universität wien

DIPLOMARBEIT

Titel der Diplomarbeit

„Targeted therapy of bladder cancer and other urothelial diseases – developing novel bioassays for analytical characterization and optimization of glyco-recognitive drug delivery systems“

verfasst von

Alexandra Hofer

angestrebter akademischer Grad

Magistra der Pharmazie (Mag.pharm.)

Wien, 2014

Studienkennzahl lt. Studienblatt:	A 449
Studienrichtung lt. Studienblatt:	Diplomstudium Pharmazie
Betreut von:	a.o. Univ.-Prof. Mag. Dr. Michael Wirth

Danksagung

Ich möchte mich bei Prof. Wirth bedanken, für die Möglichkeit auch einen Teil zu seiner interessanten Forschung im Bereich der Therapie von Blasenerkrankungen beitragen zu dürfen.

Außerdem möchte ich mich bei der gesamten Arbeitsgruppe um Prof. Wirth und Prof. Gabor am Department für pharmazeutische Technologie für die nette Zusammenarbeit bedanken. Mein besonderer Dank geht hierbei an Dr. Lukas Neutsch, der immer Zeit gefunden hat für Fragen und Probleme und mit seiner Begeisterung für die Wissenschaft eine Bereicherung für jeden darstellt, der mit ihm zusammen arbeiten darf.

Meinen Eltern und meiner Schwester gilt auch besonderer Dank für die Unterstützung während der arbeitsintensiven Zeit des Studienabschlusses.

Danke an meinen Mann Ludwig, ohne dessen guten Zuspruch ich die Zeit der Diplomarbeit wohl nicht so gut gemeistert hätte.

Abstract

Bladder diseases like bladder cancer (BCa) or urinary tract infection are prevalent and show severe shortcomings in the localized therapy, e.g. due to the elution of the utilized drugs by constant urine production. Furthermore, bladder diseases show high relapse incidents which might be overcome by new receptor-targeted drug delivery systems that are able to overcome the bladder urothelium, which is one of the hardest biological barriers to penetrate. In malignant urothelial cells (5637) the lectin wheat germ agglutinin (WGA) has found to be a promising candidate to trigger receptor-targeted cytoinvasion of conjugated cargo like nano- or microparticles (NP/MP). In order to prove successful cellular uptake of particles, which are loaded with the drug needed for therapy, a robust method for detection and characterization of endocytosis is necessary. For this, three different methods were evaluated in this work either with bioconjugates (e.g. WGA labelled with a fluorescent molecule) or with NP/MP, which were previously conjugated to labelled WGA.

The TMB assay was evaluated using a panel of MP variants, which differed concerning the amount of particle-associated Biotin, aiming to improve and intensify the interaction between Avidin, conjugated to horseradish peroxidase (HRP) for further detection via the TMB assay, and Biotin, immobilized at the MP surface. The MP variants consisted of PLGA MP either modified with Biotin-labelled WGA (b-WGA) only, additionally biotinylated with BIO C5 NHS (bb-WGA) or hyperbiotinylated (bbb-WGA) MP. Although the constant increase in the Avidin-accessible Biotin with every MP variant resulted in an improvement of the intensity of the recorded signals, the overall signal level remained too low to allow for a clear analysis of a possible cellular uptake of the particles. Direct modification of the MP surface with HRP or HRP/WGA was successful and showed an increase of the signal level with TMB to the required level for endocytosis detection. However, potential saturation phenomena or other disturbances of the enzyme reaction point to additional processes that may go on during TMB development and require further research.

For the second method a tyramide signal amplification (TSA) kit was used, which should visualize HRP (conjugated to WGA or MP surfaces) through an enzymatic reaction with fluorescence-labelled tyramide and successive covalent binding of this tyramide to nearby accessible reactive groups at the cell. Assessment of this method for bioconjugates (hrp-WGA) resulted in a successful discrimination between cytoadhesive and cytoinvasive cargo due to the impenetrability of the cell membrane for the tyramide, but only for qualitative detection (fluorescence microscopy). Unfortunately, the TSA assay showed limitations concerning the

applicability for modified MP (HRP MP) and quantitative detection (fluorimetry via Tecan Infinite® 200 Reader).

The last evaluated method, based on the quenching ability of TCEP for several fluorescent molecules, turned out to be the most promising detection method assessed in this work. The assay could be applied and optimized for different detection devices, and for generating both qualitative and quantitative results (fluorescence microscopy, fluorimetry via Tecan Infinite® 200 Reader and flow cytometry). Moreover, applicability for bioconjugates (a647-WGA) as well as NP could be demonstrated. The discrimination between cytoadhesive and cytoinvasive cargo could be achieved due to the impenetrability of the cell membrane for TCEP, which was verified in control assays via cell permeabilization. Nevertheless a clear prove of successful internalization of NP in 5637 BCa cells could not be achieved yet, because of difficulties in the surface modification of NP. However, the established detection method via TCEP quenching is suitable for discrimination between cytoadhesive and cytoinvasive cargo and therefore, might be applied for further research in endocytosis analysis.

Zusammenfassung

Erkrankungen der Blase, wie Blasenkrebs oder Harnwegsinfektionen, sind weit verbreitet und zeigen ernstzunehmende Mängel bei lokaler Therapie, zum Beispiel aufgrund von Auswaschungen der eingesetzten Medikation durch konstante Urinproduktion. Außerdem zeigen Erkrankungen der Blase eine hohe Wahrscheinlichkeit für die Ausbildung von Rezidiven, welchen man mit neuen Systemen zur zielgerichteten Medikamentenabgabe über einen Rezeptor entgegenwirken will. Obwohl das Urothelium der Blase eine der stärksten biologischen Barrieren darstellt, konnte bereits anhand von malignen Urothelzellen (5637) eine erfolgreiche Zelladhesion bzw -invasion des Lektins WGA festgestellt werden. Eine Oberflächenmodifikation von PLGA Nano- oder Mikropartikeln (NP/MP) mit WGA könnte somit eine geeignetes System für die gezielte Aufnahme in die Zelle über einen Rezeptor darstellen. Um eine erfolgreiche Zellinvasion dieser modifizierten Partikel, welche zuvor mit der geeigneten Medikation beladen wurden, beweisen zu können, braucht es eine robuste Detektionsmethode, welche eine eindeutige Unterscheidung zwischen intrazellulärer und extrazellulärer Fracht ermöglicht. Um dies zu erreichen, wurden in dieser Arbeit drei verschiedene Methoden evaluiert, entweder mit Hilfe von Biokonjugaten (z.B. WGA gebunden an ein fluoreszierendes Molekül) oder NP/MP, welche vorher mit WGA oberflächenmodifiziert wurden.

Der TMB Assay wurde mit Hilfe einer Vielzahl an MP evaluiert, welche sich durch die Menge an oberflächlich-gebundenem Biotin unterschieden. Diese unterschiedlichen Biotinmengen sollten zu einer verbesserten und intensivieren Interaktion zwischen Avidin – gebunden an Meerrettichperoxidase (HRP) für die spätere Detektion mittels TMB – und dem an der MP-Oberfläche assoziiertem Biotin führen. Die MP wurden entweder nur mittels Biotin-markiertem WGA (b-WGA), oder zusätzliche mit BIO C5 NHS in geringerer (bb-WGA) oder höherer (bbb-WGA) Konzentration modifiziert. Die Ergebnisse zeigten eine Verbesserung des erreichten Signals über den stetigen Anstieg von Avidin-zugänglichem Biotin auf der MP Oberfläche. Insgesamt blieb die erreichte Signalhöhe jedoch stark unter dem Level, welches zur Unterscheidung zwischen Zelladhäsion und Zellinvasion benötigt wird. Weiters wurden MP nun direkt mit HRP und HRP/WGA konjugiert, was sich sowohl in erfolgreicher MP Oberflächenmodifikation also auch in einer Steigerung des TMB Signals als vielversprechend erwies. Dennoch stellte sich diese Methode als mangelhaft heraus, da ein nicht näher untersuchtes Phänomen auftrat, welches man eventuell als Enzymsättigung beschreiben könnte.

Diese Störung der enzymatischen Reaktion bedarf weiterer Nachforschungen, die in diesem Zusammenhang nicht durchgeführt wurden.

Für die zweite Detektionsmethode wurde ein sogenanntes Tyramid Signal Amplification (TSA) Kit verwendet, welches dazu dient HRP (konjugiert an WGA oder MP) sichtbar zu machen. Dabei kommt es zu einer enzymatischen Reaktion zwischen einem fluoreszenz-markiertem Tyramide und HRP, welche zur Bildung eines Tyramid-Radikals führt, das nun eine kovalente Bindung mit nukleophilen Bereichen auf der Zelle eingeht und somit detektierbar wird. Die Analyse dieser Methode mittels Mikroskop zeigte sich erfolgreich unter Verwendung eines Biokonjugates (hrp-WGA); erlaubte also eine klare Unterscheidung zwischen intrazellulär und extrazellulär gebundenem Lektin, welche durch die Undurchlässigkeit der Zellmembran für Tyramid ermöglicht wurde. Leider erwies sich diese Methode als unzureichend für die Anwendung an modifizierten MP (HRP MP) und für quantitative Detektionsmethoden (Fluorimetrie via Tecan Infinite® 200 Reader).

Die letzte Methode, die in dieser Arbeit evaluiert wurde, basiert auf der Fähigkeit von TCEP verschiedene fluoreszierende Moleküle zu quenchen, und erwies sich auch als die vielversprechendste Detektionsmethode. Der TCEP Assay wurde unter Verwendung verschiedener Detektionsapparaturen (Fluoreszenzmikroskop, Fluorimetrie via Tecan Infinite® 200 Reader und Flowcytometer) erfolgreich angewendet und optimiert. Außerdem konnte die Anwendbarkeit des Assays sowohl auf Biokonjugate (a647-WGA) als auch NP demonstriert werden. Das Prinzip der Unterscheidung zwischen zelladhärenter und zellinvasiver Fracht beruht auf der Undurchlässigkeit der Zellmembran für TCEP und konnte durch Permeabilisation der Zelle als Kontrollversuch verifiziert werden. Aufgrund von Schwierigkeiten bei der Oberflächenmodifikation der NP konnte auch mit Hilfe des TCEP Assays keine klare Aussage über Internalisation der Partikel in Blasenzellen (5637) erreicht werden. Nichtsdestotrotz stellt die neu etablierte Detektionsmethode via TCEP Quenching eine geeignete Möglichkeit zur Unterscheidung von Fracht innerhalb und außerhalb der Zelle dar und könnte somit für weitere Untersuchungen auf dem Gebiet der Analyse von Endocytose hilfreich sein.

Index of contents

1. Introduction and purpose

Bladder diseases are prevalent with bladder cancer (BCa) being amongst the seven most common neoplastic malignancies in men [1] and urinary tract infection being one of the most common bacterial infections in women [2, 3]. Both diseases are known for high relapse incidences [4]. Therefore, new drug therapies with minor toxicity and the ability to target residual cancer cells or bacteria (UPEC) are urgently sought after. Although the human urothelium is one of the hardest biological barriers to penetrate, the approaches so far to recover new receptor-targeted drug delivery systems were fruitful. Wheat germ agglutinin (WGA) could be established as a promising candidate for glycorecognitive cell adsorption and further internalization via active targeting of endogenous uptake pathways [5]. Furthermore, the capability to immobilize WGA at the surface of PLGA microparticles (MP) has successfully been shown, which offers application as drug vehicle e.g. for GEM-C18, in order to improve intravesical chemotherapy for BCa [6]. The specific interaction between the MP surface and the glycocalyx mediated by WGA, should increase the residence time and with it the therapeutic success. However, cell adhesion of the drug and its vehicle alone still shows some difficulties with bladder diseases: During an instillation the active component and its carrier will be diluted by constant urinary production and therefore washed out. Furthermore, intracellularly stored bacteria or residual cancer cells are not accessible for the drug, which may lead to a relapse. An ideal solution in order to prevent these incidents would not only be cellular adhesion but also internalization of the whole drug delivery vehicle. Surface modification of MP with WGA already enables a stable cytoadhesion via cellular carbohydrate chains, which may function analogously to the specific mechanism uropathogenic bacteria utilized for cytoadhesion and further cytoinvasion [7]. However, endogenous uptake of entire PLGA MP in bladder cells has not been confirmed yet. Conclusive evidence for successful internalization of nanoparticles (NP) in resorptive cells such as CaCo-2 (heterogeneous human epithelial colorectal adenocarcinoma cells) has been provided in several studies [8, 9] but PLGA NP internalization has not been proven in bladder cells.

The current state of the art for analysis of endocytosis is mainly based on fluorescence detection with fluorescein or similar stains and their specific (pH-dependent) behaviour in case of cellular uptake. Fluorescein is a fluorescent dye, which is quenched by endogenous acidic substrates when accumulated in intracellular lysosomes. Indication of successful internalization through RFI decrease can be corroborated by achieving a dequench via Monensin treatment,

which works via equilibration of the pH in the lysosomes and the cytoplasma. Although this detection method is commonly used, it entails a lot of disadvantages in terms of feasibility and its range of uses: It is time-critical and only applicable for viable cells, i.e. fixation of the cells – and with it an immobilization of associated cargo – after internalization is not possible. Besides, this detection method does not yield marked results irrespective of the implemented cell line. Especially for bladder cells the Fluorescein/ Monensin method could not provide constant results for successful or non-successful internalization, which may be due to the specific cellular architecture of this tissue. It is known that urothelial cells possess multiple different, yet still largely unexplored endocytic pathways [10, 11]. This is in contrast to resorptive human tissues, which have been investigated in more detail. However, UPEC bacteria, which have an average diameter of approximately 3 µm, are obviously able to penetrate urothelial cell membranes through an endogenous uptake pathway. In general, the urinary tract is featured with formidable host defences, such as the constant urine production and its microbe cleansing flux. In any case, the ability of UPEC bacteria to effectively invade bladder cells is mediated by a specific interaction between the bacterial pili and a number of host glycoproteins and non-glycosylated peptide epitopes. In studies it could be shown that the FimH lectin adhesion domain at the distal end of the bacterial pili is essential and sufficient to initiate intracellular uptake of UPEC bacteria in bladder cells. The specialized knowledge of UPEC being an opportunistic intracellular pathogen also alters the view of medical treatment of urinary tract infections. Current antibiotical treatment only reaches extracellular bacteria and does not affect intracellular bacterial reservoirs. Hence, enhanced delivery strategies for antibiotics without emerging resistant strains are desired [12]. The same penetration-capability – as described for UPEC bacteria – in urothelial tissue has been demonstrated for particles up to 10 µm [13]. So, in theory, an internalization of nano- to microparticles might be possible, but for verifying this assumption, a refined and more powerful detection methodology is required.

So the prior objective of this work was to establish a robust method for detection and characterization of endocytosis. This method should not depend on subjective estimation and should be compatible with different cell lines and conditions. In addition, an easy handling should be ensured, as well as applicability for both qualitative and quantitative detection systems. Beneficial features would be time-independency, applicability for viable and fixed cells as well as feedback in form of easily quantifiable – that also means specific – signals via a distinctive stain or a staining reaction. In order to evaluate whether these goals are met, all methods were first assessed for fluorescence-labelled bioconjugates, which have previously been shown to be internalized by bladder cells [5]. Subsequently the same method was applied

for surface modified PLGA micro- or nanoparticles. Given that the specific characteristics of the urothelium (concerning endocytic pathways) should be in focus of the research work, all assays were performed with 5637 malignant bladder cells. Applicability and assessment for other cell lines was not part of the present work and still requires further research. In sum, three different detection methods were tested:

(i) The TMB assay is based on the quantification of HRP via a colorimetric enzyme reaction with TMB. HRP can be coupled to Avidin, using the principle of the very strong non-covalent binding between Biotin and Avidin in the detection process, or directly immobilized at a MP surface (Figure 1, Figure 2).

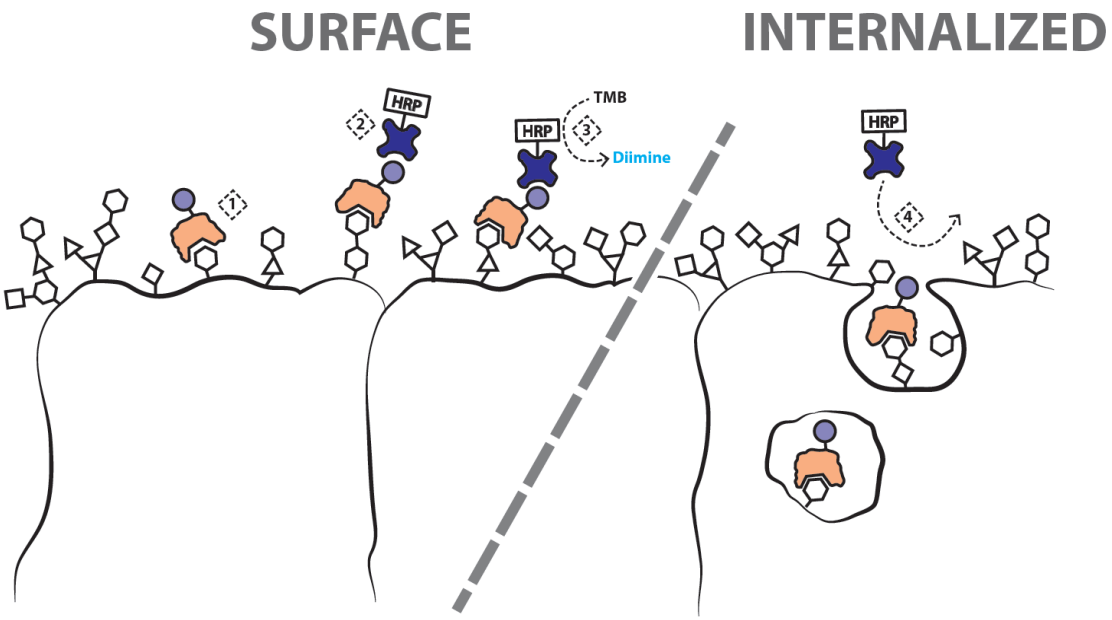


Figure 1: The image shows the principle of an internalisation assay for glyco-mediated delivery, based on a TMB reaction. *(1)* biotin-labelled, lectin-modified cargo (cargo = orange, [e.g. WGA/ MP];,Biotin = lila) specifically binds to the cell surface through sugar residues (e.g. GlcNAc, sialic acid or other glycans). *(2)* Due to the very strong non-covalent bond between Biotin and Avidin, a stable linkage is established between accessible biotin groups and hrp-Avidin (Avidin = blue, labelled with HRP). *(3)* The enzyme HRP converts TMB to a coloured diimine, with strong absorption in the blue band *(4)* Internalized biotinylated cargo is no longer accessible for hrp-Avidin, and the enzymatic reaction is precluded.

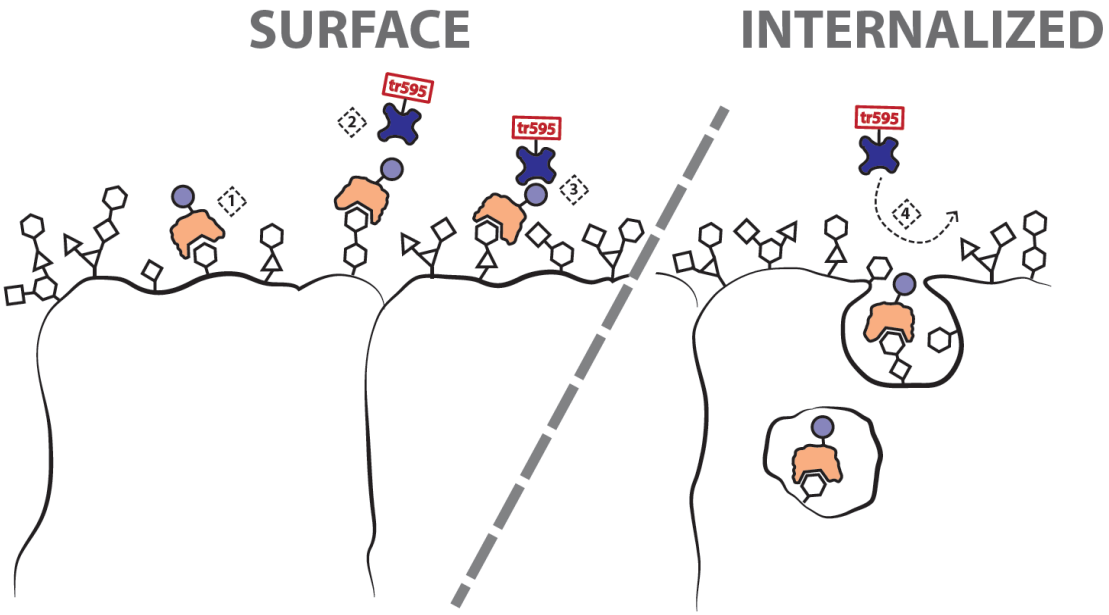


Figure 2: The image shows the principle of an internalisation assay for glyco-mediated delivery, based on fluorescence-labelled Avidin as a detection agent. *(1)* biotinylated cargo (= orange [e.g. WGA/ MP], labelled with Biotin = lila) specifically binds to the cell surface through sugar residues (e.g. GlcNAc, sialic acid or other glycans). *(2)* Due to the very strong non-covalent bond between Biotin and Avidin, a stable linkage is established between biotinylated cargo and tr-Avidin (Avidin = blue, labelled with the fluorescent molecule tr595). *(3)* The amount of cell-associated fluorescence can readily be assessed via a plate reader. *(4)* Internalized biotinylated cargo will no longer be accessible for tr-Avidin, and a lower amount of cell-associated fluorescence will be detectable.

(ii) The TSA assay, which also detects HRP linked either to a bioconjugate or particle surfaces, is based on an enzyme-triggered covalent coupling of the dye to nearby nucleophilic centers (Figure 3).

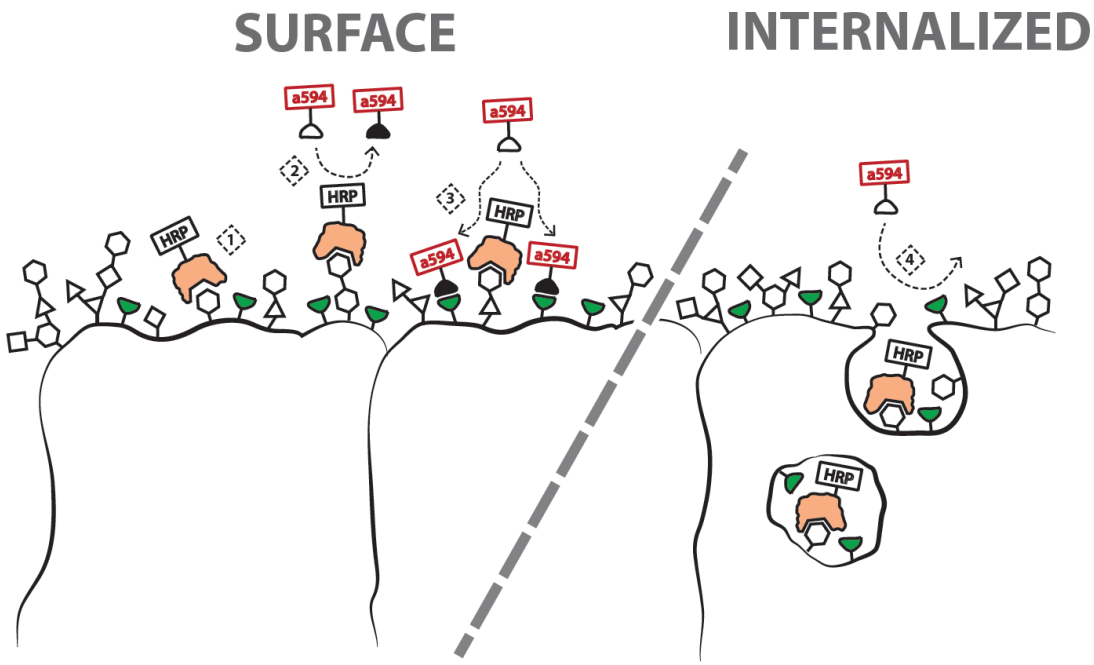


Figure 3: The image shows the principle of an internalisation assay for glyco-mediated delivery, based on the enzyme-triggered TSA assay. *(1)* HRP-labelled cargo (= orange, e.g. WGA/ MP labelled with HRP) specifically binds to the cell surface through sugar residues (e.g. GlcNAc, sialic acid or other glycans). *(2)* The a594-tyramide dye (tyramide = white, labelled with a594) is activated by HRP to highly reactive but short-lived radicals (tyramide radicals = black, still labelled with a594). *(3)* These radicals can covalently bind to nearby nucleophilic binding sites (= green), which results in stably fluorescence-labelled regions of the cell surface. *(4)* If the HRP-labelled cargo is internalized, it will no longer be accessible for the a594-tyramide dye. No labelling will occur and a lower fluorescence readout will be obtained.

(iii) The TCEP assay utilizes the reversible quenching ability of Tris(2-carboxyethyl)phosphine hydrochloride for different fluorophores, in our case Alexa Fluor® 647. This fluorescent molecule can again be part of a bioconjugate or be immobilized on micro- or nanoparticles of interest (Figure 4).

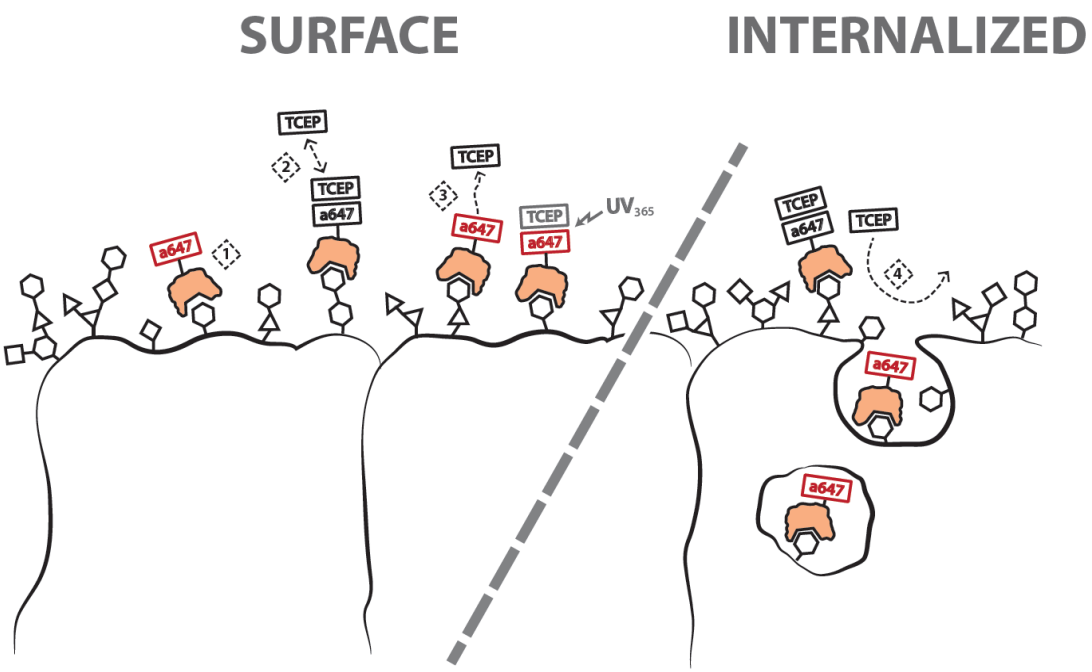


Figure 4: The image shows the principle of an internalisation assay for glyco-mediated delivery, based on the TCEP quenching assay. *(1)* Alexa-647-labelled cargo (= orange, e.g. a647-WGA/ MP) specifically binds to the cell surface through sugar residues (e.g. GlcNAc, sialic acid or other glycans). *(2)* TCEP forms a covalent adduct with the fluorescence molecule a647, resulting in a near-complete fluorescence quench. *(3)* This quench is reversible either by elution of TCEP with repeated washing or via UV (365 nm) exposure. *(4)* If Alexa-647-labelled cargo is internalized, it will no longer be accessible for the TCEP quencher. Accordingly, higher fluorescence levels from intracellular dyes will be observed.

2. The Biotin-Avidin system

Biotin, also known as Vitamin H, and Avidin, a natural glycoprotein, are characterized by their chemical bond – one of the strongest non covalent chemical bonds in nature – which is highly specific and shows a high degree of affinity [14]. It is used for various applications such as protection of pest infestation in agriculture [15] or affinity chromatography [16] and can also be effective to detect the cytoadhesive and cytoinvasive capacity of bioconjugates, or, in the present case, MP. For this purpose, Biotin or Biotin-WGA is conjugated to the MP surface and Avidin, which is either labeled with a fluorescent component or an enzyme, acts as detectable agent (For a summary of all Biotin-modified MP see Table 1).

2.1. Evaluation of covalent particle surface modification

PLGA MP were prepared via sonication as detailed in *6.2 Preparation of PLGA nano- to microparticles* and contained Bodipy®, a fluorescent dye, which allowed to calculate the particle amount used in experiments. For covalent surface modification 1 ml of these particles was stirred for 30 min at RT with 281 mg EDAC and 11 mg NHS in 500 µl of 20 mM HEPES/NaOH pH = 7,0 to activate accessible carboxyl groups at the particle surface. After removing the remaining activating reagents in the supernatant via two cycles of centrifugation (14000 rpm, 3 min, 4°C) and resuspension in 20 mM HEPES/NaOH pH = 8,0, 1 ml of the activated MP suspension was treated either with 45,0 µl of a 5,0 mg/ml WGA-solution and 25,0 µl of a 1,0 mg/ml Biotin WGA (b-WGA) -solution, which equates to a ratio of 10:1, or 50,0 µl of a 5,0 mg/ml WGA-solution or 45,8 µl of a 10,0 mg/ml human serum albumin (HSA) -solution for 18 hours during constant agitation. After 2 hours of saturation of non-reacted vinyl groups with 242 µl of a 100 mg/ml glycine solution in 20 mM HEPES/NaOH pH = 8,0 the particles were washed three times with 20 mM HEPES/NaOH pH = 7,4 by centrifugation (14000 rpm, 2 min, 4°C) and subsequently resuspended in 1ml 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68, which acts as a stabilizer.

The plant lectin WGA was used to increase the association between MP and the cells because of its carbohydrate-binding characteristics – the specific interaction with N-acetyl-D-glucosamine (GlcNAc), sialic acid and other glycans at the cell surface. WGA was chosen due to the fact that a strong cytoadhesive capacity could already been demonstrated on urothelial cells [5].

In the current experimental approach, the cytoadhesive potential or the possible endocytic uptake of the modified MP should be detected via a primary/secondary labelling system based on the Biotin-Avidin bonding. Prior to performing binding and endocytic uptake assays, an assessment of the MP modification as described above was necessary. Therefore, the modified particles were incubated with Texas red® 595-labeled Avidin (tr-Avidin), mostly for qualitative but also quantitative detection, and thereby validation of the coupling-protocol.

specification	*modification procedure*		
	step 1	*step 2*	*step 3*
b-WGA MP	conjugation of 25 µl of 1 mg/ml Biotin-WGA to the MP surface	/	/
bb-WGA MP	conjugation of of 25 µl of 1 mg/ml Biotin-WGA to the MP surface	incubation with of 65 µl of 1 mg/ml BIO C5 NHS	/
bio WGA MP	conjugation of of 50 µl of 5 mg/ml WGA to the MP surface	incubation with 65 µl of 1 mg/ml BIO C5 NHS	/
bbb-WGA MP	conjugation of of 25 µl of 1 mg/ml Biotin-WGA to the MP surface	incubation with 65 µl of 1 mg/ml BIO C5 NHS	further incubation with 65 µl of 5 mg/ml BIO C5 NHS

Table 1: Specification of particle suspensions used in the current study.

2.2. Detection of b-WGA conjugated MP via tr-Avidin

For the validation of successful MP surface modification, 1 ml particle suspension of both variants, WGA/b-WGA (10:1) modified MP (designated 'b-WGA MP') and HSA modified MP (designated 'HSA MP') were incubated with 500 µl of 0,7 nmol/ml tr-Avidin (30 min, RT) during constant agitation. For the removal of unbound Avidin, the particles were washed twice with 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68 by centrifugation and resuspension and diluted 1:50 before settling in coverslip-mounted flexiPERM® chambers. The subsequent detection via fluorescence imaging confirmed successful particle surface modification: A homogenous red surface stain of b-WGA MP due to the Biotin/-tr-Avidin bonding in comparison to no staining of the HSA MP corroborated the successful surface modification in case of b-WGA.

