency as already observed for WGA-PLGA nanoparticles (*Figure 5*). Surface modification of the F-PLGA nanoparticles with up to 75 µg WGA followed by incubation with Caco-2 cells at 37°C provoked an increase

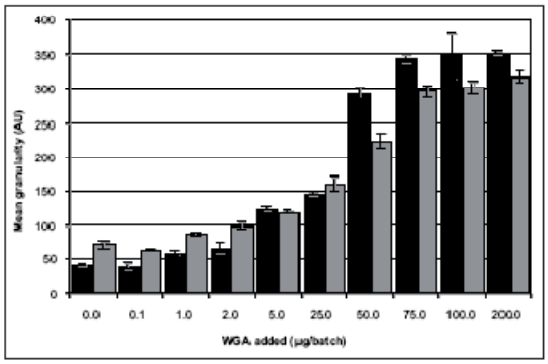

Figure 4 - Mean granularity of Caco-2 cells after incubation with WGA-grafted non-labeled PLGA nanospheres at 4°C (black columns) and 37°C (grey columns) (mean ± SD, n = 4).

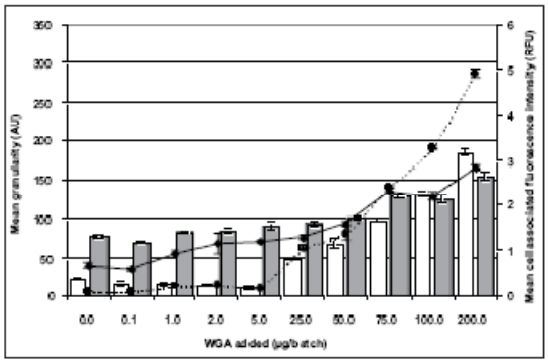

Figure 5 - Mean granularity and mean cell-associated fluorescence intensity of Caco-2 cells after incubation with WGA-F-PLGA nanospheres at 4°C (granularity, open columns; mean cell-associated fluorescence intensity, dotted line with circles) and 37°C (granularity, hatched columns; mean cell-associated fluorescence intensity, line with diamonds). The nanospheres were surface modified with increasing amounts of WGA (mean ± SD, n = 4).

in granularity amounting to 54.1 ± 17.7 AU at the mean as compared to incubation at 4°C. At 100 µg WGA the side scatter of the cells was independent of the temperature level. Surface modification with 200 µg WGA resulted in a higher granularity at 4 than at 37°C with a difference amounting to at least 31.2 ± 6.3 AU on average. When considering the mean cell-associated fluorescence intensities determined at both temperature levels, the trend was even more pronounced. Whereas the mean cell-associated fluorescence intensity of cells associated with nanoparticles modified with up to 50 µg WGA incubated at 37°C exceeded that incubated at 4°C by 0.6 ± 0.3 RFU on average, temperature independent cell-associated fluorescence intensity was observed at 75 µg WGA. A further increase of the WGA amount for surface modification provoked an inversion of the temperature dependency of the mean cell-associated fluorescence intensity. Ongoing from 100 µg WGA, the mean fluorescence intensity of the cells incubated at 4°C exceeded that incubated at 37°C by 1.6 ± 0.7 RFU.

III. DISCUSSION

In order to obtain evidence that not only the cell-associated fluorescence intensity is altered upon adhesion of fluorescent nanoparticles to the cell surface but also the roughness of the cells, the correlation of these two parameters needs to be investigated [18]. To confirm this concept, non-labeled biomimetic PLGA-nanoparticles for the detection

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of changes in granularity as well as labeled ones for simultaneous detection of both, changes in granularity as well as cell-associated fluorescence were prepared.

The surface of the nanoparticles was rendered biomimetic by covalent coupling of WGA using water-soluble carbodiimide. Although the active-ester method was originally reported to yield high coupling densities [19], the immobilization of F-WGA in the presence of the water soluble sulfo-NHS yielded no detectable lectin corona of the particles which might be attributed to the hydrophobicity of the polymer. Similar surface modification rates were observed independent of presence and absence of poorly water-soluble NHS as indicated by lectin densities of 1.94 ± 0.07 (2.0% EDAC), 50.31 ± 7.65 (3.5% EDAC), and 62.58 ± 0.07 μg WGA/mg nanospheres (7.0% EDAC) on average. Moreover, the particle size distribution increased with the concentration of the cross-linker which was more pronounced in the case of EDAC alone. Considering the size distribution, which is likely to influence the side scatter as well as the lectin density, which is assumed to influence the adhesivity of the nanoparticles, the most appropriate coupling procedure was selected.

To estimate the mean cell associated fluorescence intensity, the WGA-F-PLGA nanospheres were prepared from a covalently fluorescein-labeled PLGA. Depending on the amount of the amino-derivative of fluorescein used for carbodiimide coupling to the free carboxylate endgroups of PLGA, fluorescent polymers were obtained with every twelfth (1.5% F-cadaverine) or every fifth molecule carrying a fluorescein label (3.1% F-cadaverine). A similar fluorescence labeled polymer synthesized by a two step procedure via an amino-intermediate has been reported in the literature [20]. The preparation of WGA-F-PLGA nanospheres from fluorescent polymer revealed that the total content of F-PLGA strongly influenced not only the extent of the mean-cell associated fluorescence intensity but also the WGA-density at the surface (*Table II*). WGA-F-PLGA nanospheres with too high fluorescein-content, referring to a molar fluorescein/PLGA ratio of 1:22 (20% 3.1% F-cadaverine-PLGA), exhibited the highest quench effects as indicated by the relatively low mean cell-associated fluorescence intensity and lowest adhesion to the cells as indicated by the low side scatter of the cells. In contrast, WGA-F-PLGA nanoparticles with lower fluorescein content as indicated by a molar fluorescein/PLGA ratio of 1:110 (10% 1.7% F-cadaverine-PLGA) elicited adequate side scattering but mean cell-associated fluorescence intensities insufficient for sensitive detection of cell-binding. Altogether this indicates that the lectin density at the surface of WGA-F-PLGA nanospheres might be lower than that at the surface of WGA-PLGA nanoparticles even upon use of the same amounts of WGA for surface modification. Considering that every fifty third PLGA molecule is end-capped with fluorescein this might explain that the number of carboxylate groups on the surface of F-PLGA nanospheres available for WGA immobilization is reduced due to occupation by the fluorescein label. Additionally, the hydrophobic fluorescein label is assumed to increase the surface hydrophobicity of the nanoparticles so that the accessibility of the hydrophilic WGA is at least partially restricted. Consequently, careful adjustment of both the amount of bioadhesive ligand as well as the fluorescence intensity of the biomimetic nanospheres is crucial for most sensitive detection of granularity and mean cell-associated fluorescence intensity. Thus, nanoparticles prepared from 3.1% F cadaverine-PLGA and PLGA (1+9) were used for all further experiments.

Upon incubation at 4°C, the fluidity of the cell membrane and the metabolism is reduced so that binding of the nanospheres to the cell membrane is prevailing [15]. The slightly increased side scatter signals of the cells observed after incubation with glycine saturated PLGA or F-PLGA nanoparticles were attributed to the low bioadhesive characteristics of the polymer matrix (*Figure 1*) [21]. With increasing lectin density at the surface of the PLGA nanospheres, the granularity of the cells increased by means of the cytoadhesive WGA corona. Concurrently with approaching saturation of the complementary carbohydrates at the cell surface, a decrease in the number of single cells was observed. This points to a lower threshold of lectin-mediated cell-cross-linking at a surface density achieved by coupling with 75 μg WGA. In the case of WGA-F-PLGA nanospheres, however, a smoother increase of side scattering concurrent with increasing lectin-density was observed. Moreover, the cell-binding became apparent upon use of considerably higher amounts of WGA for surface modification. Since the surface modification of PLGA nanospheres with 5 μg WGA elicited the same side scatter as 100 μg WGA in the case of F-PLGA nanospheres, this difference of side scattering might be due to the lower WGA-density at the surface of F-PLGA nanospheres. This assumption was additionally confirmed by no appearance of cell agglomerates up to 200 μg WGA. Simultaneous detection of the mean cell-associated fluorescence intensity enabled the correlation of the side scatter signals with the fluorescence signals. Thus, the relationship between side scatter and fluorescence is characterized by a correlation factor of 48.7 ± 7.5 over the range from 5 to 100 μg WGA which confirms the utility of the side scatter signal to estimate the cell-binding of nanoparticles independent of labeling.

Upon incubation at 37°C, the cells are metabolically fully active and binding as well as energy consuming transport processes can take place. Thus, WGA-mediated binding and uptake of nanoparticles can occur at this temperature level [14]. The interdependence between the side scatter and the mean cell-associated fluorescence intensity of WGA-PLGA as well as WGA-F-PLGA nanospheres including the onset of the slope was comparable to that already observed at 4°C but the signal intensities were different. Again, there was a good correlation between the side scatter and the fluorescence intensity signals of cell-associated WGA-F-PLGA nanospheres corresponding to a factor of 57.3 ± 3.7 over the range from 50 to 200 μg WGA. Consequently, monitoring the side scatter offers reliable information about the cell-association, meaning binding and uptake of non-labeled biomimetic nanospheres.

As depicted in the plots comparing the data acquired at 4 and 37°C, which derive from side scatter or fluorescence intensities upon incubation of the cells with either WGA-PLGA or WGA-F-PLGA nanospheres, the interaction between the biomimetic nanoparticles and the cells is strongly guided by the WGA-density at the surface and the temperature level. Accordingly, three different types of interactions can be distinguished (*Figures 4 and 5*): i) Within the first section, which corresponds to a low WGA density at the surface of the nanospheres, the side scatter values and the fluorescence intensities acquired upon incubation at 37°C are higher than those at 4°C. Considering that about 30% of the WGA added is finally coupled to the surface of the nanoparticles, the real lectin-density is considerably lower than that indicated by the amount of lectin added. Thus, due to the sparse lectin-corona, the contact between the cells and the nanoparticles is governed by the Brownian motion of the nanoparticles. Due to the increased movement at 37°C more nanoparticles come into close contact with the cells and adhere to the cell surface. In contrast, at 4°C the probability of contact between the nanoparticles and the cells is reduced so that the side scatter as well as the cell-associated fluorescence intensities are lower than those at 37°C. ii) The second domain is characterized by equalization of the 37 and 4°C data. Interestingly, this second domain was observed in the range of 5-25 μg WGA in the case of WGA-PLGA-nanospheres but it was shifted to 75 μg WGA in the case of WGA-F-PLGA-nanospheres. As mentioned above, this delay might be due to the lower lectin-density of the WGA-F-PLGA nanoparticles. iii) Within the third domain the granularity is only slightly higher at 4 than at 37°C, while the mean cell-associated fluorescence intensity at 4°C strongly exceeds that at 37°C. Obviously, the bioadhesivity of WGA nanoparticles predominates diffusional processes due to the higher lectin density at the surface. Since WGA is not only cytoadhesive but also cytoinvasive, the lower side scatter values observed upon incubation at 37°C indicate that the WGA-grafted

nanoparticles initially bound to the cell surface are taken up into the cytoplasm. Thus, the contribution of these nanoparticles to the side scattering is reduced provoking a decrease in the cellular granularity at 37°C compared to 4°C. This hypothesis is in accordance with the temperature dependent behavior of WGA-grafted nanoparticles incubated with Caco-2 monolayers [7]. This effect was even more pronounced upon monitoring the cell-associated fluorescence intensity. This is probably due to possible shielding of the fluorescein label by the cell membrane and/or reduction of the quantum yield upon uptake into acidic lysosomal compartments. Consequently, the collection of side scatter data actually enables elucidation not only of the binding, but also of the uptake of non-labeled nanoparticulate matter into the cells.

As opposed to already established methods to elucidate the nanoparticle-cell interaction the monitoring of side scattering offers certain advantages. Firstly, there is no need for labeling of the nanospheres or the biorecognitive molecule. Some methods such as incorporation of dyes into the core of the nanospheres suffer from leakage of the label especially upon prolonged incubation. Consequently, preparation of nanoparticles from covalently labeled polymers seems to be preferable. When fluorescence labeling the ligands, however, their biological activity may be reduced or altered by the attached dye molecules as well as the derivatisation procedure. Secondly, the method is simple to handle and yields reliable results. The characteristics of at least 3×10^3 cells are monitored and possible artifacts due to fixation and staining procedures necessary for microscopic techniques are excluded [22]. Thirdly, flow cytometry does not only offer information about the roughness of the cell surface but also about the size of the cells. Thus, other parameters such as viability or agglomeration of the cells by multivalent nanoparticles can be monitored at the same time.

The overall applicability of the side scatter for analysis of cell-associated nanoparticles might be limited by the volume diameter of the nanoparticles since the contribution of the colloidal drug carrier to the side scattering is a key for tracking any changes in roughness of the cell surface. In general, the sensitivity of methods detecting cell-associated fluorescence intensity is assumed to be higher than that of monitoring granularity.

All in all, the determination of granularity is a useful tool for collecting valuable information on the interaction between biomimetic nanoparticles and single cells when labeling is too skilful or not possible.

REFERENCES

1. Peppas N.A. - Intelligent therapeutics: biomimetic systems and nanotechnology in drug delivery. - Adv. Drug Del. Rev., **56**, 1529-1531, 2004.
2. Nahar M., Dutta T., Murugesan S., Asthana A., Mishra D., Rajkumar V., Tare M., Saraf S., Jain N.K. - Functional polymeric nanoparticles: An efficient and promising tool for active delivery of bioactives. - Crit.Rev. Ther. Drug Carrier Syst., **23**, 259-318, 2006.
3. Hildebrand H.F., Blanchemain N., Mayer G., Chai F., Lefrebvre M., Boschin F. - Surface coatings for biological activation and functionalisation of medical devices. - Surface & Coatings Technol., **200**, 6318-6324, 2006.
4. Venkatesh S., Byrne M.E., Peppas N.A., Hilt J.Z. - Applications of biomimetic systems in drug delivery. - Expert Opin. Drug Del., **2**, 1085-1096, 2005.
5. Gu F.X., Karnik R., Wang A.Z., Alexis F., Levy-Nissenbaum E., Hong S., Langer R.S., Farokhzad O.C. - Targeted nanoparticles for cancer therapy. - Nanotoday, **2**, 14-21, 2007.
6. Qian Z.M., Li H., Sun H., Ho K. - Targeted drug delivery via the transferrin receptor-mediated endocytosis pathway. - Pharmacol. Rev., **54**, 561-587, 2002.
7. Gabor F., Bogner E., Weissenboeck A., Wirth M. - The lectin-cell interaction and its implications to intestinal lectin-mediated drug delivery. - Adv. Drug Del. Rev., **56**, 459-480, 2004.
8. Nguyen C.A., Allémann E., Schwach G., Doelker E., Gurny R. - Cell interaction studies of PLA-MePEG nanoparticles. - Int. J. Pharm., **254**, 69-72, 2003.
9. Newman K.D., Gwon G.S., Miller G.G., Chlumecky V., Samuel J. - Cytoplasmatic delivery of a macromolecular fluorescent probe by poly(d,l-lactic-co-glycolic acid) microspheres. - J. Biomed. Mater. Res., **50**, 591-597, 2000.
10. Yi Y., Kim J.H., Kang H.W., Oh H.S., Kim S.W., Seo M.H.- A polymeric nanoparticle consisting of mPEG-PLA-Toco and PLMA-COONa as a drug carrier: improvements in cellular uptake and biodistribution. - Pharm. Res., **22**, 200-208, 2005.
11. Shapiro H.M. (Ed.) - Practical Flow Cytometry. - Alan R. Liss Inc., New York, 1988.
12. Panyam J., Labhasetwar V. - Biodegradable nanoparticles for drug and gene delivery to cells and tissue. - Adv. Drug Del. Rev., **55**, 329-347, 2003.
13. Anderson J.M., Shive M.S. - Biodegradation and biocompatibility of PLA and PLGA microspheres. - Adv. Drug Del. Rev., **28**, 5-24, 1997.
14. Weissenboeck A., Bogner E., Wirth M., Gabor F. - Binding and uptake of wheat germ agglutinin-grafted PLGA-nanospheres by Caco-2 monolayers. - Pharm. Res., **21**, 1919-1925, 2004.
15. Wirth M., Kneuer C., Lehr C.M., Gabor F. - Lectin-mediated drug delivery: discrimination between cytoadhesion and cytoinvasion and evidence for lysosomal accumulation of wheat germ agglutinin in the Caco-2 model. - J. Drug Targ., **10**, 439-448, 2002.
16. Weissenboeck A., Wirth M., Gabor F. - WGA-grafted PLGA-nanospheres: Preparation and association with Caco-2 single cells. - J. Contr. Rel., **99**, 383-392, 2004.
17. Shah P., Jogani V., Bagchi T., Misra A. - Role of Caco-2 cell monolayers in prediction of intestinal drug absorption. - Biotechnol. Prog., **22**, 186-198, 2006.
18. Darzynkiewicz Z., Crissman H.A. (Eds.) - Methods in Cell Biology, Vol. 33. Flow Cytometry, Academic Press, New York, 1990.
19. Staros J.V., Wright R. W., Swingle D.W. - Enhancement by N-hydroxysulfosuccinimide of water-soluble carbodiimide-mediated coupling reactions. - Anal. Biochem., **156**, 220-222, 1986.
20. Kim S.H., Jeong J.H., Chun K.W, Park T.G. - Target-specific cellular uptake of PLGA-nanoparticles coated with poly(L-lysine)-poly(ethylene glycol)-folate conjugate. - Langmuir, **21**, 8852-8857, 2005.
21. Mathiowitz E., Jacob J. S., Jong Y.S., Carino G.P., Chickering D.E., Chaturvedi P., Santos C.A., Vijayaraghavan K., Montgomery S., Bassett M., Morrell C. - Biologically erodable microspheres as potential oral drug delivery systems. - Nature, **386**, 410-414, 1997.
22. Watson P., Jones A.T., Stephens D.J. - Intracellular trafficking pathways and drug delivery: imaging of living and fixed cells. - Adv. Drug Del. Rev., **57**, 43-61, 2005.

MANUSCRIPT

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3.2 Second Part

NONIONIC SURFACTANTS INCREASE CELL-BINDING OF NANOPARTICLES BY MODULATION OF MEMBRANE STIFFNESS

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Submitted to *Jr of Nanobiotechnology*

THE INTERACTION OF POLOXAMER 188 WITH THE CELL MEMBRANE INCREASES THE CELL-ASSOCIATION OF NANOPARTICLES

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To be submitted to *Langmuir*

Nonionic surfactants increase cell-binding of nanoparticles by modulation of membrane stiffness

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Abstract

Background

Non-ionic surfactants such as Polysorbate 20 and 80 are frequently required for preparation, storage and coating of nanoparticles (NP). Since non-ionic surfactants can be adsorbed and/or incorporated into the cell-membrane they might represent more than only inert ingredients of NP-preparations.

Results

According to Caco-2-binding studies using fluorescent NPs the amount of cell-bound NPs increases in presence of both surfactants to reach the maximum at 0.05%. Concurrently, the cell membrane gains in stiffness as confirmed by the microaspiration-technique and no cytotoxic effects were observed. As rigidity strongly influences membrane dynamics, especially the undulations of the lipid-bilayer, the increase in cell-bound NPs might result from reduced repulsive forces due to suppressed undulatory movements. At concentrations ≥ 0.1% Polysorbate 20 increasingly reduces the number of viable cells and Polysorbate 80 softens the cell membrane more and more resulting in decreased NP-binding.

Conclusions

Apart from size, surface charge and modification of NPs, rigidity of the cell membrane is identified as another important parameter influencing the NP-cell interaction. Provided that viability of cells is not affected, both, Polysorbate 20 and Polysorbate 80 are useful excipients to enhance the NP-

cell interaction and to improve colloidal drug delivery by shortening the diffusional pathway of the drug and reducing the time of drug exposure to sometimes harmful environment in the body.

Background

In recent years the "nanohype" also gained a foothold in pharmaceutical sciences because nanoparticles (NP) can provide controlled or targeted drug delivery and thus can lead to increased therapeutic efficacy together with reduced side effects. Interestingly, surfactants play a key role in the field of pharmaceutical nanotechnology: (i) Even during preparation some techniques require surfactants to yield NPs e.g. polycaprolactone-NPs are prepared by nanoprecipitation using even 0.17% Polysorbate 20 or 0.40% Polysorbate 80 as an aqueous phase.[1] Surfactants are applied not only for production of solid lipid nanoparticles,[2, 3] cationic nanoparticles [4] but also for deformable liposomes [5] and proliposomal beads.[6] (ii) For storage addition of surfactants to NP-suspensions is almost always necessary to avoid agglomeration e.g. Polysorbate 80 stabilized hesperetin nanosuspensions for dermal use.[7] In case of lyophilized NP-powders most frequently surfactants are added during sometimes problematic resuspension by ultrasonication to avoid coarse aggregates.[2, 8] (iii) Moreover, physicochemical studies revealed that coating of NPs with surfactants such as Polysorbate 80 can minimize rapid elimination of intravenously administered NPs from the blood stream by cells of the reticuloendothelial system located mainly in the liver and the spleen.[9] That way the so-called "stealth coatings" can prolong residence in circulation and enhance considerably therapeutic efficacy.

Besides that there are also hints towards distinct effects of the surfactants on viable cells. Polysorbate 20 and Polysorbate 80 increase the transepithelial transport of drugs representing P-glycoprotein-substrates by inhibition of this or other transport proteins of the multiple drug resistance protein family.[10,

11, 12, 13] In addition, surfactants are reported to increase viability of cells and protect cells from stress.[14]

Ongoing from these reported effects and being aware of the fact that surfactants are present in NP-preparations at considerable amounts, their influence on the NP-cell interaction remains to be elucidated.

In this study, the effect of the non-ionic surfactants Polysorbate 20 (Tween® 20) and 80 (Tween® 80) on the interplay between Caco-2 cells and NP is investigated. Both surfactants are widely applied as emulsifiers representing monoesters of polyethoxylated sorbitan with either lauric acid (Polysorbate 20) or oleic acid (Polysorbate 80). The daily dietary intake of Polysorbate 80 is about 100 mg at the mean as opposed to 25 mg Polysorbate 20 at the most as recommended by the WHO.[15] In addition, both surfactants are approved by the FDA for parenteral administration amounting to 10% (i.v.) and 12% (i.m.) Polysorbate 80 or 0.4% (i.v.) up to 2.4% (infusion) and 0.01% (i.m.) Polysorbate 20. At the cells, the most popular model in drug discovery and development, the human colon carcinoma cell line Caco-2 was chosen, which exhibits structural and functional features similar to intestinal epithelial cells.[16]

The aim of this work is to examine the influence of both nonionic surfactants on the association of fluorescent labeled NP with 217 nm in diameter with Caco-2 cells by flow cytometry. Considering possible dose-dependent toxic effects of the surfactants and according to results from determination of the stiffness of the Caco-2 cell membrane by the microaspiration technique an approach is presented to explain the effects of tensides on the NP-cell association.

Results

Cell-association of NPs in presence of Polysorbates

In order to detect any influence of the different concentrations of surfactants on fluorescence emission of the labelled NPs and to guarantee comparability of the results, the fluorescence intensity of the NP suspensions was determined prior to flow cytometry. Independent from the amount and type of surfactant, the fluorescence intensity of all NP-suspensions amounted to 23,354 ± 507 FI (fluorescence intensity) as determined at 480/525 nm (Spectrafluor-reader, Tecan, Groedig, Austria) at room temperature.

As temperature was supposed to influence the extent of NP-cell association, the experiments were performed at 4°C and 37°C. As opposed to 37°C, at 4°C the metabolic activity and the fluidity of the cell membrane is reduced thus active transport processes are unlikely to occur. Although the quantum yield of fluorescent NPs was independent from the incubation temperature, the fluorescence intensity of Caco-2-associated NPs was 1.4 FI at 4°C and 2.6 FI at 37°C in absence of surfactant.

Independent from incubation temperature, the amount of cell-associated NPs steeply increased ongoing from 0.0005% to 0.05% Polysorbate 20 as compared to surfactant-free incubation (Fig. 1). The maximum of cell-associated NP fluorescence was observed at 0.05% Polysorbate 20 referring to 6.50±0.06 RFI at 37°C or 4.29±0.01 RFI at 4°C. As the content of surfactant increased the amount of cell-associated NPs decreased and was comparable to the batches without surfactant at about 0.5% Polysorbate 20. Finally, at 2.5% Polysorbate 20 the fluorescence intensity of cell-associated NP decreased even to 27% (4°C) and 51% (37°C) of the reference.

In case of Polysorbate 80 the NP-cell association pursued a similar tendency but with distinctive differences between both temperature levels in the range of about +1.4 to +1.7 RFI upon incubation at 37°C in comparison to 4°C (Fig. 2). Again, the maximum of cell-associated NP occurred at 0.05% surfactant as indicated by 4.86±0.02 RFI at 37°C and 3.17±0.05 RFI at 4°C. Subsequently,

the reversal of the trend led to a rate of cell-associated NPs comparable to the reference in the range of 0.25% and 0.5% Polysorbate 80, further inhibiting association to end up in 10% (4°C) and 11% (37°C) of the reference at 2.5% surfactant.

Cytotoxicity of Polysorbates

In accordance with the cell-association studies, the viability of the Caco-2 cells was examined within the same concentration range of the surfactants. As viability of the cells in presence of surfactant and NPs was determined by setting gate A for viable cells and gate B for dead cells, the appropriate setting was confirmed by staining dead cells with propidium iodide in absence of NPs. Accordingly, no cell staining was observed in gate A (0.1 RFI) in comparison with dead cells in gate B yielding 8 – 10 RFI in case of the negative control.

The Caco-2 cell preparations applied for the experiments at 4°C contained 94% viable and 5% dead cells in comparison to 92% viable and 6% dead cells for the assays at 37°C (Fig. 3). Upon addition of increasing amounts of Polysorbate 20 the fraction of viable cells was ≥ 90% up to 0.1% surfactant at both temperature levels. A further increase in Polysorbate 20-content strongly decreased the percentage of viable cells with concurrently increasing percentage of dead cells and non-gated cells representing cell debris and aggregates. At 2.5% Polysorbate 20, the percentage of viable cells was reduced to 35.22±1.78% (4°C) and 13.63±2.81% (37°C), respectively.

In contrast, Polysorbate 80 revealed only moderate Caco-2 cytotoxicity. Interestingly, the threshold of 90% viable cells was again surpassed at surfactant concentrations higher than 0.25% (Fig. 4). Nevertheless, the percentage of viable cells was higher than 82.43±0.92% upon incubation at 4°C and 74.48±3.14% upon incubation at 37°C in presence of NPs at 2.5% surfactant at the most.

Stiffness of cell membrane in presence of Polysorbates

To elucidate the influence of the Polysorbates on the extensibility of the cell membrane, the microaspiration technique was applied. It relies on the fact that the cell attached to the orifice of a micropipette is sucked into the lumen of the micropipette. Consequently, the cylindrical volume in the micropipette increases with decreasing stiffness of the cell membrane. As compared to cells pre-incubated without surfactant, presence of 0.05% tenside reduced extensibility of the cells by 44% (Polysorbate 20) and 69% (Polysorbate 80) (Fig. 5). Increasing the surfactant concentration to 0.5% Polysorbate 20 resulted in 36.27±0.54% dead cells, which preferably adsorbed to the orifice, to viable cells or at the outside of the micropipette. In case of cells pre-incubated with 2.5% Polysorbate 80, the cell membrane lost extremely in stiffness so that vesicles were pinched off upon application of negative pressure (Fig. 5). Both experimental conditions rendered a reliable measurement impossible.

In order to describe the gain in stiffness also quantitatively, the data from the image's pixels were calculated and the stiffness is compared by the slope of the regression line. At the apparent strain = 1, the slope of non-treated cells was 195 at the mean. In contrast, the presence of Polysorbate 20 the slope increased from 220 at 0.0005% to 251 at 0.05% surfactant. In case of Polysorbate 80 a stronger increase in stiffness was observed amounting to 381 at 0.0005% and 471 at 0.05% tenside. Interestingly, rising the Polysorbate 80 concentration to 0.25% during preincubation of Caco-2 cells resulted in a decrease of stiffness as indicated by a slope of 206.

Discussion

For elucidation of the cell-NP interaction in presence of Polysorbates fluorescein-loaded Polystyrene-NPs were used. Due to lack of any biorecognitive moiety exclusively non-specific interactions can provoke any cell-association i.e. binding and/or uptake of the NPs. In addition, the NPs do not contain any surfactant according to the manufacturer.

When the NPs were allowed to interact with the cells in absence of Polysorbates, the cell-associated RFI revealed a 1.8-fold increase of cell-associated NPs at 37°C as compared to 4°C. The higher cell-association at 37°C is due to higher fluidity of the cell-membrane[17] and full metabolic activity which facilitates non-specific adhesion. Although flow cytometry can not discriminate between binding and uptake of fluorescent NPs, it is most unlikely that NPs are intracellularily accumulated by endocytotic mechanisms. Trafficking to acidic vesicles is part of the endocytosis so that the acid-sensitive label of the NPs is usually quenched therein indicated by higher RFI at 4°C than at 37°C. Since a this inversion of RFI was not observed, it is supposed that mainly binding to the cell membrane and negligible uptake of NPs is monitored throughout the experiments.[18]

In presence of only 0.0005% Polysorbate the amount of cell-bound NPs increases up to the maximum at 0.05% surfactant independent from the Polysorbate type and the temperature level (Fig. 1, 2). Upon incubation at 37°C, however, the NP-binding rate increases steeper than at 4°C and the total amount of cell-bound NPs in presence of Polysorbate 20 exceeds that of Polysorbate 80 about 1.35-fold at both temperature levels. A further increase in concentration of both Polysorbates decreased the amount of cell-bound NPs to meet that of surfactant-free cells and even to drop below.

According to toxicity studies with both surfactants in absence of NPs, the descending part of the binding curve is due to cytotoxicity which increases with higher surfactant concentration. In case of Polysorbate 20, the viability is strongly reduced in a concentration dependent manner below the apparent threshold of ≤ 80% viable cells between 0.1% and 0.25% surfactant (Fig.3). In accordance with the literature,[19, 20] the detected cytotoxicity of Polysorbate 80 was quite lower than that of Polysorbate 20 (Fig. 4). Obviously, the decrease in cell-bound NPs in presence of Polysorbate 80 is not due to loss of viability. Nevertheless, the cytotoxicity of both Polysorbates at 37°C exceeds that at 4°C which reflects the higher fluidity of the cell membrane at elevated

temperature and indicates for facilitated incorporation of both non-ionic surfactants into the cell membrane.

Although the fluidity of the cell membrane changes concurrently with the tenside concentration due to interplay of the non-ionic surfactants and the cell membrane, the increase in cell-bound NPs cannot simply be explained by changes in membrane fluidity and cytotoxic effects of surfactants.[13] According to laser scattering studies on cell membranes[21] and neutron spin-echo investigations on lipid bilayers,[22] not only temperature dependent fluidity but also undulations contribute to the dynamics of the cell membrane. Since rigidity of the cell membrane governs the flexibility of the cell membrane, the micro-aspiration technique of cells pre-incubated with both non-ionic surfactants was applied to assess their effect on the stiffness of the cell membrane. Within the concentration range of both Polysorbates without any negative effect on viability of cells, even lowest concentrations of surfactants increased the rigidity of the cells as compared to non-pretreated cells (Fig. 5, 6). Interestingly, this result coincides with higher cell-bound NPs at both temperature levels. At 0.05% Polysorbate 20 or 80 and >80% cell-viability not only the peak of cell-bound NPs was observed but also the highest gain in stiffness. In contrast, further increasing the surfactant concentration led to decreasing cell-binding of NPs. In case of Polysorbate 20 this is due to the affected viability of cells, whereas in case of Polysorbate 80 increasing incorporation of the tenside into the cell membrane an inverted effect on the membrane stiffness was observed: At 0.25% Polysorbate 80 the rigidity of the cell membrane matched that of non-treated cells and further softened that much that finally vesicles were formed at 2.5% Polysorbate 80 most likely due to uncoupling of the lipid-bilayer from the underlying cytoskeleton (Fig. 5, 7).

Thus, NP-binding to the cells increases with rigidity of the cell membrane. Ongoing from these results it is suggested that in presence of up to 0.05% Polysorbate the surfactants are increasingly adsorbed and/or incorporated into the cell membrane to gain in stiffness and to round the cell. In response

to the increasing stiffness the transverse undulations of the cell membrane with amplitudes in the nanometer scale and Hz frequency are reduced.[23] This facilitates approaching to as well as interaction of NP with the cell membrane leading to enhanced binding even in absence of bioadhesive interactions.

Although some issues, such as the fact that the gain in stiffness by Polysorbate 80 provokes a higher rigidity of the cell membrane but a lower binding rate of NPs as compared to Polysorbate 20, remain to be elucidated, undulation of the cell membrane is identified as another important parameter determining the NP-cell interaction apart from size, surface charge and modification of the particles.

Conclusions

Non-ionic surfactants such as Polysorbate 20 and 80 are frequently used for preparation, storage and coating of NPs, however, they also influence the interplay between NPs and cells due to incorporation into the cell membrane. Increasing the surfactant concentration in the non-cytotoxic range from 0.0005% to 0.05% stiffens the cell membrane. Apparently, this gain in rigidity reduces the undulations of the lipid-bilayer and thus repulsive forces towards deposit of NPs at the cell surface. At concentrations beyond 0.1% Polysorbate 80 softens the cell membrane and increasingly reduces NP-adsorption whereas Polysorbate 20 exerts considerably increasing cytotoxic effects.

Provided that viability of cells is not affected, both, Polysorbate 20 and Polysorbate 80 are useful excipients to enhance the NP-cell interaction and to improve delivery of incorporated drugs by shortening the diffusional pathway and reducing the time of drug exposure to harmful environment.

Methods

Chemicals

Plain fluorescent nanoparticles (FluoresbriteTM YG 0.20 µm microspheres 2.54% solids, polystyrene, 0.217 ± 0.015 µm) were purchased from

Polysciences, Inc. (Warrington, USA). Polysorbate 20, Polysorbate 80, and 2-(4-(2-Hydroxyetyl)-1-piperazinyl)-ethanesulfonic acid (HEPES) were obtained from Sigma-Aldrich GmbH (Vienna, Austria). All other chemicals in use were of analytical grade.

Cell culture

The human intestinal epithelial cell line, Caco-2, was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The cells (passage number 39-81) were grown in RPMI-1640 cell culture medium supplemented with 10 % fetal bovine serum, 4 mmol L-glutamine, and 150 µg/ml gentamycine in a humidified atmosphere of 5 % CO_2 and 95 % air at 37°C. Upon reaching 70-80% confluence, the cells were subcultured with TrypLE® select.

Flow cytometry

The amount of cell-associated NPs was determined by mixing 50 µl cell suspension (2.5 x 10^5 Caco-2 cells in cultivation medium) with 50 µl NP suspension (15.9 µg/ml in 20 mM isotone HEPES/NaOH buffer pH 7.4 containing 0.00, 0.001, 0.01, 0.10, 0.20, 0.50, 1.00, 2.00, and 5.00% w/v Polysorbate 20 or Polysorbate 80) and incubation for 60 min at 4°C or 37°C. In order to remove free or weakly bound NPs, the cells were centrifuged (5 min, 1000 rpm, 4°C) and washed twice with 150 µl PBS buffer each. The cells were resuspended in 1000 µl particle-free PBS buffer and analysed by flow cytometry (Epics XL MCL Flow cytometer, Coulter, Miami, USA) using a forward versus side scatter gate to include the single-cell population and exclude debris and aggregates. Single viable cells with associated NPs were detected in gate A, whereas dead cells were accumulated in gate B. The fluorescence label of NPs attached to cell membrane was detected at 525 nm. For analysis data of 3000 cells was accumulated. All the tests were performed at least in triplicate.

Toxicity tests

The impact of both surfactants on the viability of cells was examined in two ways: (i) the amount of live cells accumulated in gate A and dead cells in gate B according to the flow cytometric histograms as well as (ii) the membrane permeability for propidium iodide and staining of the nucleus. To exclude any interference between the fluorescence emission of propidium iodide and that of the fluorescent NPs upon flow cytometric analysis, the latter assay was performed in absence of NPs. At this, 50 µl cell suspension (2.5 x 10^5 Caco-2 cells in cultivation medium) and 50 µl 20 mM isotonic HEPES/NaOH-buffer pH 7.4 containing increasing amounts of surfactant as above were incubated for 1h at 4°C or 37°C, followed by washing, incubation with 2.7 µl propidium iodide solution (0.1 mg/ml in PBS) for 2 min and flow cytometry. For control, reference samples were treated with 100 µl methanol pre-cooled at –20°C to yield 100% dead cells.

Membrane tension determination

The membrane tension was determined by the micropipette aspiration technique.[24] 50 µl cell suspension (2.5 x 10^5 Caco-2 cells in cultivation medium) was incubated with 50 µl of the surfactant solution (0.0005, 0.05, 0.5 and 2.5% of Polysorbate 20 and 0.0005, 0.05, 0.25 and 2.5% of Polysorbate 80 in isotone PBS buffer) for 60 min at 37°C. The cells and the patch pipette were viewed during pressure application on a Zeiss Axiovert 200 microscope (Zeiss, Munich, Germany) using a 40x objective and a Hamamatsu camera (Hersching, Germany) for acquiring of images. Borosilicate micropipettes were filled with isotone PBS buffer, mounted on a syringe and brought into close vicinity of a cell until the pipette touched the membrane. A moderate negative pressure was applied, just enough for attachment of the cell to the orifice of the micropipette. This membrane apex was set as zero-point of membrane tension. The suction pressure was stepwise (ΔP = 0.1 kPa) increased until the membrane was not expanding any more. An image was acquired per each pressure change. Micromanipulations were performed at

room temperature and repeated at least eight times at each concentration level.