For further validation/alteration of these results, plain PLGA/Bodipy® MP were modified with a mix of WGA and Alexa Fluor® 594 WGA (a594-WGA) (applied in a ratio of 10:1) as described above and directly detected via fluorescence imaging. The a594-WGA modified MP (designated 'a594-WGA MP') also showed a homogenous red surface staining. This direct staining of the cells with a594-WGA (in comparison to the two step staining via b-

WGA and tr-Avidin) even resulted in more intensive surface staining than that of the b-WGA MP.

2.3. Detection of bb-WGA conjugated MP via tr-Avidin

For optimization of the MP surface modification (and with it the Avidin-based detection system), it was necessary to increase the intensity of the fluorescent stain. If there would be more Biotin at the particle surface, more binding sites for Avidin should be available and an enhancement of the fluorescence intensity should be possible. For this, the b-WGA modified PLGA/Bodipy® MP were additionally biotinylated with Biotinamidocaproate-N-hydroxysuccinimideester (BIO C5 NHS). After washing the particles three times with 20 mM HEPES/NaOH pH = 7,4 by centrifugation and resuspension to remove the possible disturbing factor Pluronic® F-68, the MP were resuspended in 1 ml of 20 mM HEPES/NaOH pH = 7,4. Subsequently, 65 µl of 1 mg/ml BIO C5 NHS in DMF were added to the particle suspension and it was incubated for 1 hour at RT during constant agitation. The MP were washed thrice by centrifugation/ resuspension to remove excessive BIO C5 NHS. The additionally biotinylated b-WGA conjugated MP (designated 'bb-WGA MP') and HSA MP were incubated with 500 µl of 0,7 nmol/ml tr-Avidin for 16 hours on the rotary shaker. The modification was verified by fluorescence imaging after purifying the particle suspension from the unbound tr-Avidin via centrifugation and resuspension. The acquired images showed that the additional biotinylation of the b-WGA MP was successful, as they showed a more intensive red surface staining than the non-biotinylated b-WGA MP. The extended incubation time with tr-Avidin had no effect on non-specific staining of the MP, demonstrated by HSA MP, which did not show any red fluorescence at all.

2.4. Detection of bbb-WGA conjugated MP via tr-Avidin

Initial results of the bb-WGA MP were promising, but with regard to detection sensitivity it was still to be evaluated whether a further fluorescence intensity enhancement is possible. For this, bb-WGA MP were incubated in a second step with a higher concentration of the BIO C5 NHS (referred to as 'hyperbiotinylated' or 'bbb-WGA MP'). The protocol involved incubation of 1 ml MP suspension with BIO C5 NHS (65 µl of 5 mg/ml BIO C5 NHS in DMF) over night on the rotary shaker. 1 ml of the surface-modified MP, either in hyperbiotinylated

form or normally biotinylated (bb-WGA MP), as well as HSA MP were incubated with 500 µl of 0,7 nmol/ml tr-Avidin for 60 minutes to allow for fluorescence imaging.

Effectiveness of the hyperbiotinylation process could be verified by a more intensive fluorescent surface staining in comparison to bb-WGA MP. Again, no red fluorescence was visible for HSA MP (Figure 5).

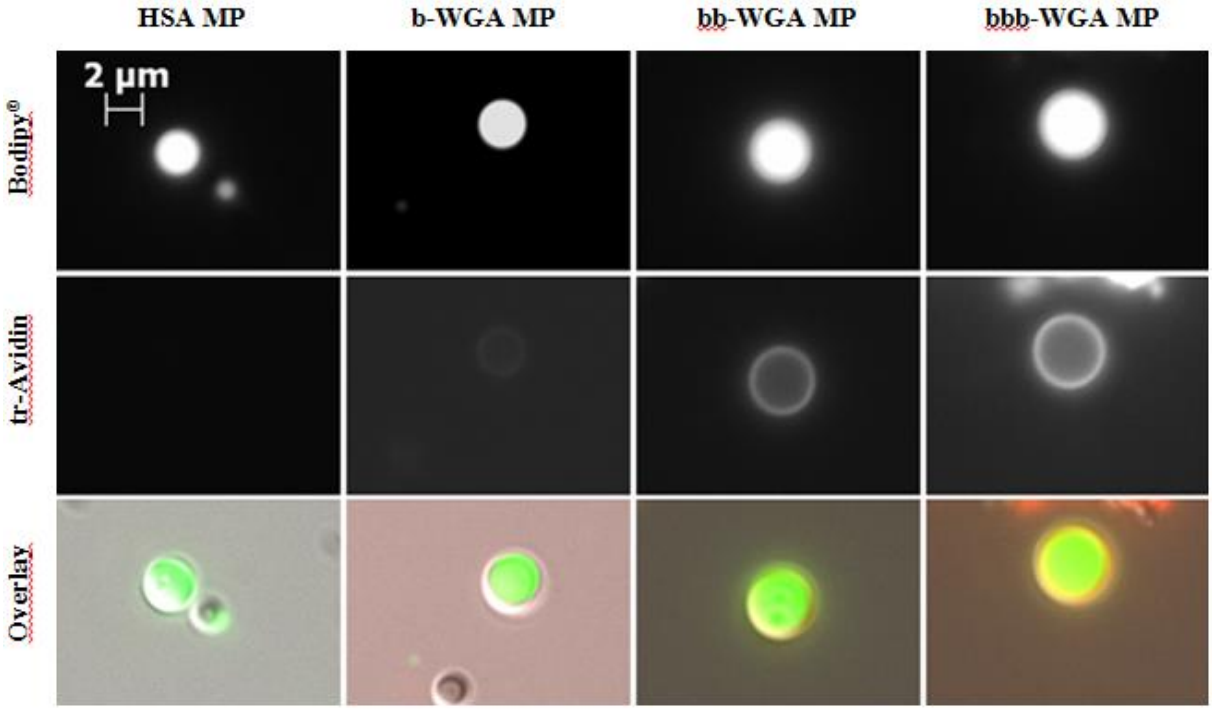


Figure 5: Microscopic analysis of the affinity of tr-Avidin to different surface-modified microparticles. HSA MP show no affinity at all, whereas the particle surface staining caused by the targeter increased gradually from b-WGA over bb-WGA and bbb-WGA MP, which corroborates the success of the particle surface modification as well as the specificity of the Biotin – Avidin interaction. Overlay images show merged channels of Bodipy®, tr-Avidin and DIC. All images were processed using the same imaging conditions to allow for direct comparison. (green channel, Bodipy®, 470nm, 100%, 1ms; red channel, tr-Avidin, 590nm, 100%, 1000ms)

Another issue for detection assays on cells is the potentially impeded accessibility of MP for Avidin if the particle is associated with the cell surface and might (entirely or in part) be covered by the contacting membrane. Hence, if attached to a monolayer, the limited accessibility of the MP surface could lead to minor binding between Avidin and Biotin molecules, which could falsify a quantitative analysis.

For the evaluation of the accessibility of MP after cytoadhesion via fluorescence-microscopic analysis confluent cell monolayers on coverslips were washed thrice with PBS ($+Ca^{2+}/Mg^{2+}$) prior to incubation with the different MP – bb-WGA and bbb-WGA MP – for 30 minutes. The MP were treated with 0,7 nmol/ml tr-Avidin for 60 minutes either before (bb-

WGA/Av MP; bbb-WGA/Av MP) or after cytoadhesion (bb-WGA MP + Av; bbb-WGA MP + Av).

Again, the assay verified successful particle surface modification and no indication for accessibility problems for tr-Avidin after cytoadhesion of the MP could be found, since no obvious difference in the fluorescence intensity between incubation with tr-Avidin before and after cell surface association of the MP was found (Figure 6).

Non-specific association of tr-Avidin to the cell surface was assessed in a control assay where a confluent 5637 monolayer was incubated with 50 µl of 0,7 nmol/ml tr-Avidin for 1, 2 or 5 hours. No non-specific staining was visible in microscopic analysis irrespective of the incubation time.

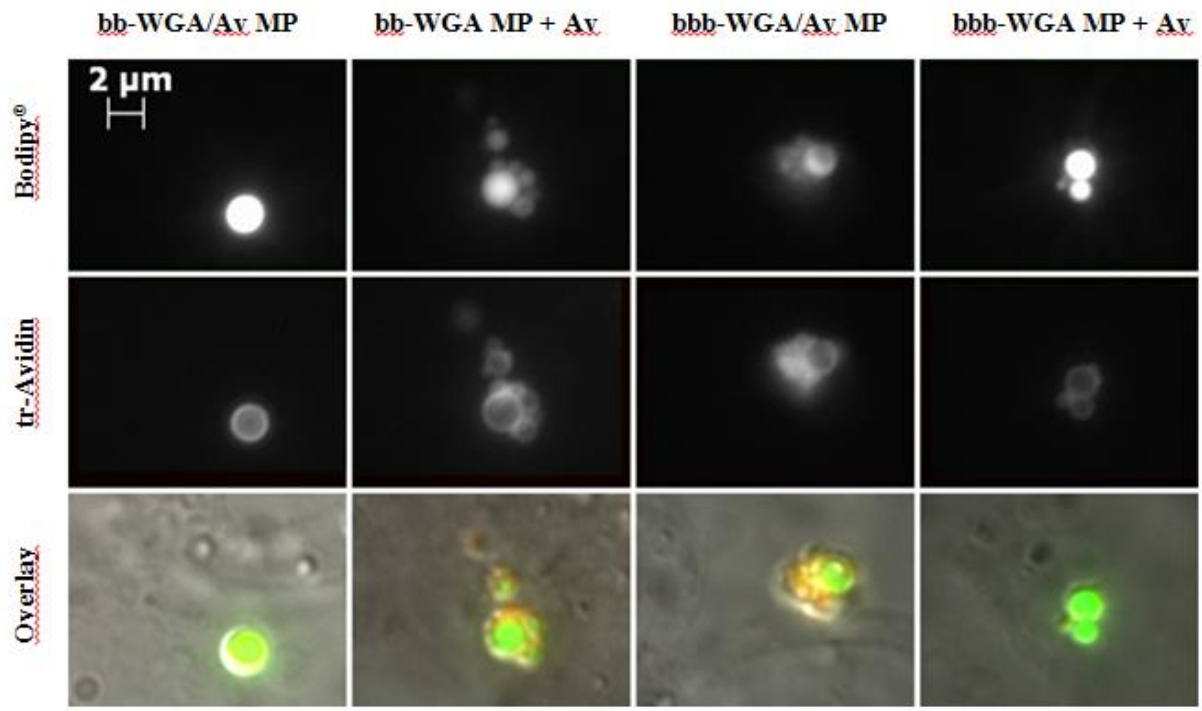


Figure 6: Microscopic analysis of the accessibility of MP for tr-Avidin after cytoadhesion. Confluent 5637 cells were incubated with different MP, which were treated with tr-Avidin either before (bb-WGA/Av MP; bbb-WGA/Av MP) or after cytoadhesion (bb-WGA MP + Av; bbb-WGA MP + Av). The differently treated MP showed no difference in their fluorescence intensities irrespective of the point of tr-Avidin incubation. Hence, no accessibility problems for Avidin after cytoadhesion could be found. Overlay images show merged channels of Bodipy®, tr-Avidin and DIC. Images showing either bb-WGA MP or bbb-WGA MP were processed using the same imaging conditions to allow for direct comparison and conclude on the accessibility of tr-Avidin. (bb-WGA MP: green channel, Bodipy®, 470nm, 100%, 17ms; red channel, tr-Avidin, 590nm, 100%, 2217ms; bbb-WGA MP: green channel, Bodipy®, 470nm, 100%, 8ms; red channel, tr-Avidin, 590nm, 100%, 270ms)

2.5. TMB Assay

After confirming successful MP surface modification, a quantitative approach on basis of the well-known TMB assay was to be evaluated (see below).

2.5.1. Basic principle of the TMB Assays

3,3',5,5'-Tetramethylbenzidine (TMB) is a chemical substrate of the benzidine family and shows chromogenic characteristics. TMB catalyses the reduction of hydrogen peroxide to water through peroxidase enzymes – in our case horseradish peroxidase (HRP) – by acting as a hydrogen donor. The resulting diimine causes the solution colour in blue, which can be determined and quantified by spectrophotometric readout (Tecan Infinite® 200 Reader). The ongoing enzymatic reaction can be stopped by adding an acid, which inactivates the enzyme and causes the solution to take on a yellow colour.

Prior to the enzyme-based quantification reaction (TMB substrate + HRP), 5637 confluent monolayers were thoroughly washed with 150 µl HEPES/NaOH pH = 7,4 + 0,1% Pluronic® F-68 and incubated for 30 min at 4°C with 100 µl surface modified MP (e.g. b-WGA MP, for concentration refer to *6.2 Preparation of PLGA nano- to microparticles*) which were treated with HRP-labelled Avidin (hrp-Avidin) either before or after incubation with the target cells. After the selective bonding had formed between Biotin and Avidin, the MP could be detected enzymatically. 70µl of TMB in PBS ($+Ca^{2+}/Mg^{2+}$) with 0,0015% hydrogen peroxide were added and the reaction mix with HRP was incubated for 10 minutes before stopping the reaction with 20µl 1M H_2SO_4.

During the enzymatic reaction, the blue colouring caused by the resulting diimine of TMB was determined every 60 seconds at 370 nm and the intensity of the yellow colour caused by the sulphuric acid was determined immediately after stopping at 450 nm.

2.5.2. Evaluation of the TMB concentration

In order to apply the enzymatic TMB reaction for quantification, some basic parameters had to be evaluated first.

Evidently, the quantity and activity of the enzyme, in our case HRP, play an important role, as well as the concentration of TMB and the incubation time with the catalytic enzyme. If the TMB concentration is too low, it is depleted too fast and the intensity of the colour change

is not in direct correlation to the quantity of the enzyme. Thus, an enhancement of the enzyme amount would not lead to an increase in the detected signal. On the other hand, if the TMB concentration is too high, the reaction could be too slow due to substrate inhibition.

The used hrp-Avidin is standardised and therefore the homogeneity of HRP molecules per Avidin and the activity should be guaranteed. In order to evaluate the ideal TMB concentration, b-WGA MP were incubated with 500 µl of 0,7 nmol/ml hrp-Avidin for 30 minutes at RT. After removing the remaining fraction of free hrp-Avidin by centrifugation and resuspension, the final volume was adjusted to 1 ml. Two TMB concentration levels were tested: TMB in PBS ($+Ca^{2+}/Mg^{2+}$) with 0,0015% hydrogen peroxide diluted 1:5 from the available stock and TMB in PBS ($+Ca^{2+}/Mg^{2+}$) with 0,0015% hydrogen peroxide diluted 1:2 from the same stock. The quantity of the processed substrate was determined every 60 seconds and the reaction was stopped after 10 minutes by 1M sulphuric acid.

The 1:5 dilution showed a rather slow development and the signal remained very low, which suggested that the substrate concentration was too low. The run of the curve of the 1:2 dilution suggested a continuous development with a signal that constantly rose and levelled out after 7 minutes. The intensity of the signal after stopping the reaction suggested that the concentration of the TMB substrate after 1:2 dilution is better suited for this assay.

2.5.3. b-WGA conjugation to MP

The conjugation of b-WGA to the MP surface was performed as detailed in *2.1 Evaluation of covalent particle surface modification.*

For the further investigation of the applicability of the TMB assay, two different experimental strategies were pursued: The modified MP were incubated with hrp-Avidin either before or after incubation with the target cells.

2.5.3.1. b-WGA MP detection via hrp-Avidin prior to cytoadhesion

For these experiments, 1ml of b-WGA MP (for concentration refer to *6.2 Preparation of PLGA nano- to microparticles*) was stirred with 500 µl of 0,2 nmol/ml hrp-Avidin for 30 minutes at RT, followed by washing the MP suspension twice and resuspension in 1ml 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68.

A confluent cell monolayer (5637) was washed thoroughly and incubated with 100µl of different dilutions of the respective particle suspension for 30 minutes at 4°C. After rinsing the cells three times with 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68, the enzymatic reaction was initiated by adding 70 µl TMB in PBS ($+Ca^{2+}/Mg^{2+}$) with 0,0015% hydrogen peroxide (1:2) and stopped after 15 minutes with 20 µl 1M H_2SO_4.

The TMB signal did not increase and the recorded signal after stopping was nearly the same as the blank value. Besides, no difference between the various MP concentrations could be seen.

2.5.3.2. b-WGA MP detection via hrp-Avidin added after cytoadhesion

Here, a washed 5637 monolayer was incubated with 100 µl of different dilutions of the b-WGA MP for 30 minutes at 4°C. After washing to remove unbound MP,100 µl of 2,67 pmol/ml hrp-Avidin were added and the monolayer was incubated for 30 minutes at 4°C, followed by rinsing again three times with 20 mM HEPES/NaOH pH=7,4 with 0,1% Pluronic® F-68. In order to estimate non-specific hrp-Avidin binding to the cell membrane, a control was implemented by incubating confluent cells with 100 µl of 2,67 pmol/ml hrp-Avidin only. For both, the control and sample wells, the enzymatic reaction was started by adding 70 µl TMB in PBS ($+Ca^{2+}/Mg^{2+}$) with 0,0015% hydrogen peroxide (1:2) and stopped after 15 minutes with 20 µl 1M H_2SO_4.

The resulting run of the curve was continuously rising, although the signal intensity remained at a very low level. However, the value of the hrp-Avidin blank was as low as the value of the PBS ($+Ca^{2+}/Mg^{2+}$) blank (< 0.1 AU, cf. Figure 7), indicating that there was no obvious non-specific interaction between hrp-Avdin and the cell surface. The recorded signal showed no marked difference between the various MP dilutions. (Figure 7)

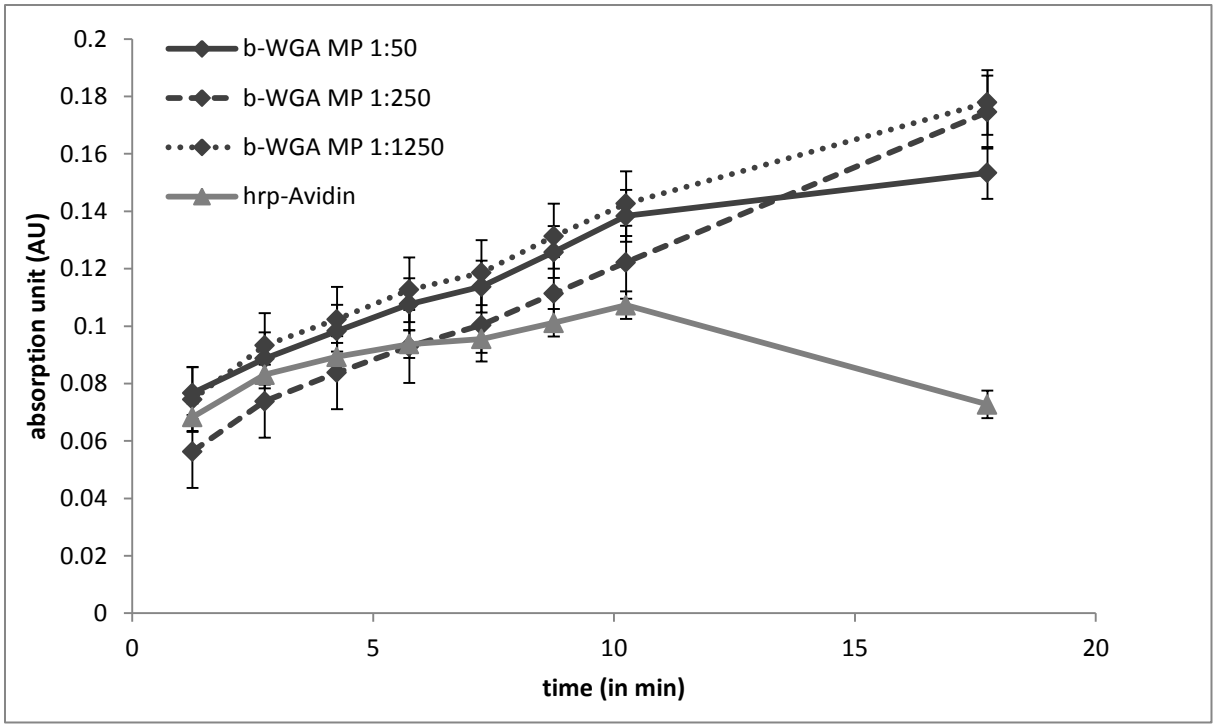


Figure 7: Association of b-WGA MP to the surface of bladder cells as detected by incubation with 2,67 pmol/ml hrp-Avidin after cytoadhesion. A confluent 5637 monolayer is incubated with 100 µl of the various dilutions of b-WGA MP (1:50; 1:250; 1:1250) for 30 minutes at 4°C. Subsequently, 100 µl of 2,67 pmol/ml hrp-Avidin are added and allowed to react for another 30 minutes at 4°C with the available Biotin on the MP surface. As control, confluent cells are also incubated with hrp-Avidin only (no MP). The monolayer is incubated with TMB for 10 minutes, followed by stopping the reaction with 1M sulphuric acid at minute 17. The enzymatic reaction with TMB showed no difference in the various MP dilutions and low signals. The values represent the mean AU ± SEM of a quadruplicate.

2.5.3.3. Discussion of the results required with b-WGA MP

In the first assessment, the application of a TMB assay for quantifying the cell adhesion of various surface-modified MP caused several problems, such as the low intensity of the recorded signal, which impeded reliable measurements. There would have been some options to improve the sensitivity but mostly with unacceptable side effects. If the MP concentration is increased, this will result in a negative influence on cell viability. An increase in the hrp-Avidin concentration could also boost the non-specific binding of Avidin to the monolayer. In order to determine the best-suited hrp-Avidin concentration – that is, the highest possible concentration with the lowest non-specific signal – a calibration line (hrp-Avidin on cell monolayers) was established with concentrations between 66,7 and 0,000034 pmol/ml. At an hrp-Avidin concentration of 2,67 pmol/ml a good signal-to-noise ratio could be obtained. However, the signal intensity when detecting b-WGA MP at this concentration was found to be too low for quantification purposes.

For experiments that involved prae-hoc incubation of MP with hrp-Avidin (prior to cytoadhesion) the hrp-Avidin and MP concentration were adapted to the approximate remaining cell-bound MP level in experiments with detection after cytoadhesion. However, satisfying results could be obtained in none of these combinations of modified MP and Avidin.

2.5.4. Additional biotinylation of b-WGA conjugated MP

Given that the problem of the low intensity of the signal cannot be solved by increasing the MP or hrp-Avidin concentration, one possible alternative strategy consisted of multiplying the available binding sites at the MP surface for Avidin, that is the amount of surface Biotin. For this reason, the b-WGA MP were additionally biotinylated as described in *2.3. Detection of bb-WGA conjugated MP via tr-Avidin* to yield bb-WGA MP.

2.5.4.1. bb-WGA MP detection via hrp-Avidin prior to cytoadhesion

Possible optimization via additional biotinylation was tested with three different MP variants: bb-WGA, b-WGA and HSA MP. In theory, the intensity of the signal should increase between the b-WGA and bb-WGA MP and the HSA MP should give no signal at all. Thus, they represent the blank.

All types of modified MP were incubated with 500 µl of 0,7 nmol/ml hrp-Avidin for 18 hours and washed twice with 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68 by centrifugation and resuspension.

In pilot tests, without using cells but detecting the MP via TMB and H_2SO_4, the HSA MP showed the same enzymatic reaction as the b-WGA MP (Figure 8). This implicates a non-specific interaction between hrp-Avidin and the modified MP, which could not be removed from the surface by the usual washing steps via centrifugation. After changing the washing protocol from centrifugation to dialysis, this problem was solved and further pilot tests showed the desired results. Generally, the run of the curve presented a continuous rise for all of the different MP. The intensity of the signal decreased gradually from the bb-WGA over the b-WGA to the HSA MP (Figure 9).

In order to verify these results on a cell surface, a confluent monolayer was washed thrice and incubated with the same selection of modified MP and two additional controls (b-WGA and HSA MP, which were not incubated with hrp-Avidin) for 30 minutes at 4°C. In order to prevent non-specific binding to the cell surface, 1% bovine serum albumin (BSA), was

added to the usual washing buffer PBS (+Ca^{2+}/Mg^{2+}). As usual, the enzymatic reaction was initiated by adding 70 µl TMB in PBS (+Ca^{2+}/Mg^{2+}) with 0,0015% hydrogen peroxide (1:2) and stopped after 20 minutes with 20 µl 1M H_2SO_4.

The signals of the applied MP had the same gradation as suggested in the pilot tests. Also the HSA MP (incubated with hrp-Avidin) behaved as expected: The intensity of the signal matched the value of the other blanks without hrp-Avidin incubation (see above) (Figure 10a); moreover, the entrapped Bodipy® indicated that the HSA MP could be rinsed away more easily because they showed no specific interaction with the glycocalyx of the cells (Figure 10b). In addition, the results suggested that no steric effects were inhibiting the specific binding of the MP: Although hrp-Avidin was already conjugated to the particle surface, cytoadhesion was still possible.

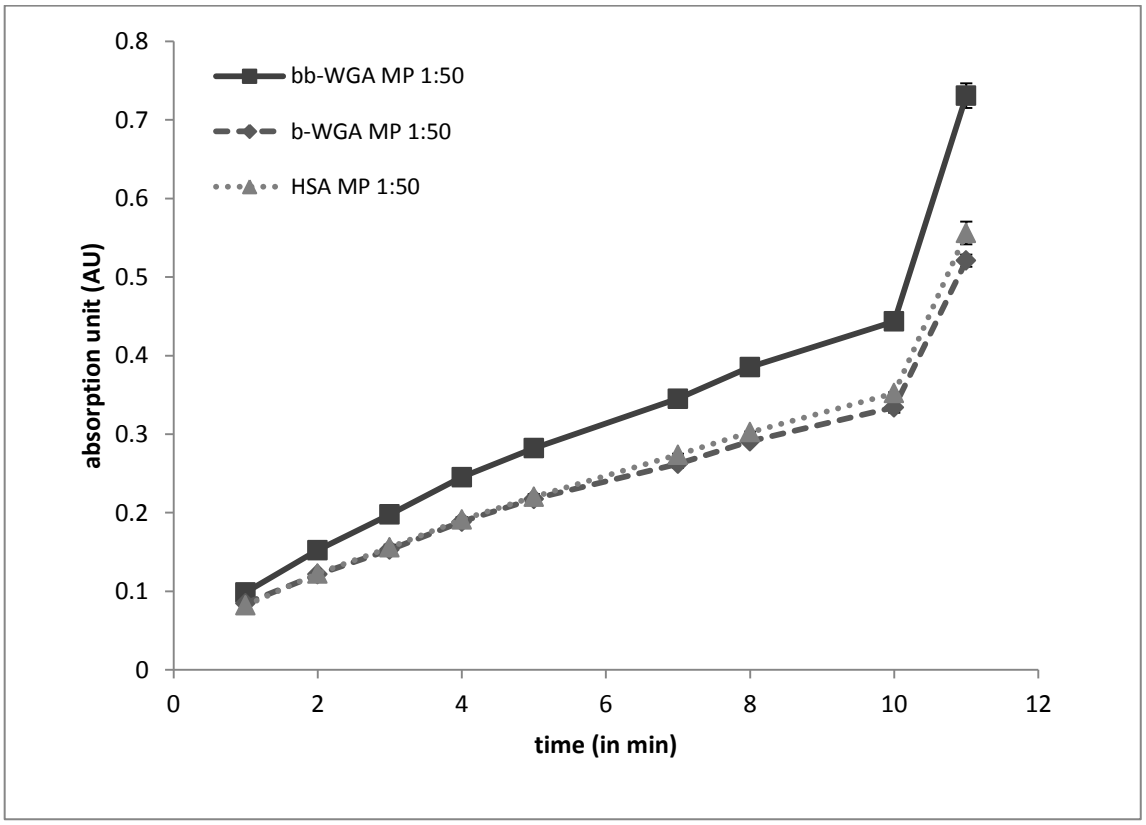


Figure 8: Evaluation of the coupling between hrp-Avidin and various MP (bb-WGA MP, b-WGA MP, HSA MP) washed via centrifugation/ resuspension without incubation on cells. All types of MP are incubated with 0,7 nmol/ml hrp-Avidin, subsequently washed via centrifugation/ resuspension and diluted 1:50 prior to TMB treatment for 10 minutes. The enzymatic reaction is stopped with H_2SO_4 after 11 minutes. Both b-WGA and HSA MP reach the same AU level, probably due to a non-specific adsorption of hrp-Avidin to the particle surface, which cannot be removed by the usual washing step via centrifugation. The values represent the average AU ± SEM. All samples are performed in triplicate.

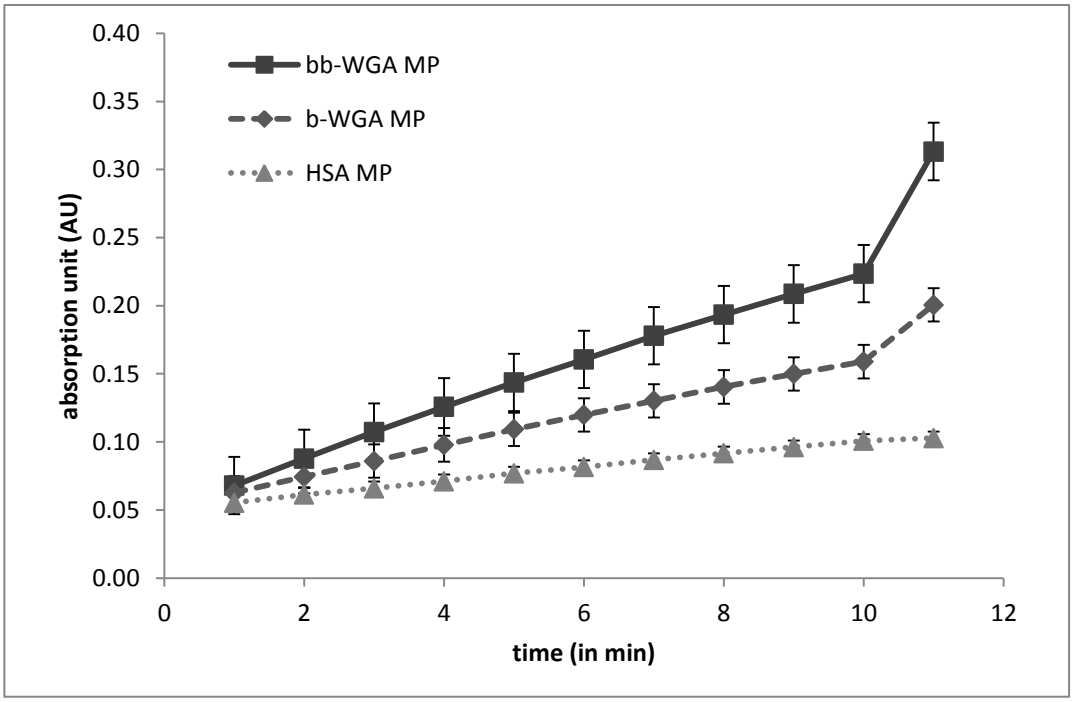


Figure 9: Evaluation of the coupling between hrp-Avidin and various MP (bb-WGA MP, b-WGA MP, HSA MP) washed via dialysis without incubation on cells. All types of MP are incubated with 0,7 nmol/ml hrp-Avidin, subsequently washed via dialysis and adjusted to the same level concerning their Bodipy® fluorescence signal (RFI approximately 180, gain 80, ex/em: 485/525). The MP are treated with TMB for 10 minutes and the enzymatic reaction is stopped with H_2SO_4 after 11 minutes. A clear distinction between the various MP can be seen according to the amount of surface associated Biotin. All values represent the average AU ± SEM of a triplicate.