The data were calculated from the pixels of the acquired images and fit into equation 1 to calculate strain (x-axis) and tension (y-axis).[25] Object's deformation in length, normalized with zero-tension state is called strain (δA), dimensionsless parameter calculated as:

$$\delta A \cong 2\pi R_p \left(1 - \frac{R_p}{R_c}\right) \Delta L \qquad \text{Eq. 1}$$

The tension (τ, Ncm^{-1}) was derived according to equation 2 and is expressed as:

$$\tau = \frac{P\, R_p}{2 - 2\frac{R_p}{R_c}} \qquad \text{Eq. 2}$$

P = suction pressure

R_p = inner radius of the pipette

R_c = radius of the cell

ΔL = apex length difference.

The inclination of the resulting graph represents the stiffness of the cell membrane. It might be specified by the slope from the general form of the equation of a straight line.

Competing interests

The authors declare that they have no competing interests.

Acknowledgements

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References

1. Vauthier C, Bouchemal K: **Methods for the preparation and manufacture of polymeric nanoparticles.** *Pharm. Res.* 2009, **26**:1025-1058
2. Müller RH, Rühl D, Runge S, Schulze-Forste K, Mehnert V: **Cytotoxicity of solid lipid nanoparticles as a function of the lipid matrix and the surfactant.** *Pharm. Res.* 1997, **14**(No 4):458–462
3. del Pozo-Rodriguez A, Delgado D, Solinis MA, Gascon AR, Pedraz JL: **Solid lipid nanoparticles: Formulation factors affecting cell transfection capacity.** *International Jr of Pharmaceutics* 2007, **339:**261–268
4. Hattori Y, Ding W, Maitani Y. **Highly efficient cationic hydroxylated cholesterol-based nanoparticle-mediated gene transfer in vivo and in vitro in prostate carcinoma PC-3 cells.** *Jr of Controlled Release* 2007, **120:**122–130
5. Oh YK, Kim MY, Shin JY, Kim TW, Yun MO, Yang SJ, Choi SS, Jung WW, Kim JA, Choi HG: **Skin permeation of retinol in Tween 20-based deformable liposomes: in-vitro evaluation in human skin and keratinocyte models [abstract].** *Jr of Pharmaceutical Pharmacology* 2006, **58:** 161–166
6. Deshmukh DD, Ravis WR, Betageri GV: **Improved delivery of cromolyn from oral proliposomal beads.** *International. Jr of Pharmaceutics* 2008, **358:**128–136
7. Mishra PR, Shaal LA, Müller RH, Keck CM: **Production and characterization of Hesperetin nanosuspensions for dermal delivery.** *Int. J. Pharm.* 2009, 371:182-189
8. Weissenböck A, Wirth M, Gabor F: **WGA-grafted PLGA-nanospheres: preparation and association with Caco-2 single cells.** *Jr of Controlled Release* 2004, **99:**383–392
9. Kreuter J: **Nanoparticulate systems for brain delivery of drugs.** *Adv. Drug Delivery Rev.* 2001, **47:**65-81

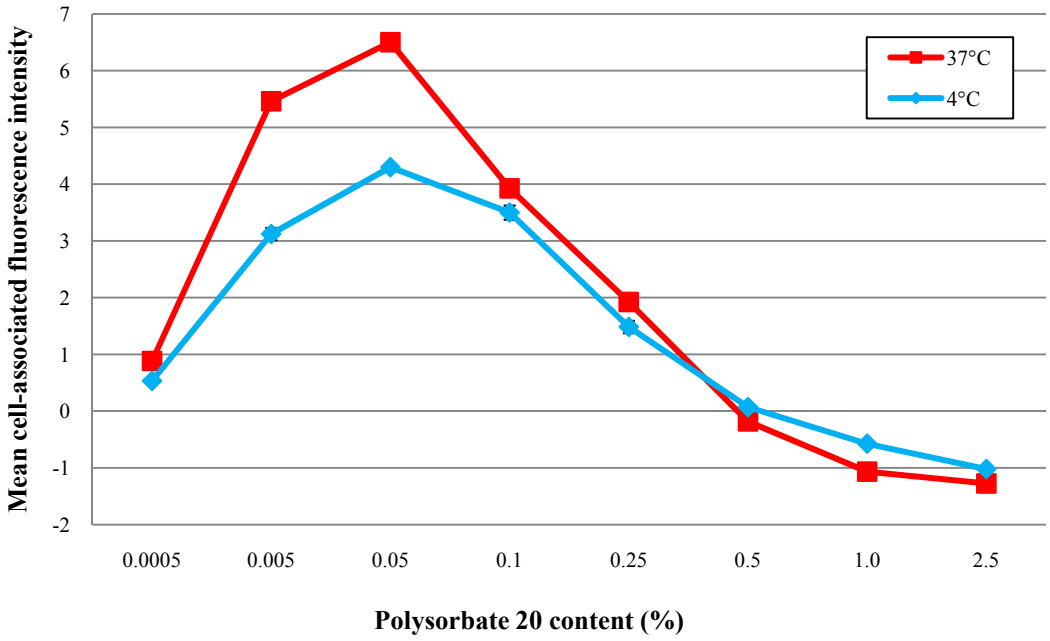
10. Yu-li L: **Relationship between the hydrophilic-lipophilic balance values of pharmaceutical excipients and their multidrug resistance modulating effect in Caco-2 cells and rat intestines.** *Jr of Controlled Release* 2003, **90:**37–48
11. Shono Y, Nishihara H, Matsuda Y, Furukawa S, Okada N, Fujita T, Yamamoto A: **Modulation of intestinal P-glycoprotein function by cremophor EL and other surfactants by an in vitro diffusion chamber method using the isolated rat intestinal membranes.** *Jr of Pharmaceutical Sciencies* 2004, **93:**877-885
12. Nerurkar MM, Burton PS, Borchardt RT: **The use of surfactants to enhance the permeability of peptides through Caco-2 cells by inhibition of an apically polarized efflux system.** *Pharmaceutical Research* 1996, **13:** 528-534
13. Rege BD, Kao JPY, Polli JE: **Effects of nonionic surfactants on membrane transporters in Caco-2 cell monolayers.** *European Jr of Pharmaceutical Sciences* 2002, **16:**237–246
14. Choi SH, Gu MB: **A portable toxicity biosensor using freeze-dried recombinant bioluminescent bacteria.** *Biosensors & Bioelectronics* 2002, **17:**433–440
15. Krantz JC: **Limiting factors in the use of antibiotics.** (*Bull. Sch. Med. Maryland* 1951, **36:**48) In *Toxicological evaluation of some food additives including anticaking agents, antimicrobials, antioxidants, emulsifiers and thickening agents: 25. June – 4. July 1973; Geneva,* World Health Organization: In *Seventeenth report of the Joint FAO/WHO expert committee on food additives:* Geneva 1974
16. Meunier V, Bourrie M, Bergerand Y, Fabre G: **The human intestinal epithelial cell line Caco-2: pharmacological and pharmakokinetic applications.** *Cell.Biol.Toxicol.* 1995, **11:**187-194
17. Wirth M, Kneuer C, Lehr CM, Gabor F. **Lectin-mediated drug delivery: discrimination between cytoadhesion and cytoinvasion and evidence for**

lysosomal accumulation of wheat germ agglutinin in the Caco-2 model. *Jr of Drug Targeting* 2002 **10:** 439 – 448

18. Trimmel K, Ratzinger G, Kerleta V, Fillafer C, Wirth M, Gabor F. **Characterization of binding and uptake of biomimetic nanoparticles by flow cytometry.** *J Drug Del. Sci. Tech.* 2008, **18**:51-57
19. Benassi L, Bertazzoni G, Seidenari S: **In vitro testing of tensides employing monolayer cultures: a comparison with results of patch tests on human volunteers [abstract].** *Contact Dermatitis* 1999, **40:**38–44
20. Weyenberg W, Filev P, Van den Plas D, Vandervoort J, De Smet K, Sollie P, Ludwig A: **Cytotoxicity of submicron emulsions and solid lipid nanoparticles for dermal application.** *International Jr of Pharmaceutics* 2007, **337:**291 – 298
21. Haidekker MA, Stevens HY, Frangos JA: **Cell membrane fluidity changes and membrane undulytions observed using a laser scattering technique**. *Ann. Biomed. Eng.* 2004, **32:**531-536
22. Takeda T, Kawabata Y, Seto S, Komura S, Shosh SK, Nagao M, Okuhara D: **Neutron spin-echo investigations of membrane undulations in complex fluids involving amphiphiles.** *J. Phys. Chem. Solids* 1999, **60:**1375-1377
23. Pierres A, Monnet-Corti V, Benoliel A-M, Bongrand P: **Do membrane undulations help cells to probe the world?** *Trends in Cell Biology* 2009, **19:**428-433
24. Hochmuth RM: **Micropipette aspiration of living cells.** *Jr of Biomechanics* 2000. **33:**15–22
25. Evans E, Rawicz W: **Entropy-driven tension and bending elasticity in condensed-fluid membranes.** *Physical Rewiev letters* 1990, **64:**2094-2097

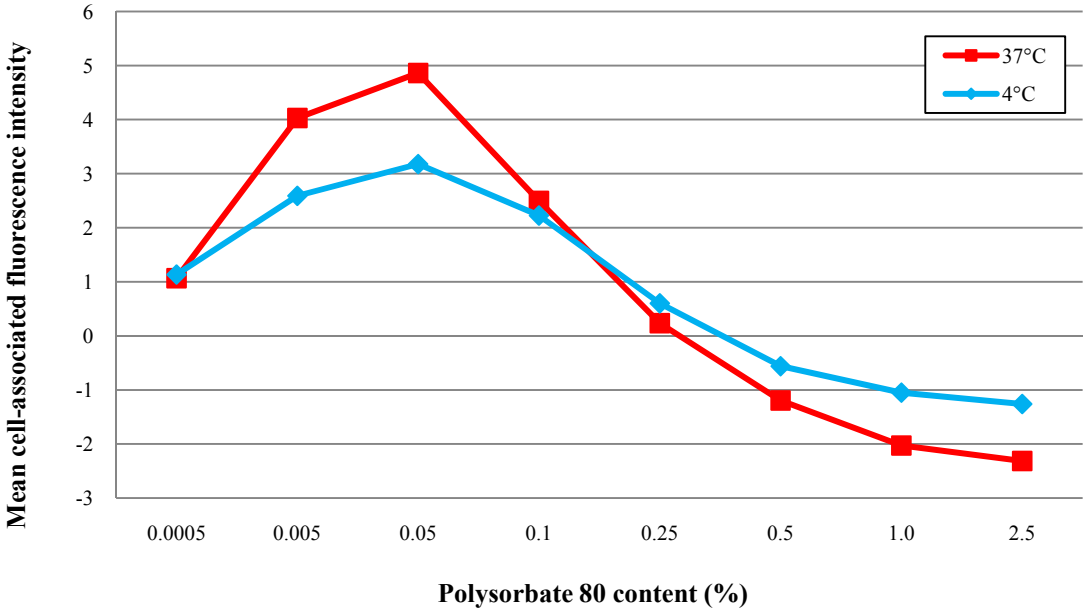
Figures

Figure 1 - Cell-association of NPs in presence of increasing amounts of Polysorbate 20.



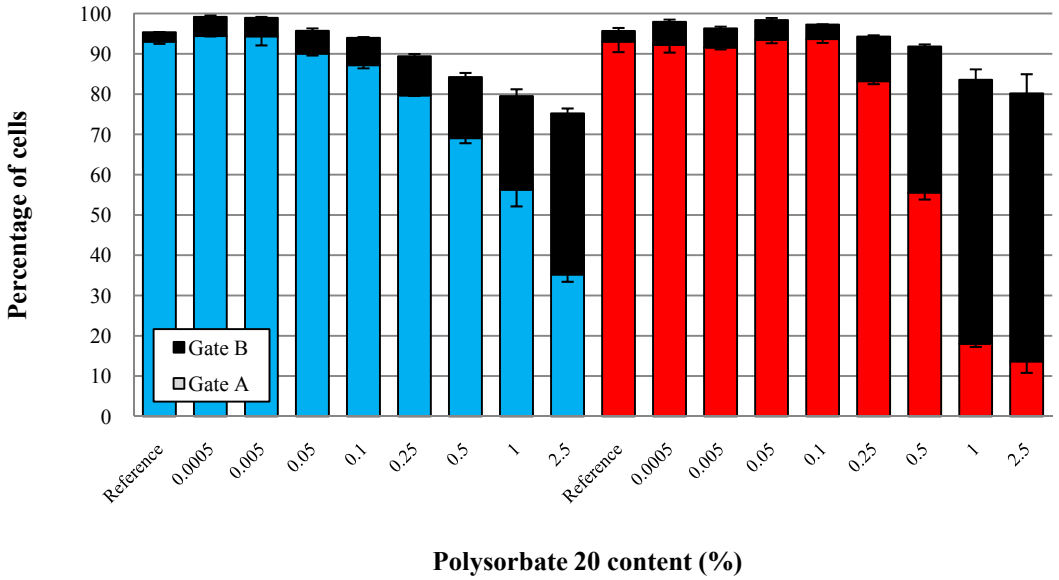
Caco-2 cells were incubated with a mixture containing a constant amount of NPs and increasing amount of surfactant at 4°C (red diamonds) or 37°C (blue squares), washed and analysed by flow cytometry (mean ± SD, n = 3)

Figure 2 - Cell-association of NPs in presence of increasing amounts of Polysorbate 80.



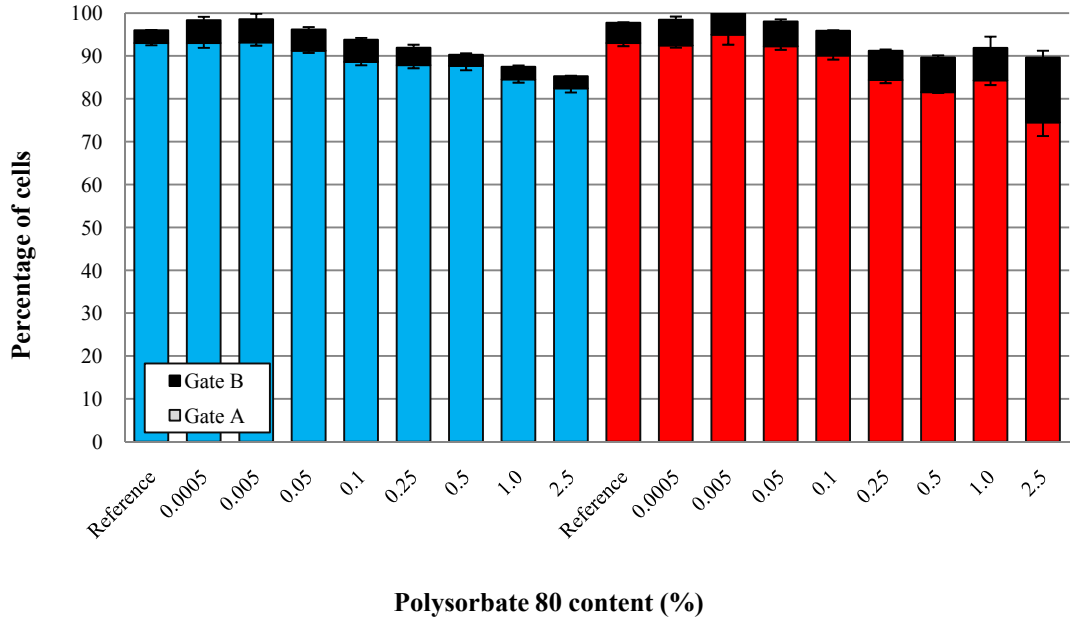
Caco-2 cells were incubated with a mixture containing a constant amount of NPs and increasing amount of surfactant at 4°C (red diamonds) or 37°C (blue squares), washed and analysed by flow cytometry (mean ± SD, n = 3)

Figure 3 - Viability of cells upon incubation with NPs and increasing amounts of Polysorbate 20.



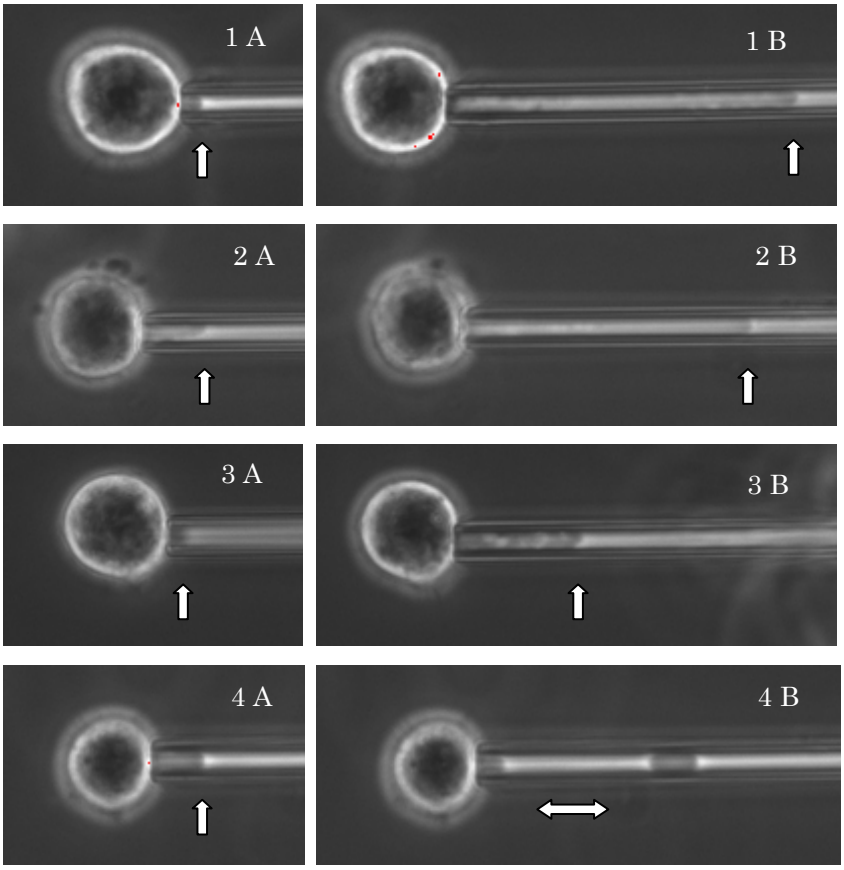
Caco-2 cells were incubated with a suspension containing constant amounts of NPs and increasing amounts of surfactant at 4°C (blue) or 37°C (red), washed and viability was analysed by flow cytometry. Viable cells were detected in gate A (coloured columns) and dead cells in gate B (black columns, mean ± SD, n = 3)

Figure 4 - Viability of cells upon incubation with NPs and increasing amounts of Polysorbate 80.