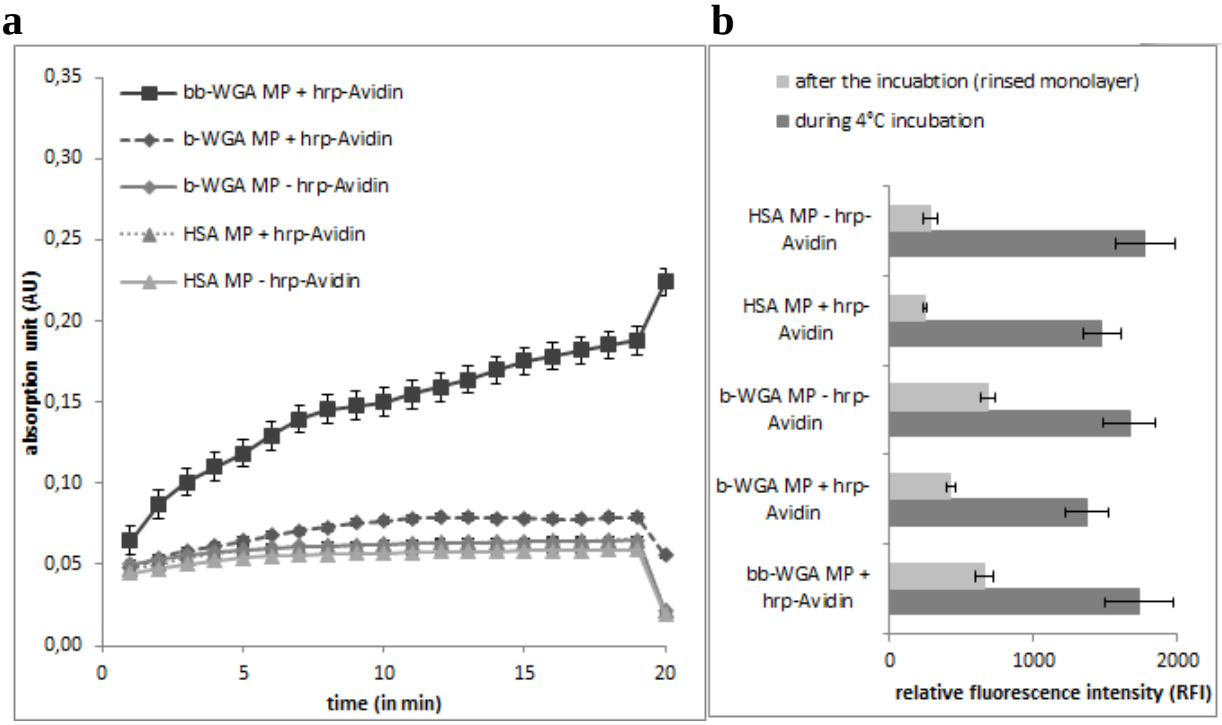


Figure 10: Evaluation of the cytoadhesive potential of various MP (bb-WGA MP, b-WGA MP, HSA MP; see table 2 for detailed specification) already conjugated with hrp-Avidin and washed via dialysis. A confluent 5637 monolayer is incubated with various MP, previously adjusted to approximately the same level via the Bodipy® fluorescence intensity, for 30 min at 4°C and subsequently rinsed with PBS (+Ca^{2+}/Mg^{2+}) + 1% BSA. **(a)** The detection with TMB (min 1-19) and H_2SO_4 (min 20) shows an enhancement of additionally biotinylated MP, visible in higher AU signals of bb-WGA MP than b-WGA MP. **(b)** The corresponding cellular Bodipy® fluorescence signals corroborate a specific interaction between WGA and the glycocalyx by higher remaining RFI

of MP with WGA immobilized at the particle surface. All samples were carried out in quadruplicate and the values represent the calculated mean AU/RFI ± SEM.

specification	*experimental procedure*
bb-WGA MP + hrp-Avidin	cells in monolayer configuration are incubated with bb-WGA MP, which were previously conjugated to hrp-Avidin
b-WGA MP + hrp-Avidin	cells in monolayer configuration are incubated with b-WGA MP, which were previously conjugated to hrp-Avidin
HSA MP + hrp-Avidin	cells in monolayer configuration are incubated with HSA MP, which were previously conjugated to hrp-Avidin
b-WGA MP - hrp-Avidin	cells in monolayer configuration are incubated with b-WGA MP only (without previous hrp-Avidin conjugation)
HSA MP - hrp-Avidin	cells in monolayer configuration are incubated with HSA MP only (without previous hrp-Avidin conjugation)

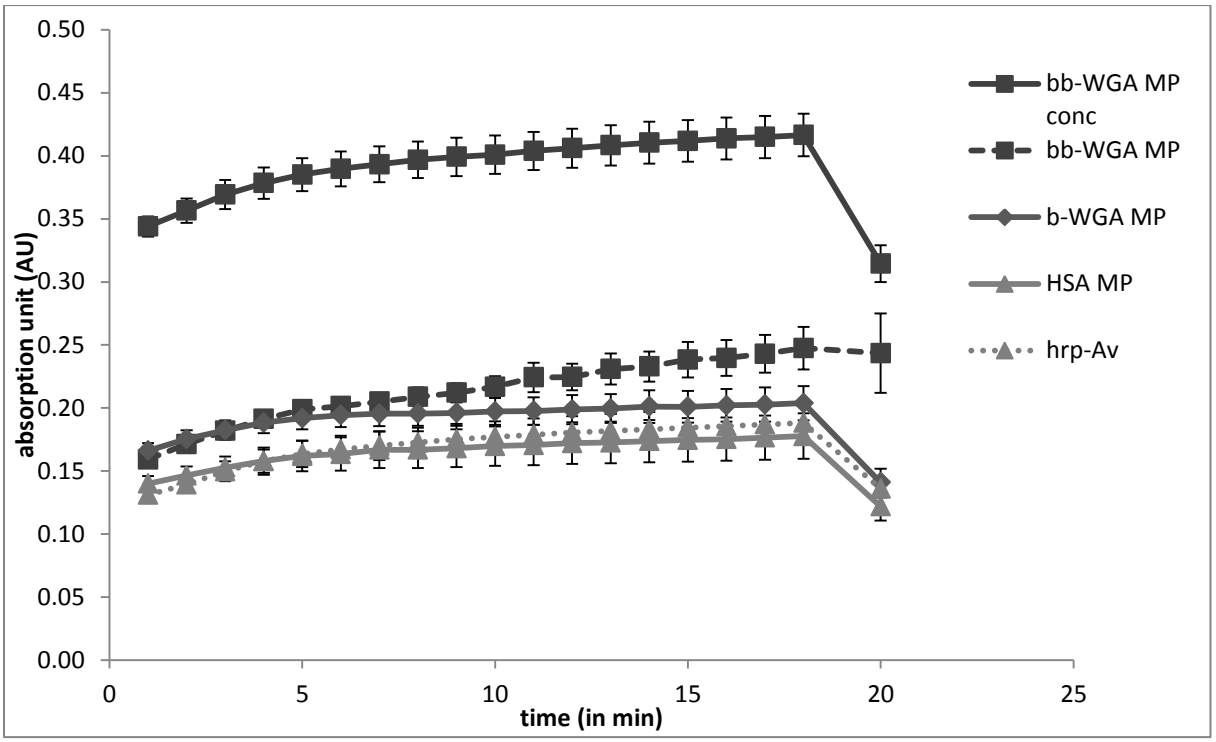
Table 2: Specification of assay conditions used for evaluation of the cytoadhesive potential (Figure 10)

2.5.4.2. bb-WGA MP detection via hrp-Avidin added after cytoadhesion

After the positive results with the bb-WGA MP which were conjugated to hrp-Avidin prior to cell contact, the same assay was repeated but with incubation with hrp-Avidin after cytoadhesion of the MP. For this, bb-WGA MP, b-WGA MP and HSA MP were incubated with the cell monolayer for 30 minutes at 4°C followed by washing with the usual protocol with 1% BSA. Only the MP remaining at the cell surface after washing got in contact with 2,67 pmol/ml hrp-Avidin. After 1 hour of incubation at 4°C the monolayer was rinsed again three times with PBS ($+Ca^{2+}/Mg^{2+}$) and 1% BSA and the enzymatic reaction proceeded as usual for 20 minutes with TMB and was stopped by 1M H_2SO_4.

The obtained curve confirmed the results shown before. The applied detection method via TMB is markedly more sensitive for the bb-WGA MP than for the b-WGA MP. (Figure 11) The controls, hrp-Avidin and HSA MP, showed no enzymatic reaction and the non-specific binding of the HSA MP could be reduced drastically by adding 1% BSA to the washing buffer.

a



b

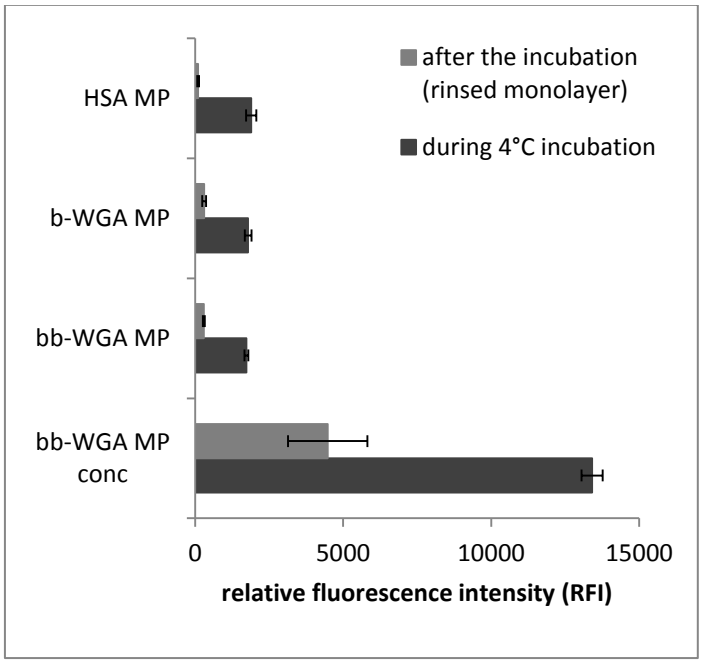


Figure 11: Detection of different cell-associated MP via hrp-Avidin and impact of additional biotinylation. Cells (5637) are incubated with 2,67 pmol/ml hrp-Avidin after cytoadhesion of various MP (bb-WGA MP, b-WGA MP and HSA MP, which are adjusted to the same Bodipy® fluorescence level, and bb-WGA in the highest possible concentration (bb-WGA MP conc).) **(a)** Development with TMB for 18 minutes, followed by an acidic stop at minute 20 suggests that the additional biotinylation step increases the recorded signal. Higher amounts of MP (bb-WGA MP conc) also increase the signal, but not in a linear manner. **(b)** The RFI of the entrapped Bodipy® shows the different amount of cell-associated cargo and with it the reduced non-specific binding of HSA MP due to BSA washing. The values represent mean AU/RFI ± SEM of a quadruplicate.

Given these results, the main part of the available Biotin at the MP surface seems to be free for Avidin binding and therefore detectable via TMB. However, the main portion of the detectable signal seems to result from interaction of hrp-Avidin with Biotin groups that were introduced via the additional biotinylation step (after b-WGA conjugation). The Biotin groups

introduced by the conjugation of b-WGA to the MP seem to play an inferior role. In order to confirm this assumption, another batch of PLGA/Bodipy®-labeled MP was produced, modified with non-biotinylated WGA and afterwards biotinylated with BIO C5 NHS for 1 hour. After cytoadhesion of these particles (designated 'bio WGA MP') and incubation with hrp-Avidin, markedly higher signal intensities were observed (Figure 12), proving the functionality and importance of the additional biotinylation step. The same Bodipy® signal levels (Bodipy® RFI of about 4500) were recorded for cell-adhesive bb-WGA MP (Figure 11) and bio WGA MP (Figure 12b). Corresponding to this same level of MP surface adhesion, also incubation with TMB gave comparable signal intensities, with bio WGA MP potentially reaching a slightly higher stopping signal after acidic treatment. Overall, Bodipy® and TMB detection seems to be well comparable.

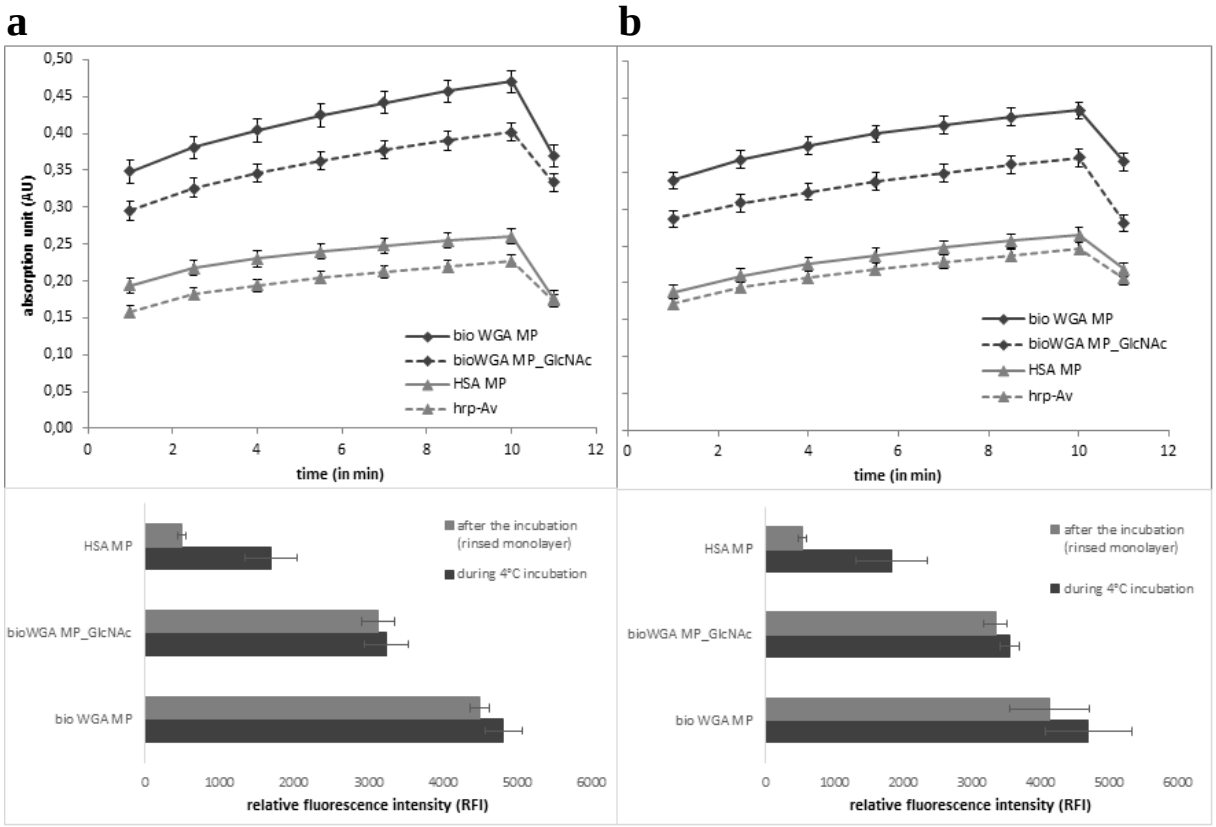


Figure 12: Evaluation of the applicability of bio WGA MP, two different hrp-Avidin concentrations as well as inhibition of WGA-cell interaction with competitive carbohydrates. A confluent monolayer (5637) is topped with 113 mM GlcNAc or buffer and bio WGA MP or HSA MP. After washing the cells they are incubated with **(a)** 8 pmol/ml or **(b)** 2,67 pmol/ml hrp-Avidin and detected via TMB (min 1-10)/ H_2SO_4 (min 11). The recorded difference of the AU signals between HSA MP and bio WGA MP corroborates the functionality of MP modification via BIO C5 NHS. In addition, the higher hrp-Avidin concentration results in an increase of sensitivity of the TMB assay without marked increase of non-specific cell binding. Inhibition with GlcNAc seems to decrease the amount of cell-associated WGA MP. All samples are performed in quadruplicate and the values symbolize the mean AU/RFI ± SEM. The bar charts show the appropriate Bodipy® fluorescence signals.

2.5.4.3. Optimization of the hrp-Avidin concentration and evaluation of specific/non-specific binding of hrp-Avidin

For further improvement of the assay, the ideal hrp-Avidin concentration was evaluated again across a restricted range of biotin using the bb-WGA MP. The former calibration line covered a concentration range from 66,7 to 0,000034 pmol/ml in dilution steps of 1:5. Best results were achieved with a concentration of 2,67 pmol/ml, whereas 13,34 pmol/ml (one level higher) already seemed to increase the non-specific Avidin binding too much. An intermediate concentration of hrp-Avidin between the two levels could hence offer a good compromise.

In order to test this, bio WGA MP and HSA MP were incubated with 2,67 and 8 pmol/ml hrp-Avidin for 60 minutes at 4°C after cytoadhesion. The TMB assay showed that the higher hrp-Avidin concentration of 8 pmol/ml had no influence on the non-specific Avidin binding but achieved a slight increase in the MP detection sensitivity (Figure 12).

The effect of this higher hrp-Avidin concentration was also tested as a function of time. Hereby, the bb-WGA MP were incubated with 8 pmol/ml hrp-Avidin between 1 and 6 hours. The increased incubation time had no effect on the non-specific cell association of hrp-Avidin, but also no marked effect on the sensitivity of the MP detection. Only the particles which were in contact with hrp-Avidin for 6 hours showed a clear improvement in signal intensity (Figure 13).

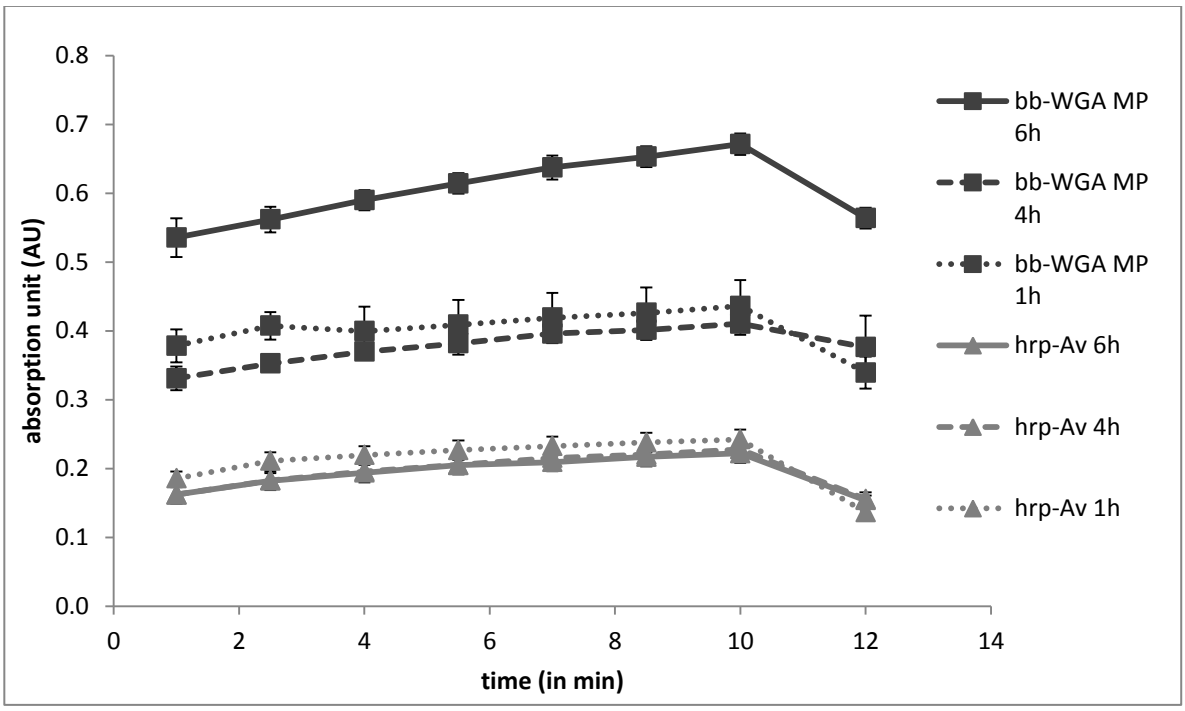


Figure 13: Time-dependency of the specific and non-specific cell-association of hrp-Avidin. A confluent monolayer (5637) is loaded with bb-WGA MP and subsequently incubated with 8 pmol/ml hrp-Avidin between 1

and 6 hours. A control without MP but with hrp-Avidin incubation is also included. HRP was detected via TMB incubation for 10 minutes, followed by an acidic stop of the reaction after 12 minutes. Except for the 6h hrp-Avidin incubation period no marked differences in the signal level can be observed. The non-specific Avidin binding seems to be unaffected by the increased contact time with the cells. The values represent mean AU ± SEM of a quadruplicate.

2.5.4.4. Inhibition of the lectin-mediated interaction between MP and the cell surface with competitive carbohydrates

In order to investigate the specific interaction between WGA on the MP surface and the cellular glycocalyx, the Bodipy® fluorescence signal can serve as an easy-to-measure indicator for MP/cell adhesion. If cells are incubated with the same amount of WGA- and HSA MP, the remaining Bodipy® fluorescence signals after washing of the cells should correlate with the amount of cell-bound particles. Due to the strong, bio-recognitive binding of WGA, it cannot be rinsed away as easily as HSA, which shows no marked binding properties in the chosen setup. Correspondingly, this results in higher RFI signals of the WGA-modified particles.

Another way to verify the lectin-mediated interaction is to test the inhibition potential of carbohydrates in a competitive inhibition assay, where the complementary glycan is applied together with the modified MP. The interference with the WGA binding caused by the competitive carbohydrate can be determined via the Bodipy® fluorescence signal or TMB. Given that WGA specifically binds to GlcNAc residues, sialic acid and some other glycans at the cell surface, 50 mM GlcNAc was used for this inhibition assay. The described effect was tested via the usual TMB assay with bio WGA MP, which were diluted 1:2 in 113 mM GlcNAc or 20 mM HEPES/NaOH pH = 7,4 and then applied on the cells, using 2,67 and 8 pmol/ml hrp-Avidin for detection. The increased TMB signal levels after development at of both hrp-Avidin concentrations indicated a decrease of the MP adhesion capacity of about 25 %, and consequently corroborated the specific binding of WGA to the glycocalyx. (Figure 12)

2.5.4.5. Evaluation of the impact of cell-fixation

All experiments mentioned so far were designed to be compatible with viable cells and have been carried out at 4°C in order to inhibit the internalisation of MP – or later of conjugates or NP. This inhibition of endocytosis could be achieved more easily by fixation of the cells prior to the enzymatic detection. If the fixation does not influence the sensitivity of the method

or enhance the non-specific Avidin binding, it could additionally simplify the handling of the assay.

For the fixation of the cell layer, freshly prepared 0,5 % glutaraldehyde (GlutAld) in aqua bidest. was applied – at particular points of time during the assay: before MP loading, after MP loading but before incubation with hrp-Avidin, and after incubation with hrp-Avidin. In every case the incubation time with 0,5 % GlutAld was 30 minutes at 4°C, followed by addition of 100 mg/ml glycine in 20 mM HEPES pH = 8,0 for 30 minutes to block unreacted aldehyde groups. The results suggested that the fixation had no effect on the sensitivity of the TMB assay, regardless of the point of time of fixation. The non-specific binding of hrp-Avidin to the cell surface remained as well unaffected. (Figure 14)

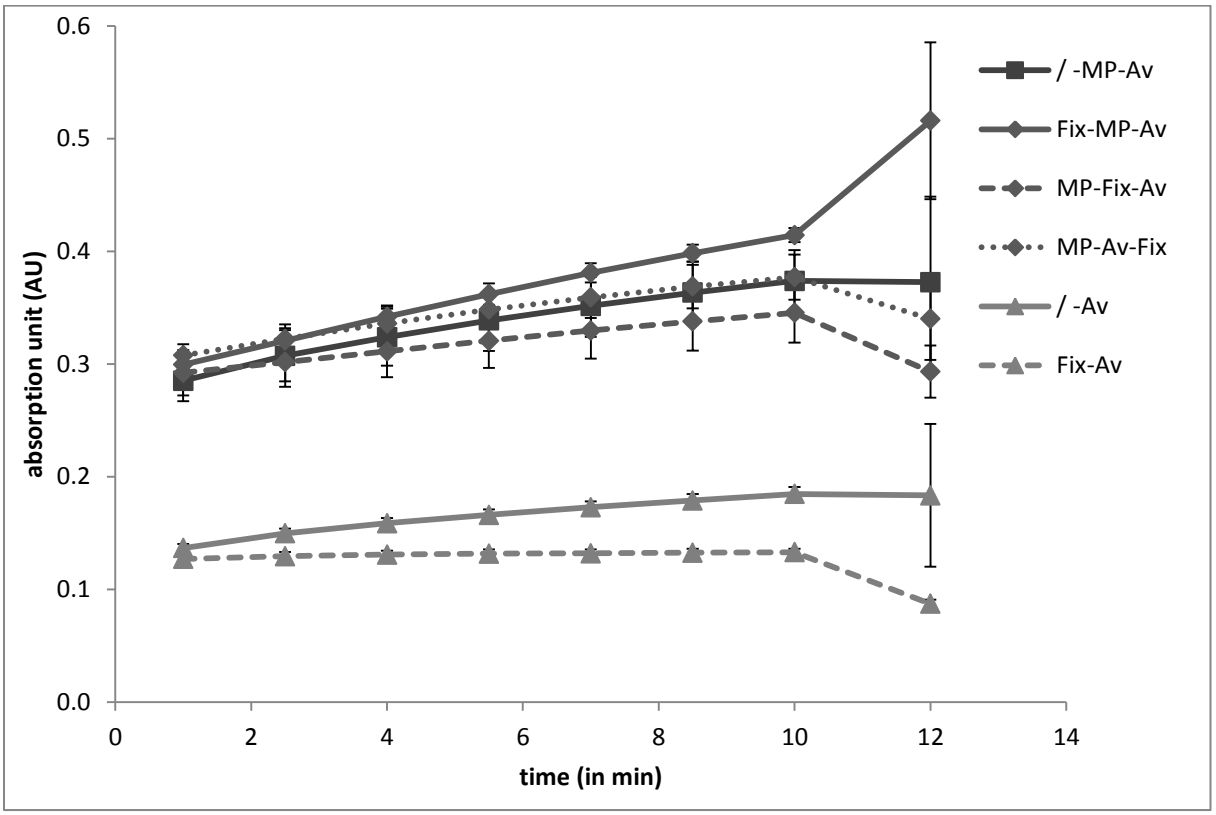


Figure 14: Evaluation of the impact of cell-fixation on specific and non-specific cell-association of MP and hrp-Avidin, performed at particular points of time during the assay. Cells in monolayer configuration (5637) were loaded with bb-WGA MP and subsequently incubated with hrp-Avidin. Fixation was performed at various points of time during the assay (see Table 3 for further specification). As control, the assay was additionally performed without fixation and with hrp-Avidin only (without MP) both with previous fixation and without fixation at all. Cell/MP-bound hrp-Avidin was detected via TMB for 10 minutes, followed by an acidic stop of the colorimetric reaction after 12 minutes. No marked differences between fixed and viable cells could be observed in the recorded AU, as well as no increase in non-specific cell-binding of Avidin due to fixation. The values represent the calculated mean AU ± SEM of a quadruplicate.

specification	*treatment of the confluent cells/ experimental procedure*		
	step 1	*step 2*	*step 3*
/ - MP - Av	no treatment	incubation with bb-WGA MP	incubation with hrp-Avidin
Fix - MP - Av	fixation with GlutAld	incubation with bb-WGA MP	incubation with hrp-Avidin
MP - Fix - Av	incubation with bb-WGA MP	fixation with GlutAld	incubation with hrp-Avidin
MP - Av - Fix	incubation with bb-WGA MP	incubation with hrp-Avidin	fixation with GlutAld
/ - Av	no treatment	no treatment	incubation with hrp-Avidin
Fix - Av	no treatment	fixation with GlutAld	incubation with hrp-Avidin

Table 3: Specification of assay conditions used for the evaluation of the impact of cell-fixation on the sensitivity of the TMB assay (Figure 14).

2.5.4.6. Discussion of the results obtained with bb-WGA/bio-WGA MP

The bb-WGA MP or bio WGA MP showed an improvement in the sensitivity of the TMB assay. All in all, dialysis seemed to represent the best washing method for particles which were incubated with Avidin before cell adhesion. That way, non-specific interaction between too much remaining free Avidin and the monolayer could be prevented. For assays in which the Avidin incubation took place on the monolayer (with prior adhesion of the MP), an increase of the hrp-Avidin concentration up to 8 pmol/ml and a fixation with 0,5% GlutAld were possible without disturbing the robustness of the assay. Besides, GlutAld fixation could be performed at different points during the assay time line without influencing the TMB reaction, even after incubation of the MP loaded cells with hrp-Avidin. It is particular surprising that the HRP seems to remain active and capable for reaction with TMB irrespective of the previous contact with the fixation agent. Inhibition with GlcNAc and the use of HSA MP combined with an additive of 1% BSA in the washing buffer proved the specific interaction between WGA present at the particle surface and the glycocalyx. However, the intensity of the signal has not yet reached an ideal level which would allow to assess subtle differences in surface-binding or internalization.

2.5.5. Hyperbiotinylation of b-WGA conjugated MP

In an attempt to further increase the sensitivity of the assay, the bb-WGA MP were hyperbiotinylated, i.e. incubated with 65 µl of 5 mg/ml BIO C5 NHS in DMF for 19 hours as described in *2.4. Detection of bb-WGA conjugated MP via tr-Avidin.*

These MP were incubated with 500 µl of 0,7 nmol/ml hrp-Avidin for 60 minutes at RT during constant agitation and subsequently washed via centrifugation and resuspension. 50 µl of bbb-WGA MP and bb-WGA MP each were adjusted to the same level via Bodipy® fluorescence intensity and applied to wells without cell layers in a 96 well microtiterplate. The enzymatic reaction was initiated as usual by addition of 70 µl TMB in PBS ($+Ca^{2+}/Mg^{2+}$) with 0,0015% hydrogen peroxide (1:2) and stopped with 20 µl 1M H_2SO_4. The results showed an enormously high sensitivity of the assay, which did not correlate with the findings of MP associated to the cell surface (Figure 15). In comparison to previous experiments with cell-associated MP (see *2.5.4.2. bb-WGA MP detection via hrp-Avidin added after cytoadhesion*), putative differences in the accessibility of particles for TMB became visible. In both experimental setups the same particles (bb-WGA-MP; same degree of biotinylation) were used. In principle, the ratio of the Bodipy® fluorescence signal to TMB development should be the same. However, for bb-WGA MP, which were incubated with hrp-Avidin after cytoadhesion (Figure 11; bb-WGA MP conc) the ratio between the Bodipy® fluorescence signal and the recorded stopping signal (treatment with 1M H_2SO_4) was about 14228:1, whereas the equivalent ratio for these particles conjugated to hrp-Avidin but without cell contact was about 525:1 (Figure 15). If the stopping signals of these two differently treated MP are normalized to the same Bodipy® fluorescence signal, a reduction of about 96 % (near complete) of the TMB stopping signal intensity of cell-associated bb-WGA MP in comparison to free bb-WGA MP can be calculated.

As already mentioned the Bodipy® signals allow for an estimation of the initially applied particle amount. So it should also be noted, that the Bodipy® fluorescence signal in case of the free bb-WGA MP conjugated to hrp-Avidin was reduced by nearly 5/6 in comparison to the Bodipy® signal recorded for the assays with hrp-Avidin incubation after cell loading. A reason for this reduction in Bodipy® signal might be the additional washing steps via centrifugation due to the hrp-Avidin conjugation prior to TMB detection. This contrast of low TMB-detectable MP concentrations/ well (at the cell surface) and high AU obtained for TMB/ H_2SO_4-incubated MP (without cells) leads to the obvious conclusion that the accessibility of the Biotin groups at the MP surface is not guaranteed if the MP are associated to a monolayer. However, this contrasts to other qualitative experiments, where a principally good accessibility was demonstrated via fluorescence imaging after counter-staining with tr-Avidin (see section *2.4 Detection of bbb-WGA conjugated MP via tr-Avidin).*

Still, due to mechanistic differences in the assays (hrp-Avidin/TMB as compared to tr-Avidin as detecting reagent), the results acquired via a qualitative detection method

(fluorescence microscopy) do not necessarily have to match the quantitative assessment via enzymatic processes (which are obtained from TMB assays).