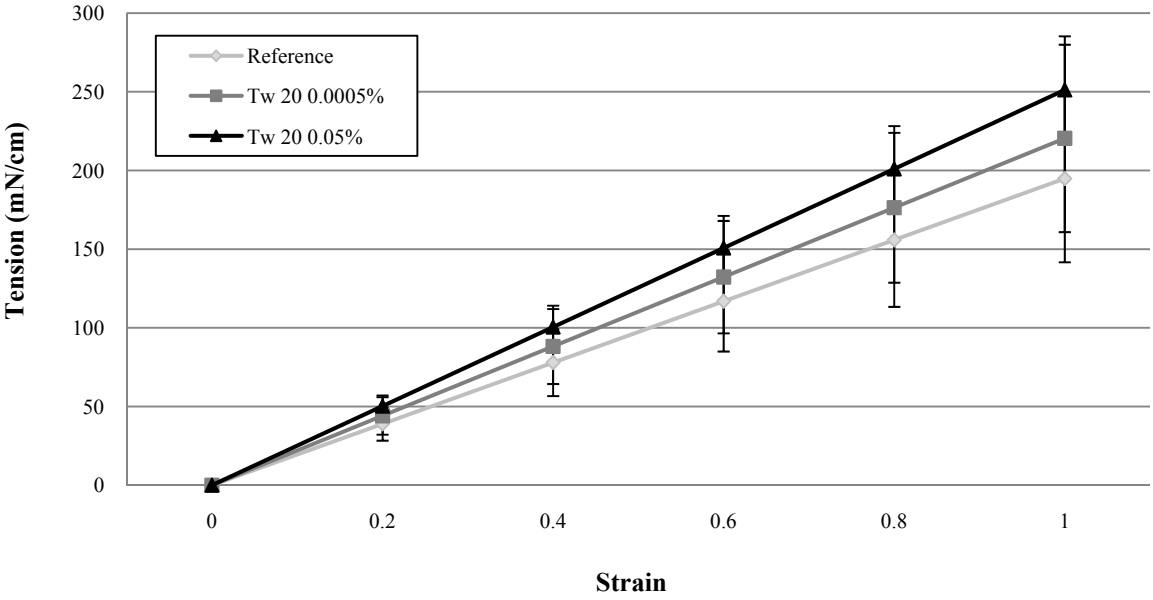
Caco-2 cells were incubated with a suspension containing constant amounts of NPs and increasing amounts of surfactant at 4°C (blue) or 37°C (red), washed and viability was analysed by flow cytometry. Viable cells were detected in gate A (coloured columns) and dead cells in gate B (black columns, mean ± SD, n = 3)

Figure 5 - Microaspiration of Caco-2 cells pre-incubated with different amounts of surfactants.



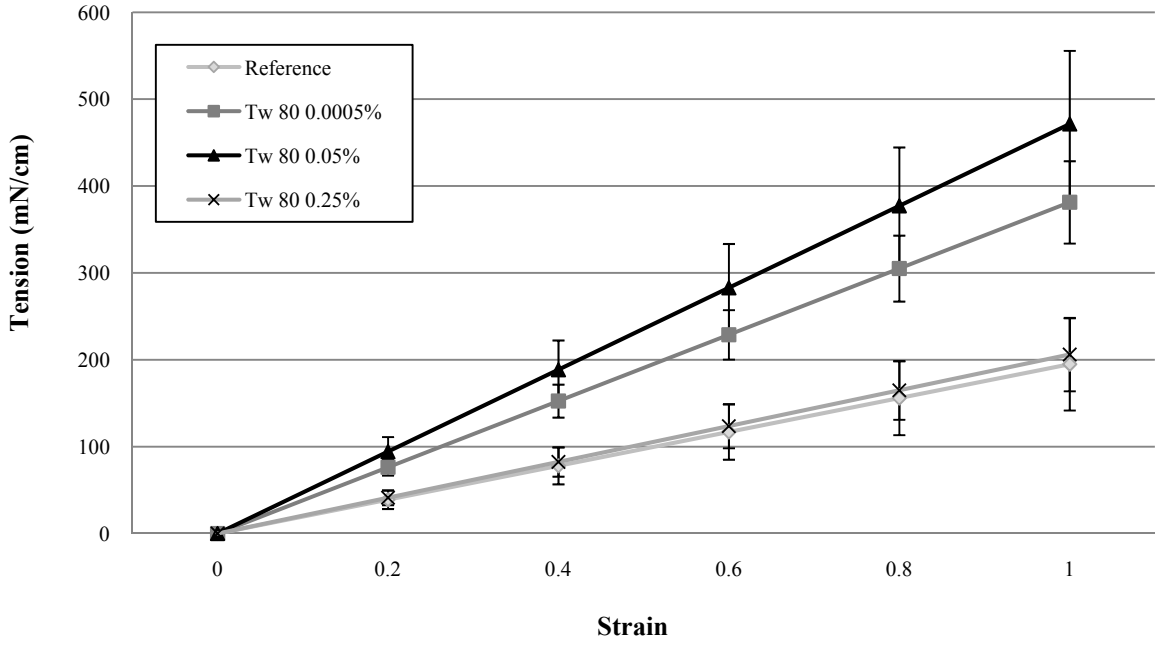
Caco-2 cells were incubated with isotone solutions of surfactants for 1h, mounted on the micropipette (column A) and a suction pressure of 1000 Pa was applied (column B), which results in differing shift of the apex of the cell membrane as indicated by the arrows. The images were acquired in absence of surfactant (A1, B1), and in presence of 0.05% Polysorbate 20 (A2, B2), 0.05% Polysorbate 80 (A3, B3) as well as 2.5% Polysorbate 80 (A4, B4; the horizontal arrow indicates the free space between the cell with extended membrane and a vesicle detached thereof). (n=8)

Figure 6 - Tension of the cell membrane in absence and in presence of varying concentrations of Polysorbate 20.



The data were calculated according to equation 1 and 2 from the pixels of the images acquired during micro-aspiration of Caco-2 cells without (diamonds) and with 0.0005% Polysorbate 20 (squares) or 0.05% Polysorbate 20 (triangles; mean ± SD, n = 8)

Figure 7 - Tension of the cell membrane in absence and in presence of varying concentrations of Polysorbate 80.



The data were calculated according to equation 1 and 2 from the pixels of the images acquired during micro-aspiration of Caco-2 cells without (diamonds) and with 0.25 % Polysorbate 80 (crosses), 0.0005% Polysorbate 80 (squares) or 0.05% Polysorbate 80 (triangles; mean ± SD, n = 8)

Poloxamer 188 interaction with cell membrane induces rigidity change; enhancement of nanoparticle-cell association

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Non-ionic surfactants such as Poloxamer 188 (Pluronic® F-68) are frequently used for preparation, stabilization or coating of nanoparticles (NP). According to reported effects on the dynamics of the cell membrane, this surfactant seems to be more than an inert excipient in Pharmaceutical Technology.

Upon incubation at 37°C, the association of fluorescent NPs (200nm) with Caco-2 cells was 2.7-fold higher in presence of 2.5% Poloxamer 188 than in surfactant absence as determined by flow cytometry. Pretreatment of the cells resulted in a further 5.3-fold increase in cell-associated NPs. This enhancing effect was also confirmed by fluorescence imaging of the cells, although some indifferent membrane staining was observed. The reason for this association-enhancing effect was elucidated by micro-aspiration of surfactant-treated cells revealing a considerable and concentration-dependent gain in stiffness of the cell-membrane. Exerting no cytotoxic but rather cytoprotective effects, the higher membrane rigidity due to adsoption and/or incorporation of Poloxamer 188 reduces undulations of the cell-membrane and thus repulsive forces against deposition of negatively charged NPs even at the negatively polarized cell membrane.

All in all, Poloxamer 188, an approved excipient for medical use, enhances the binding as well as uptake of NPs into cells and is expected to further pave the way towards successful therapy with nanoparticulate formulations.

Keywords: Caco-2, micro-aspiration, nanoparticle, Poloxamer 188, undulation.

Introduction

Nanoparticles (NPs) made from either drugs or drug-polymer mixtures attract increasing interest in pharmaceutical sciences as well as industry due to promising perspectives for overcoming several hurdles towards successful therapy. In case of nanoparticulate drugs, enhanced solubility of poorly absorbable compounds and thus increased bioavailability, improved stability as well as reduction of intra-individual fasted-fed differences were reported. Nano-scaled matrix systems can protect the incorporated drug from harmful biological environment, allow controlled release of the drug, overcome barriers against absorption and offer passive and/or active targeting of diseased tissue. Both subtypes of NPs share the common feature that preparation as well as stabilization usually requires addition of surfactants which can substantially modify influence the NP-surface on one hand.[1] On the other hand, the question arises whether some of the advantages of NPs, especially their binding to and uptake by cells, is influenced by presence of these amphiphilic excipients.

To elucidate this issue, the interaction between fluorescent NPs and cells in presence and absence of the commonly applied surfactant Poloxamer 188 (Pluronic® F-68, PF-68, Synperonic® PE/F68) is investigated. Poloxamers represent tri-block copolymers consisting of a hydrophobic poly(propylene oxide) (PPO) core and two hydrophilic poly(ethylene oxide) (PEO) branches arranged according to the structure $(PEO)_{n/2}$ – $(PPO)_m$ – $(PEO)_{n/2}$. In case of Poloxamer 188, 30 PO-units build up the core, while the total number of EO-groups ranges 152 yielding a mean molecular weight of 8400 Da.[2,3,4,5] This non-ionic surfactant with hydrophilic-lipophilic balance of 29[6,7] is approved by the FDA for various application routes e.g. for topical application in emulsions and for oral administration in suspensions up to 2.50 %.[8] It is widely used in nanoparticle production

as stabilizer due to adsorption on NP surface.[9] Poloxamer 188 represents not only an inert excipient but also a drug candidate. Administered i.v. as RheothRx in clinical trials it lowered intensity and duration of pain during vaso-occlusive crisis in sickle cell disease most likely due to reducing the viscosity of blood and enhancing microvascular flow.[10] It succeeded as well in reduction of myocardial infarct size.[11]

To assess the influence of the surfactant on cells, Caco-2 single cells were used. Although derived from a human colon carcinoma, these cells morphologically and functionally differentiate similar to human intestinal epithelial cells. The Caco-2 model is well established in pharmaceutical technology to study the binding, cellular uptake, and transport of drugs. This cell line is also recommended by the FDA for prediction of permeability according to the Bio-classification System of drug substances.[15] Applying this ex-vivo model, Poloxamer 188 was reported to significantly increase the apical to basolateral transport and to reduce the basolateral to apical efflux of epirubicin across Caco-2 monolayers by inhibition of intestinal P-glycoprotein.[12,13] The latter effect is exploited to enhance the absorption of immuno-suppressive sirolimus-NPs marketed as Rapamune®.[14]

Commercially available surfactant-free, well standardised fluorescent polystyrene NPs were used throughout the assays to exclude any size-related effects, to guarantee comparable particle-concentrations and to allow detection of cell-associated NPs by flow cytometry. Since more than 90 % all produced nanoparticles are negatively charged, carboxylated NPs exhibiting a zeta potential of -55mV were applied in this work.

The objective of this work was to elucidate the complex interplay between NPs, cells, and the approved nonionic surfactant by flow cytometric analysis at two different temperature levels to modulate the functionality of cells as well. Applying the micro-

aspiration technique, additional information was collected to conclude that Poloxamer 188 increases the stiffness of the cell membrane which is suggested to contribute to higher cell-association of NPs.

Experimental Section

Chemicals. Carboxylated fluorescent NPs (Fluoresbrite™ Carboxy YG microspheres 0.20 µm, 2.61 % solids, polystyrene, 0.210 ± 0.013 µm) were purchased from Polysciences, Inc. (Warrington, USA). Poloxamer 188 and 2-(4-(2-Hydroxyetyl)-1-piperazinyl)-ethanesulfonic acid (HEPES) were obtained from Sigma-Aldrich Chemie GmbH (Vienna, Austria). All other chemicals in use were of analytical grade.

Cell Culture. The human intestinal epithelial cell line Caco-2, was obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The cells (passage number 39-61) were grown in RPMI-1640 (Rosewell Park Memorial Institute) cell culture medium containing 10 % fetal calf serum (FCS), 4 mmol L-glutamine, and 150 µg/ml gentamycine in a humidified atmosphere of 5 % CO_2 and 95 % air at 37°C. Upon reaching ~ 70-80 % confluence, the cells were sub-cultured with TrypLE® select.

Fluorimetry. To detect any influence of the surfactant on the quantum yield of the NPs, the fluorescence intensity of all NP-suspensions (100 µl) was determined at 485/525 nm on a microplate reader (Infinite M200i, Tecan, Groedig, Austria).

To quantify free fluorophore in the supplied NP-suspension or its leakage due to presence of Poloxamer 188, NP-suspensions containing 0% or 5% surfactant (500 µl) were incubated with RPMI-medium (500 µl) for 60 min at 4°C or 37°C. The NPs were spun down (90 min, 14000 rpm, 4°C) and free/released fluorophore was determined in 100 µl supernatant as described above.

Flow Cytometry. The supplied NP suspension was diluted 1:80 with 20 mM isotonic HEPES/NaOH buffer pH 7.4 containing 0.0, 0.2, 1.0, 2.0 or 5.0 % (w/v) Poloxamer 188 to yield suspensions of 0.326 mg/ml carboxyl NPs.

To study the NP-cell interaction two different experimental setups were followed:

(i) Simple incubation: 50 µl cell suspension (5 x 10^6 cells/ml culture medium) and 50 µl NP-suspension (in isotonic HEPES/NaOH buffer pH 7.4 containing 0.0, 0.2, 1.0, 2.0 or 5.0 % (w/v) Poloxamer 188) were mixed and incubated for 60 min at 4°C or 37°C. Caco-2 cells incubated with a NP-suspension without any surfactant served as a reference. In order to remove non-bound NPs, the cells were centrifuged (5 min, 1000 rpm, 4°C) and washed twice with 150 µl PBS buffer. The cell pellet was resuspended in 1000 µl PBS buffer and analysed by flow cytometry (Epics XL MCL Flow cytometer; Coulter, Miami, USA) using a forward versus side scatter gate to include the single-cell population and to exclude cell debris and aggregates. Single live cells with membrane bound NPs were detected in gate A, whereas dead cells were accumulated in gate B. The cell associated fluorescence intensity elicited by bound NP was detected at 485/525 nm (Ex/Em). For each run, analysis data of 3000 cells was accumulated. All experiments were performed at least in triplicate.

(ii) Pulse-chase incubation: A mixture of 50 µl cell suspension (5 x 10^6 cells/ml culture medium) with 50 µl isotonic HEPES/NaOH buffer pH 7.4 containing 0.0, 0.2, 1.0, 2.0 or 5.0 % (w/v) Poloxamer 188 was pulse-incubated for 60 min at 4°C followed by centrifugation (5 min, 1000 rpm, 4°C), removal of the supernatant (70 µl) and addition of 20 µl isotonic HEPES/NaOH buffer pH 7.4. For the chase-incubation, the cell suspension (50 µl) was mixed with 50 µl NP-suspension in absence or presence of serial dilutions of Poloxamer 188 (0.0, 0.2, 1.0, 2.0 or 5.0 % (w/v)) and incubated for further 60 min at

either 4°C or 37°C. After a washing step, the cells were analysed by flow cytometry as described above.

Determination of Membrane Tension. The effect of Poloxamer 188 on tension of the cell membrane was investigated by the micropipette aspiration technique.[16] 50 µl cell suspension (5 x 10^6 cells/ml culture medium) was incubated with 50 µl 0.0%, 1.0 % or 5.0 % surfactant solution in 20mM isotonic HEPES/NaOH buffer pH 7.4 for 60 min at 37°C. Micropipettes pulled from borosilicate glass capillaries were filled with 20mM isotonic HEPES/NaOH buffer pH 7.4, mounted on a syringe and brought into close vicinity of a cell until the pipette touched the membrane. Subsequently, a moderate negative pressure was applied, just enough to attach the cell to the orifice of the micropipette and the resulting membrane apex was set as "zero tension state". Then, the suction pressure was increased stepwise (ΔP = -100 Pa) until the membrane was not expanding any more. At each step the cells and the patch pipette were imaged by a Zeiss Axiovert 200 microscope (Zeiss, Munich, Germany) using a 40x objective and a Hamamatsu camera (Hersching, Germany). All micromanipulations were performed at room temperature and each experiment was repeated at least eight times.

The apex length of aspirated cells was measured from the acquired images. These values were used to calculate strain (x-axis) and tension (y-axis) according to equation 1 and 2.[17] The areal strain (δA), relative to the "zero tension state", is a dimensionsless parameter and represents the object's deformation in one direction such as the amount of stretch or compression. It is calculated according to

$$\delta A \cong 2\pi R_p \left(1 - \frac{R_p}{R_c}\right) \Delta L \qquad \text{Eq. 1}$$

The tension (τ) is expressed as:

$$\tau = \frac{P\,R_p}{2 - 2\frac{R_p}{R_c}} \qquad \text{Eq. 2}$$

P = suction pressure

R_p = inner radius of the pipette

R_c = radius of the cell

ΔL = apex length difference.

The inclination of the resulting graph indicates the stiffness of the cell membrane. It is described by either the angle between the curve and the x-axis or the slope "m" from the general form of the equation for a straight line.

Fluorescence Microscopy. To visualize cell-associated NPs, 100 µl cell suspension (5 x 10^6 cells/ml culture medium) was incubated with 100 µl NP-suspension (in isotonic HEPES/NaOH buffer pH 7.4 containing 0.0 and 5.0 % (w/v) Poloxamer 188) for 60 min at 4°C. After removal of non-bound NPs by centrifugation (5 min, 1000 rpm, 4°C) and washing twice with 150 µl PBS buffer the cells were mounted for microscopy using a NIKON Eclipse 50i microscope (Nikon Corporation, Japan) at 40x magnification, equipped with EXFO-Xcite 120 fluorescence illumination system and NIKON camera Digital Sight DS-SM (Nikon Corporation, Japan).

Toxicity Tests. The influence of the surfactant on the viability of cells was examined by two methods: (i) the ratio between live/dead cells accumulated in gate A/gate B according to the flow cytometric histograms and (ii) the membrane permeability for propidium iodide which results in staining of the nucleus of only dead cells. In order to avoid any interference of the fluorescence emission of propidium iodide with that of the fluorescent NPs upon flow cytometric analysis, the viability assay was performed in absence of NPs. In brief, 50 µl cell suspension and 50 µl isotonic HEPES/NaOH-buffer pH 7.4 containing increasing amounts of Poloxamer 188 (0.0, 0.2, 1.0, 2.0 or 5.0 % (w/v))

were incubated for 1h at 4°C or 37°C, followed by washing, addition of 2.7 µl propidium iodide solution (0.1 mg/ml in PBS) and flow cytometry after incubation for 2 min. For control, reference samples were treated with 100 µl methanol pre-cooled at –20°C to yield 100% dead cells.

Results and Discussion

Fluorimetry. Since flow cytometry was applied to elucidate the influence of Poloxamer 188 on the particle-cell interaction, any interference between the quantum yield of fluorescence labelled NPs and the surfactant had to be excluded to guarantee comparability of the results. According to preliminary assays the fluorescent NP-suspension yielded 23560 ± 540 RFI (relative fluorescence intensity) which remained unchanged upon addition of the surfactant in the concentration range investigated.

Additionally, to take into account that flow cytometric analysis cannot discriminate between cell-associated fluorescence due to attachment of fluorescent nanoparticles and cell-associated fluorescence due to cellular uptake of excessive free dye present in the stock NP-preparation or leaked from the stock preparation by time, the amount of free fluorophore in NP-suspension was determined. Only small amounts of free dye were detected in the supernatant of the NP-suspension amounting to 0.64 % (4°C) or 0.81 % (37°C) of the total fluorescence signal in absence of surfactant after incubation for 60 min. In presence of 2.5 % Poloxamer 188 and the same incubation time this percentage slightly increased to 1.08 % (4°C) or 1.52 % (37°C), presumably due to improved solubilization of the hydrophobic dye by the non-ionic surfactant.

Particle-Cell Interaction in Absence of Poloxamer 188. The NP-cell interaction is reported to be determined by the size, the charge and the surface modification of the

particles. Since carboxyl-NP with zeta potential of -55mV were applied in this study, the interaction between negatively charged NPs and the negatively charged cell surface might be hindered due to electrostatic repulsion.[18,19] In addition, according to the results, the incubation temperature represents another important parameter influencing the association of NPs with cells (Table 1).

Upon incubation at 4°C the fluidity of the cell membrane and the metabolism is reduced and energy consuming transport processes such as endocytosis are repressed. In contrast, at the physiological temperature of 37°C the metabolism of the cells reaches its optimum activity and multiple uptake processes can occur. Consequently, NP-binding to the cell membrane prevails at 4°C, whereas incubation at 37°C allows both binding and uptake, referred to as association of NPs.[20] Additionally, accelerated Brownian motion of particles at higher temperature is supposed to increase the contact events between NPs and cells. In accordance with these parameters, the cell associated fluorescence intensity upon incubation at 37°C exceeded that at 4°C 1.2-fold indicating uptake of negatively charged NPs.

Particle-Cell Interaction in Presence of Poloxamer 188. At both temperature levels investigated, the amount of cell-associated NPs as indicated by the mean cell-associated fluorescence intensity increased concurrent with increasing Poloxamer 188 concentration (Table 1). But the slope of the binding curve was dependent on the temperature level: As compared to the reference without surfactant, the mean cell-associated fluorescence intensity increased from 1.6-fold (0.5 % tenside) to 2.4-fold (2.5 % surfactant) at 4°C, but from 1,8-fold (0.5 % tenside) to 3.7-fold (2.5 % surfactant) at 37°C. This discrepancy points to binding followed by uptake of carboxyl-NP at 37°C higher temperature in spite of repulsively charged cell membrane and NPs.

Interestingly, the NP-association was improved even at 4°C although the metabolism of the cell should reach a minimum and it increased with surfactant concentration too. According to the literature this phenomenon might be explained by the fact that Poloxamer 188 might be incorporated into the cell membrane so that the characteristics or even the strength of the cells is altered.[21] There are also reports that Poloxamers induce drastic changes in microviscosity of the cell membrane microviscosity.[22,23]

Tension of Cell Membrane. To elucidate basically the effects of Poloxamer 188 on membrane stiffness, the micropipette aspiration technique was chosen. This technique provides information about the mechanical properties of living cells[16] and allows calculation of membrane stiffness. The underlying principle is that the cell membrane and the cytoskeleton are sucked into a pipette by stepwise increasing negative pressure. The shift of the apex of the cell and its length correlate with the stiffness of the cell membrane (Figure 1). The analysis of the data presented as inclination gradients of the regression lines at each pressure applied (Figure2) show a steeper slope in case of Poloxamer 188-treated cells as compared to non-treated cells. This clearly points to the fact that the cell membrane gained in stiffness after contact with surfactant in a concentration dependent manner. Extrapolation of the data resulted in a tension of 243.95 ± 38.73 mN/cm (0.5% Poloxamer 188) and 264.28 ± 58.09 mN/cm (2.5% Poloxamer 188) at the apparent strain = 1.0.

According to the literature the membrane of eucaryotic cells is not a static envelope but exhibits wave-like movements, the so-called undulations. They occur over large cell membrane areas, with low amplitude and high frequency and contribute to cell motility as well as physical sensing of the environment.[24] As Poloxamer 188 is reported to adsorb onto the cell membrane, to be incorporated into the cell membranes or even accumulate

inside the cells,[21,22] Poloxamer 188 exerts protective effects on cells in agitated bioreactors by hydrodynamic modulation. In addition, resealing of the cell membrane and repair is increased by the surfactant.[25]

Interestingly, the NP-association increased with the surfactant concentration, which is direct proportional to the increase in membrane stiffness. Thus it is suggested that adsorption and/or incorporation of the surfactant reduce the undulatory movements of the cell membrane. This assumption is also confirmed by the observation that cells round up in presence of Poloxamer 188 and the cell volume is increased by about 5%[26] due to strengthening of the cell membrane.[27] Considering the available literature and the results of micro-aspiration it is suggested that the interaction of the cell membrane with Poloxamer 188 reduces the undulations of the cell membrane and thus repulsive forces against deposition of the NPs. It is likely that suppression of membrane oscillations facilitates the adsorption and/or uptake of the NPs into the cell as reflected by the results of the NP-cell association studies.

Pulse-Chase Incubation. In order to further confirm the proposed mechanism, the cell membranes were first loaded with Poloxamer 188 followed by incubation with NP-surfactant suspension according to a pulse-chase protocol. In order to allow comparison of the results of the simple incubation and the pulse-chase protocol they are presented in one figure (Figure 3).

According to flow cytometric analysis, upon incubation at 4°C even at the lowest surfactant concentration of 0.1% the amount of cell-associated NPs increased 1.6-fold upon pre-treating the cell-membrane with surfactant followed by incubation with NP-Poloxamer 188 suspension as compared to one-step incubation. This tendency further increased throughout all the tenside concentrations to end up in a 4.9-fold increase in NP-

association at 2.5% Poloxamer 188. A similar trend was observed upon pulse-incubation at 4°C and chase-incubation at 37°C (4°C / 37°C). At 0.1% Poloxamer 188 pre-loading of the cell-membrane with surfactant even doubled the amount of cell-associated NPs upon incubation at 37°C as compared to incubation without pre-loading the cell-membrane with surfactant (Figure 3). Again, the difference in amount of cell-associated NPs increased with the Poloxamer 188 concentration as indicated by a five-fold amount of cell-associated NPs at 2.5% surfactant. Surfactants can modify not only physical properties of NPs[1,29] but also physiological ones of the cells. In culture medium containing Poloxamer 188, the membrane tension, the strength of the membranes and the mean elastic area compressibility modulus of cells were significantly higher.[21] As pre-treatment of the cell-membrane with Poloxamer 188 further increased the association of NPs, the theory of "suppressed undulation" and facilitated association of NPs is underlined.

Fluorescence Microscopy. Imaging of cells incubated with fluorescent NPs in presence of Poloxamer 188 further confirmed increased cell-association of NPs as compared to incubation without surfactant (Figure 4). Nevertheless, the images show some staining of the cell membrane which might be due to incorporation of minimal amounts of hydrophobic dye deriving from free fluorophore in the NP-preparation, dye leakage from the NPs or uptake of the dye released from adsorbing NPs. As only 0.64 – 1.52% free dye was present in the NP-preparations, the first case is unlikely to occur. Although the reason for indifferent membrane staining could not be clarified, the contribution of Poloxamer 188 to indifferent membrane staining should not be underestimated since amphiphilic polymers are known to disturb lipid bilayers and to

accelerate the flip-flop movements in liquid phospholipid membranes[2,3] which enhanced permeability of the cell-membrane for biologically active substances.

Cytotoxicity of Poloxamer 188. To omit any false conclusions from the experiments, the cytotoxicity of Poloxamer 188 was assessed. As healthy cells have distinct shape and size, the gates in flow cytometer's histogram were set to accumulate viable cells gate A, whereas damaged cells exhibit deformations and altered size, form cell-aggregates and debris. Consequently, gate B was set to display dead cells and debris. The Caco-2 toxicity of the surfactant is indicated by the percentage of live and dead cells upon incubation at the two temperature levels applied (Table 2). Independent from the protocol and the temperature level of incubation, the number of viable cells differed 3% at the maximum at all applied Poloxamer 188 concentrations as compared to non-treated cells. In addition, the cytoprotective properties of the surfactant became apparent upon pulse-chase incubation. At this, all applied surfactant concentrations except for the highest, retained more viable cells in comparison to the reference. The preserved vitality of the cells due to presence of Poloxamer 188 can be explained by the cytoprotective effects against physical damages caused by shear stress at high agitation speed.[29,30] In this experiment, the cells seem to be protected by the surfactant against damage due to experimental handling or shear stress in flow chamber of the cytometer.[31]

In addition, propidium iodide staining of dead cells was negligible amounting to 0.1 RFI as compared to even 8 – 10 RFI in case of the negative control which also confirmed that exclusively viable cells were accumulated in gate A. Thus, both viability tests proved that neither NPs nor Poloxamer 188 exert any toxic effect to the cells within the applied concentration levels.

Conclusion

This study presents a new aspect of Poloxamer 188 potentially beneficial for improved colloidal drug delivery. Being non-toxic up to 2.5%, the surfactant is adsorbed and/or incorporated into the cell-membrane. Resulting in gain in stiffness, the undulations of the cell membrane and thus repulsive forces against NP-deposition at the cell-membrane are reduced. The cell-association of NPs increased with surfactant concentration and was observed at 4°C as well as at 37°C, even in spite of negatively charged NPs being opposite to that of the cell membrane. As the effect of enhancing the cell-association of NPs was pronounced at even low concentrations, it is supposed that the presence of Poloxamer 188 at concentrations approved for intravenous or peroral administration might increase NP-binding and uptake at least at the site of administration or even improve systemic uptake of drug loaded NPs until dropping below the lower effective Poloxamer 188 concentration due to dilution with body fluids. All in all, Poloxamer 188 might represent a key-component in NP preparations to approach the aim to attain therapeutically relevant plasma levels with drug-loaded nanoparticulate formulations.

Acknowledgements. We thank Mrs. Susanne Braunmueller for helping us learn the micropipette aspiration technique and Mr. Daniel Steppich.

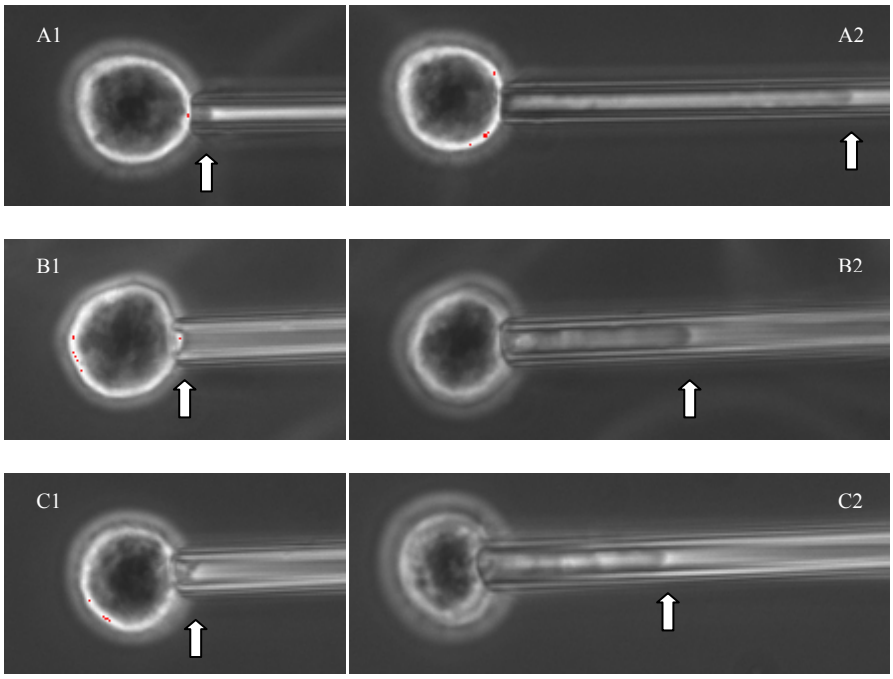


Figure 1: Images of microaspiration of the cells in Poloxamer 188 absence (A) and with 0.5% (B) and 2.5% (C) Poloxamer 188 presenting different cell membrane extension as response to "zero tension state", 0 Pa (A1, B1, C1) as well as negative pressure of 1 kPa (A2, B2, C2).

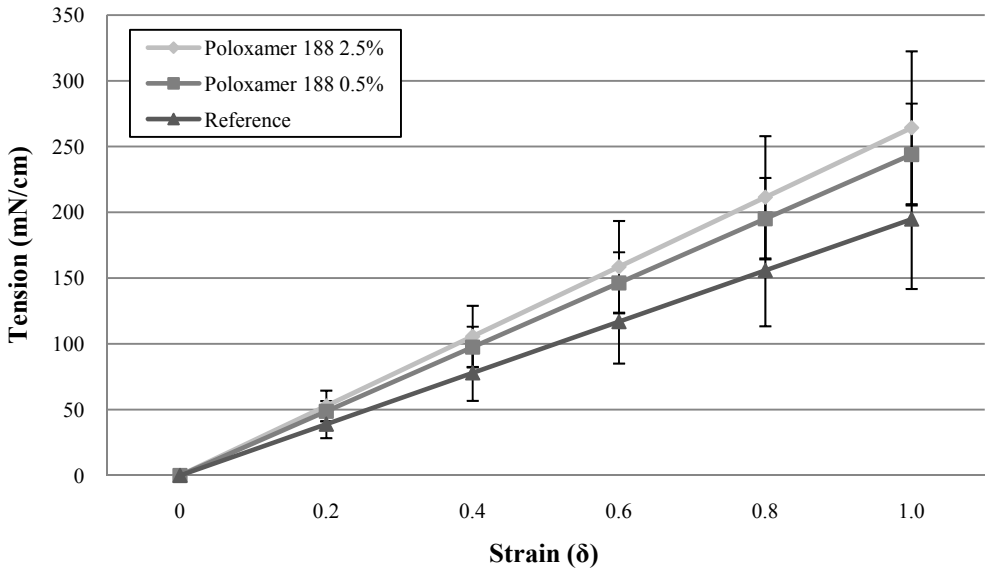


Figure 2: The inclination gradients of cell membrane aspiration, after 60 minutes incubation upon 37°C with 0.5% Poloxamer 188 (squares) and 2.5% Poloxamer 188 (diamonds); reference is marked with triangles. (n= 9; mean ± SD)

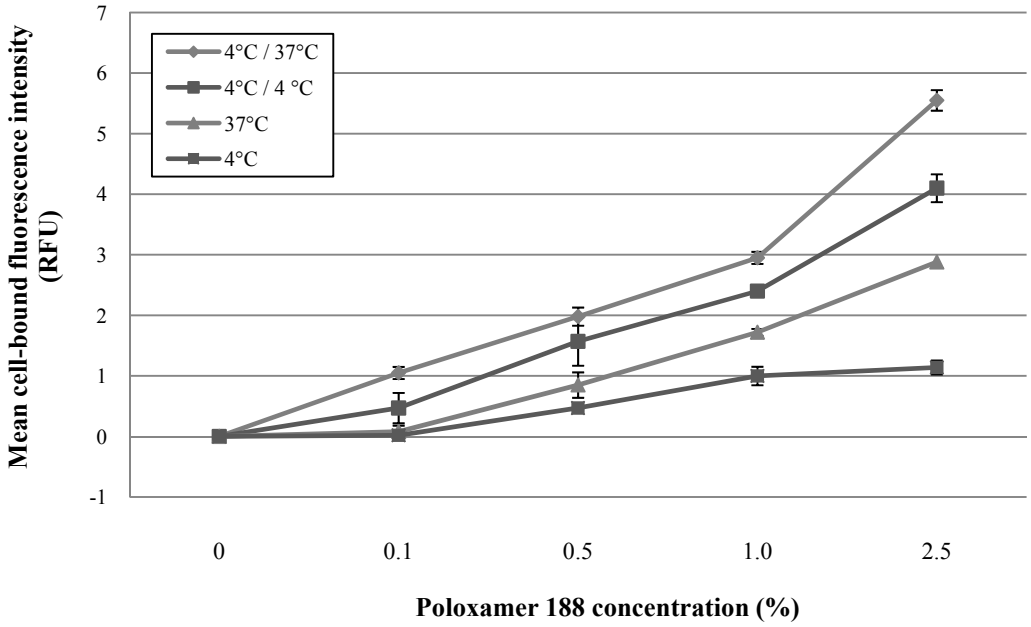


Figure 3: Flow cytometric readings of mean fluorescence intensity of interaction between carboxyl-NP and single cells upon incubation with Poloxamer 188 at 4°C (crosses) and 37°C (triangles) and the mean fluorescence intensity with preincubated cells at 4°C and main incubation at 4°C (squares) and at 37°C (diamonds). The reference values are subtracted. (n= 3; mean ±SD)

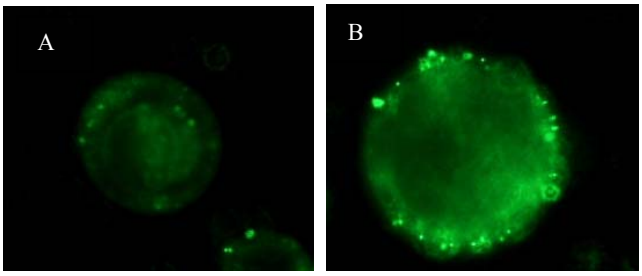


Figure 4: Fluorescent microscopy images of the NP-cell interaction after the cells were incubated with NP-suspensions 60 min at 4°C with 0.0% (A) and 2.5% (B) Poloxamer 188.

Table 1: Mean cell-associated fluorescence intensities due to interaction of carboxyl-NP with Caco-2 single cells upon incubation with Poloxamer 188 at 4°C and 37°C, respectively. (n=3, mean ± SD)

Poloxamer 188 concentration (%)	Mean fluorescence intensities of cell bound carboxyl NP (RFU)	
	4°C	37°C
0.00	0.83 ± 0.01	1.05 ± 0.06
0.10	0.85 ± 0.01	1.13 ± 0.06
0.50	1.30 ± 0.01	1.90 ± 0.10
1.00	1.83 ± 0.15	2.75 ± 0.21
2.50	1.97 ± 0.12	3.93 ± 0.06

Table 2: Flow cytometric readings of cell viability upon incubation with carboxyl-NP-Poloxamer 188 suspension at 4°C and at 37°C, and upon pulse chase incubation with a carboxyl-NP-Poloxamer 188 suspension at 4°C and at 37°C presented through distribution of live cells (Gate A) and dead cells (Gate B) at different surfactant concentrations. (n= 3, mean ± SD)

Incubation temperature (°C)	Poloxamer 188 concentration (%)	Carboxyl NP		Carboxyl NP (pulse chase)	
		Live cells	Dead cells	Live cells	Dead cells
4°C (4°C/4°C)	0.00	88.94 ± 0.90	4.15 ± 0.16	78.10 ± 2.50	10.57 ± 0.47
	0.10	88.12 ± 0.89	3.67 ± 0.46	80.25 ± 0.63	5.60 ± 0.63
	0.50	87.29 ± 0.64	3.57 ± 0 56	80.85 ± 0.58	7.36 ± 0.42
	1.00	87.56 ± 0.92	3.58 ± 0.53	80.70 ± 1.46	7.30 ± 0.65
	2.50	85.86 ± 0.71	4.07 ± 0.35	77.13 ± 1.26	6.49 ± 0.87
37°C (4°C/37°C)	0.00	90.54 ± 0.13	2.96 ± 0.14	79.13 ± 3.75	8.14 ± 2.42
	0.10	88.23 ± 0.98	2.58 ± 0.23	83.00 ± 0.60	4.97 ± 0.86
	0.50	89.11 ± 0.87	2.52 ± 0.14	84.41 ± 0.90	4.06 ± 0.45
	1.00	88.90 ± 1.43	2.97 ± 0.25	82.36 ± 4.13	5.13 ± 1.44
	2.50	87.88 ± 1.45	3.29 ± 0.29	76.00 ± 1.04	5.64 ± 0.48

References:

(1) Ratzinger, G.; Laenger, U.; Neutsch, L.; Pittner, F.; Wirth, M.; Gabor, F. *Langmuir* **2010**, *26*, 1855-1859.