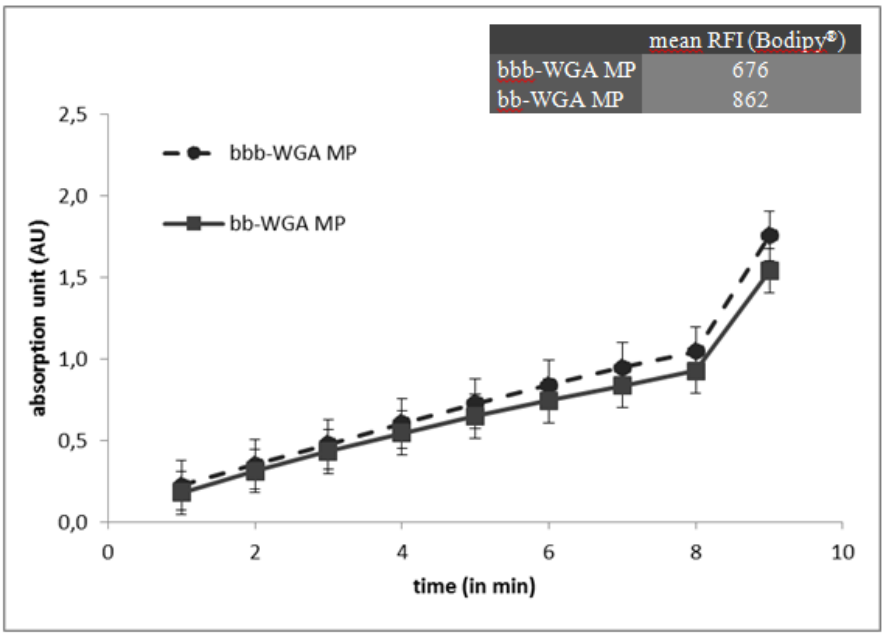


Figure 15: Investigation of bbb-WGA MP and bb-WGA MP conjugated to 0,7 nmol/ml hrp-Avidin without a cell layer. The MP are detected via TMB (min 1 – 8) and H_2SO_4 (min 9). The high signals do not correlate to the minor sensitivity of the assay when detecting MP that are bound to a monolayer. Therefore, a sterically impeded accessibility of Biotin might be resulting from MP cytoadhesion. The samples were performed in quadruplicate and the mean AU ± SEM was evaluated.

2.5.5.1. Discussion of the results obtained with bbb-WGA MP

Although the hyperbiotinylation of bb-WGA MP achieved positive results concerning the sensitivity of the assay, the intensity of the signal has still not reached the desired level. Hence, a possible endocytosis might not be detectable. Since all the parameters which could improve the results, such as the amount of Biotin at the particle surface or the hrp-Avidin concentration, had already been optimized, the final conclusion is that the Biotin-Avidin system might not be suitable for the detection of endocytosis.

2.5.6. HRP or HRP/WGA conjugation to MP

The detection of MP or conjugates by an enzymatic reaction via TMB and HRP is a well-established method. Unfortunately, integration of the Biotin-Avidin system led to some difficulties which rendered this method unsuited for the detection of endocytosis. However, if the Biotin-Avidin system could be replaced by another functional step, the TMB assay might still be a promising detection method.

For this reason PLGA/Bodipy®-labeled MP were modified directly with HRP (HRP MP) or with HRP and WGA (HRP/WGA MP). After activation of the MP with EDAC and

NHS, as described above *(2.1. Evaluation of covalent particle surface modification)*, they were incubated either with a mixture of 25 µl of a 5,0 mg/ml WGA-solution and 25 µl of a 9,8 mg/ml HRP-solution, which equates to a molar ratio of 1:1, or with 50 µl of a 9,8 mg/ml HRP-solution for 16 hours. After blocking unreacted binding sites with 242 µl of a 100 mg/ml glycine solution in 20 mM HEPES/NaOH pH = 8,0 for 2 hours, the particles were washed three times with 20 mM HEPES/NaOH pH = 7,4 by centrifugation and resuspension. Finally, the modified MP were adjusted to a volume of 1 ml in 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68.

2.5.6.1. Evaluation of the particle surface modification directly with HRP

In order to distinguish if the observed TMB traces back to HRP, which is immobilized at the particle surface, or HRP that is still floating free in the solution, two different washing methods were compared. On the one hand the HRP/WGA MP were washed via centrifugation as established in the current protocol, on the other hand the washing was carried out by dialysis (MWO 8341). After adjusting the approximate MP concentration to the same level via the Bodipy® fluorescence signal (RFI about 800, gain 80, ex/em: 485:525), the particles were reacted with TMB 1:5 followed by addition of 1M H_2SO_4 to stop the enzymatic reaction. The resulting run of the curve indicated no marked difference in the signal development, which led to the conclusion that the HRP is indeed stably linked to the MP surface.

2.5.6.2. Quantitative assessment of HRP-modified MP using the TMB assay

The verification of successful MP surface modification with HRP allowed for further investigation of the applicability of these MP for analysis of endocytosis. For this, a 5637 confluent monolayer was thoroughly washed and incubated with 100 µl of HRP/WGA MP, HRP MP and plain MP for 30 minutes at 4°C followed by rinsing seven times with PBS ($+Ca^{2+}/Mg^{2+}$) and 1% BSA to remove any non-specific adsorption. The enzymatic reaction was initiated by adding 70 µl TMB in PBS ($+Ca^{2+}/Mg^{2+}$) with 0,0015% hydrogen peroxide (1:5) and stopped after 10 minutes with 20µl 1M H_2SO_4.

The resulting Bodipy® signal intensities showed that seven washing steps with BSA are required for marked distinction of specific and non-specific cell-association of the MP. The recorded signals indicated that approximately 30 % of the original MP payload remained associated to the cells when modified with WGA. In contrast, less than 10 % of MP remained

stably bound at the cell membrane without the specific WGA targeter (Figure 16b). The intensity of the TMB signal after stopping (i.e. stop of the enzymatic reaction with sulphuric acid) increased from plain MP over HRP MP to HRP/WGA MP. The low Bodipy® signal intensity of stably cell-associated HRP MP (Figure 16b) would suggest that only a minor amount of HRP MP durably remains in the system. The fact that, nevertheless, considerable colour development was observed in the TMB assay (Figure 16a) could indicate a higher molar amount of HRP is immobilized at HPR MP as compared to HRP/WGA MP. Finally, the intensity of the signal had reached a level that could be high enough for distinction of possible endocytosis/ non-endocytosis (Figure 16).

Another TMB assay was performed with the same suspensions of MP (adjusted according to the Bodipy® fluorescence) but diluted 1:3 with 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68 and without prior incubation of the MP with the target cells. The run of the curve of these free MP (without cytoadhesion) at early time points showed a steep increase. However, after 4 minutes a stagnation in the signal increase occurred, which might be due to an inhibition of the enzyme reaction or substrate depletion (Figure 17).

In order to verify the results, a similar TMB assay was performed, after incubation of the target cells for 30 minutes at 4°C with HRP MP, HRP/WGA MP and WGA MP which were previously adjusted to the same level concerning their Bodipy® fluorescence signal. Directly before TMB treatment, cells incubated with HRP/WGA MP or HRP MP were treated with 50 µl of 3 % H_2O_2 in PBS ($+Ca^{2+}/Mg^{2+}$) to block the enzyme reaction. After 30 minutes of incubation these cells were washed thrice followed by TMB development for every kind of applied MP. The enzymatic reaction was initiated with TMB (proprietary solution Sciotec, Vienna, Austria, used as supplied) and stopped with 1 M sulphuric acid.

By using the TMB solution in the undiluted form, the intensity of the signal could be increased. The solution of 3 % H_2O_2 in PBS ($+Ca^{2+}/Mg^{2+}$) should have inactivated the HRP and thus decrease the recorded signals to blank values. The results showed that both, HRP/WGA MP and HRP MP, were affected by the quenching buffer, although the signals indicate that there was still some HRP activity left. (Figure 18)

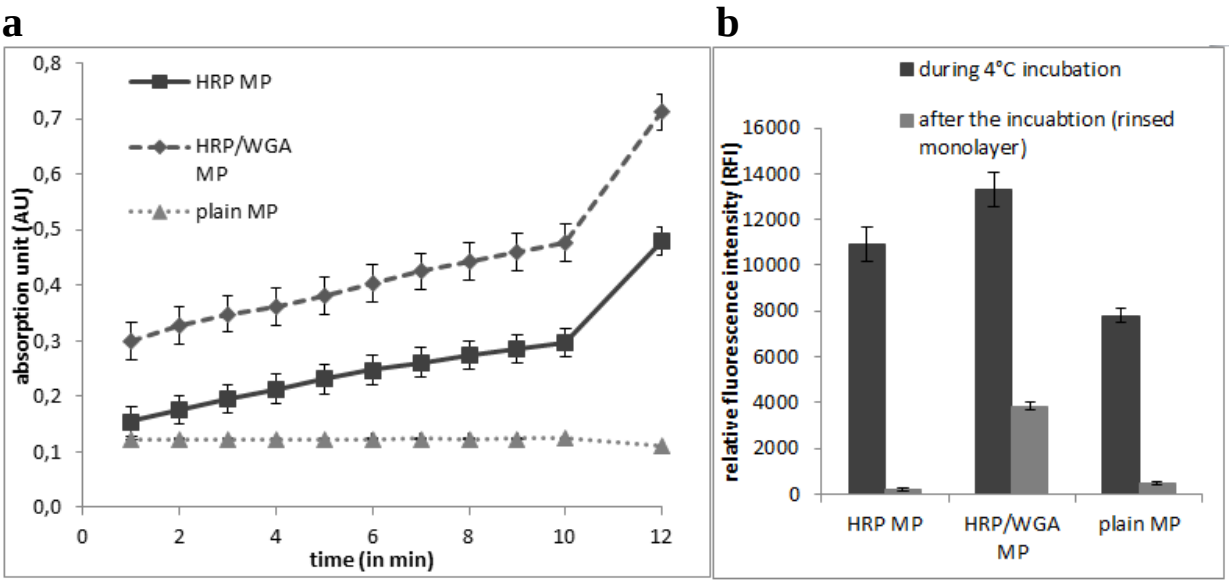


Figure 16: Evaluation of the cytoadhesive potential of differently modified MP. For this, a 5637 monolayer was incubated with HRP MP, HRP/WGA MP, WGA MP and plain MP at 4°C and subsequently rinsed 7 times with PBS ($+Ca^{2+}/Mg^{2+}$) and 1% BSA. **(a)** Development with TMB (min 1-10)/ H_2SO_4 (min12) shows signals, which might be high enough for distinction of possible endocytosis, HRP/WGA MP reach a higher stopping signal (signal after acidic treatment) than HRP MP. **(b)** The seven washing steps result in a higher amount of cytoadhesive MP when coupled to the specific targeter WGA. All values represent the mean AU/RFI ± SEM of a triplicate.

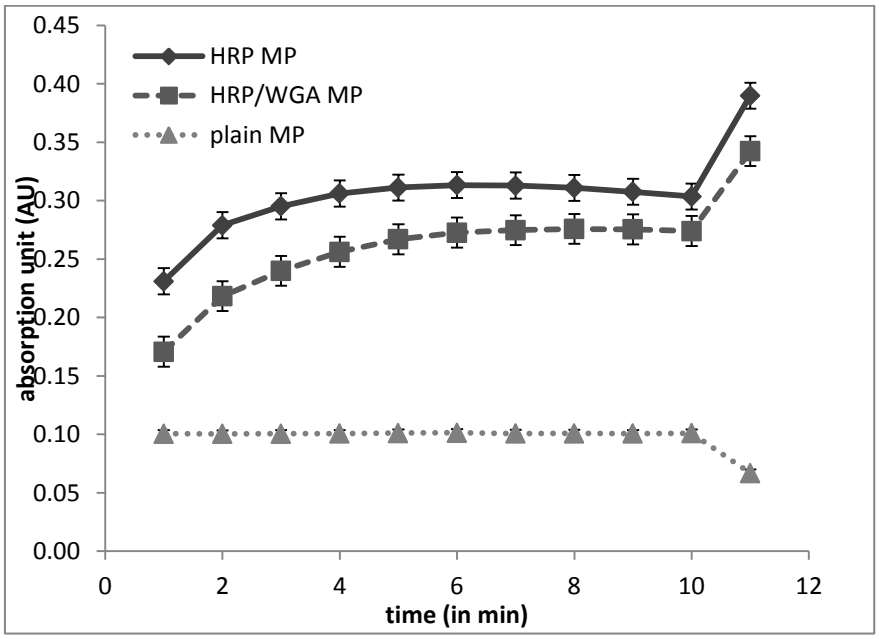


Figure 17: Assessment of the signal intensity development of HRP MP, HRP/WGA MP and plain MP without previous incubation with the target cells. The MP are detected via TMB (min 1-10) and the stopping agent H_2SO_4 (min 11). After 4 minutes the AU evens out, which may be due to an inhibition of the enzyme reaction or depletion of substrate. The values represent the mean AU ± SEM of a triplicate.

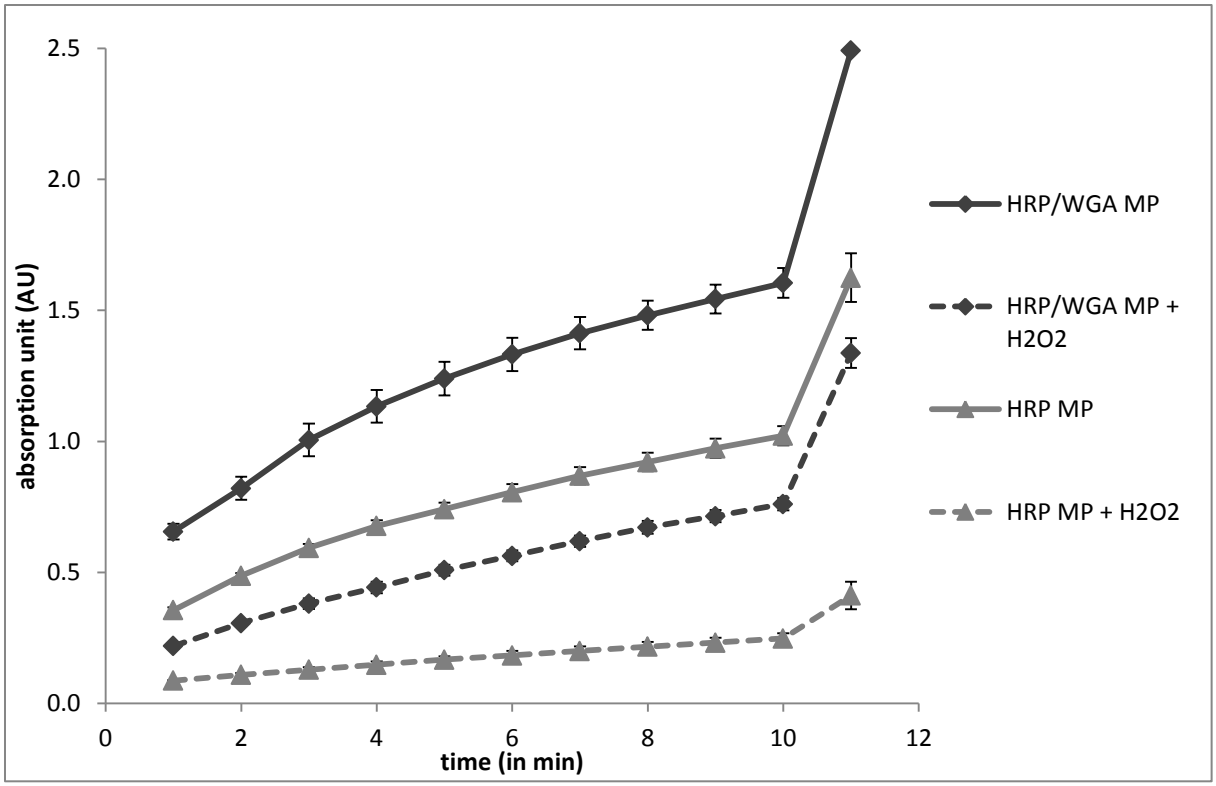


Figure 18: Enhancement of the sensitivity of the TMB assay by using concentrated TMB. A 5637 monolayer is incubated with HRP MP and HRP/WGA MP at 4°C for 30 minutes, rinsed, partly incubated with H_2O_2 (to block HRP activity) and treated with TMB (min 1-10) and sulphuric acid (min 11). The run of the curve shows an increase in signal intensity due to the utilization of concentrated TMB. Besides, treatment with H_2O_2 results in a partial block of HRP, visible in a marked decrease of the signal intensity. All values symbolize mean AU ± SEM evaluated of a triplicate performance.

2.5.6.3. Discussion of the results obtained with HRP-modified MP

Direct modification of the MP surface with HRP or HRP/WGA increased the intensity of the signal to a suitable level. Besides, the difference in cell adhesion between WGA and non-WGA modified MP could be demonstrated by extending the washing protocol by 4 rinsing steps. However, MP without incubation with a cell layer showed a time-dependent saturation/inhibition of the enzyme reaction; the reason is still unknown.

3. Tyramid Signal Amplification (TSA)

Because of the too low signal intensities, the Biotin-Avidin system was not found to be an ideal detection method to monitor the internalization process of either WGA conjugates or nano- to microparticles. An alternative approach consisted of modifying the MP directly with HRP and WGA to avoid the Biotin-Avidin intermediate step. This strategy led to an increase in the signal intensity but created other problems, such as enzyme saturation. The detection limitation (weak signals) could be overcome by using enzymatic reaction-based fluorescent staining systems that allow for amplifying the signal, which would result in a sensitivity enhancement of the entire test. Due to the already successfully established modification protocol of the MP surface with HRP (chapter 2.5.6) , as well as the widespread use in protein bioconjugates, an HRP/tyramide signal amplification kit, (which works due to the catalytic activity of this enzyme) was chosen as a potentially interesting system.

The expected outcome would be a simple detection method, which increases the sensitivity and allows discrimination between cytoadhesion and cytoinvasion for MP using qualitative and quantitative approaches.

3.1. Basic principle of the TSA assay

The TSA assay is – similar to the TMB assay – an enzyme mediated detection method which is based on the catalytic activity of HRP. However, in this case the assay is based on the gradual accumulation of an already fluorescent dye molecule, rather than the conversion of a non-fluorescent to a fluorescent molecule. The substrate tyramide is labelled with a fluorescence agent, in our case Alexa Fluor® 594, and is activated by HRP to highly reactive but short-lived radicals, which can covalently bind to nearby nucleophilic centers [17]. These binding sites can be either proteins at a cell surface or parts of the surface (e.g. carboxy groups) of PLGA MP. In this work, this assay was performed with HRP MP, as well as with HRP-labelled WGA (bioconjugates) after cytoadhesion or cytoinvasion at/in bladder cancer cells.

3.2. Evaluation of the TSA assay using hrp-WGA and f-WGA

In order to explore the possibilities and limitations of this detection method, HRP- and fluorescein-labelled WGA (hrp- and f-WGA) were used in parallel. The staining pattern of f-

WGA after cytoadhesion or cytoinvasion is well known, and could be correlated with the pattern resulting from hrp-WGA and the TSA dye, respectively. This should allow for concluding on the ability of the tyramide dye to target HRP after endogenous uptake. For a more detailed analysis, the assay was performed using viable, fixed or fixed/ permeabilized cells as well as blocked HRP.

For the optimization of the assay different parameters were tested such as

- the incubation time with TSA
- the concentration of TSA
- the concentration of hrp-WGA and
- the incubation time at 37°C.

All experiments were carried out on confluent 5637 monolayers in FlexiPERM® insets mounted on glass coverslips, which were thoroughly washed and incubated for 30 minutes with 50 µl of a mixture of hrp- and f-WGA (see below) at 4°C and subsequently rinsed three times with 150 µl PBS ($+Ca^{2+}/Mg^{2+}$). Depending on the specific aim of the assay, the cells were either warmed up to 37°C to initiate energy-dependent uptake, or kept at 4°C in a metabolically quiescent state. Directly after the incubation at 4°C or after the internalization at 37°C the monolayer was either immediately incubated with 50 µl of the tyramide reagent solution at RT, or fixed first for 20 minutes with 150 µl of 0,5 % GlutAld and saturated for another 20 minutes with 100 µl of 100 mg/ml glycine in 20 mM HEPES/NaOH pH = 8,0 at RT prior to the dye incubation step. Besides, if the accessibility of intracellular cargo was to be investigated, a permeabilization step was implemented before the tyramide treatment, by incubating fixed cells with 100 µl of 0,15 % Triton X-100 (in H_2O) for 20 minutes at RT. After the incubation with the tyramide dye, the cells were washed three times with 150 µl PBS and the volume was adjusted to 100 µl PBS per well, followed by the detection of the resulting staining pattern via fluorescence imaging. A control was implemented using f-WGA with identical processing for the rest of the assay. In the last step, which is the tyramide dye incubation, 5 µg/ml HOECHST 33342 DNA stain were added to label the cell nuclei. Hence, the incubation time of the DNA stain was always varied analogously to the tyramide incubation time. The imaging parameters were kept constant to allow for direct comparison.

Tyramide incubation time

The evaluation of the tyramide incubation time was performed with viable or fixed cells, both after incubation at 4°C and after internalization at 37°C for 30 minutes, using a mixture of 12,5 pmol/ml hrp-WGA and 12,5 pmol/ml f-WGA. Afterwards, 50 µl of the

tyramide working solution (equating a 1:100 dilution of the tyramide stock solution in amplification buffer/0,0015% H_2O_2; supplied by Invitrogen (Carlsbad, CA, USA; see reference [18]) were added and the reaction was led to proceed for 5, 10 or 15 minutes at RT. Subsequently, the monolayer was rinsed three times with PBS, adjusted to a volume of 100 µl per well (in the FlexiPERM®) and detected via fluorescence-microscopic analysis. The acquired images showed no marked difference in the intensity of the staining pattern or any non-specific staining (Figure 19).

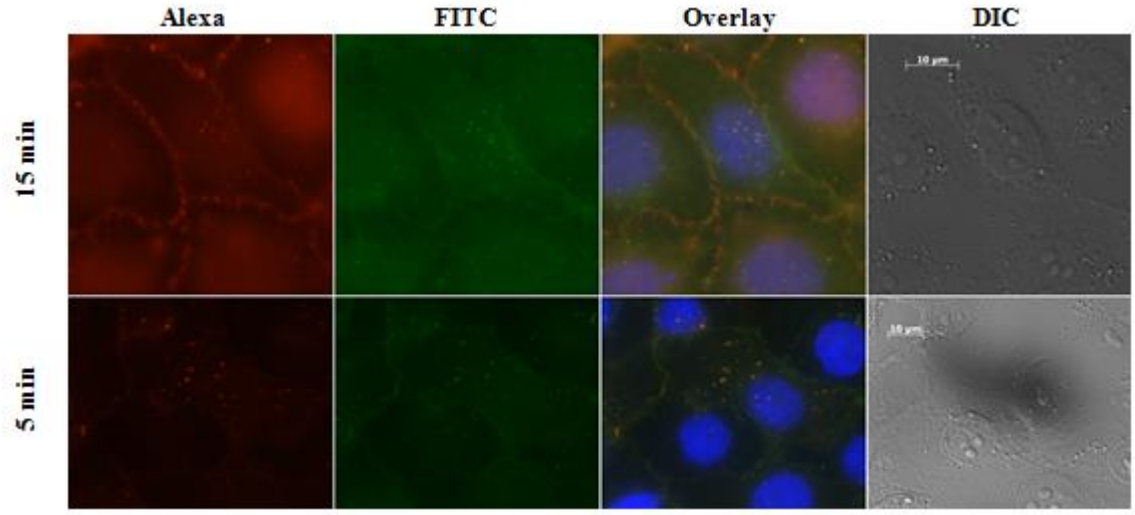


Figure 19: Microscopic analysis of internalized hrp-WGA and f-WGA using the TSA dye as detecting agent. 5637 cells are pulse/chase incubated with 12,5 pmol/ml hrp-WGA and 12,5 pmol/ml f-WGA, fixed and subsequently stained with tyramide for 5 or 15 minutes. The non-specific staining seems to be less distinct by decreasing the TSA incubation time but the different exposure times should be considered. (15 min: green channel, *FITC*, 470nm, 100%, 350 ms; red channel, *Alexa*, 590nm, 100%, 500 ms; 5 min: green channel, *FITC*, 470nm, 100%, 91 ms; red channel, *Alexa*, 590nm, 100%, 84 ms)

hrp-WGA concentration

For the optimization of the quality and intensity of the resulting staining pattern and the minimization of non-specific staining, three different hrp-WGA concentrations (2,7 pmol/ml, 12,5 pmol/ml and 250 pmol/ml) were tested, again on viable and fixed cells after incubation at 4°C or pulse/chase incubation (4°C for 30 minutes and 37°C for 30 minutes). f-WGA, the agent used for colocalization, was applied simultaneously but always at a constant concentration of 12,5 pmol/ml, which is known to result in good detectability.

The generated fluorescence images indicated that a concentration of 2,7 pmol/ml hrp-WGA is far too low to achieve good optical visibility. Nevertheless, a colocalization of the green staining pattern of f-WGA and the red staining pattern caused by the tyramide dye illustrated the principal functionality of the TSA assay and could already be found at a hrp-

WGA concentration of 2,7 pmol/ml. An increase of the hrp-WGA concentration to 12,5 pmol/ml enhanced the intensity of the resulting images but still the applicability of the TSA assay was ambiguous due to non-specific staining, especially at the cell borders and cell nuclei (Figure 20). Partially, the non-specific red fluorescence outshone the signal of the HRP, especially when originating from small vesicular compartments, which may correspond to peroxisomes. Finally, the usage of 250 pmol/ml hrp-WGA led to the desired results. The exposure time could be reduced and therefore the non-specific staining, particularly the signal putatively caused by the peroxisomes, could be minimized. A definitive colocalization with f-WGA could be corroborated (personal observation).

There were no differences in the quality and intensity of the images between the experiments with fixed and viable cells. A differentiation between the states with surface-bound WGA and internalized WGA could always be made using either fixed or viable cells.

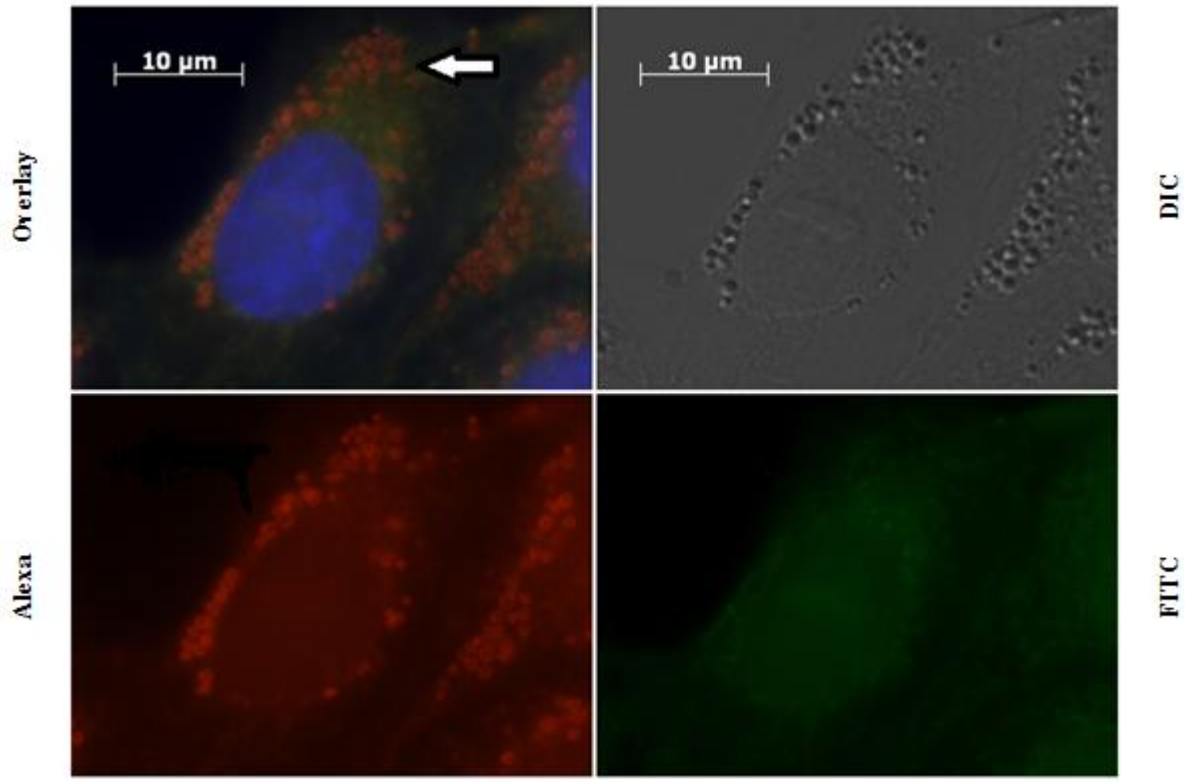


Figure 20: Microscopic demonstration of non-specific TSA-staining in 5637 cells. Cells are treated with hrp- and f-WGA and incubated with tyramide solution. Due to an accumulation of small vesicular departments, which can be stained by tyramide and therefore are considered to be peroxisomes (arrow), a possible accumulation region of HRP cannot be clearly identified. (green channel, 470nm, 100%, 641 ms; red channel, 590nm, 100%, 847 ms; blue channel, 365 nm, 100%, 269 ms)

Incubation time at 37°C to allow for energy-dependent uptake

The evaluation of the ideal internalization time was performed by pulse/chase incubation of a confluent 5637 monolayer with 50 µl of a mixture of 250 pmol/ml hrp-WGA

and 12,5 pmol/ml f-WGA, fixation with 150 µl of 0,5 % GlutAld (in aqua bidest) and subsequent permeabilization with 100 µl of 0,15% Triton X-100 (in aqua dest) for 20 minutes at RT. The permeabilization was implemented to guarantee the detection of intracellular hrp-WGA because intact cell membranes showed an impenetrability for the tyramide dye, as illustrated in the following. The energy-dependent uptake was determined for an incubation time of 0, 10, 30 and 60 minutes at 37°C. A control was made using fixed but non-permeabilized cells, which should result in no dying of intracellular hrp-WGA.

The resulting fluorescence images showed a marked anchoring effect and colocalization of f- and hrp-WGA at the cell surface irrespective of the incubation time at 37°C, but no signs of endocytosis after 0 and 10 minutes. However, an incubation time of only 30 minutes allowed endogenous uptake of both hrp- and f-WGA, which could be seen as the typical intracellular clustering around the cell nuclei. The amount of internalized WGA increased further when incubating the monolayer for 60 minutes at 37°C (Figure 21).

As expected, the fixed (and non-permeabilized) control wells showed no red fluorescence staining caused by an interaction of tyramide and intracellular hrp-WGA, but the typical intracellular staining pattern of f-WGA could clearly be seen. This corresponded to the expected inability of the tyramide dye molecules to reach intracellular hrp-WGA. Only after 60 minutes of incubation a hint of clustering around the cell nuclei, caused by the tyramide dye, could be seen, which could not clearly be identified as intracellular. If this clustering was indeed intracellular, it would indicate that the fixating agent GlutAld partially disrupted the membrane integrity of the cells, which then of course results in limited access for the tyramide dye to intracellular hrp-WGA.