(2) Yaroslavov, A. A.; Melik-Nubarov, N. S.; Menger, F. M. *Accounts of Chemical Research* **2006**, *39*, 702–710.

(3) Demina, T.; Grozdova, I.; Krylova, O.; Zhirnov, A.; Istratov, V.; Frey, H.; Kautz, H.; Melik-Nubarov N. S. *Biochemistry* **2005**, *44*, 4042–4054.

(4) Gigout, A.; Buschmann, M. D.; Jolicoeur, M. *Biotechnology and Bioengineering* **2008**, *5*, 975-987.

(5) Kabanov, A. V.; Batrakova, E. V.; Miller, D. W. *Advanced Drug Delivery Reviews* **2003**, *55***,** 151–164.

(6) Blanco, D.; Alonso, J. D. *Eur.J.Pharm.Biopharm* **1998**, *45*, 285-294.

(7) Moghimi, S. M.; Hunter, A. C. *Trends in Biotechnology* **2000**, *18*, 412–420.

(8) www.accessdata.fda.gov/scripts/cder/iig/getiigWEB.cfm (23.04.2009)

(9) Vauthier, C.; Bouchemal, K. *Expert Review* **2009**, 26, 1025-1058

(10) Adams-Graves, P.; Kedar, A.; Koshy, M.; Steinberg, M.; Veith, R.; Ward, D.; Crawford, R.; Edwards, S.; Bustrack, J.; Emanuele, M. *Blood* **1997**, *90*, 2041–2046.

(11) Schaer, G. L.; Spaccavento, L. J.; Browne, K. F.; Krueger, K. A.; Kirchbaum, D.; Phelan, J. M.; Fletcher, W. O.; Grines, C. L.; Edwards. S.; Jolly, M. K.; Gibbons, R. J. *Circulation* **1996**, *94***,** 298–307.

(12) Batrakova, E. V.; Han, H. Y.; Alakhov, V. Yu.; Miller, D. W.; Kabanov, A. V. *Pharmaceutical Research* **1998**, *15,* 850-855.

(13) Batrakova, E. V.; Li, S.; Alakhov, V. Yu.; Elmquist, W. F.; Miller, D. W.; Kabanov, A. V. *Pharmaceutical Research* **2003**, *20,* 1581–1590.

(14) www.hippocratesforum.de/pharmazie-DE-13364-drug-rapamune_1mg_tabletten.html, 22.02.2010

(15) Shah, P.; Jogani, V.; Bagchi, T.; Misra, A. *Biotechnol. Prog.* **2006**, *22,* 186-198.

(16) Hochmuth, R. M. *Jr of Biomechanics* **2000**, *33***,** 15–22.

(17) Evans, E.; Rawicz, W. *Physical Rewiev Letters* **1990**, *64*, 2094-2097.

(18) Shinkai, M.; Yanase, M.; Honda, H.; Wakabayashi, T.; Yoshida, J.; Kobayashi, T. *Jpn Jr of Cancer Research* **1996**, *87*, 1179–1183.

(19) Ito, A.; Shinkai, M.; Honda, H.; Kobayashi, T. *Jr of Bioscience and Bioengineering* **2005**, *100*, 1-11.

(20) Wirth, M.; Kneuer, C.; Lehr, C. M.; Gabor, F. *Jr of Drug Targeting* **2002**, *10*, 439–448.

(21) Zhang, Z.; Al-Rubeai, M.; Thomas, C. R. *Enzyme Microb. Technol.* **1992**, *14,* 980–983.

(22) Melik-Nubarov, N. S.; Pomaz, O. O.; Dorodnych, T. Yu.; Badun, G. A.; Ksenofontov, A. L.; Schemchukova, O. B.; Arzhakov, S. A. *FEBS Letters* **1999**, *446,* 194–198.

(23) Batrakova, E. V.; Li, S.; Vinogradov, S. V.; Alakhov, V. Y.; Miller, D. W.; Kabanov, A. V. *The Jr of Pharmacology and Experimental Therapeutics* **2001**, *299,* 483–493.

(24) Partin, A. W.; Schoeniger, J. S.; Mohler, J. L.; Coffey, D. S. *Cell Biology* **1989**, *86* 1254-1258.

(25) Togo, T.; Alderton, J. M.; Bi, G.; Steinhardt, R. A. *Jr of Cell science* **1999**, *112,* 719–731.

(26) Hellung-Larsen, P. *Jr of Biotechnology* **2005**, *115,* 167–177.

(27) Raucher, D.; Sheetz, M. P. *The Jr of Cell Biology* **1999**, *144*, 497–506.

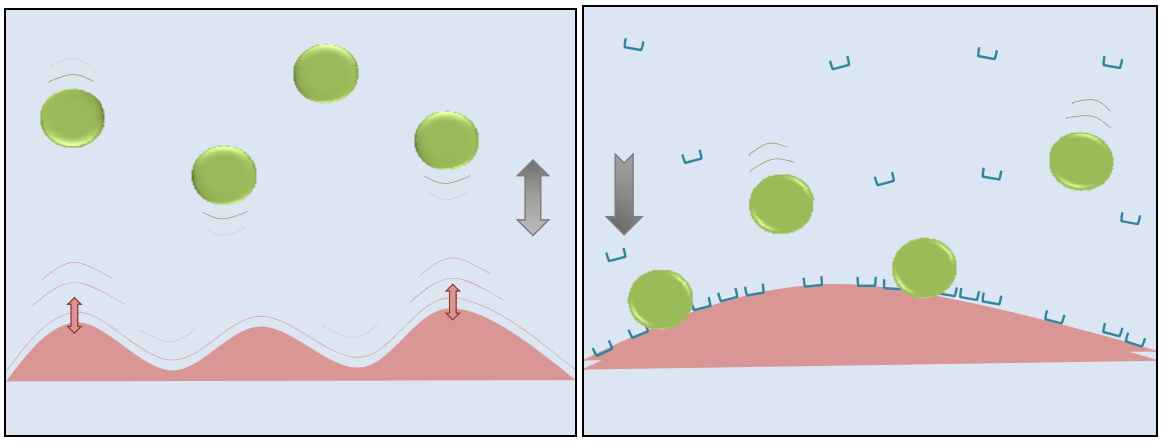
(28) Fillafer, C., Wirth, M.; Gabor, F. *Langmuir* **2007**, *23,* 8699-8702.

(29) Hellung-Larsen, P.; Assaad, F.; Pankratova, S.; Saietz, B. L.; Skovgaard, L. T. *Jr of Biotechnology* **2000**, *76,* 185–195.

(30) Xu, D.; Wu, T.; Zhang, Y.; Chen, Y. *Chinese Jr of Biotechnology* **1995**, *11,* 101–107.

(31) Al-Rubeai, M.; Emery, A. N.; Chalder, S.; Goldman, M. H. *Jr of Biotechnology* **1993**, *31,* 161–177.

Synopsis:



3.3 Third Part

POLOXAMER 188 SUPPLEMENTED CULTURE MEDIUM INCREASES THE VITALITY OF CACO-2 CELLS AFTER SUBCULTIVATION AND FREEZE/THAW CYCLES

V. Kerleta, I. Andrlik, S. Braunmüller, T. Franke, M. Wirth, F. Gabor

Submitted to *ALTEX*

Poloxamer 188 supplemented culture medium increases the vitality of Caco-2 cells after subcultivation and freeze/thaw cycles

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Abstract:

Cryoconservation media containing 1% of the non-ionic surfactant Poloxamer 188 provided full recovery of mammalian cells (Gonzalez Hernandez, 2006), but the effects during thawing of cryostored cells and proteolytic subcultivation are still unknown. At this, the proliferation and viability of pre-confluent Caco-2 monolayers cultivated in media supplemented with the non-ionic surfactant were investigated. The results revealed that the addition of 0.5% Poloxamer 188 increases proliferation of subcultivated cells 1.5 fold and that of thawed cells about twofold. According to microaspiration experiments the non-ionic surfactant increases the tension of the cell membrane most notably at concentrations ≤ 0.5% because of adsorption and incorporation into the phospholipid bilayer. Thus, the performance of the cells is suggested to be improved. Since vitality of cells is a prerequisite for reproducibility and reliability of cell models for absorption studies at early stages of drug development, usage of Poloxamer 188 supplemented cultivation media will help to refine cell culturing to further reduce animal trials in preclinical investigations.

Keywords: Poloxamer 188; Caco-2 cells; proliferation; cell membrane undulation; micropipette aspiration

1 Introduction

In order to reduce animal trials during preclinical evaluation of APIs (active pharmaceutical ingredient) as well as to circumvent clinical verification of the bioequivalence of certain formulations, the biopharmaceutics classification system (BCS) is recommended by the FDA (Food and Drug Administration) as well as the EMEA (European Medicines Agency) (Kim et al., 2006). The monolayer forming Caco-2 cell line is an integral part of this biowaiver to assess the permeability of APIs across artificial human intestinal epithelium mimicking the process of absorption (Artursson et al., 2001). The accuracy of predicting human absorption is about 60% (Sachan et al., 2009).

The reproducibility and reliability of such assays strongly depend on the viability of the cells. Routine cultivation, however, requires procedures which are unfavourable to live cells: Subcultivation of cells might harm or damage the cell membrane in course of the proteolytic detachment of adherent cells. Even mild manipulation of cells might be injurious because of exposition to shear forces during pipetting. Cryostorage of cells comprises freezing and formation of ice crystals inside and outside the cell which may disrupt cell membranes. In turn, recrystallisation during thawing can also be detritimental for cell survival (Woods et al., 2004). Out of these shortcomings and to strengthen the relevancy of cell models the currently used media should be improved.

In terms of cryostorage, all BALB/c myeloma cells stored in medium containing 1% Poloxamer 188 survived freezing/thawing as compared to 80% without supplement (Hernandez and Fischer, 2007). Meanwhile, this cryomedium is commercially available (Filoceth™-media, procryotect GmbH, Ruedlingen, Switzerland) and it was suggested that the surfactant stabilizes the cell membrane.

Poloxamer 188 (Pluronic® F-68, PF-68) is a non-ionic surfactant with an average molecular weight of 8400 Da built up of EO (polyoxyethylene) and PO (polyoxypropylene) units being arranged in a basic triblock structure according to EO_{76}-PO_{30}-EO_{76}. The non-toxic surfactant is approved by the

FDA (www.accessdata.fda.gov/scripts/cder/iig/getiigWEB.cfm, 2009), and has been reported to exert cytoprotective effects e.g. higher viability of mammalian cells under high agitation (Al-Rubeai et al., 1993) and multiple protective effects on Tetrahymena cells exposed to various physical and/or chemical stress parameters (Hellung-Larsen et al., 2000). The cytoprotective effects were explained by a complex interaction between the cell membrane and Poloxamer 188 (Al-Rubeai et al., 1993). However, sometimes contradictory findings were reported in terms of the tension of the cell membrane (Togo et al., 1999; Zhang et al., 1992).

The aim of this work was to elucidate some additional advantageous effects of Poloxamer 188 supplemented media on subcultivated and thawed cells after cyrostorage using proliferation and viability as indicative parameters for cytoprotection. Furthermore, to shed some light on the mechanisms of cytoprotection as well as to confirm the explanation of cryoprotective effects of Poloxamer 188 cell membrane tension measurements were performed.

2 Materials and methods

2.1 Chemicals

Poloxamer 188 was obtained from Sigma-Aldrich Chemie GmbH (Vienna, Austria). All other chemicals in use were of analytical grade.

2.2 Cell culture techniques

Caco-2 cells were obtained from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). The cells were grown in RPMI-1640 (Rosewell Park Memorial Institute) cell culture medium containing 10% fetal bovine serum (FBS), 4 mmol L-glutamine, and 150 µg/mL gentamycine in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Upon reaching ~ 80 - 90% confluence the cells were subcultured with TrypLE® Select and seeded for the proliferation and viability studies.

For cryopreservation 2.5 x 10^6 cells were suspended in 1 ml cryo-medium (RPMI-medium supplemented as above but with additional 10% FBS and 10% DMSO (dimethyl sulphoxide)). After equilibration in a Nalgene® Mr.Frosty Cryobox (Thermo Fisher Scientific, Roskilde, Denmark) for 25 min. at -20°C the cryovials were stored at -80°C for at least one week. For thawing, the cryovials were warmed up as fast as possible in water at 37°C. Then, the cell suspension was transferred into 10 ml cell culture medium of 37°C, spun down (1000 rpm, 4°C, 5 min.), and the supernatant containing cytotoxic DMSO was discarded. After resuspension in fresh cell culture medium, the cells were seeded for proliferation and viability studies.

2.3 Proliferation and viability tests

The proliferative activity of the cells was determined using the BrdU cell proliferation ELISA test kit (Roche diagnostics GmbH, Vienna, Austria) according to the manufacturer's instructions. Immediately after splitting or thawing the cells were seeded in a 96-well microplate (Iwaki, Bertoni, Vienna, Austria) at a density of 8 x 10^3 cells in 200 µl medium per well and cultivated for 2 or 3 days under cell culture conditions. The medium consisted of 20 µl sterile-filtered (0.22 µm pore diameter) surfactant solution in 20 mM isotone HEPES/NaOH buffer pH 7.4 or buffer alone and 180 µl cell culture medium resulting in 0, 0.2, 0.4, 0.6, 0.8 or 1.0% Poloxamer 188 content. The DNA-incorporation of 5-brom-2-desoxyuridine (BrdU) into proliferating cells was quantified using a microplate reader (Spectrafluor-reader, Tecan, Grödig, Austria) at 450 nm.

The viability of cells was analyzed via the EZ4U test (Easy for you, Biomedica, Vienna, Austria) following the manufacturer's instructions. At this, the splitted cells were seeded in 96-well microplates at a density of 3000 cells/well in 200 µl medium as above and cultivated for 3 days in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

2.4 Cell membrane tension determination

The micropipette aspiration technique was applied to determine the stiffness of the cell membrane (Hochmuth, 2000). Micropipettes were pulled from borosilicate glass, filled with PBS, mounted on a syringe and connected with a fluid reservoir. 50 µl cell suspension in culture medium containing 2.5 x 10^5 cells was incubated with 50 µl 0%, 1 % or 5 % Poloxamer 188 in 20mM isotone HEPES/NaOH pH 7.4 for 60 minutes at 37°C. Under microscopic inspection (Zeiss Axiovert 200 microscope (Zeiss, Munich, Germany) equipped with 40x objective and a Hamamatsu camera (Hersching, Germany)) the micropipette was moved to touch a cell and a distinct negative pressure was applied to fix the cell at the orifice. The apex of the cell membrane was set as the zero-point for evaluation of the membrane extension. Equal steps of -100 Pa were applied until the cell membrane stopped expanding.

At each pressure change an image was acquired and the position of the apex of the aspirated cell was calculated. These data were fit into equation 1 and 2 to calculate strain and tension (Evans and Rawicz, 1990). The length deformation of an object caused by stretching or compression is called areal strain (δA) ((delta A)). It has no dimension and can be calculated from:

$$\delta A \cong 2\pi R_p \left(1 - \frac{R_p}{R_c}\right) \Delta L \qquad \text{Eq. 1}$$

The tension (τ) ((tau)) of the membrane represents the force against the suction pressure and can be expressed as:

$$\tau = \frac{P\, R_p}{2 - 2R_p/R_c} \qquad \text{Eq. 2}$$

P = suction pressure

R_p = inner diameter of the pipette

R_c = diameter of the cell

ΔL = length difference.

3 Results

3.1 Influence of Poloxamer 188 on cell proliferation and viability

The proliferative activity of subcultivated Caco-2 cells in presence of Poloxamer 188 was elucidated by incorporation of BrdU into the DNA of

dividing cells in pre-confluent monolayers (see Fig. 1). Independent from cultivation time and the concentration range under investigation, the addition of the non-ionic surfactant increased the proliferation. Two days after seeding, the mean Caco-2 proliferation rate increased from 1.25 fold at 0.2% surfactant to a maximum of 1.5 fold at 0.6 – 0.8% Poloxamer 188 as compared to the control without surfactant. Finally, the effect of 1.0% Poloxamer 188 was found to be similar to that of 0.2% surfactant. Three days post-seeding, the proliferation exceeded that of the control 1.26 fold and the maximum proliferation was observed at 0.4 – 0.6% Poloxamer 188 amounting to a 1.45 fold increase as compared to the reference. Again, the proliferative activity at 0.8 and 1.0% Poloxamer 188 was similar to that at 0.2%.

Using cells after one freeze/thaw cycle, the same assay revealed that the mean proliferation increases concurrently with the amount of Poloxamer 188 added (see Fig. 2). After two days in culture, already 0.2% surfactant enhanced the proliferation 1.20 fold up to 2.1 fold at 1.0 % Poloxamer 188 in comparison to the control. After cultivation for three days this effect was even more pronounced ranging from a 1.25 fold increase at 0.2% surfactant to a 2.5-fold enhancement at 1.0% Poloxamer 188 as compared to cultivation without surfactant.

In order to assess possible toxic effects of Poloxamer 188, the viability of Caco-2 cells as indicated by their mitochondrial activity was tested after a 3-days cultivation (see Tab. 1). As compared to cultivation without surfactant, the presence of Poloxamer 188 increased the viability by about 70% in the range of 0.2 – 0.8% and still 42% at 1.0% tenside. Thus, Poloxamer 188 not only proved to be non-toxic within this period, but it even improved cells' viability.

3.2 Membrane aspiration test

To elucidate the effect of Poloxamer 188 on the physical characteristics of Caco-2 cells, membrane aspiration tests were performed with cells preincubated with culture medium containing 0.0%, 0.5% or 2.5% surfactant.

Basically, when a cell is mounted at the mouth of a micropipette and a negative pressure is applied through the micropipette, the membrane together with the underlying cytoskeleton is sucked into the pipette mouth. Thus, the length of the apex depends on the cell's rigidity i.e. the stiffer the cell the shorter is the extended part of the cell (see Fig. 3).

For quantitative description of the results, the strain was set at 1.0 (equation 1) and the tension was calculated from equation 2. Accordingly, the tension meaning the negative pressure necessary to extend the cell volume to the same point in the micropipette like untreated cells was 49 mN/cm (0.5% Poloxamer 188) or 70 mN/cm (2.5% Poloxamer 188) at the mean (see Tab. 2). Thus, the stiffness of the cell membrane increases with the concentration of Poloxamer 188.

4 Discussion

According to the literature and the marketed Filoceth™-media the non-ionic surfactant Poloxamer 188 proved to be a useful compound in cryopreservation media to increase viability of cells after thawing (Hernandez and Fischer, 2006). In order to detect further advantages for the cultivation of human cell lines, varying amounts of the surfactant were added to commonly applied cell culture media and used during cultivation of Caco-2 cells. Proliferation as well as viability of cells served as decisive parameters.

In presence of Poloxamer 188, the proliferative activity of cells stressed by either proteolytic subcultivation or freezing/thawing was considerably higher than without supplementation. For subcultivated cells, the most pronounced effect on pre-confluent monolayers was observed upon addition of 0.4 - 0.6% Poloxamer 188 as represented by a 1.5 fold increase (see Fig. 1). In case of Caco-2 cells after one freeze/thaw cycle, the proliferative activity steeply increased with the Poloxamer 188 concentration exceeding that of the Poloxamer-free medium 2.1 – 2.5 fold (see Fig. 2).

Interestingly, the proliferation of cells after one freeze/thaw cycle in cell culture medium without Poloxamer 188 is 56% lower than that of non-frozen

cells most probably due to cytotoxic effects of DMSO (Guell et al., 2009) and damaging ice crystals (see Fig. 1 and 2). Thus, the steeper increase in proliferation rates post thawing especially at surfactant concentrations higher than 0.4% might be attributed by part to the lower number of seeded viable cells as well as a considerable number of cells which would have been damaged upon cryostorage and thawing but could be rescued by the resealing properties of the surfactant (Togo et al. 1999). In addition, the enhanced viability of the cells after cultivation in presence of surfactant for three days confirmed the cytoprotective effect of 0.2 – 0.8% Poloxamer 188 (see Tab. 1). These results are in line with the literature reporting that Poloxamer 188 protects cells against chemical and physical stress (Hellung-Larsen et al., 2000) and facilitates cell membrane resealing after wounding by decreasing the cell surface tension (Togo et al. 1999). In contrast, another study revealed that the mean membrane bursting tension as well as the mean elastic compressibility modulus of cells increases in presence of Poloxamer 188 as a consequence of a supposed increase in membrane tension (Zhang et al., 1992).
In order to get an idea of the impact of Poloxamer 188 on the membrane tension of a single cell, the micropipette aspiration technique was applied. For this assay two surfactant concentrations were chosen: (i) 0.5% Poloxamer 188 as it exerted an optimum impact on cell proliferation and considerably increased viability, and (ii) 2.5% Poloxamer as an extreme exceeding the most useful investigated concentration for post-thaw proliferation (1.0%).
The micropipette aspiration assays revealed that the tension of the plasma membrane concurrently increased with the amount of surfactant added (see Tab. 2, Fig. 3). Interestingly, the difference in tension between 0% and 0.5% Poloxamer 188 was 49 mN/cm as necessary to achieve strain 1, whereas only 21 mN/cm was measured upon increasing the Poloxamer 188 concentration from 0.5 % to 2.5%. Additionally, as depicted from Figure 3, the horizontal shift of the cell's apex between 0% and 0.5% Poloxamer 188 is 42%, while further increasing the Poloxamer content from 0.5% to 2.5% provoked only a shift of 8% in comparison to the untreated cell (100%). Consequently, the

effect of Poloxamer 188 is more pronounced in the range below 0.5% implying that marked changes in membrane rigidity occur already in presence of low amounts of the non-ionic surfactant.

It is most unlikely that micellar effects contribute to changes in membrane tension since the critical micelle concentration of Poloxamer 188 is about 1 mM (Batrakova et al., 1998). However, Poloxamer 188 not only adsorbs to the cell membrane as confirmed by rheogoniometric investigations (Al-Rubeai et al., 1993), but it also incorporates into the phospholipid-bilayer and decreases the fluidity of the cell membrane as indicated by fluorescence polarization experiments (Ramirez and Mutharasan, 1990). Thus, it is supposed that the increase in membrane tension is due to adsorption and incorporation of Poloxamer 188 into the cell membrane.

Apparently, this gain in stiffness also reduces the "Brownian" shape low amplitude and high frequency fluctuations of large areas of the cell membrane, the so-called undulations (Partin et al., 1989). In addition, the cell volume increases by 5% in presence of Poloxamer 188 and the cell rounds up (Raucher and Sheetz, 1999) increasing the contact area of the cell membrane with the support (Hellung-Larsen, 2005). Altogether the observed and reported effects of Poloxamer 188 including the gain in stiffness are supposed to enhance attachment of adherent Caco-2 cells and that way to increase the proliferation of subcultivated and thawed cells. These findings are in accordance with the results of viability assay relying on the overall mitochondrial activity. Consequently, 0.5% Poloxamer 188 might be added to cell culture media to exploit the stabilizing effects during cell cultivation. Interestingly, 1% Poloxamer 188 is already a cryoprotective compound of a marketed freezing medium.

All in all, the non-ionic surfactant Poloxamer 188 represents a valuable cytoprotective supplement for cell culture media not only for cryoprotection of cells but also to improve cell viability and proliferation after subcultivation and freezing/thawing procedures. That way, the reproducibility and the reliability of cell culturing at early stages of drug and formulation

development might be improved hence reducing and consequently at least partly replacing animal and human experiments as required for research and approval of drug formulations by legal authorities.

References

Al-Rubeai, M., Emery, A. N., Chalder, S., Goldman, M. H. (1993) A flow cytometric study of hydrodynamic damage to mammalian cells. *J. Biotechnol. 31,* 161–177.

Artursson, P., Palm, K., Luthman. K. (2001) Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Del. Rev. 46,* 27–43.

Woods, E. J., Benson, J. D., Agca, Y. Critser, J. K. (2004) Fundamental cryobiology of reproductive cells and tissues. *Cryobiology 48,* 146–156.

Batrakova, E.V., Han, H. Y., Alakhov, V. Yu., Miller, D. W., Kabanov, A. V. (1998). Effects of Pluronic block polymers on drug absorption in Caco-2 cell monolayers. *Pharm. Res. 15,* 850-855.

Evans, E., Rawicz, W. (1990) Entropy-driven tension and bending elasticity in condensed-fluid membranes. *Phys. Rev. Lett. 64*, 2094-2097.

Gonzalez Hernandez, Y., Fischer, R. W. (2007) Serum-free culturing of mammalian cells – Adaptation to and cryopreservation in fully defined media. *ALTEX 24*, 110–116.

Guell, I., Alves, P. M., Gabor, F., Wirth, M. (2009) Viability of the human adenocarcinoma cell line Caco-2: Influence of cryoprotectant, freezing rate and storage temperature. *Scientia Pharmaceutica 77*, 133–141.

Hellung-Larsen, P. (2005) Cloning of Tetrahymena cells in a chemically defined medium is possible in the presence of surfactants or at reduced temperature. *J. Biotechnol. 115*, 167-177.

Hellung-Larsen, P., Assaad, F., Pankratova, S., Saietz, B. L., Skovgaard, L. T. (2000) Effects of Pluronic F-68 on Tetrahymena cells: protection against chemical and physical stress and prolongation of survival under toxic conditions. *J. Biotechnol. 76,* 185–195.

Hochmuth, R. M. (2000) Micropipette aspiration of living cells. *J. Biomech. 33*, 15– 22.

Kim, J. S., Mitchell, S., Kijek, P., Tsume, Y., Hilfinger, J., Amidon. G. L. (2006) The suitability of an insitu perfusion model for permeability

determinations: Utility for BCS Class I Biowaiwer requests. *Mol. Pharmaceutics 3,* 686–694

Partin, A. W., Schoeniger, J. S., Mohler, J. L., Coffey, D. S. (1989) Fourier analysis of cell motility: Correlation of motility with metastatic potential. Proc. Natl. Acad. Sci. U.S.A. *86*, 1254–1258.

Ramirez, O. T., Mutharasan. R. (1990) The role of the plasma membrane fluidity on the shear sensitivity of hybridomas grown under hydrodynamic stress. *Biotechnol. Bioeng. 36,* 911–920.

Raucher, D., Sheetz, M. P. (1999) Membrane expansion increases endocytosis rate during mitosis. *J Cell Biol. 144*, 497–506.

Sachan, N. K., Bhattacharya, A., Pushkar, S., Mishra, A. (2009) Biopharmaceutical Classification system: A strategic tool for oral drug delivery technology. *Asian J. Pharm., 3,* 76-81.

Togo, T., Alderton, J. M., Bi, G., Steinhardt, R. A. (1999) The mechanism of facilitated cell membrane resealing. *J. Cell Sci. 112*, 719–731.

www.accessdata.fda.gov/scripts/cder/iig/getiigWEB.cfm (23.04.2009)

Zhang, Z., Al-Rubeai, M., Thomas, C. R. (1992) Effect of Pluronic F-68 on the mechanical properties of mammalian cells. *Enzyme Microb. Technol. 14*, 980–983.

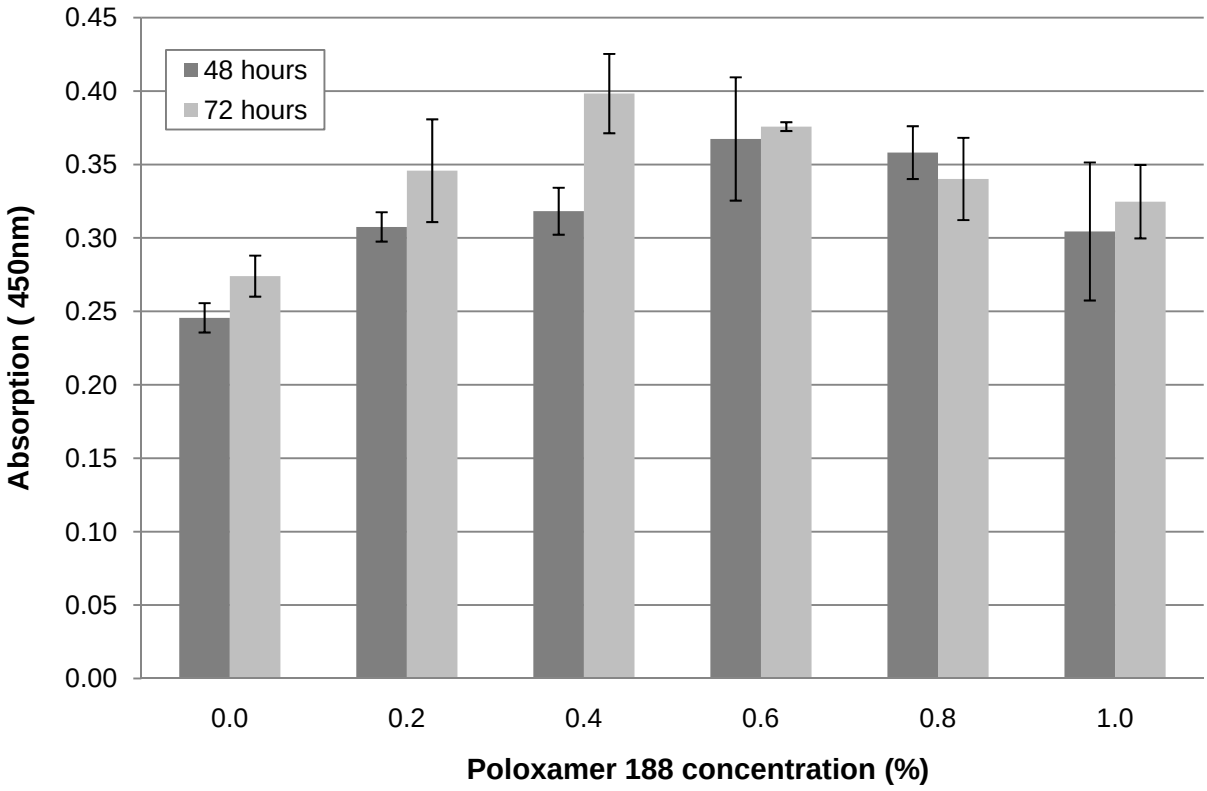

Figure 1: Proliferative activity of Caco-2 cells after subcultivation and propagation in presence of Poloxamer 188 on day 2 and 3 post seeding (n=6, mean ± SD).

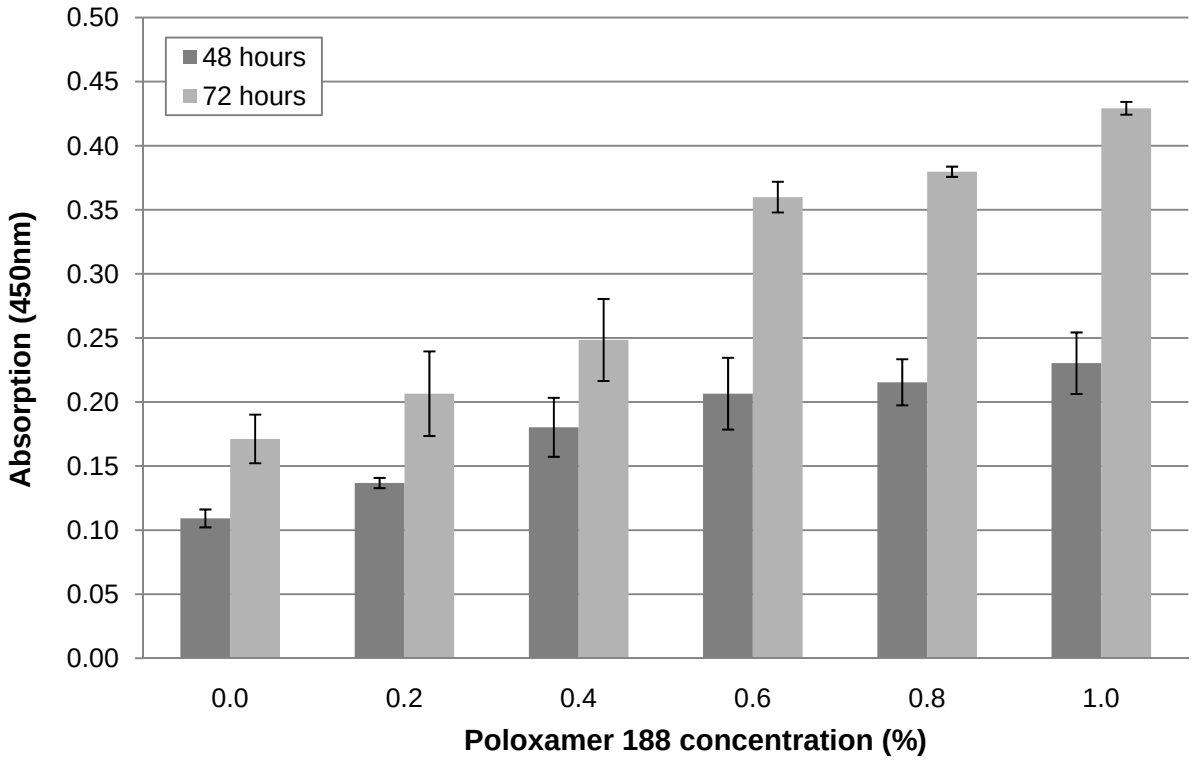

Figure 2: Proliferative activity of Caco-2 cells after one freeze/thaw cycle and cultivation in presence of Poloxamer 188 on day 2 and 3 post seeding (n=6, mean ± SD).

Table 1: Viability of Caco-2 cells.

Poloxamer 188 conc (%)	**0.0**	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**
Absorption (450 nm)	1.07±0.01	1.74±0.04	1.68±0.06	1.68±0.05	1.71±0.09	1.53±0.02

Viability of Caco-2 cells after splitting and cultivation in cell culture medium containing Poloxamer 188 for 3 days (n=6, mean ± SD).

Table 2: Cell-membrane tension.

Poloxamer 188 conc (%)	Tension (mN/cm)
0.0	194.81 ± 53.10
0.5	243.95 ± 38.73
2.5	264.28 ± 58.09

Tension (set at strain 1) of Caco-2 cell membranes after incubation for 60 min at 37°C without and with 0.5% or 2.5% Poloxamer 188 (n = 9, mean± SD).

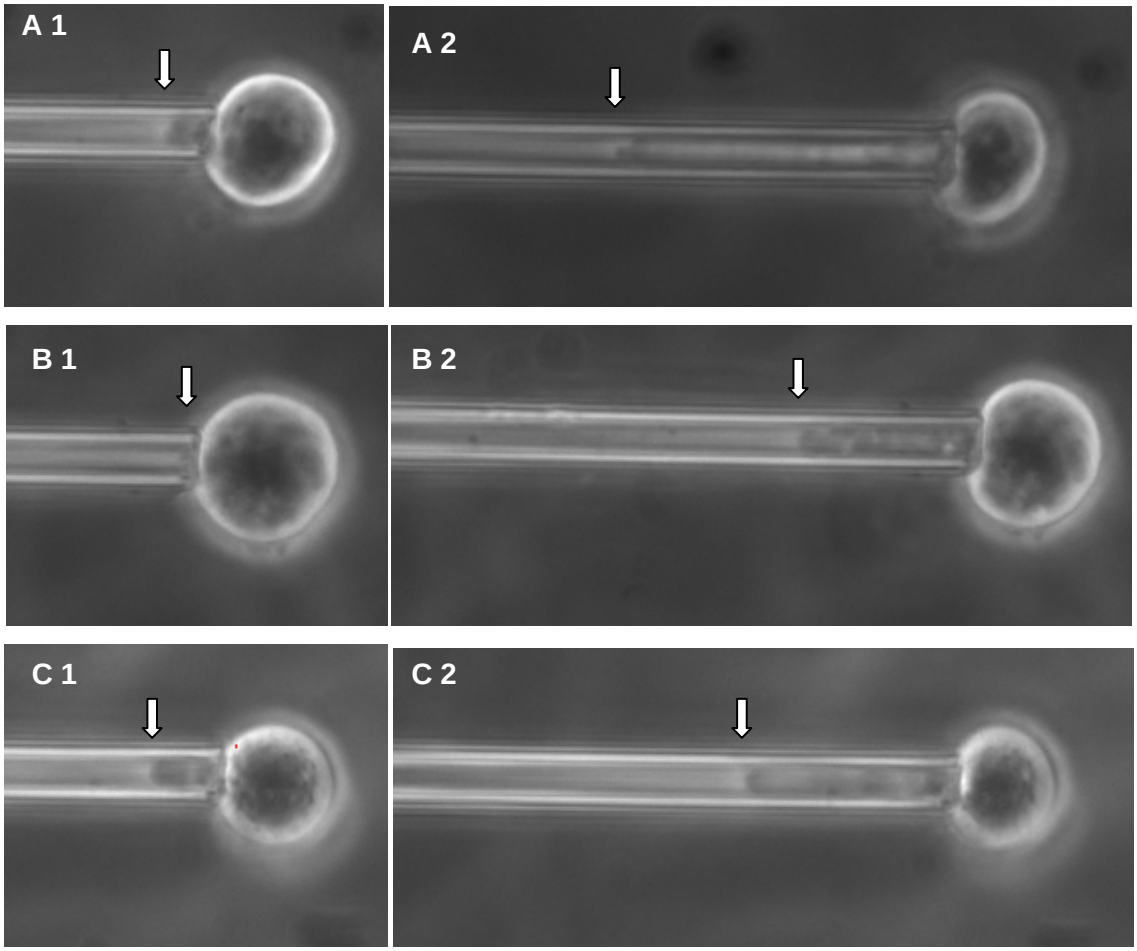

Figure 3: Microaspiration of Caco-2 cells in absence (A) and presence (0.5% B and 2.5% C) of Poloxamer 188 without applying pressure (A1, B1, C1) and a suction pressure of 1 kPa (A2, B2, C2). The arrows indicate apex shift of the cell membrane in response to the applied pressure and presence of Poloxamer 188.