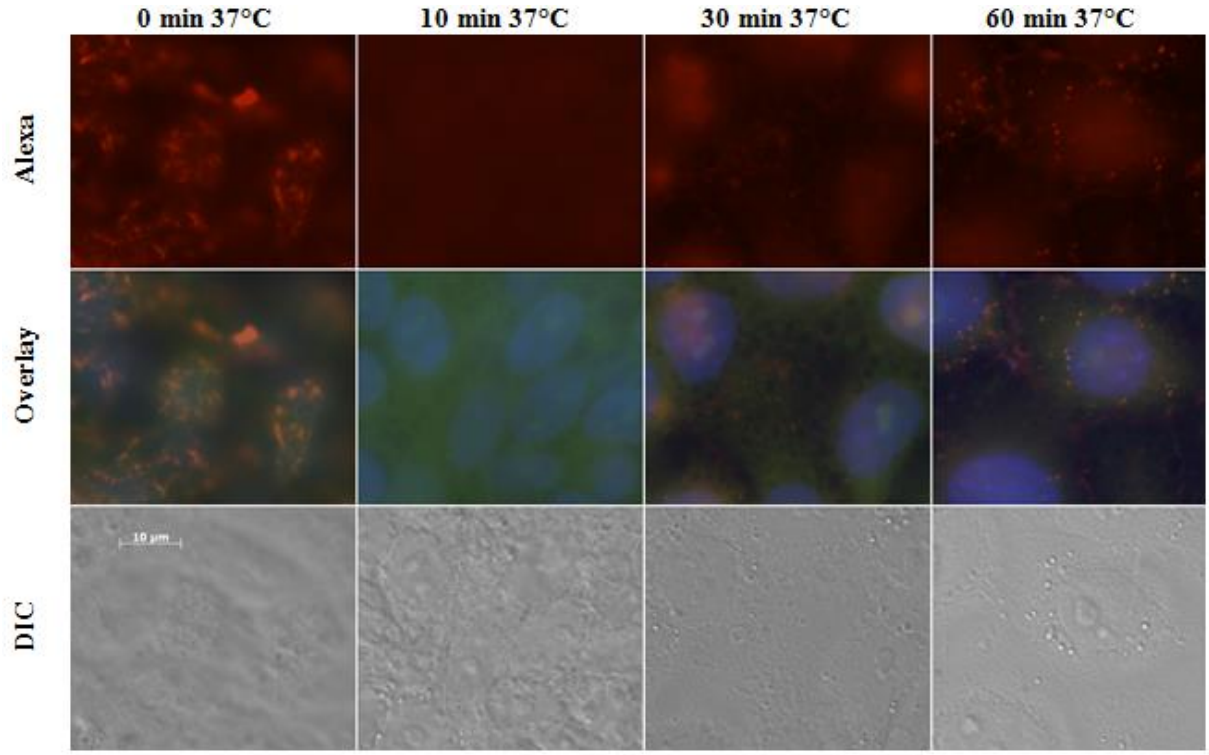


Figure 21: Internalization time course (0, 10, 30, 60 minutes at 37°C) of hrp- and f-WGA, detected via the TSA dye. 5637 cells are pulse/chase incubated with 250 pmol/ml hrp-WGA and 12,5 pmol/ml f-WGA, fixed, permeabilized and stained with fluorescence labelled tyramide. The images show that an incubation time of at least 30 minutes is necessary to achieve intracellular uptake of the cargo. Samples incubated for 0 or 10 minutes at 37°C only show extracellular but no intracellular staining. The overlay images show merged channels of *Alexa*, *FITC* and *DAPI*. All images are captured using the same imaging parameters to allow for direct comparison. (green channel, *FITC*, 470nm, 100%, 50 ms; red channel, *Alexa*, 590nm, 100%, 100 ms; blue channel, *DAPI*, 365 nm, 100%, 300 ms)

Concentration of the tyramide working solution

A disadvantage of this detection method is the non-specific staining caused by the tyramide dye, whose activity depends on the incubation time but also on its concentration. In order to achieve a reduction of the background fluorescence signal, not only the recommended tyramid working solution (corresponding to a 1:100 dilution of the stock) but also a more diluted version (1:500 dilution of the stock solution) was tested at an incubation time of 15 minutes. The experiments were performed at 4°C but also – in a following incubation period – at 37°C with fixation after the cytoadhesion and cytoinvasion of the WGA conjugates, respectively.

Via fluorescence-microscopic analysis, a decrease of non-specific staining, especially at the cell borders, could be observed using the 1:500 dilution of the stock tyramide solution in comparison to a 1:100 dilution.

Overall results of the optimization process

In summary the preferable parameters for the TSA assay turned out to be a concentration of 250 pmol/ml hrp- and 12,5 pmol/ml f-WGA for surface loading; a subsequent energy-dependent uptake (37°C) is well detectable after 30 minutes and the enzymatic reaction between HRP and the tyramide dye should be performed with a 1:500 dilution of the tyramide stock solution. The incubation time of the tyramide reaction can be adjusted to the desired outcomes: If a clear distinction between intracellular and extracellular WGA is required, an incubation time of 15 minutes with tyramide should be favoured to guarantee marked results. However, if the main aim is obtaining clear images with a minimum of non-specific staining, 5 minutes of incubation should be chosen.

3.3. Application of the TSA dye to evaluate internalization of WGA conjugates

In order to investigate the suitability of the tyramide dye as a detecting agent to discriminate between cytoadhesion and cytoinvasion, the TSA assay was performed on viable, fixed or fixed/permeabilized cells. Again, the assay was carried out on confluent cell monolayers in FlexiPERM® insets mounted on glass coverslips (using 5637 cells) to facilitate fluorescence imaging as detection method. The cells were incubated either at 4°C for 30 minutes or via pulse-chase incubation (4°C for 30 minutes and 37°C for 120 minutes) with 250 pmol/ml hrp- and 12,5 pmol/ml f-WGA. Fixation, permeabilization, staining of the cell nuclei and the incubation with the tyramide dye (1:500 over a period of 5 or 15 minutes) were performed as described above. As a control, the cells were loaded with 12,5 pmol/ml f-WGA only, followed by the same assay.

The acquired images showed an impenetrability of the tyramide dye for intact cell membranes, therefore the dye could not create a staining pattern of internalized hrp-WGA when used on viable cells and – with certain limitations – on fixed cells (detailed in the following). The control f-WGA of course showed the typical intracellular clustering after energy-dependent uptake, as its fluorescence emission is independent of the tyramide staining. Surface-adherent hrp-WGA was accessible for the tyramide, and colocalization with surface adherent f-WGA could be demonstrated (Figure 22a).

Viable cells appeared to be completely impenetrable for the tyramide molecules, whereas a fixation with GlutAld seemed to cause slight damages of the cell membrane and therefore let a small amount of the dye reach intracellular hrp-WGA. In any case a marked difference between fixed and permeabilized cells could be shown (Figure 22b).

Expectedly a fixation and subsequent permeabilization of the monolayer using 0,15% Triton X-100 allowed for diffusion of tyramide molecules through the damaged cell membrane. The resulting images showed a perfect colocalization of the intracellular staining patterns of f- and hrp-WGA.

These results suggest the notion that the TSA assay is applicable for the differentiation of cytoadhesion and cytoinvasion of WGA conjugates by utilizing the characteristics of the tyramid molecules concerning cell membrane permeability.

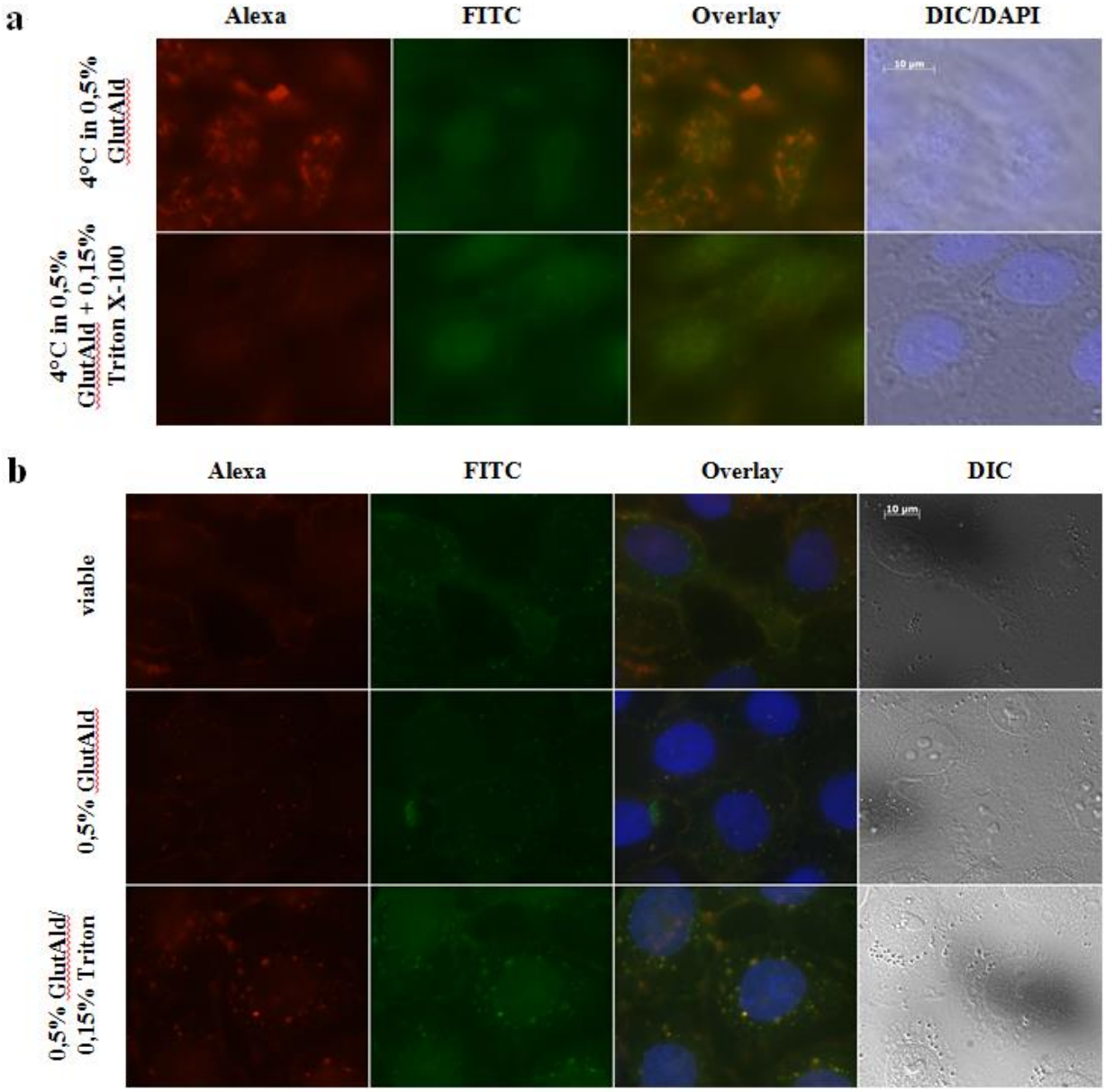


Figure 22: Microscopic analysis of hrp-WGA and f-WGA binding and internalization using the TSA dye as detecting agent and the impact of pre-assay cell permeabilization. **(a)** Cells (5637) kept in a metabolically quiescent state while incubated with 250 pmol/ml hrp-WGA and 12,5 pmol/ml f-WGA (4°C for 30 minutes) show a typical red surface staining due to the reaction between surface-accessible HRP and the tyramide dye. f-WGA and tyramide dye produce identical staining patterns visible as yellow staining in the *Overlay* images (merged channels of *FITC*, *Alexa* and HOECHST 33342 DNA stain). As long as the conjugates are only at the surface no difference between fixed (0,5% GlutAld) and fixed/permeabilized (0,5% GlutAld/ 0,15% Triton X-100) cells can

be seen. **(b)** On the contrary, the impenetrability of viable cells for tyramide can be seen when comparing images of viable, fixed (0,5% GlutAld) and fixed/permeabilized (0,5% GlutAld/ 0,15% Triton) cells, all pulse/chase (30 min at 4°C and 120 min at 37°C) incubated with 250 pmol/ml hrp-WGA and 12,5 pmol/ml f-WGA. All images in the *FITC* channel show an intracellular staining of f-WGA, whereas the *Alexa* channel shows an increase of intracellular staining from viable over fixed to fixed/permeabilized cells, due to an increase in cell membrane permeability. All images were processed using the same imaging conditions to allow for direct comparison. (green channel, *FITC*, 470nm, 100%, 100 ms; red channel, *Alexa*, 590nm, 100%, 300 ms; blue channel, *DAPI*, 365 nm, 100%, 396 ms)

Blocking of the HRP

In order to confirm the previous results, another control assay was performed by including an additional blocking step prior to the incubation with the tyramide dye. The cells, already loaded with 250 pmol/ml hrp-WGA, fixed and permeabilized, were incubated with 150 µl of the blocking agent (3 % solution of H_2O_2 in PBS) for 60 minutes and subsequently rinsed three times with PBS ($+Ca^{2+}/Mg^{2+}$). Then the cells were incubated with tyramide 1:500 and 5 µg/ml HOECHST 33342 DNA stain for 15 minutes. The control was performed using 12,5 pmol/ml f-WGA only.

The high concentration of H_2O_2 resulted in an irreversible inactivation of the peroxidase; hence no active enzyme was left to interact with the tyramide and therefore create reactive radicals to label the surrounding nucleophilic binding sites. Via fluorescence imaging the successful blocking of HRP either anchored intracellularly or surface-adherent could be demonstrated. No red staining at the surface or around the cell nuclei due to hrp-WGA was visible, whereas the typical staining pattern of f-WGA was still prominent (Figure 23). This confirms the principle mechanisms of the tyramide dye labelling of cells.

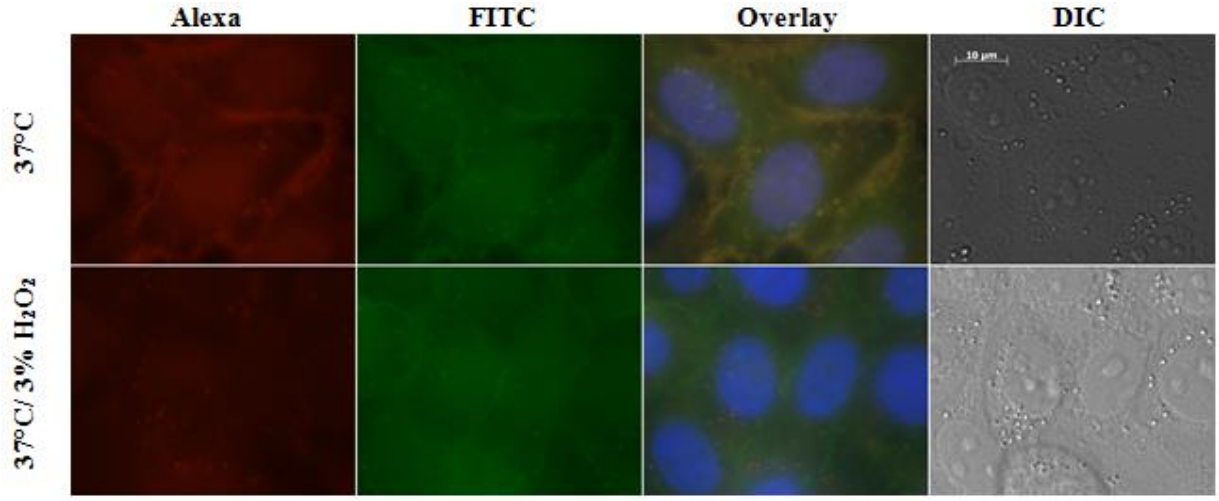


Figure 23: Microscopic analysis of internalized hrp-WGA and f-WGA using the TSA dye as detecting agent and the impact of HRP blocking. The target cells (5637) are treated with 250 pmol/ml hrp-WGA and 12,5 pmol/ml f-WGA, pulse/chase (4°C 30 minutes and 37°C 60 minutes) incubated to allow endocytosis and fixed/permeabilized to impair the membrane integrity. Cells additionally treated with 3% H_2O_2 still produced green intracellular

staining of f-WGA, whereas the HRP was completely quenched, i.e. no red staining is visible at all. The left red stains belong to small vesicular compartments, which may correspond to Peroxisomes and could not be quenched completely (note that their localisation is different from that of f-WGA-containing vesicles). In contrast, cells which were not treated with the quenching agent showed both intracellular staining of f-WGA and hrp-WGA, with identical staining patterns best seen in the *Overlay* images. All images were captured using identical exposure conditions to allow for direct comparison. (green channel, *FITC*, 470nm, 100%, 203 ms; red channel, *Alexa*, 590nm, 100%, 600 ms; blue channel, *DAPI*, 365 nm, 100%, 308 ms)

Elution of surface-bound WGA with N-acetyl-D-glucosamin

In contrast to the inhibition assay performed to prove the specific interaction of WGA-modified microparticles (described in *2.5.4.4. Inhibition of the lectin-mediated interaction between MP and the cell surface with competitive carbohydrates*), an elution assay is based on the disruptive potential of complementary glycans after cytoadhesion of WGA. In quantitative preliminary experiments it was shown that 80 - 90 % of surface associated WGA could be removed by elution with 50 mM GlcNAc [5, 7].

The elution assay was carried out by inverse loading (60, 30, 10 or 0 minutes incubation at 37°C followed by 30 minutes incubation at 4°C) of a confluent 5637 monolayer with 250 pmol/ml hrp- and 12,5 pmol/ml f-WGA, which was subsequently rinsed three times with 150 µl PBS ($+Ca^{2+}/Mg^{2+}$) or 150 µl of 50 mM GlcNAc allowing the competitive agent to interact for 10 minutes at 4°C between each washing step. Prior to the incubation with the tyramide dye (1:500 for 15 minutes at RT) the monolayer was fixed with 0,5 % GlutAld, saturated and permeabilized with Triton X-100 for 20 minutes at RT. Then, images for fluorescence microscopic analysis were generated, i.e. a qualitative detection method was chosen.

Coverslips with 5637 cells that were incubated for 0 or 10 minutes at 37°C showed neither a sign of hrp-WGA nor of f-WGA associated to the cell surface, which indicated an substantial elution effect of the GlcNAc. Cells which were in contact with the WGA conjugates for 30 or 60 minutes at 37°C showed an analogue intracellular staining pattern of hrp- and f-WGA, but no cytoadhesion at the cell surface could be determined any more, probably due to the removal of WGA by the eluting GlcNAc.

Staining of intracellular glycans with WGA conjugates after fixation and permeabilization of the target cells

In order to perform a general assessment of available binding sites throughout the entire cytosol, confluent 5637 glass coverslips were incubated with 12,5 pmol/ml f-WGA at 4°C for 30 minutes, rinsed three times with PBS ($+Ca^{2+}/Mg^{2+}$), fixed, saturated and permeabilized. Prior to the staining, using tyramide 1:500 of the stock, the cells were again incubated for 60 minutes at RT with a mixture of 250 pmol/ml hrp-WGA and 12,5 pmol/ml f-WGA and rinsed

three times with PBS (+Ca^{2+}/Mg^{2+}). The resulting fluorescence images suggested that f-WGA was able to invade the fixed and permeabilized cells, whereas hrp-WGA was not, which may lead to the conclusion that the HRP/WGA molecule is too bulky to be transported into the cytosol without using active endogenous pathways (Figure 24). Alternatively, it cannot be excluded that f-WGA and hrp-WGA feature slightly different epitope specificities (different isolectins, or as a result of the conjugation to fluorescein or hrp).

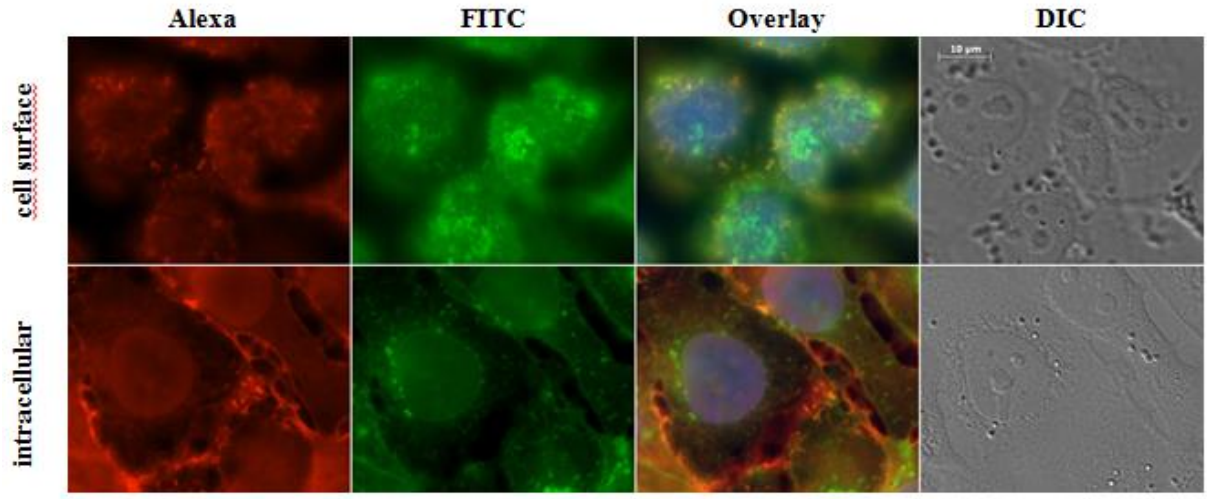


Figure 24: Microscopic analysis of the behaviour of different WGA conjugates when applied on fixed and permeabilized cells. 5637 cells were incubated with 12,5 pmol/ml f-WGA at 4°C and subsequently fixed and permeabilized. Afterwards the dead cells are again incubated with 250 pmol/ml hrp-WGA and 12,5 pmol/ml f-WGA at RT and the HRP was stained by a fluorescence labelled tyramide. The images demonstrate that the cell surface (focus plane set to the cell surface) is stained both by f- and hrp-WGA, whereas intracellularly (focus set to the level of the nuclei) only green staining patterns are visible. hrp-WGA may be too bulky to cross the permeabilized membrane without using endogenous transport pathways. All images were captured using identical exposure conditions to allow for direct comparison. (green channel, *FITC*, 470nm, 100%, 726 ms; red channel, *Alexa*, 590nm, 100%, 239 ms; blue channel, *DAPI*, 365 nm, 100%, 250 ms)

3.4. Application of the TSA dye to detect HRP-modified MP

PLGA/Bodipy®-labeled MP were activated with EDAC/ NHS and modified either with HRP or HRP/WGA in a molar ratio of 1:1 as detailed in *2.5.6. HRP or HRP/WGA conjugation to MP*. An evaluation of the association of HRP to the MP surface was performed via TMB assay as shown in *2.5.6.1. Evaluation of the particle surface modification directly with HRP*. The applicability of the TSA dye for modified MP was assessed by comparing the fluorescence staining of HRP/WGA MP in PBS or in quenching buffer and plain MP, all without incubation with target cells. For this, the various MP were diluted 1:40 either with PBS or 3 % H_2O_2 in PBS to a final volume of 50 µl and incubated with 50 µl of the tyramide dye 1:500 for 15

minutes at RT. After washing the particles two times via centrifugation and resuspension in PBS, the volume was adjusted to 50 µl and analysed via fluorescence imaging (Figure 25).

HRP/WGA MP in PBS developed a homogenous but pale red surface stain evidently caused by the tyramide dye, whereas HRP/WGA MP diluted in the quenching buffer, which inactivated the HRP, showed no red fluorescence at all. The plain MP were partially covered in a hint of a red surface stain or completely free of red fluorescence (Figure 25). This suggests a limited non-specific staining of the particle surface even in the complete absence of the peroxidase, which should be considered during assay interpretation.

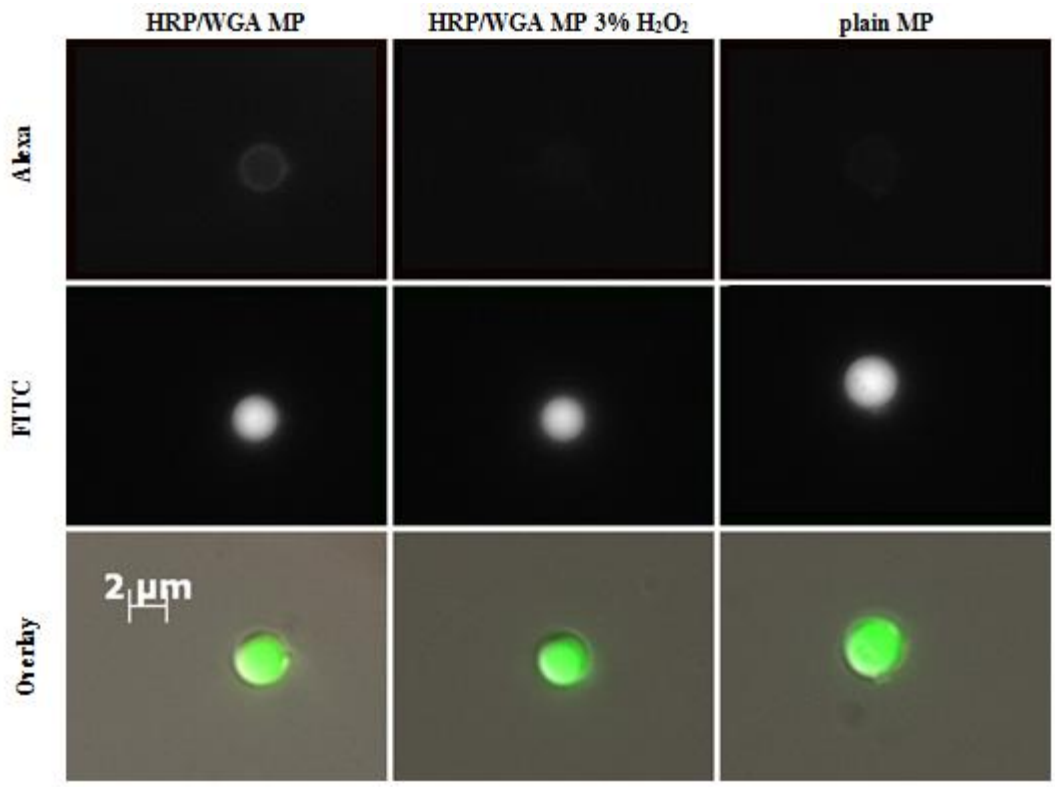


Figure 25: Microscopic analysis of the affinity of the tyramide dye to modified or plain MP. HRP/WGA MP diluted 1:40 in PBS show a marked surface staining due to an anchoring effect between HRP and the tyramide, which can be almost completely blocked by dilution in 3% H_2O_2. Plain MP, which should not be able to bind tyramide and its fluorescence label, partially show a slight surface staining which indicates a non-specific interaction of the dye and the particle surface. All images were processed using the same imaging conditions to allow for direct comparison. (green channel, *FITC* 470nm, 100%, 1 ms; red channel, *Alexa*, 590nm, 100%, 2000 ms)

3.4.1. Qualitative detection of surface modified MP using the tyramide dye

The first TSA assay using modified MP on biological samples was carried out on confluent 5637 glass coverslips in metabolically quiescent state, i.e. by keeping the cells at 4°C. For this, HRP MP, HRP/WGA MP and plain MP were adjusted to the same level concerning their Bodipy® fluorescence signal and afterwards diluted 1:2 in 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68. The target cells were first loaded with 50 µl PBS ($+Ca^{2+}/Mg^{2+}$) followed by an addition of 50 µl of the particle suspensions to enhance the compatibility between the cells and the particle suspension. After an incubation period of 30 minutes at 4°C, the cells were rinsed three times with PBS ($+Ca^{2+}/Mg^{2+}$) and 1 % BSA to diminish non-specific cell adhesion of the MP. Afterwards, the cells were either fixed with 150 µl of 0,5 % GlutAld for 30 minutes at 4°C and subsequently saturated with 100 µl of 100 mg/ml glycine solution for another 30 minutes at 4°C prior to TSA incubation or the viable cells/particles were directly stained with tyramide 1:500 for 30 minutes at 4°C. As a second control supplementary to the plain MP, three wells containing cells which were surface loaded with HRP MP and fixed, were incubated with 3 % H_2O_2 in PBS to block the HRP activity, and then stained with tyramide and analysed analogously via fluorescence imaging.

Viable as well as fixed cells brought about similar results (personal observation), except that viable cells required an increased exposure time to achieve the same signal intensity as fixed cells. As expected, HRP MP seemed to most strongly react with the dye and showed most intensively a red particle surface dye, followed by HRP/WGA MP. In all assays, however, a high non-specific staining was visible, and also plain MP seemed to induce a slight homogenous red lining around the cell nuclei. This allowed no clear conclusion on the success of the assay because of the difficulty in differentiating between non-specific background staining and a possible red surface of the plain particles. The MP whose HRP was blocked by H_2O_2 showed an increase of red fluorescence all over the cells and the cell-associated MP. This finding differs from the details obtained in *3.4. Application of the TSA dye to detect HRP-modified MP*, which showed that blocked HRP of modified MP without previous incubation with cells resulted in no recordable signal. This phenomenon could be explained by a different effect of H_2O_2 in the presence cells. Apparently, the quenching buffer allows the tyramide to anchor non-specifically to the cells and cell-associated MP but not to the MP surface in the absence of cells.

A similar assay was performed by incubating the target cells with HRP/WGA MP in a dilution of 1:20 for 120, 60, 30 or 10 minutes at 37°C to allow for energy-dependent uptake.

After three washing steps with PBS (+Ca^{2+}/Mg^{2+}) the viable cells were directly incubated with tyramide 1:500 for 5 minutes at RT and analysed via fluorescence imaging. In theory, only cell-surface adherent MP should be dyed by the tyramide because this substrate is not able to penetrate the cell membrane, so it should be possible to discriminate between extracellular and intracellular located MP.

Unfortunately, it was not possible to make a clear statement on (potential) endocytosis because of the very high exposure time required, and consequently the dominant non-specific staining. Besides, the probable red surface dye of MP did not co-localize with the green fluorescence of the entrapped Bodipy®.

3.4.2. Quantitative detection of surface-modified MP using the tyramide dye

For a final clarification on the applicability of the TSA dye in endocytosis detection, a quantitative approach was tested using the TMB assay as control. A 5637 confluent monolayer was thoroughly washed and loaded with 50 µl PBS (+Ca^{2+}/Mg^{2+}) and 50 µl of HRP/WGA MP, HRP MP, WGA MP - adjusted to a similar level via their Bodipy® fluorescence signal - or no MP at all. The cells were incubated for 30 minutes at 4°C and subsequently rinsed seven times with PBS (+Ca^{2+}/Mg^{2+}) and 1 % BSA to diminish non-specific cell association of the MP. The enzymatic reaction was either initiated by adding 70 µl of concentrated TMB and stopped after 10 minutes with 20µl 1 M H_2SO_4 or by incubation with the tyramide dye 1:500 for 11 minutes. The enzymatic conversion processes were monitored every 60 seconds at 370 nm (for TMB) or via RFI (ex 595 nm/ em 625 nm, for tyramide) whereas the stopping signal (corresponding to the signal after addition of 1 M sulphuric acid) and the signal of the washed monolayer, after tyramide incubation were determined at 450 nm (TMB + acid) and via RFI (ex 525 nm/ em 625 nm, for tyramide) using a Tecan Infinite® 200 Reader.

The recorded signal using TMB as detecting agent showed a continuous increase of the TMB signal and a high stopping signal (1 M sulphuric acid) analogous to the already performed experiments in *2.5.6.2. Quantitative assessment of HRP-modified MP using the TMB assay*. The TSA dye detection did not match the results of the enzymatic detection method. The fluorescence signal remained at a very low level and it was not possible to discriminate between the various applied MP and the cells, which were not in contact with MP at all.

3.5. Discussion of the results obtained via the TSA assay

In summary, it is important to note that the TSA assay shows some useful characteristics to assess endocytosis (e.g. the impenetrability of the cell membrane for the dying agent as long as the cells are viable and intact). Both of the counter evidences using permeabilized cells and the blocking of the peroxidase activity worked well when using WGA conjugates and a qualitative detection method. Unfortunately, these mechanisms did not work properly for HRP-modified MP in neither a qualitative nor quantitative fashion. Therefore, the desired final goal of establishing a simple but successful method to discriminate between cytoadhesion and cytoinvasion of MP could not be fully met.

It should be noted that this research task brought some interesting side findings. 5637 cells seemed to possess small vesicular compartments, which could be dyed by the fluorescence labelled tyramide, and were therefore considered to most probably be peroxisomes. An additional quenching step of 3 % H_2O_2 was able to block HRP, conjugated to WGA or particle surfaces, but could not completely inhibit the interaction between the tyramide dye and the peroxisomes.

Furthermore, it was discovered that hrp-WGA is not able to interact with intracellular glycans if applied after fixation and permeabilization of the target cells. Given that f-WGA was still able to penetrate the membrane and access intracellular binding sites, the most obvious explanation for that is that hrp-WGA is too bulky to traffic through the damaged cell membrane without the support of active endogenous pathways.

Another promising side finding was established by proving a successful linkage of the tyramide dye to the MP surface via HRP. If a tyramide-dye conjugate can be enzymatically linked to the surface of PLGA MP, this may also be the case for other tyramide-protein conjugates, and hence be of use for developing new coupling protocols for particle surface modification. However, in order to actually take advantage of tyramide-based coupling procedures for particle modification, some additional research will be necessary.

4. Tris-2-carboxyethylphosphine hydrochloride

Tris-2-carboxyethylphosphine hydrochloride (TCEP) is a reducing agent commonly used in biochemistry for the reduction of disulfide bonds of proteins or small molecules [19]. It has already been revealed to be a reversible quenching agent for Cy 5 and similar fluorescence agents, which makes it an interesting tool for the detection of endocytosis [19].

A first try to use TCEP for the differentiation between surface-loaded and internalized cargo gave quite successful results [19], which offers the prospect that TCEP is a satisfactory and less limited alternative to assays with fluorescein-labeled cargo and Monensin.

4.1. Basic principle of the TCEP assay

The quenching ability of TCEP was already tested and characterized using the cyanine dye Cy 5 as model [19]. It was shown that TCEP is able to reversibly quench the fluorescence by 1,4-addition of the phosphine to the polymethine bridge of Cy 5, resulting in a covalent adduct (for detailed reaction schemes, the reader is referred to [19]). This chemical bond can be dissociated by exposure to UV light – a process which is also reversible, that means fluorescence quenching, dequenching and requenching can be repeated several times by applying or removing an UV source [19]. The TCEP-mediated quench can also be restored by removing excessive quenching reagent via repeated washing. In addition, treatment of TCEP quenched Cy 5 with the disulfide cystamine results in a complete fluorescence dequench, which corroborates the reversibility of the quenching process [19].

The quenching ability of TCEP was proven for Cy 5, but also Alexa 647, Alexa 750 and IRdye 800cw has been tested for an internalization assay using Alexa 647-labeled transferrin in A549 cells [19]. Via confocal microscopy, a differentiation between cargo outside and inside the cells was possible, due to the inability of TCEP to cross biological membranes.

These findings, however, have to be validated for bladder cells and WGA-conjugates to exploit the applicability of this agent for clear qualitative but also quantitative detection of lectin-mediated cytoinvasion and later on implementation in particle assays.

4.2. Evaluation of the TCEP assay using WGA conjugates

In order to exploit the potential of this new assay the ideal parameters, such as the optimal TCEP concentration or the most effective dequenching strategy, have to be evaluated. This was in the present study done by using a647-WGA as fluorescence agent and later adaptation of the strategy to surface-modified NP.

Both the quenching agent and the recommended buffer turned out to be detrimental for cell viability, and thus suitable alternatives had to be assessed. The evaluation assays were performed with or without target cells using qualitative as well as quantitative detection methods.

4.2.1. Qualitative approach for assay assessment

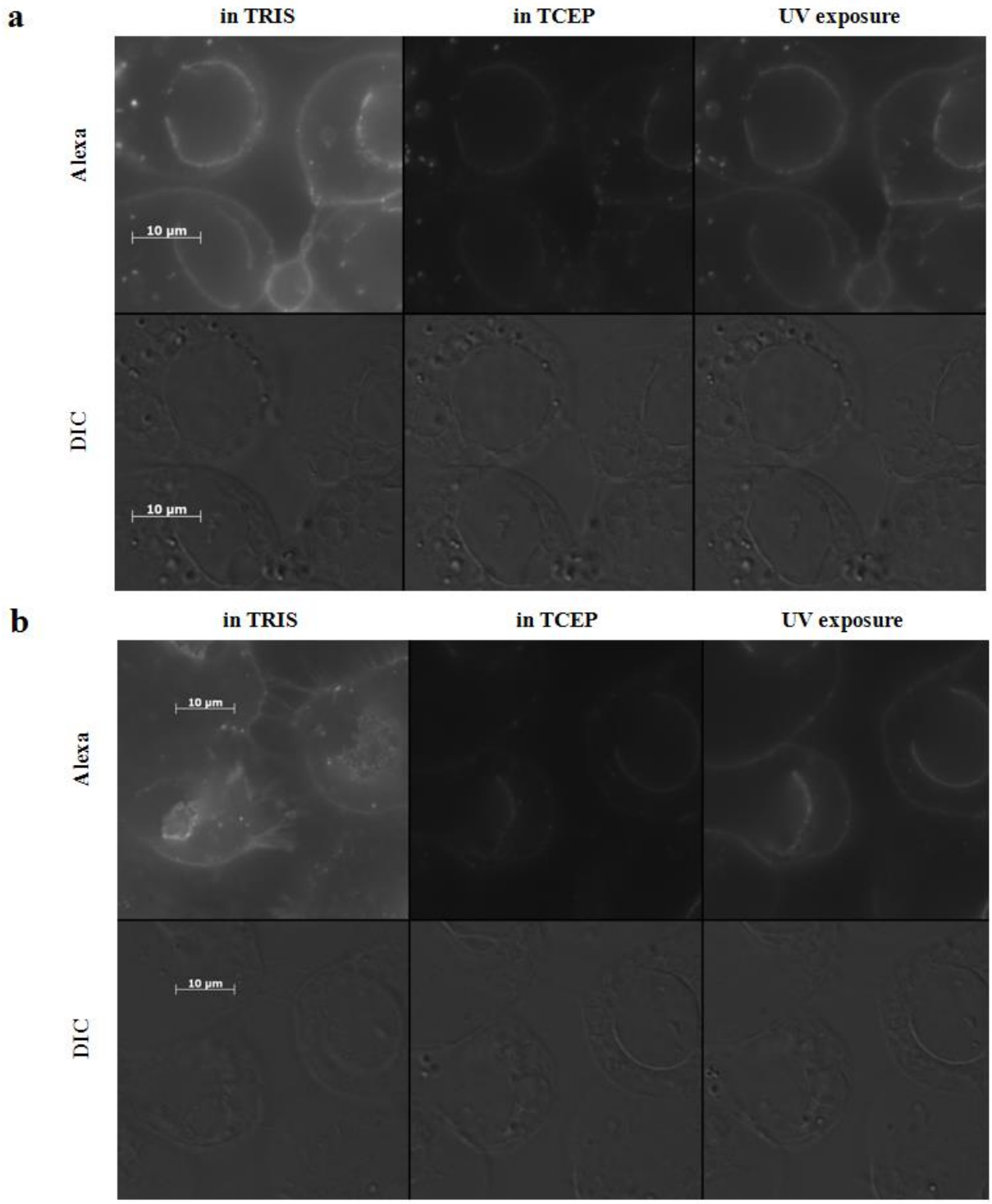
In order to evaluate qualitatively the feasibility of the phosphine quench for the desired conditions, that means for a647-WGA and 5637 cells, fluorescence microscopy was chosen as detection method.

For this, confluent 5637 cells on glass coverslips were thoroughly washed and incubated with 50 µl a647-WGA and f-WGA – for colocalization analysis – at 4°C for 30 minutes only or followed by an internalization period at 37°C. After three rinsing steps using 150 µl PBS ($+Ca^{2+}/Mg^{2+}$), quenching was performed testing various concentrations and dilution buffers of TCEP and subsequently both UV and cell washing (e.g. three wash steps with 150 µl of a 0.4 M TRIS/HCl buffer, pH = 9,0) were evaluated as dequenching strategies. In detail, UV dequench was performed by exposing the samples for 10 seconds to an irradiation at a wavelength of 365 nm with an intensity of 100 % (AxioVision Colibri light source, Zeiss). Fluorescence images were recorded before and during the quench as well as at the dequench at constant imaging conditions to allow for direct comparison.

The first assay was performed with 100 pmol/ml a647-WGA, 60 minutes of incubation at 37°C to allow for energy-dependent uptake, and six different concentrations of TCEP in 0,25 M TRIS/HCl buffer pH = 9,0 (20,800 M; 12,500 M; 6,250 M; 3,125 M; 1,563 M; 0,781 M).

The recorded images substantiated the strong and immediate quenching effect on surface-adherent fluorescent cargo and the inability of TCEP to penetrate intact cell membranes. In general, in cells kept at 4°C the quenching effect decreased with decreasing TCEP concentrations. The difference between surface-adherent and internalized WGA could be

seen more clearly at lower TCEP concentrations, with the best results at a concentration of 0,781 M TCEP (Figure 26c). However, UV dequenching, which was quite effective at high TCEP concentrations, lost its functionality at a TCEP concentration of 1,563 M and below (Figure 26). UV dequench and subsequent requench by removing the UV source could be achieved and repeated several times, but the initial fluorescence intensity – prior to TCEP treatment – could not be restored.



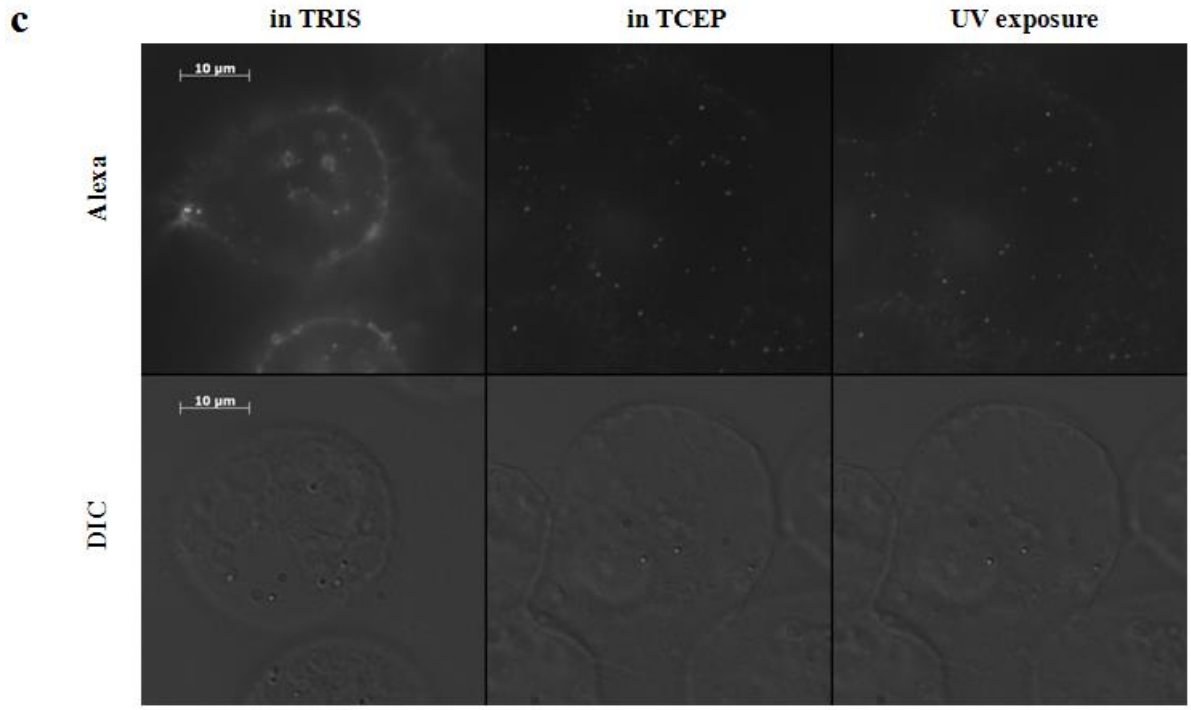


Figure 26: Microscopic analysis of a647-WGA binding and internalization using TCEP as quenching agent. Confluent cells (5637) are pulse/chase incubated with 100 pmol/ml a647-WGA and its fluorescence signal is quenched with **(a)** 6,250 M TCEP, **(b)** 1,563 M TCEP or **(c)** 0,781 M TCEP. The quench is efficient for every concentration, but 0,781 M TCEP brings about best results in the differentiation of cytoadhesive or cytoinvasive cargo. Subsequent dequench ability via UV exposure decreases from high to low TCEP concentration. All concentrations show an incompatibility of the mammalian cells and the phosphine and the TRIS/HCl buffer, respectively, demonstrated in a rounding up of the cells visible in the DIC channel. In order to allow for direct comparison, all images were captured using identical imaging conditions. (red channel, *Alexa*, 590nm, 100%, 2900 ms)

It should be noted that an incompatibility of the quenching agent and its buffer, respectively, with the mammalian cells could be observed, i.e. some cells showed apoptotic behavior (Figure 27 and Figure 28). Based on this finding, a variation of the dilution buffer of TCEP was evaluated, applying the most effective TCEP concentration of 20,8 M and 3 mM PBS ($+Ca^{2+}/Mg^{2+}$) pH = 7,2 – a buffer which is known to be well-tolerated by mammalian cells (as compared to the normal dilution agent 0,25 M TRIS/HCl buffer pH = 9,0). For this assay, the concentration and composition of the fluorescence agents was changed to a mixture of 50 pmol/ml a647-WGA and 12,5 pmol/ml f-WGA, granting the cells 5 hours for energy-dependent uptake at 37°C.

As expected, the captured images showed both at the cell surface and intracellularly a colocalization of the red staining pattern of a647-WGA, which could be quenched when accessible, and of f-WGA, which was nearly completely resistant to TCEP treatment. A difference in the quenching ability of TCEP concerning the buffer system used could be clearly seen. The mammalian cell "friendly" buffer PBS ($+Ca^{2+}/Mg^{2+}$) was able to quench surface-adherent cargo markedly but to a lesser extent than TRIS/HCl pH = 9,0 also leading to a poorer

signal difference between cytoadhesive and cytoinvasive a647-WGA in PBS (+Ca^{2+}/Mg^{2+}) as compared to TRIS/HCl pH = 9,0 (Figure 27). However, the UV dequench showed slightly better results with PBS (+Ca^{2+}/Mg^{2+}) than with TRIS/HCl pH = 9,0 treatment, as was the case for cell viability.

In summary it is to say that TRIS/HCl pH = 9,0 buffer – even at the rather low concentration of 0,25 M – is no suitable option for viable cells but may be used for immobilized/ fixed samples, whereas an ideal buffer for viable cells is still to be determined due to the unsatisfactory quenching activity of TCEP when diluted in PBS (+Ca^{2+}/Mg^{2+}).

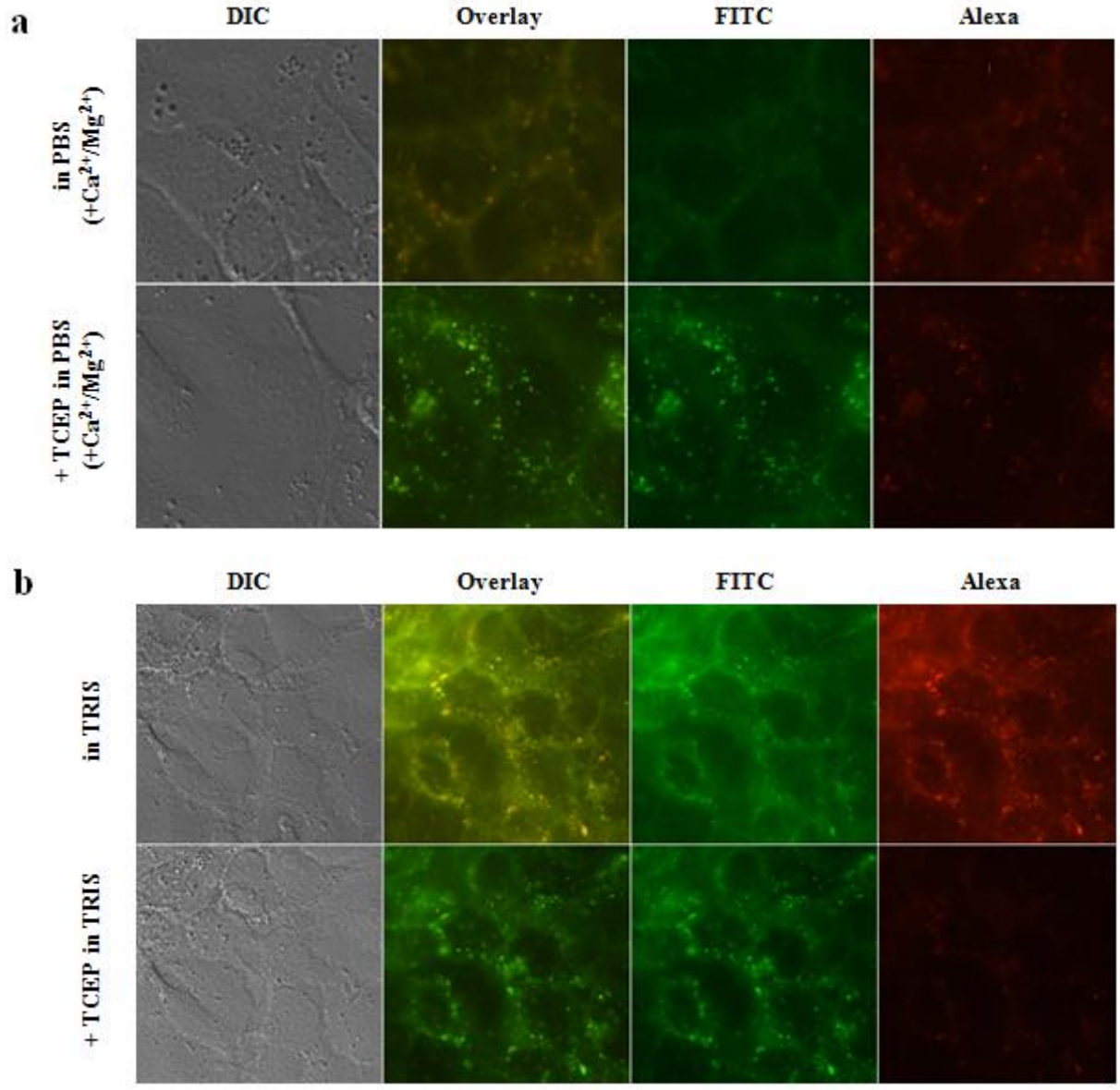


Figure 27: Evaluation of the impact of different buffers on the cell viability and the TCEP quench via microscopic analysis. Cells (5637) are loaded with 50 pmol/ml a647-WGA and 12,5 pmol/ml f-WGA, incubated for 5 hours at 37°C and kept either in **(a)** PBS (+Ca^{2+}/Mg^{2+}) or **(b)** 0,4 M TRIS/HCl pH = 9,0. Both buffers show a marked quenching effect but the differentiation of intra- and extracellular cargo appears more obvious in cells treated with TRIS/HCl. However, cell viability is markedly improved using an isotonic buffer, i.e. PBS (+Ca^{2+}/Mg^{2+}). All

images were processed using the same imaging conditions to allow for direct comparison. (green channel, *FITC* 470nm, 100%, 800 ms; red channel, *Alexa*, 590nm, 100%, 1500 ms)

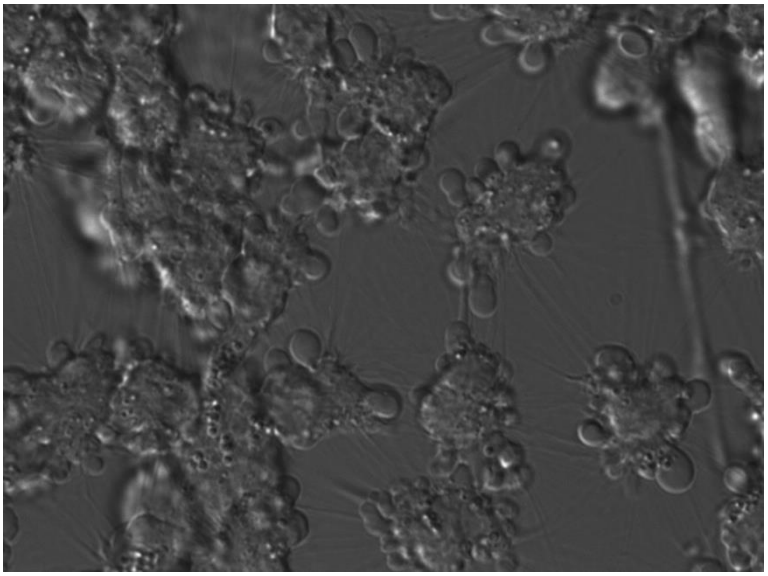


Figure 28: Viable cells (5637) treated with 100 mM TCEP in 0,4 M TRIS/HCl pH = 9,0 showed unambiguous apoptotic behaviour.

4.2.2. Optimization of the TCEP assay with regard to the sensibility of the mammalian cells

The hitherto conducted assays showed an incompatibility of the TRIS/HCl buffer with a pH of 9,0 or the TCEP itself, which is toxic, and mammalian cells. For further experiments, an optimization of the buffer compatibility with the target cells was carried out, with focus on osmolarity and pH, while pilot tests were initially performed without cells.

Analysis of different buffers and different concentrations of TCEP concerning the quenching effectiveness

With regard to the further usage with mammalian cells, which are highly sensitive to osmotic and pH parameters, five different TCEP concentrations (1 mM/ 5 mM/ 25 mM/ 125 mM/ 500 mM), each diluted in different buffers (3 mM PBS ($+Ca^{2+}/Mg^{2+}$) pH = 7,2; 0,25 M TRIS/HCl pH = 9,0; 3 mM iso HEPES/NaOH pH = 7,4; 3 mM iso HEPES/NaOH pH = 8,13) were tested.

50 µl of 1,25 pmol/ml a647-WGA were applied per well in 96 well microtiterplates, the RFI (ex 647 nm/ em 675 nm) was determined via Tecan Infinite® 200 Reader and subsequently 50 µl of the various TCEP concentrations in different buffers were added per well. Again, the RFI was determined and with it the extent of the quenching effect could be evaluated.

The pilot tests showed that a minimal concentration of 125 mM TCEP is necessary to observe a decrease in the fluorescence signals and that the pH of the used buffer for dilution is important for the quenching effect. PBS ($+Ca^{2+}/Mg^{2+}$) and iso HEPES/NaOH pH = 7,4 showed a less pronounced decrease in fluorescence intensity, whereas TRIS/HCl pH = 9,0 and iso HEPES/NaOH pH = 8,13 had a slightly higher quenching effect. (Figure 29)

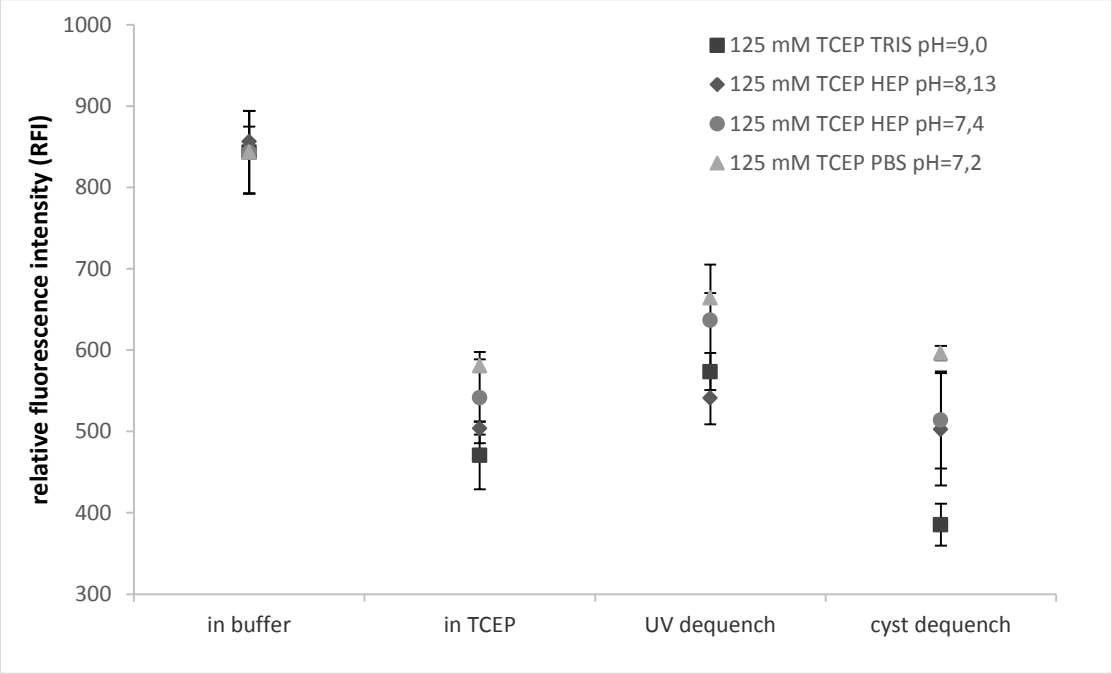


Figure 29: Analysis of the ideal buffering system for fluorescence quenching of a647-WGA, evaluated in the absence of cells. When comparing the most efficient TCEP concentration of 125 mmol/l in different buffers, a correlation between the pH and a notable quenching effect can be determined. Both 0,4 M TRIS/HCl pH = 9,0 and iso HEPES/NaOH pH = 8,13 achieved higher RFI (ex/em: 647/675 nm) decreases than PBS ($+Ca^{2+/}Mg^{2+}$) pH = 7,2 and iso HEPES/NaOH pH = 7,4, starting from nearly identical initial RFI levels. A dequench via UV exposure was visible but not satisfying in every buffer and cystamine as dequenching agent shows no effect at all. Every variation was performed in triplicate and the values represent the mean ± SEM.

Investigation of different dequenching strategies

Two different approaches were tested with regard to the capability of restoring the quenched a647-WGA signal. First, a dequench with UV exposure was assessed, which led to an increase in the fluorescence signal of only 10%, far beyond the level that would be sufficient for control purposes. Second, addition of 250 mM cystamine – diluted in each of the different buffers (3 mM PBS ($+Ca^{2+}/Mg^{2+}$) pH = 7,2; 0,25 M TRIS/HCl pH = 9,0; 3 mM iso HEPES/NaOH pH = 7,4; 3 mM iso HEPES/NaOH pH = 8,13) – should disrupt the covalent bonding between TCEP and the fluorescent agent and thus result in a dequench of the fluorescence signal [19]. Nevertheless, cystamine did not dequench the system at all – the RFI remained almost unchanged (Figure 29).

Fluorescence interference of f-WGA and a647-WGA

For cell studies, it would be useful to be able to determine the quenching effect of a647-WGA via fluorimetry and in parallel monitor the fluorescein signal of e.g. f-WGA. In order to evaluate a possible interference of these two fluorescence agents, another pilot test was performed. For this, the RFI of three different solutions was assessed via Tecan Infinite® 200 Reader: 50 µl of 12,5 pmol/ml a647-WGA mixed either with 50 µl of 12,5 pmol/ml f-WGA or 50 µl PBS ($+Ca^{2+}/Mg^{2+}$) and 50 µl of 12,5 pmol/ml f-WGA diluted with 50 µl PBS ($+Ca^{2+}/Mg^{2+}$). The recorded signals suggested no interference in the fluorescence signals of the two fluorescent agents.

4.2.3. Optimization of the TCEP assay for fixed cells

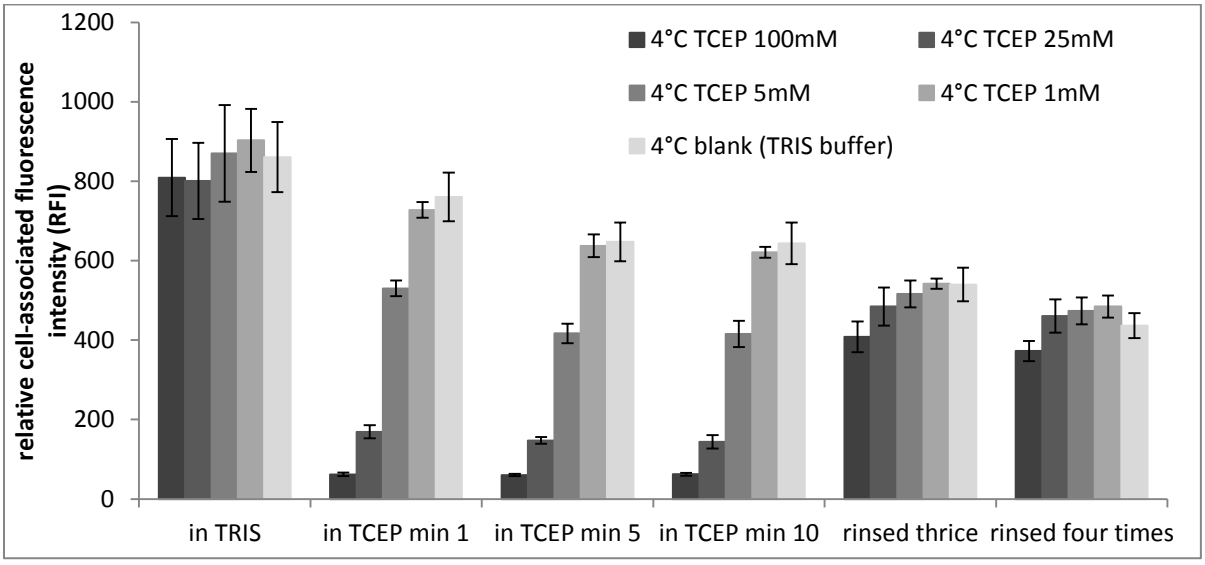
In order to circumvent problems with cell apoptosis due to the quenching agent, the TCEP assay was adopted for application on fixed cells. Four different concentrations of TCEP (1 mM, 5 mM, 25 mM, 100 mM) diluted in 0,4 M TRIS/HCl pH = 9,0 were tested, as the sensitivity of the cells towards an adverse buffer system has no longer to be taken into account here.

A confluent 5637 monolayer was thoroughly washed and incubated with 50 µl of 12,5 pmol/ml a647-WGA either for 30 minutes at 4°C or via pulse/chase incubation (30 min at 4°C followed by 60 min at 37°C). After rinsing the cells three times with 150 µl PBS ($+Ca^{2+}/Mg^{2+}$), 150 µl of 4% Paraformaldehyd (PFA) in PBS were added followed by incubation for another 20 minutes at RT. The washed cells were topped with 100 µl of 0,4 M TRIS/HCl buffer per well and the RFI was determined via Tecan Infinite® 200 Reader. Prior to the next RFI measurement the cells were incubated for 1, 5 or 10 minutes with 100 µl of TCEP in the various concentrations. After rinsing the cells three times with 150 µl of 0,4 M TRIS/HCl buffer, the volume was again adjusted to 100 µl and another RFI detection was performed.

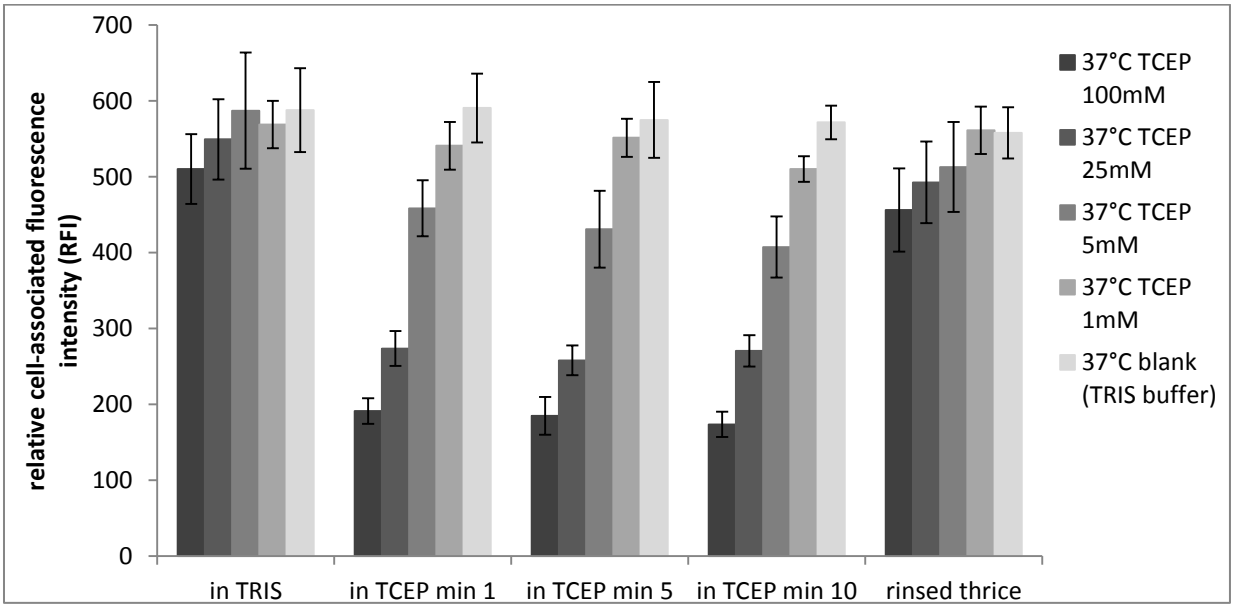
The recorded results showed a successful quench (remaining RFI between 7 and 80 % of the original signal) depending on the extent of internalization and the TCEP concentration (Figure 30a). The most evident quench was achieved at 100 mM TCEP, followed by 25 mM and 5 mM. At 1 mM, almost no quench was visible. As expected, surface-attached a647-WGA was almost completely quenched, whereas prolonged incubation at 37°C (to initiate energy-dependent uptake) decreased the accessibility of the fluorescence agent for TCEP and hence resulted in higher RFI signals under TCEP (Figure 30b). A fixation using 0,5 % GlutAld can be expected to disrupt to a certain degree the integrity of the cell membrane (as shown in *3.2*

Evaluation of the assay using hrp-WGA and f-WGA), which might possibly be avoided by the usage of 4 % PFA in PBS. Although the difference in the quenched RFI signals between metabolically quiescent cells and those that were stimulated to endocytosis was obvious, the absolute integrity of the cell membrane could not be guaranteed. The dequench by rinsing the cells (detailed above) thoroughly also showed no consistent results. After the dequench of cells incubated at 4°C the RFI stayed quite low (nearly 50 % of the original RFI), whereas cells enabled to perform endocytosis showed a dequenched RFI of about 90 % of the original signal. A possible loss of surface-associated a647-WGA due to the repeated washing steps, despite prior fixation with a cross-linking agent, should be considered as a potential cause for these results. RFI values should thus always be interpreted with reference to the respective controls, as illustrated in Figure 30c+d.