4. Conclusion

Since a couple of years enormous efforts were made to discover and estimate the potential of nanoparticles for pharmaceutical applications. To shed some light on the characteristics of these nano-scaled formulations, a multidisciplinary approach exceeding by far the classical techniques of pharmaceutical technology was inevitably necessary to disclose unique features of nanoparticles such as the so-called EPR-effect beneficial for therapy of cancer or inflammation, the exciting possibility to cross barriers towards absorption, which cannot be surmounted by conventional formulations so far, or even the successful introduction of active pharmaceutical ingredients into the market suffering either from insufficient solubility or overwhelming toxicity. Nevertheless, all aspects of colloidal formulations are not yet elucidated to date. Accordingly, this thesis is dedicated to contribute basic knowledge on the interaction between nanoparticles and cells requiring established techniques for cell cultivation and characterization as well as inclusion of new biophysical approaches.

In the **first part**, the review being prepared in collaboration with other members of the working group "*The role of surface functionalization in the design of PLGA micro- and nanoparticles"* summarizes different aspects of particle surface modification and offers a comprehensive overview about the state of the art. Representing a kind of theoretical background for the practical work of this thesis, even during workup of the currently available literature it became evident that surfactants play a key role in pharmaceutical nanotechnology.

The first of the practical work reported in this thesis deals with "*Characterization of binding and uptake of biomimetic nanoparticles by flow cytometry".* Although flow cytometry represents a well-established technique

and allows even multi-parametric analysis of the nanoparticle-cell interaction, it also inevitably requires fluorescent labeling of the nanoparticles. Labeling with fluorescent dyes, however, bears the risk of dye leakage in case of adsorptive loading or incorporation probably falsifying the results. In case of covalent labeling, skillful techniques for labeling as well as removal of excessive dye are required. Additionally, incorporation as well as grafting with dyes alters the physicochemical characteristics of the nanoparticles. Alternatively, the utility of the side scatter signal, which monitors the granularity of the cell and increases upon binding of nanoparticles, was proposed as a label-free technique to track the nanoparticle-cell interplay. Applying biomimetic lectin-grafted nanospheres and their non-labelled counterparts, the reliability of this new flow cytometric technique was confirmed by a temperature-dependent linear correlation between side scatter data and cell-associated fluorescence intensities of Caco-2 cells loaded with fluorescent biomimetic nanoparticles. Moreover, not only the binding to but also the uptake of the nanoparticles into the cells could be detected. In addition to the amount of cell-associated nanoparticles, the multi-parametric character of flow cytometric analysis provides additional useful information about viability and agglomeration of cells in even one run. In this study and throughout the thesis Caco-2 cells were used which represent a well established cell line mimicking intestinal epithelial cells. According to the literature, this label-free technique has been applied meanwhile for cell-interaction studies by foreign research groups too. The nanoparticles used in this study were 600nm in diameter and the lower limit of nanoparticles' diameter for reliable detection still remains open. Great efforts were made to prepare similar biomimetic nanoparticles but with a few 100nm in diameter and especially monomodal size distribution were less successful until now.

The **second part** focuses on the influence of surfactants on the interaction between nanoparticles and Caco-2 cells. Surfactants are commonly required

for nanoparticle preparation and stabilization. Consequently, nearly all nanoparticle preparations contain surfactants even at remarkable concentration levels. In addition, surfactants proved to be useful for coating of nanoparticles to mediate the so-called "stealth-effect" for prolonged residence in circulation. Some clinical data available and studies with tensides and cells, however, revealed some influence on the cell membrane. Thus, the influence of commonly applied surfactants on the characteristics of the nanoparticle cell interaction was elucidated in the second part.

The research article "*Nonionic surfactants increase cell-binding of nanoparticles by modulation of membrane stiffness"* reports about the interplay between nanoparticles, Caco-2 single cells and non-ionic surfactants Polysorbates. Applying flow cytometry to detect cell-associated fluorescent nanoparticles and viability of Caco-2 cells at the same time, increasing nanoparticle binding up to the maximum of 0.05% Polysorbate 20 or 80 was observed. Additionally, this concentration range of tensides provoked no cytotoxic effects. According to the literature the Polysorbates are adsorbed and/or incorporated into the cell membrane. In order to detect changes in membrane dynamics, a biophysical method, the so-called micropipette aspiration technique was applied. The results clearly revealed that the stiffness of the cell-membrane increases concurrent with the concentration of surfactants. Since increasing rigidity of the cell membrane lowers its undulation, repulsive forces against adsorption of particulate matter are reduced. Consequently, even lowest amounts of Polysorbates starting at a concentration of 0.0005% represent an active constituent increasing the deposition and thus the adsorption of nanoparticles at the cell membrane. It is most likely that the amount of surfactant present in nanoparticle preparations due to manufacturing and/or stabilization needs are sufficient to provoke such effects even in vivo at least locally at the site of administration. At higher concentrations ≥ 0.1%, however, the beneficial effect is inverted: Polysorbate 20 increasingly provokes cytotoxic effects whereas Polysorbate 80

softens the cell membrane so much that membrane vesicles are pinched off. According to our opinion this negative effect on the cells as well as the cell-association of nanoparticles will be hardly observed in vivo due to tremendous dilution of the preparations in body fluids. Interestingly, the concentrations of both tensides approved for administration meet and exceed the concentration range where toxic effects were observed.

Second study in this part entitled with "*The interaction of Poloxamer 188 with the cell membrane increases the cell-association of nanoparticles"* is focused on another, even more frequently used surfactant. Poloxamer 188 is reported to influence cell physiology, to inhibit P-glycoprotein mediated efflux of drugs and thereby enhancing the transcellular transport, to exert cytoprotective effects and exhibits a broader safe therapeutic range as compared to the Polysorbates. As confirmed by cell-association studies in presence of the surfactant and imaging, also Poloxamer 188 increases the Caco-2 association of nanoparticles in the concentration range between 0.5% and 2.5% but without any negative effects on viability of cells. Since Poloxamer 188 pretreated cells adsorb more nanoparticles than non-pretreated ones, the observed effect is exclusively due to modulation of the cell-membrane. Micro-aspiration experiments in presence of Poloxamer 188 clearly revealed a concentration-dependent gain in stiffness of the cell-membrane and confirmed the proposed "undulation theory" to enhance the nanoparticle-cell interaction. As opposed to the Polysorbates, Poloxamer 188 offers the advantage to be more efficient in stabilizing the cell-membrane and by far less toxic. Thus, Poloxamer 188 is preferable for preparation, stabilization and coating of nanoparticles over Polysorbates, even upon administration in-vivo.

In the **third part** according to the membrane-stabilizing effect and the low toxicity of Poloxamer 188, another study was aimed to investigate any beneficial effects on cultivation of cell lines. The experimental work of the

study *"Poloxamer 188 supplemented culture medium increases the vitality of Caco-2 cells after subcultivation and freeze/thaw cycles"* revealed that medium supplemented with 0.5% Poloxamer 188 increased proliferation freshly subcultivated cells 1.5 fold and that of thawed cells about 2-fold in addition to improved viability. As mentioned above, the beneficial effect on cell cultivation is also due to the adsorption and/or incorporation of the surfactant into the cell-membrane. The higher rigidity of the membrane leads to rounding up of the cell and is suggested to facilitate the adhesion on the substrate. As the Caco-2 cell line is widely used in preclinical biopharmaceutics for drug evaluation, any step forward to improve cell cultivation and thus reliability of ex-vivo models is expected to further reduce the number of necessary animal trials and probably also clinical trials with humans.

All in all, this thesis describes a mechanism to enhance the cell-association of nanoparticles, which was not reported until now. The reduction of undulations of the cell membrane and thus that of repulsive forces against deposition of nanoparticles improves the contact between colloidal formulations and the absorptive cell. Apart from size and surface modification, this effect is supposed to increase and accelerate the cellular uptake of drugs or nanoparticulate drug delivery systems. Consequently, the diffusional pathway is shortened and the exposition of the colloidal formulation to sometimes harmful environment in the body is reduced. Interestingly, this effect is mediated by approved surfactants at lowest concentrations that are already contained in nanoparticle preparations to take account for preparation, stabilization or biodistribution issues. Lowering the undulatory movements of the cell membrane is also beneficial for ex-vivo models in Pharmaceutical Technology. At this, the use of Poloxamer 188 supplemented cultivation media will help to refine and make cell culturing more reliable to further reduce animal trials in preclinical investigations.

5. Appendix

5.1 Abstract

Roughly, nanoparticles (NP) are 10 – 100fold smaller than an eucarytic cell thus opening the possibility to be taken up into a cell. To date, labeled NP are used for uptake-studies with the risk of dye-leakage and false positive results. As an alternative avoiding labeling, the changes in roughness of the cell surface upon binding and uptake of NP was monitored by side scatter data using flow cytometry. Based on comparative studies with fluorescent labeled NP, granularity of cells was established as an additional parameter indicating cytoadhesion as well as cytoinvasion of biomimetic NP.

Although NP can be taken up into cells, the uptake rate is usually too low for successful therapy. Facing this challenge, the influence of nonionic surfactants such as Poloxamer 188 and Polysorbates on association of fluorescent NP with Caco-2 single cells was investigated flow cytometrically. Poloxamer 188 proved to be non-toxic and increased NP-association even at amounts that are contained in NP due to the preparation process. In presence of free Poloxamer 188, the NP-association considerably increased with concentration. In contrast, presence of Polysorbate 20 and Polysorbate 80 provoked either improved or inhibited NP-cell interaction in a concentration-dependent manner. In addition, cytotoxic effects of Tweens were observed. Applying a microaspiration technique, a gain in stiffness of the cell membrane in presence of Poloxamer 188 was observed. Thus, impairing undulation of the cell membrane increases the contact frequency with NPs facilitating cytoassociation.

This increasing stiffness of the cell membrane and non-toxicty of Poloxamer 188 was also supposed to be beneficial for cryo-storage of sensitive cell lines.

According to higher proliferation rates of Caco-2 cells post thawing, Poloxamer 188 improved viability in comparison to usually applied freezing protocols.

5.2 German abstract

Zusammenfassung

Nanopartikel (NP) sind etwa 10- 100mal kleiner als eine Eukaryontenzelle und können in diese aufgenommen werden. Bei Aufnahmestudien werden derzeit werden Fluoreszenz-markierte NP eingesetzt, die durch Ausbluten des Farbstoffes falsch positive Ergebnisse vorpsiegeln können. Als Alternative zur Markierung wurde die Änderung der Rauheit der Zelloberfläche durch Bindung und Aufnahme von NP im Durchflußzytometer untersucht. Basiertend auf Vergleichsstudien mit fluoreszenz-markierten NP konnte die Zellgranularität als ein zusätzlicher Parameter für Zytoadhäsion und Zytoinvasion von biomimetischen NP etabliert werden.

Obwohl NP in Zellen aufgenommen werden können ist die Aufnahmerate für einen therapeutischen Nutzen oftmals zu gering. Um diesem Problem Rechnung zu tragen wurde der Einfluss der nichtionogenen Tenside Poloxamer 188, Polysorbat 20 und Polysorbat 80 auf die Wechselwirkung von fluoreszierenden NP und Caco-2 Einzelzellen im Durchflusszytometer untersucht. Poloxamer 188 konnte als atoxisch bewertet werden und erhöhte die NP-Zell Assoziation sogar in Mengen, die produktionsbedingt in NP enthalten sind. In Gegenwart von freiem Poloxamer 188 nahm die Zellassoziation der NP mit steigender Konzentration zu. Im Gegensatz dazu verbesserten oder inhibierten Polysorbat 20 und Polysorbat 80 die NP-Zell Wechselwirkung in Abhängigkeit von deren Konzentration. Darüber hinaus wurden zytotoxische Effekte der Polysorbate beobachtet. Mit Hilfe der Mikroaspirationstechnik konnte eine zunehmende Steifigkeit der Zellmembran in Gegenwart von Poloxamer 188 nachgewiesen werden. Die erhöhte NP-Zell Assoziation dürfte daher auf der Unterdrückung der Wellenbewegung der Zellmembran und der damit erhöhten Kontaktfrequenz zwischen NP und Zelloberfläche beruhen.

Inwieweit die erhöhte Steifigkeit der Zellmembran in Gegenwart von Poloxamer 188 die Kryokonservierung von empfindlichen Zelllinien vorteilhaft ist, wurde durch Proliferationstests nach dem Tauen von kryokonservierten Caco-2 Zellen untersucht. Verglichen mit herkömmlichen Frier-Tau-Protokollen bewirkte Poloxamer 188 eine deutlich erhöhte Wachstumsrate von Caco-2 Zellen.

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Univ. Prof. Dr. Michael Wirth

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PhD students: **Christian Fillafer, Iris (Güll) Krejci, Lukas Neutsch, Claudia Piana, Verena Plattner, Gerda Ratzinger** as well as **all associates** of the Deptartemnt of Pharmaceutical Technology and Biopharmaceutics

Thank you for all those memorable moments we had together.

Diploma students: **Nadira Ibrisimović, Isabella Andrlik, Cornelia Teichmann**

Thank you for enjoyable teamwork.

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Thank You for Your endless love, patience and support I always had in all of You and the confidence You always had in me.

Admir Tuzović

Thank You for being by my side in all these years!

5.4 Curriculum Vitae

Personal Data

Name	Vera KERLETA
Date of Birth	14.07.1980
Place of Birth	Jajce, Bosnia and Herzegowina
Nationality	Bosnian
Marital status	Single

Education

since 03.2006	PhD studies at Institute of Pharmaceutical Technology and Biopharmacy, University of Vienna
12. 2005	Mag. pharm. (MpharmSC) degree awarded with distinction
03.2005 – 12.2005	Diploma thesis at Institute of Pharmaceutical Technology and Biopharmacy, University of Vienna
10.2001 – 12.2005	Studies of Pharmaceutical sciences at the University of Vienna
10.1999 - 09.2001	Studies of Pharmaceutical sciences at the University of Sarajevo
06.1999	Secondary school graduation Summa cum Laude
09.1995 – 06.1999	Secondary Nursing School, Catholic School Centre, Sarajevo

Professional Experience

Since 03.2006	University assistant in the practical course of Advanced industrial pharmacy, Institute of Pharmaceutical Technology and Biopharmacy, University of Vienna
Since 03.2007	Associate lecturer in the practical course of Basics in industrial pharmacy, Institute of Pharmaceutical Technology and Biopharmacy, University of Vienna
09.2006 – 02.2008	Associate lecturer in the practical course of Cell culture in pharmaceutical technology, Institute of Pharmaceutical Technology and Biopharmacy, University of Vienna
06.2009 – 09.2009	Research associate at Institute of Pharmaceutical Technology and Biopharmacy, University of Vienna for Baxter, Austria
09.2006 – 02.2008	Research associate at Institute of Pharmaceutical Technology and Biopharmacy, University of Vienna within the framework of CellPROM project
09.2005 – 02.2006	Tutor in the practical course on industrial pharmacy, Institute of Pharmaceutical Technology and Biopharmacy, University of Vienna

Book

V. Kerleta. Flowcytometrische Untersuchungen zur Interaktion von Lektin-funktionalisierten Nanopartikeln mit Caco-2 Zellen *Verlag Dr. Müller*; 2009

Publications

K. Trimmel, G. Ratzinger, **V. Kerleta**, C. Fillafer, M. Wirth, F. Gabor. Characterisation of binding and uptake of biomimetic nanoparticles by flow cytometry. *Journal of Drug Delivery Science and Technology* 2008

H. Al-Dubai, G. Oberhofer, **V. Kerleta**, H. H. Hinterwirth, M. Strobl, F. Gabor. Cleavage of antibodies using dihydrolipoamide and anchoring of antibody fragments onto biocompatible coated carriers. *Chemical Monthly* 2010

G. Ratzinger, C. Fillafer, **V. Kerleta**, M. Wirth, F. Gabor. The role of surface functionalization in the design of PLGA micro- and nanoparticles. *Critical Reviews* 2010

Submitted research articles (03.2010)

V. Kerleta, I. Andrlik, M. Wirth, F. Gabor. Nonionic surfactants ameliorate and inhibit nanoparticle-cell interaction through cell membrane stiffness modulation Submitted to *Jr of Nanobiotechnology*

V. Kerleta, C. Fillafer, M.F. Schneider, T. Franke, M. Wirth, F. Gabor. Poloxamer 188 interaction with cell membrane induces rigidity change; enhancement of nanoparticle-cell association
To be submitted to *Langmuir*

V. Kerleta, I. Andrlik, S. Braunmüller, T. Franke, M. Wirth, F. Gabor. Poloxamer 188 supplemented culture medium increases the vitality of Caco-2 cells after subcultivation and freeze/thaw cycles
Submitted to *ALTEX*

Congress contributions

Kerleta V, Piana C, Güll I, Wirth M, Gabor F. Biocompatibility and coating of the GeSiM cell carrier. *Poster Presentation* at C_21/CellPROM Annual Meeting, 03.2007, Lisbon

Kerleta V, Trimmel K, Ratzinger G, Wirth M, Gabor F. Flow Cytometric Side Scatter Analysis as a Directive for Cell-Nanoparticle Interaction; *Oral presentation* at Pre-Satelite Meeting, Pharmaceutical Sciences World Congress; 04.2007 Amsterdam

Kerleta V, Trimmel K, Ratzinger G, Wirth M, Gabor F. Flow Cytometric Side Scatter Analysis as a Directive for Cell-Nanoparticle Interaction; *Poster presentation* at, Pharmaceutical Sciences World Congress; 04.2007 Amsterdam

Guell I, Piana C, **Kerleta V**, Neutsch L, Howitz S, Gabor F, Wirth M. Handling and biocompatibility of magnetic cell carriers. *Poster presentation* at CP_28/CellPROM Final Meeting, 02.2008, Sulzbach

Piana C, Guell I, Neutsch L, **Kerleta V**, Toegel S, Gerbes S, Wirth M, Gabor F. Proliferation and differentiation of Caco-2 cells on surface engineered nanoscapes. *Poster presentation* at CP_28/CellPROM Final Meeting, 02.2008, Sulzbach

V. Kerleta, C. Fillafer, M. Wirth, F. Gabor. Dynamic light scattering of nanoparticles: Influence of the surfactant viscosity on particle size; *Poster presentation* at World Meeting; 04.2008 Barcelona

V. Kerleta, C. Fillafer, M. Wirth, F. Gabor. Influence of the surfactant content on nanoparticle size determination by DLS; *Poster presentation* in Graz, 05.2008

V. Kerleta, I. Andrlik, M. Schneider, T. Franke, M. Wirth, F. Gabor. Pluronic® F-68 enhances the nanoparticle-cell interaction; *Oral presentation* at 21st Scientific Congress of the Austrian Pharmaceutical Society; 04.2009 Vienna