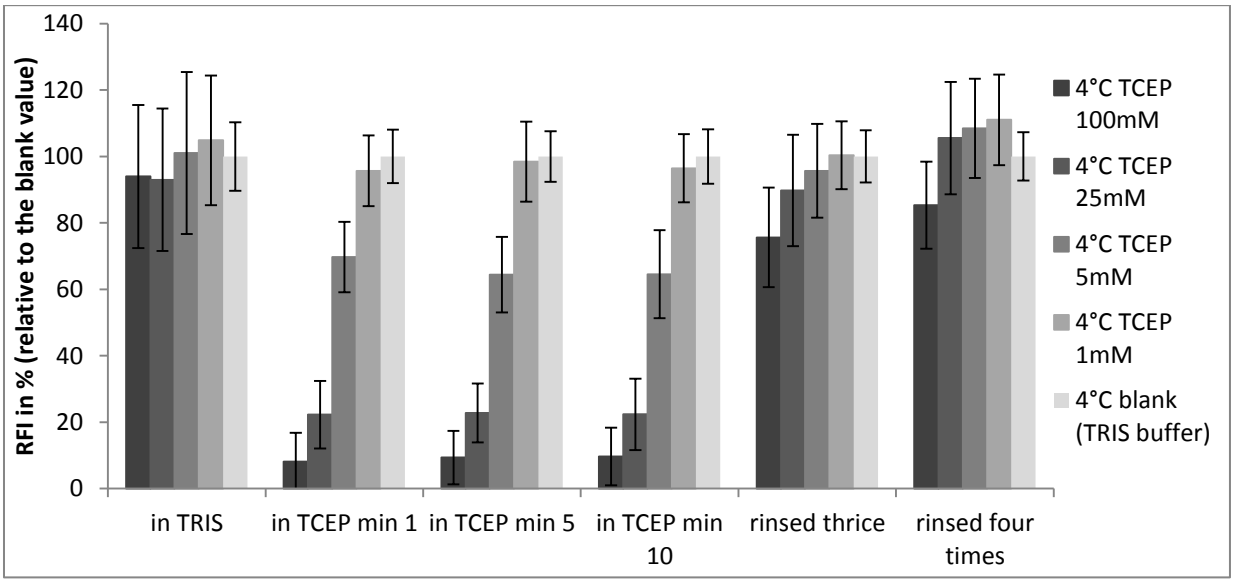
a



b


relative cell-associated fluorescence intensity (RFI)
700
600
500
400
300
200
100
0
in TRIS
in TCEP min 1
in TCEP min 5
in TCEP min 10
rinsed thrice
37°C TCEP 100mM
37°C TCEP 25mM
37°C TCEP 5mM
37°C TCEP 1mM
37°C blank (TRIS buffer)

c


RFI in % (relative to the blank value)
140
120
100
80
60
40
20
0
in TRIS
in TCEP min 1
in TCEP min 5
in TCEP min 10
rinsed thrice
rinsed four times
4°C TCEP 100mM
4°C TCEP 25mM
4°C TCEP 5mM
4°C TCEP 1mM
4°C blank (TRIS buffer)

d

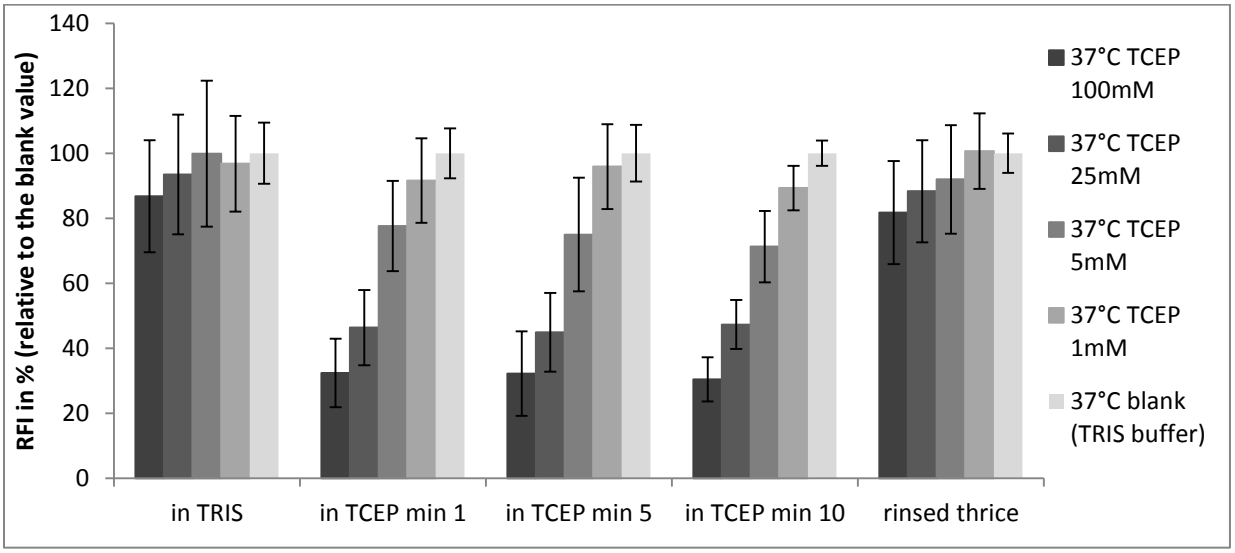


Figure 30: Analysis of the quenching ability of different TCEP concentrations when applied to fixed cells. Confluent 5637 monolayers are either **(a) (c)** surface-loaded with 12,5 pmol/ml a647-WGA or **(b) (d)** endocytosis is initiated via pulse/chase incubation. For both extra- and intracellular cargo, a TCEP concentration of 100 mM yields the best quenching results, with a decrease of the fluorescence signal of about 93% at 4°C and about 63% at 37°C. Dequench seems more effective in 37°C samples, but in 4°C samples a loss of extracellular a647-WGA should be taken under consideration. This can be demonstrated in the control samples, which are also treated with a647-WGA (blank (TRIS/HCl buffer)) but are not quenched at all. Nevertheless, these controls show a decrease in the RFI after the additional washing steps (for dequenching) **(a)**. In order to show the results without the surface elution of WGA, figure **(c)** and **(d)** demonstrate the RFI signals in percent ± SEM relative to the control sample (set as 100 %). Every TCEP concentration was performed in triplicate and for figure **(a)** and **(b)** the values represent the mean ± SEM.

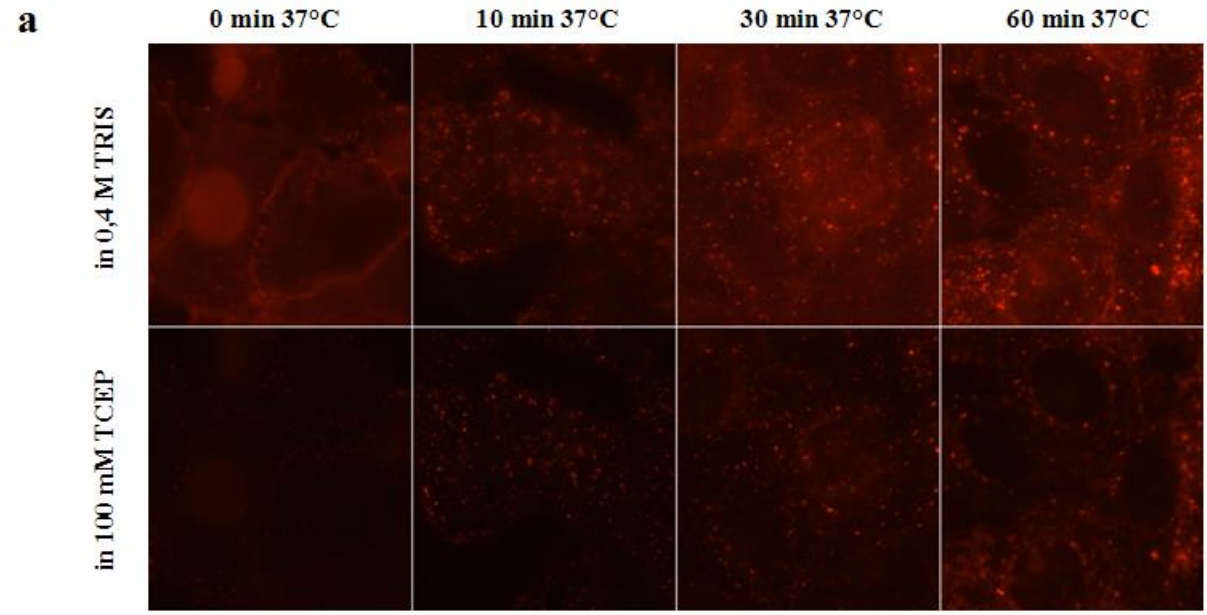
4.3. Application of the TCEP assay for qualitative detection of WGA conjugates

The qualitative assays were carried out using confluent 5637 monolayers on glass coverslips mounted in FlexiPERM®s, which were loaded with 50 µl of 12,5 pmol/ml a647-WGA or f-WGA (as a control) at 4°C for 30 minutes, rinsed three times with 150 µl PBS ($+Ca^{2+}/Mg^{2+}$) and subsequently incubated at 37°C (time course see below) to initialize endocytosis. The fixation was performed with 4 % PFA for 20 minutes at RT either directly after the 4°C incubation or after the internalization period. The monolayer was rinsed again and the volume adjusted to 100 µl with 0,4 M TRIS/HCl pH = 9,0. Fluorescence quenching of the cargo was initiated by applying 100 µl of 100 mM TCEP in 0,4 M TRIS/HCl pH = 9,0 instead of buffer. Dequenching was performed via UV exposure or/and washing of the monolayer. Fluorescence images were captured before and during the quench and after dequenching the fluorescence signal of the cargo.

Internalization time course

In order to explore the specificity and sensitivity of the TCEP assay, an internalization time course for a647-WGA was performed incubating the target cells for 0, 10, 30 or 60 minutes at 37°C and implementing a control by adding 50 µl of 12,5 pmol/ml f-WGA and 5 µg/ml HOECHST 33342 DNA stain after the fixation to one well of each of the different incubation times. The dequench was performed via UV exposure.

The recorded images showed an increase in the remaining fluorescence staining over the time when treated with 100 mM TCEP, which illustrated the functionality of the assay for detecting endocytosis of the assay (Figure 31a). However, UV exposure showed no effect on the fluorescence signal, maybe due to an interference of the fixation with 4 % PFA. The double stained wells showed red and green fluorescence staining patterns but no colocalization, which could be a result of the non-simultaneous cell loading and/ or already eluted a647-WGA (Figure 31b). As expected, f-WGA was not quenched by TCEP, whereas a647-WGA was almost completely quenched when located at the cell surface.



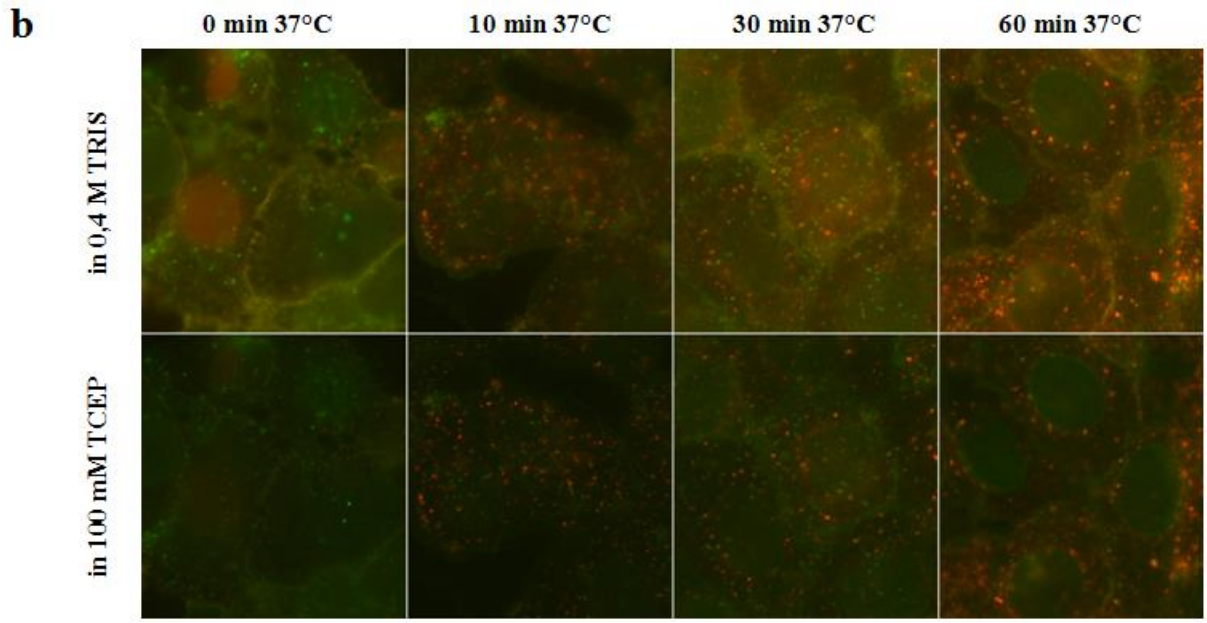


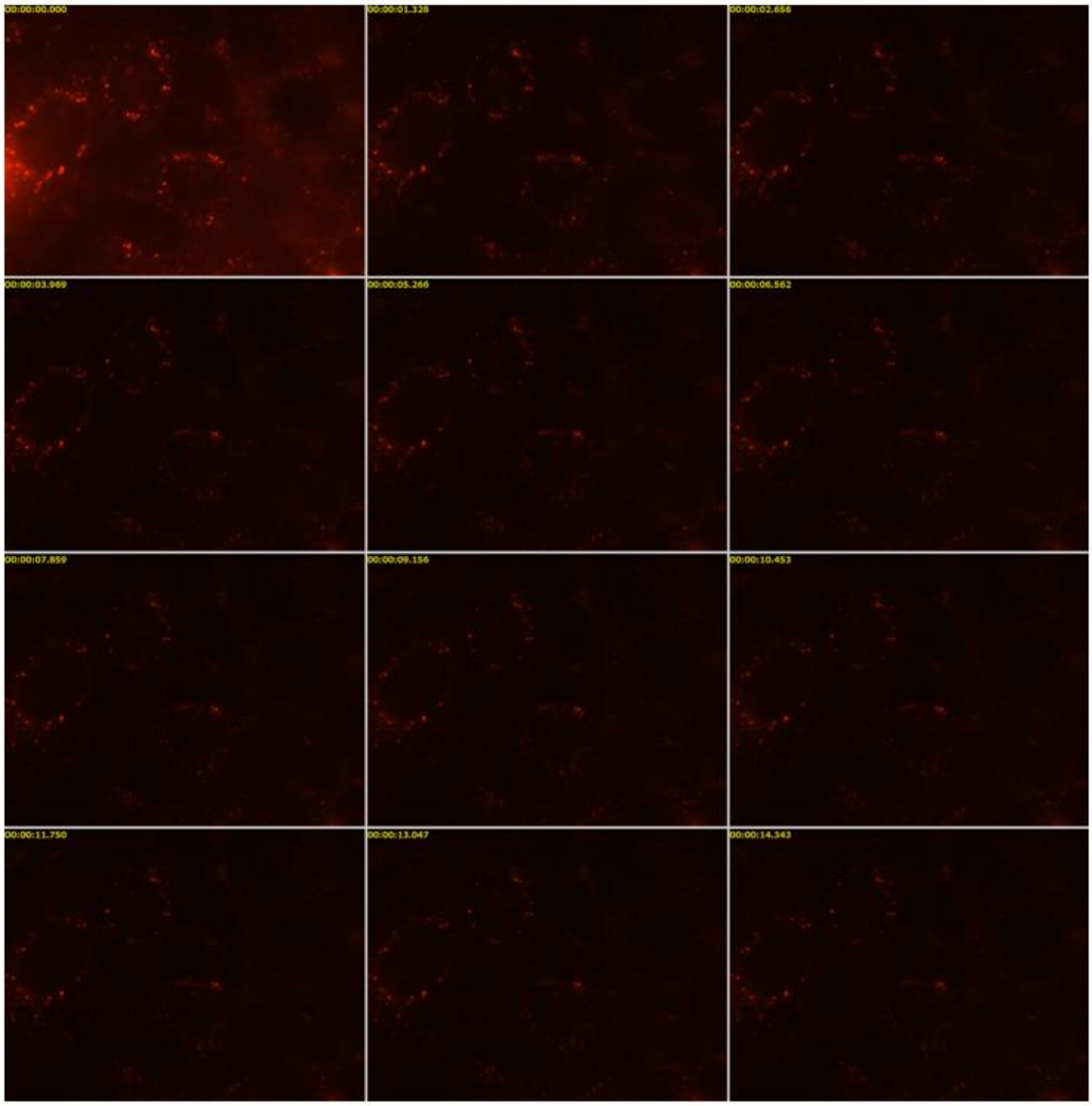
Figure 31: Microscopic analysis of time course internalization studies via the TCEP assay. A 5637 monolayer is incubated with 12,5 pmol/ml a647-WGA via pulse/chase incubation, i.e. 30 minutes at 4°C followed by 0, 10, 30 or 60 minutes at 37°C. The cells are immobilized using 4 % PFA, and subsequently 12,5 pmol/ml f-WGA are added. **(a)** Images captured using the *Alexa* channel show an increase of intracellular cargo demonstrated both by typical staining around the cell nuclei in 0,4 M TRIS/HCl buffer and by a decrease of the quenching ability of the TCEP. **(b)** Merged images (*FITC* and *Alexa* channel) show a double staining of a647- and f-WGA, but no identical staining patterns, which may be due to non-simultaneous cell loading and already eluted a647-WGA. All images are displayed with the same imaging conditions. (green channel, *FITC* 470nm, 25%, 1000 ms; red channel, *Alexa*, 590nm, 100%, 1500 ms)

Elution assay via competitive GlcNAc

The elution of cell surface-associated WGA via competitive carbohydrates was already established in previous experiments, but the compatibility of this approach with the TCEP assay was yet to be tested. For this, the cells were loaded with 12,5 pmol/ml a647-WGA via pulse/chase incubation (30 min at 4°C and 60 min at 37°C), then rinsed either with PBS ($+Ca^{2+}/Mg^{2+}$) or 50 mM GlcNAc in PBS ($+Ca^{2+}/Mg^{2+}$), fixed and quenched as usual.

As expected, a marked difference (compare Fig. 32 a+b, upper left image) between the particular rinsing protocols could be observed in non-quenched cells, with GlcNAc eluting most of the a647-WGA cargo at the cell surface. Treatment with TCEP confirmed these findings as shown by a decrease in its quenching ability for GlcNAc-treated cells in comparison to PBS ($+Ca^{2+}/Mg^{2+}$) rinsed ones, based on the decrease of the amount of surface-adherent and, therefore, accessible cargo. The overall quench is higher in case of PBS-rinsed samples (Figure 32 b). However, also a slight loss of intracellular fluorescence was observed after initial quenching, which may be due to a cell membrane that is not completely impenetrable for the glycan (Figure 32 a+b, upper row). No further quenching could be observed at prolonged exposure times (Figure 32 a+b, lower two panels).

a


00:00:00.000
00:00:01.328
00:00:02.656
00:00:03.969
00:00:05.266
00:00:06.562
00:00:07.859
00:00:09.156
00:00:10.453
00:00:11.750
00:00:13.047
00:00:14.343

b

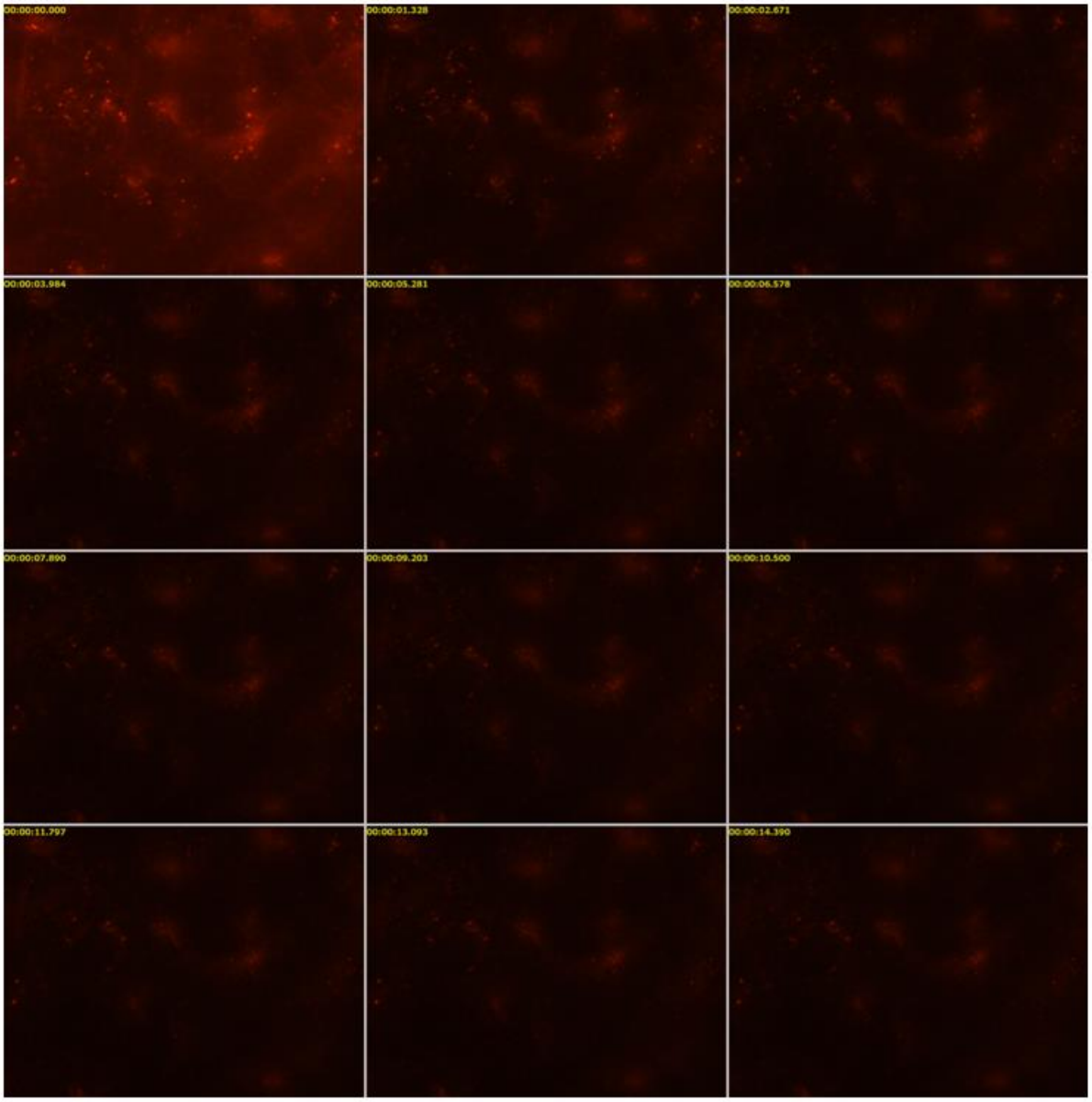


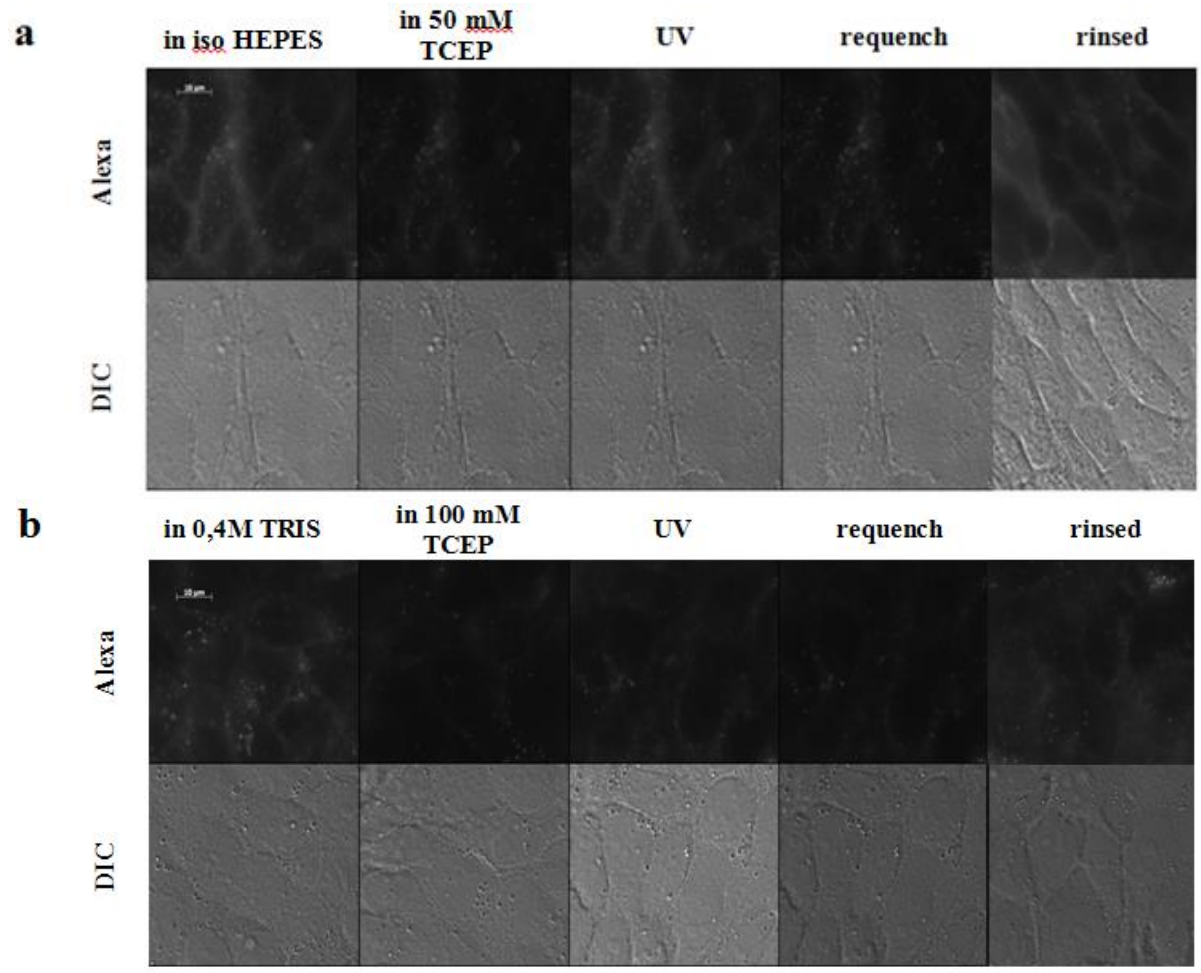
Figure 32: Microscopic analysis of competitive elution of WGA- mediated cytoadhesion using GlcNAc and the TCEP assay. Confluent cells (5637) with intra- and extracellular a647-WGA are rinsed with **(a)** 50 mM GlcNAc or **(b)** PBS ($+Ca^{2+}/Mg^{2+}$) and fixed with 4 % PFA. The captured images show cells in TRIS/HCl buffer and in 100 mM TCEP, demonstrating the gradual quenching process over a time period of 14 seconds. Glycan-treated cells show a lower fluorescence intensity before the quench, due to a smaller amount of WGA at the cell surface, and a lower decrease of the fluorescence during the treatment with 100 mM TCEP - both confirming the successful a647-WGA elution. In order to allow for direct comparison all images were captured using identical imaging conditions. (red channel, *Alexa*, 590nm, 100%, 1000 ms)

Applicability of the TCEP assay for fixed, fixed/permeabilized and viable cells

In order to prove the feasibility of this assay for differently treated cells, cells were loaded via pulse/chase incubation as described above and subsequently kept in buffer or fixed or fixed and permeabilized with 0,15 % Triton X-100 for 20 minutes at RT. In order to guarantee viability of non-fixed cells, iso HEPES/NaOH pH = 8,15 was used instead of 0,4 M

TRIS/HCl pH = 9,0, and the quench was performed using only 50 mM TCEP in iso HEPES/NaOH pH = 8,15 instead of 100 mM TCEP in 0,4 M TRIS/HCl pH = 9,0. Dequenching was performed via UV exposure as well as via an additional washing protocol either with iso HEPES/NaOH pH = 8,15 or 0,4 M TRIS/HCl pH = 9,0. Dequenching was reversible as demonstrated by a requench of the fluorescence signal via removing the UV source, performed between the UV dequench and the washing step.

The fluorescence quenching worked well for all the differently treated cells and the different TCEP concentrations (50 or 100 mM final concentration), i.e. nearly all of the extracellular cargo was quenched (Figure 33). However, the impact of the different dequenching strategies turned out to be non-homogenous. UV exposure increased the fluorescence staining of fixed and viable cells, but was variable for fixed/permeabilized cells. The washing protocol resulted in a dequench of all the differently treated wells, yet not of consistent extent (Figure 33).



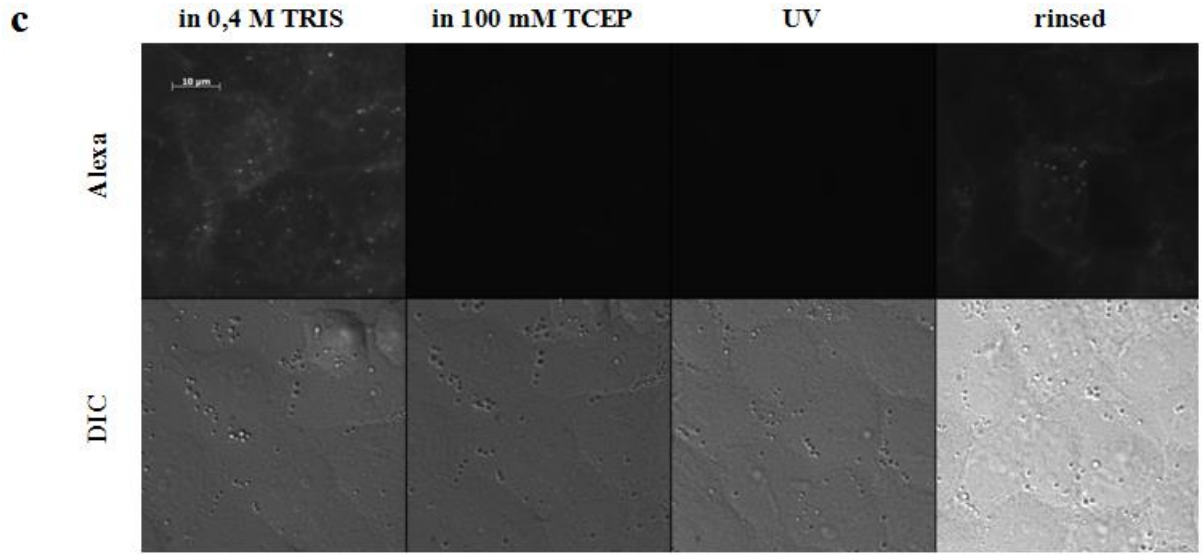


Figure 33: Microscopic analysis of the feasibility of the TCEP assay for differently treated cells. A 5637 monolayer is pulse/chase incubated with 12,5 pmol/ml a647-WGA and subsequently **(a)** kept in buffer, **(b)** fixed or **(c)** fixed/permeabilized. Fluorescence quenching with TCEP is feasible for all the differently treated cells, but the impact of various dequenching strategies are not consistent. UV exposure only worked for viable and fixed cells and the regained signal intensity due to cell washing does not show the same strength in the various samples. Requench is performed after UV dequench but before cell washing by removing the UV source. All images were exposed to identical imaging conditions. (red channel, *Alexa*, 590nm, 100%, 1000 ms)

In summary, the TCEP assay seems to be useful for the differentiation of cytoadhesive and cytoinvasive cargo when assessed via a qualitative detection method. It is applicable for viable, fixed and fixed/ permeabilized cells and GlcNAc elution. A dequench, either via UV exposure or cell rinsing, and requench is feasible but not consistent in case of this detection method.

4.4. Quantitative assessment of the TCEP/WGA assays via fluorescence readout

Documentation of successful internalization via fixation, fixation/permeabilization and an internalization time course

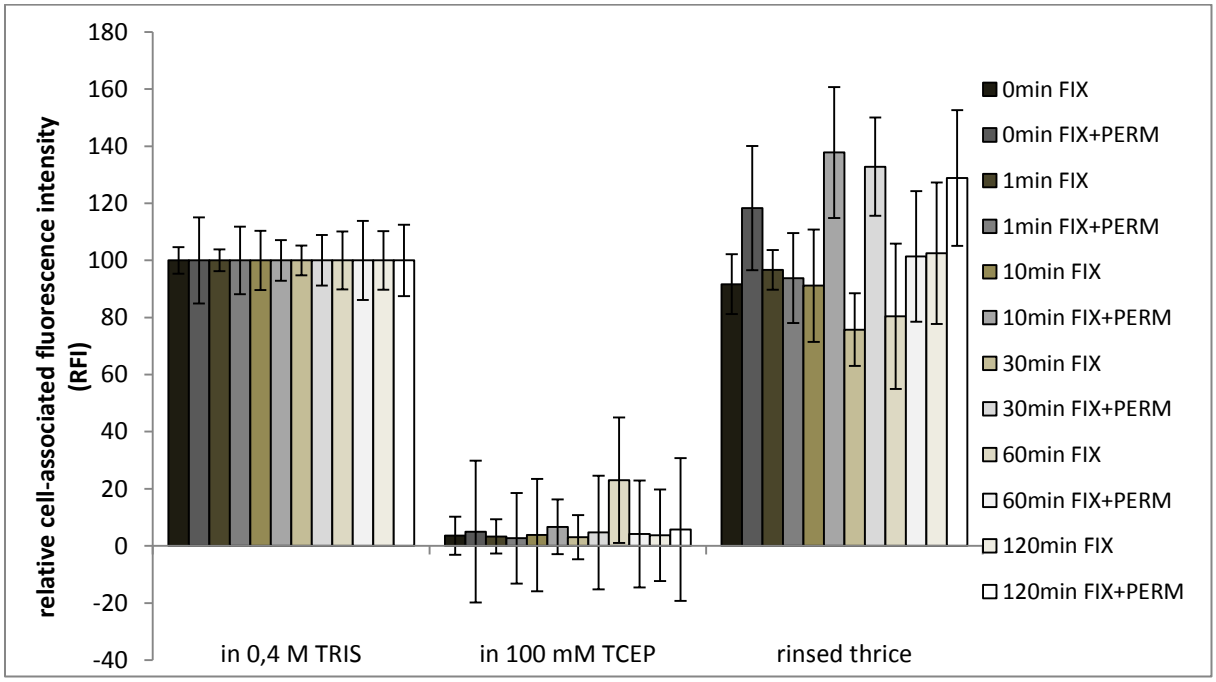
In order to explore the possibilities of the TCEP assay for quantitative studies, the ideal conditions evaluated with regard to the quenching agent, i.e. using 100 mM TCEP in 0,4 M TRIS/HCl buffer, were applied to monitor the internalization time course of fixed and fixed/ permeabilized cells. The latter case should allow the quenching agent to reach internalized WGA. Additionally, the robustness of the process was tested using varying concentrations of a647-WGA and different cell lines, malignant 5637 and healthy Sv-Huc cells.

After loading the cells with different concentrations of 50 µl a647-WGA and incubation for 30 minutes at 4°C, energy-dependent uptake was initiated by incubating the cells for 0, 1, 10, 30, 60 and 120 minutes at 37°C. In order to avoid endocytosis of WGA after the set

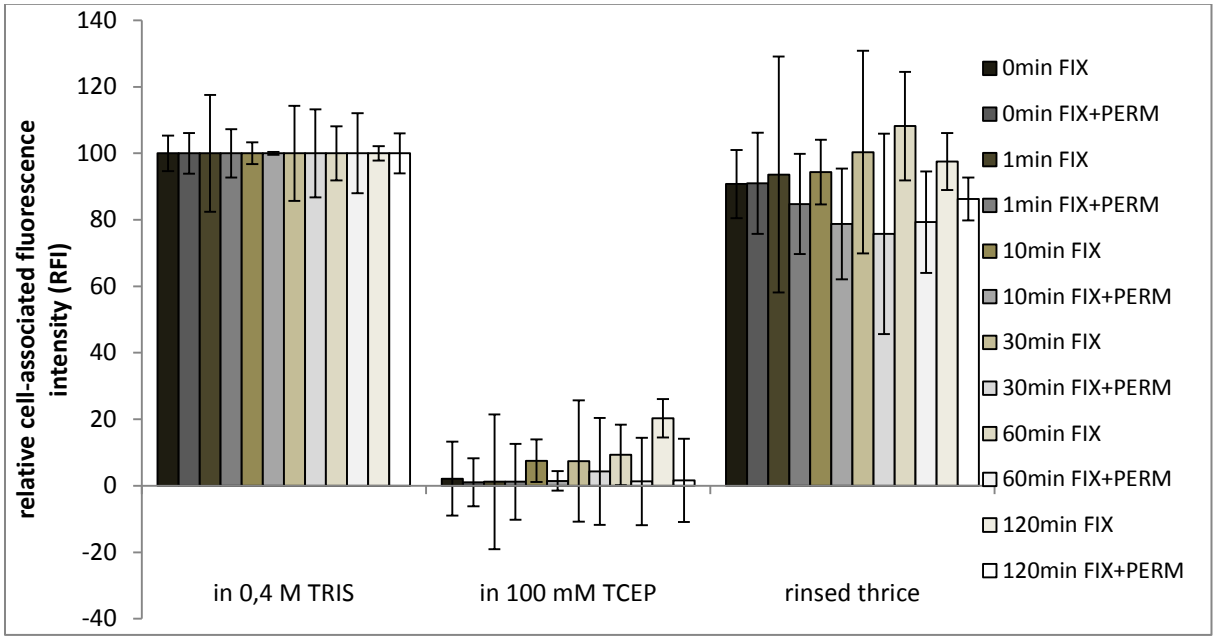
incubation time, the cells were immediately immobilized with 150 µl/well of 4 % PFA in PBS and optionally permeabilized with 100 µl/well of 0,15 % Triton X-100 in aqua dest. Quenching was performed using 100 µl of 100 mM TCEP in 0,4 M TRIS/HCl buffer pH = 9,0 and dequenching was achieved by rinsing the cells three times with 150 µl 0,4 M TRIS/HCl buffer pH = 9,0 including a 5 minutes incubation time of the eluent on the shaker to make sure that the dequench was also working for permeabilized cells. As a control, an analogous incubation with 50 µl of 12,5 pmol/ml f-WGA was performed, either leaving the cells viable and treating them with 100 µl of 20 nmol/ml Monensin as dequenching agent or fixing and fixing/permeabilizing them, respectively, without further treatment. The RFI was detected via a Tecan Infinite® 200 Reader not only after incubation at 4°C, but also after incubation at 37°C, during incubation with PFA (and optionally Triton X-100), directly before the quench, during the quench and after the dequench. Additionally, 50 µl of the supernatants of the 37°C and PFA incubation period were investigated with regard to their fluorescence at 647 nm and 488 nm, respectively.

The recorded signals supported the notion that the amount of intracellular a647-WGA increased along with the incubation time at 37°C. Fixed cells showed an increase in fluorescence intensity after the quench depending on the incubation time at 37°C, whereas fixed/permeabilized cells showed the same quenching effect irrespective of incubation at 4°C or 37°C. These effects, however, were not completely identical using different a647-WGA concentrations. A cell-loading concentration of 4,2 pmol/ml a647-WGA seemed to be too low to achieve a marked differentiation of the various incubation times at 37°C, only reaching a clearly visible internalization at an incubation time of 60 minutes (Figure 34a). Both other a647-WGA concentrations – 12,5 pmol/ml and 100 pmol/ml – showed the desired gradation of the RFI after quenching as described above, with 100 pmol/ml a647-WGA providing the most consistent and reproducible results (Figure 34b/c). The dequenching via rinsing the cells worked well for both fixed and fixed/permeabilized cells and seemed to be equally effective for extra- and intracellular WGA – an average of 80 % of the original RFI could be regained (Figure 34).

a


relative cell-associated fluorescence intensity (RFI)
180
160
140
120
100
80
60
40
20
0
-20
-40
in 0,4 M TRIS
in 100 mM TCEP
rinsed thrice
0min FIX
0min FIX+PERM
1min FIX
1min FIX+PERM
10min FIX
10min FIX+PERM
30min FIX
30min FIX+PERM
60min FIX
60min FIX+PERM
120min FIX
120min FIX+PERM

b


relative cell-associated fluorescence intensity (RFI)
140
120
100
80
60
40
20
0
-20
-40
in 0,4 M TRIS
in 100 mM TCEP
rinsed thrice
0min FIX
0min FIX+PERM
1min FIX
1min FIX+PERM
10min FIX
10min FIX+PERM
30min FIX
30min FIX+PERM
60min FIX
60min FIX+PERM
120min FIX
120min FIX+PERM

c

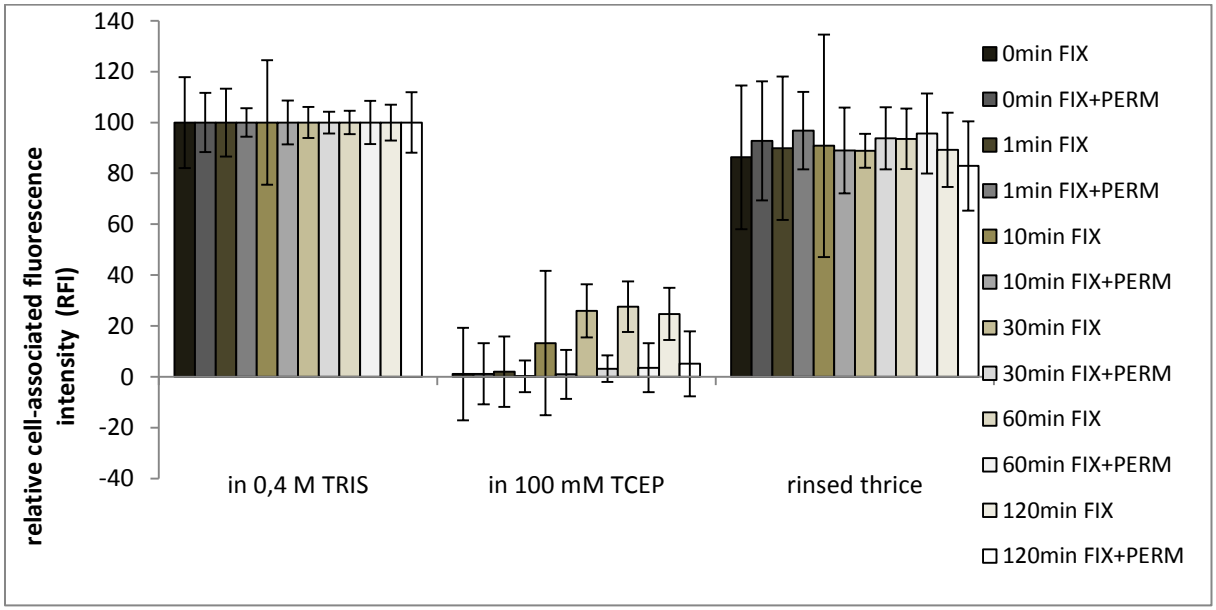


Figure 34: Analysis of the consistency and reliability of the TCEP assay for differentiation between cargo inside and outside the cells. 5637 monolayers are loaded with **(a)** 4,2 pmol/ml, **(b)** 12,5 pmol/ml or **(c)** 100 pmol/ml a647-WGA at 4°C for 30 minutes and subsequently incubated at 37°C for 0, 1, 10, 30, 60 and 120 minutes to allow for endocytosis. The cells are either fixed or fixed/permeabilized to prove the inability of TCEP to cross biological membranes. A successful internalization is visible by an increase of the RFI (of fixed cells) according to the incubation time for cells loaded with 12,5 or 100 pmol/ml a647-WGA. Cells loaded with 4,2 pmol/ml only let assume an internalization after 60 minutes of incubation. Fixed/permeabilized wells show nearly a total quench irrespective of the adopted cargo concentration. A restorage of the fluorescence intensity by washing the cells is achieved at every WGA concentration. The values represent the mean RFI of a triplicate performance converted in percent, taking the RFI directly before quench as the 100 % reference.

In spite of these positive results, some irregularities when using PFA were noticable, which were not observed previously. Directly before the treatment with TCEP all different samples (4°C, 1 min 37°C, etc) showed about the same RFI, thus a similar amount of a647-WGA should be associated with the cells, either intra- or extracellularly. These comparatively similar signal levels were observed irrespective of the treatment of the cells (different incubation times and PFA); hence, the effects described in the following seemed to be quite universal. As shown in Figure 35, an increasing incubation at 37°C correlated with an increasing loss of a647-WGA into the supernatant. During incubation with PFA, the RFI altered quite unpredictably – although a correlation to the temperature incubation could be established – and the PFA supernatants (see above) showed an inverse behavior to the 37°C supernatants (i.e. lower fluorescence intensity for longer incubation time at 37°C), which may have led to the rather uniform signals before quench (see Figure 35 *directly before quench – in TRIS/HCl*). Although these findings of the elution of a647-WGA at 37°C or due to PFA seemed to explain the equalized RFI of differently treated samples/wells, this could not yet be

substantiated on a quantitative level. Besides, no explanation could be found why the RFI signals behave differently after PFA addition. It seems that especially at 4°C, a high fraction of lectin is lost from the surface due to the change(s) induced by PFA treatment. This fraction decreases with longer incubation time at 37°C, since here a significant portion of lectin might already be lost due to other reasons (off-diffusion, internalization).

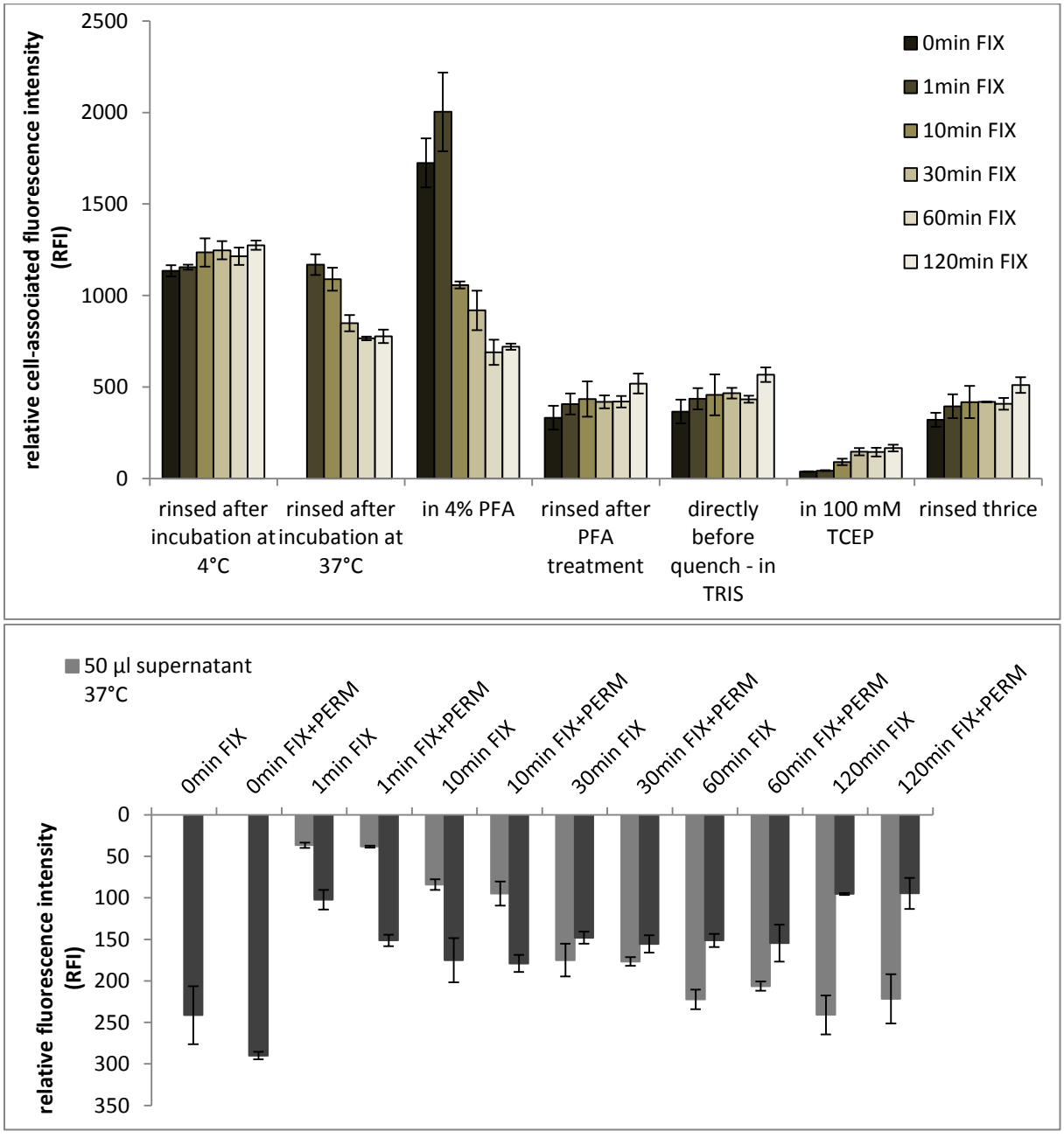
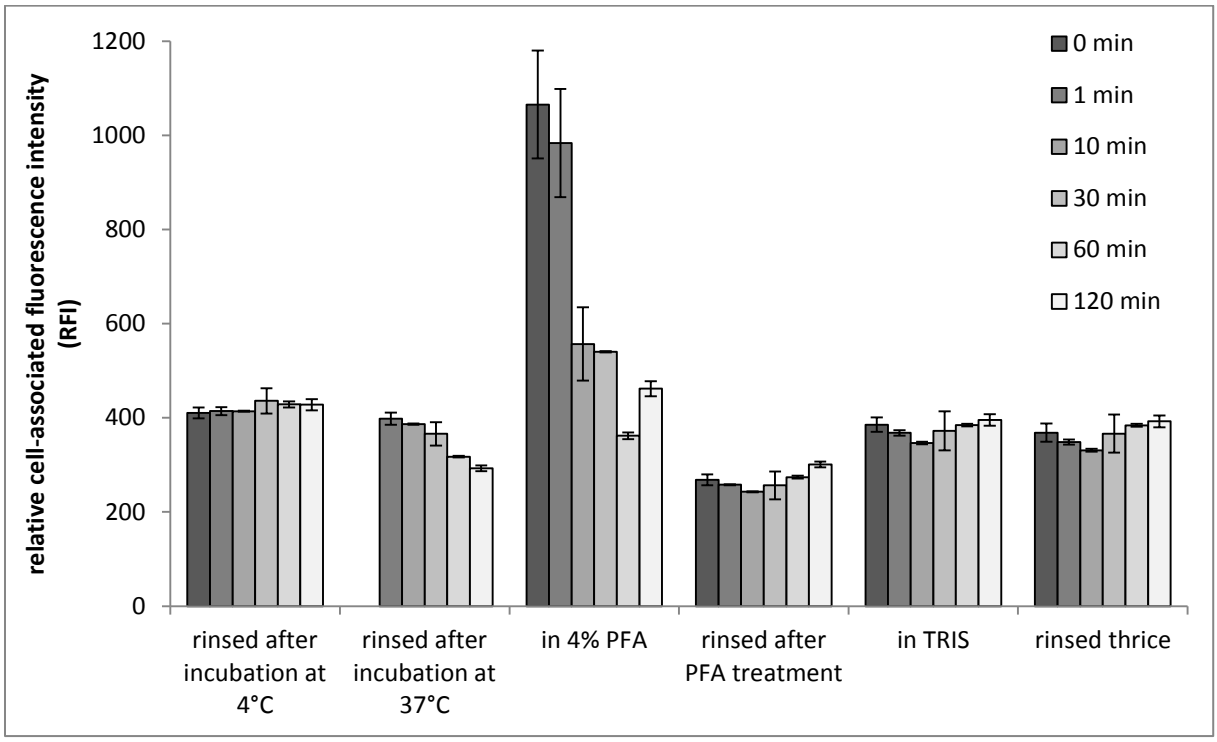


Figure 35: Analysis of the change in the fluorescence signal and supernatants during the TCEP assay with regard to the incubation time and the immobilization with PFA. This assay is performed with 100 pmol/ml a647-WGA payload, the incubation time course and 4 % PFA for fixation of the cells (5637). The cells that are rinsed after incubation at 37°C show a loss of cargo over time, which is the higher the longer the incubation at 37°C lasted. The partial increase of the RFI while treated with PFA cannot be explained as well as the equalization of the RFI signals after PFA incubation. Supernatants *(lower panel)* are sampled as follows: The volume per well is adjusted to 100 µl before RFI detection and 50 µl thereof are sampled for RFI detection of the supernatant either after the incubation at 37°C or after incubation with PFA for immobilization of the cells. Obviously, the elution of a647-

WGA on the cell surface is associated with the internalization period, with more cargo being lost with longer incubation times at 37°C. The PFA supernatants show inverse behaviour concerning their RFI, which cannot be explained yet. All values symbolize the mean ± SEM of a triplicate performance.

The results of the controls using f-WGA varied with regard to the treatment. No clear statement can be made regarding potential internalization. Treatment with 4 % PFA led to changed signals, but allowed no further conclusion on successful endocytosis (Figure 36). For the 37°C supernatants of f-WGA, no indication for a loss of surface-associated cargo could be detected, but the PFA supernatants behaved analogously to the a647-WGA PFA supernatants supporting the notion of a PFA-specific, and not a WGA-specific phenomenon (since it was found for both WGA types).

a



b

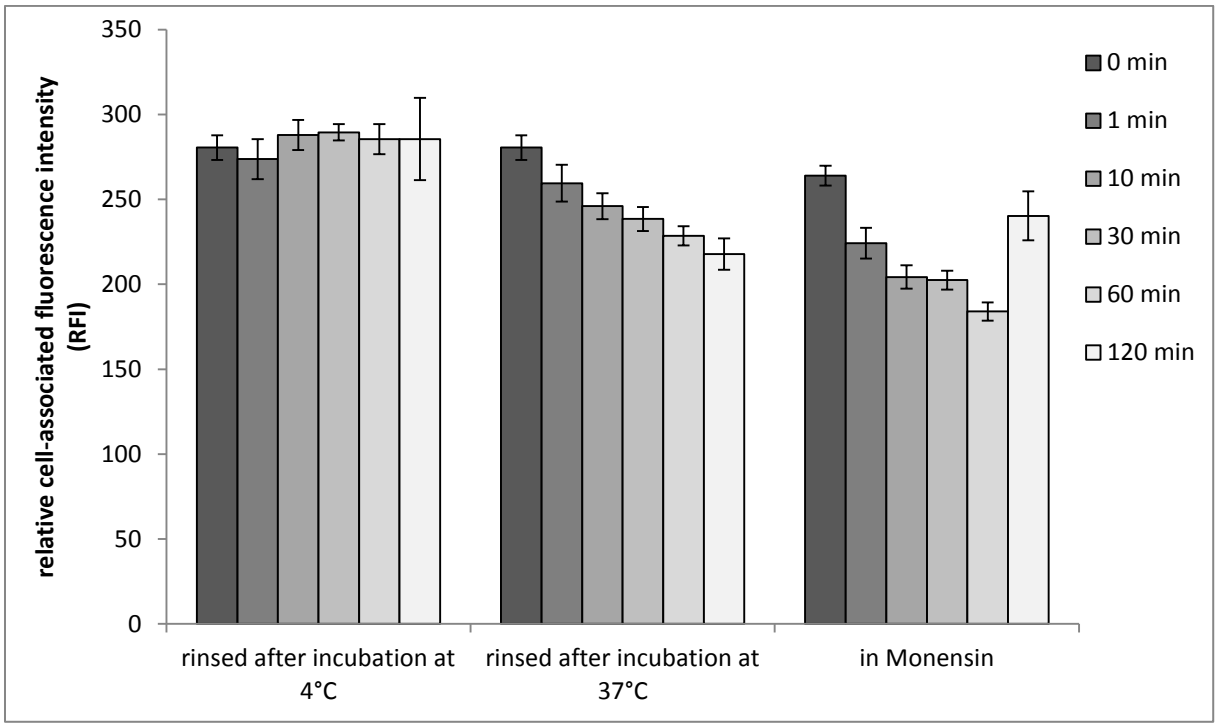


Figure 36: Analysis of the change in the fluorescence signal of viable or fixed cells treated with f-WGA in an internalization time course. **(a)** A monolayer (5637) is treated with 100 pmol/ml f-WGA and subsequently fixed with 4 % PFA. After the different duration of the incubation period at 37°C, endocytosis is visible through a quench of the intracellular f-WGA, which wears off when treated with PFA. **(b)** The target cells are incubated with 12,5 pmol/ml f-WGA, followed by Monensin treatment. Again the quench of intracellular f-WGA is visible, but dequench via Monensin did not achieve satisfying results. All values represent the mean RFI ± SEM of a triplicate.

Competitive elution of cytoadhesive WGA with GlcNAc

The elution assay was carried out by pulse/chase incubation (30 min 4°C, 60 min 37°C) with 50 µl of 12,5 pmol/ml a647-WGA on a confluent 5637 monolayer, which was subsequently rinsed three times with 150 µl PBS ($+Ca^{2+}/Mg^{2+}$) or 150 µl of 50 mM GlcNAc allowing the competitive agent to interact for 10 minutes at 4°C between each washing step. Prior to the treatment with 100 mM TCEP in 0,4 M TRIS/HCl buffer pH = 9,0, the monolayer was fixed with 4 % PFA and rinsed again with PBS ($+Ca^{2+}/Mg^{2+}$). The RFI was assessed via Tecan Infinite® 200 Reader (ex/em: 647/675 nm) before and during the quench, as well as after the dequench via additional washing steps. A control was implemented using f-WGA (ex/em: 485/525 nm), viable cells and Monensin as dequenching agent.

A substantial elution effect could be determined before quenching or after dequenching for a647-WGA (showing a decrease of the RFI of 51 %) and for f-WGA (with a slightly lower decrease of 37 %). The addition of TCEP confirmed these results, as the amount of quenchable, that is surface-adherent, a647-WGA was markedly higher for cells without GlcNAc treatment.

The dequench with Monensin for f-WGA treated cells was quite weak and not conclusive (Figure 37).

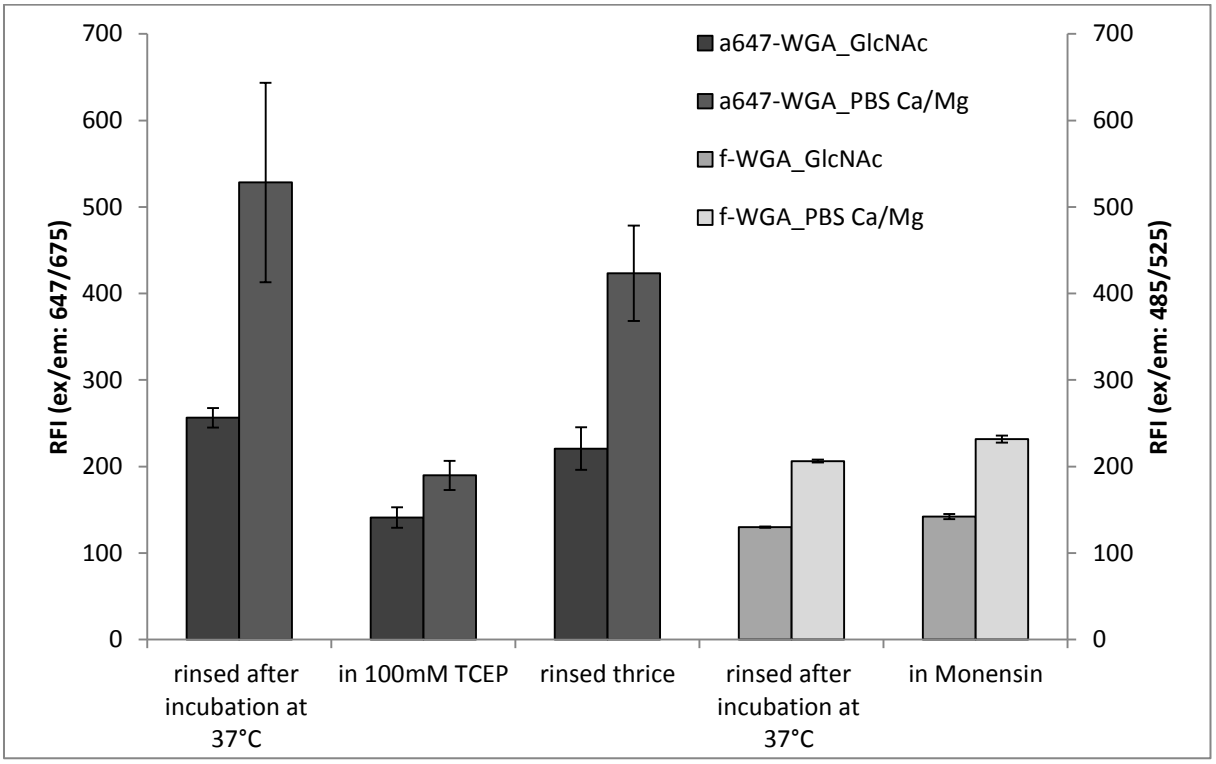


Figure 37: Applicability of the TCEP assay for the detection of surface-adherent cargo using GlcNAc or PBS ($+Ca^{2+}/Mg^{2+}$) as eluting agent. A 5637 monolayer is pulse/chase incubated with 12,5 pmol/ml a647- or f-WGA, followed by rinsing the cells either with PBS ($+Ca^{2+}/Mg^{2+}$) or a competitive carbohydrate. For both a647-WGA (*black bars*) and f-WGA (*light grey bars*) an eluting effect of surface-associated cargo by the glycan is visible in form of lower RFI values as compared to commonly treated cells. Correspondingly, a higher quenching ability of TCEP for cells rinsed with PBS ($+Ca^{2+}/Mg^{2+}$) is observed (for cells treated with GlcNAc 45% of the original signal can be quenched in comparison to a quench of 64% of the signal intensity when treated with PBS ($+Ca^{2+}/Mg^{2+}$)). Unfortunately, treatment with Monesin for f-WGA loaded cells shows no marked dequenching effect. RFI is represented by mean RFI ± SEM and the assay was operated in triplicate.

4.5. Flow cytometric detection of glycotargeted bioconjugates using the TCEP assay

Based on the promising results generated on monolayers, the applicability of the TCEP assay for single cell flow cytometric assays was evaluated. For this, confluent 5637 cells were passaged, counted and adjusted with iso HEPES/NaOH pH = 7,4 to a value between 6 and 25 million cells/ml. The initial cell concentration was chosen as required for the respective assay conditions, and with aim of compensating cell loss during sample workup (repeated centrifugation and resuspension steps). Afterwards, 50 µl of the cell-suspension were mixed thoroughly with 50 µl a647-WGA (the WGA concentration was chosen to match 12,5 pmol/ml a647-WGA for 6 million cells/ml) and incubated for 30 minutes at 4°C to allow for a surface-

adhesion between cells and the fluorescent cargo. The cell-suspension was centrifuged twice (1500 rpm, 15 min, 4°C) to remove unbound a647-WGA in the supernatant and subsequently resuspended in 150 µl iso HEPES/NaOH pH = 7,4. If intended, a further incubation at 37°C was performed to initiate endocytosis. The cells were washed again via centrifugation and were resuspended either in 200 µl iso HEPES/NaOH pH = 8,13 for direct analysis with the flow cytometer or in 150 µl iso HEPES/NaOH pH=7,4 for a following fixation. In order to prevent the cells from agglomeration, the fixation was performed by dripping 10 µl of the cell-suspension into 900 µl of 4 % PFA during constant vortexing of the solution and subsequent incubation for 20 minutes on the rotary shaker. Prior to the single cell analysis, the fixed cell-suspension was washed twice via centrifugation (5000 rpm, 5 min, 4°C) and resuspension in 0,4 M TRIS/HCl pH = 9,0 and adjusted to a volume of 200 µl with 0,4 M TRIS/HCl pH = 9,0. All parameters for the centrifugation steps were adjusted as to minimize cell loss with regard to the high amount of required washing steps.

Each sample was analyzed before the quench in its particular buffer, during quench i.e. after adding 200 µl of 100 mM or 200 mM TCEP – again in the respective buffer – and after the dequench i.e. after two more washing steps via centrifugation (5000 rpm, 5 min, 4°C)/resuspension. In every case the detection was performed with a final volume of 400 µl of the cell-suspension. If the cells also had to be permeabilized, this was performed after and in analogy to the fixation using 0,15 % Triton X-100.

Comparison of viable, fixed and fixed/permeabilized cells

In order to confirm the versatile applicability of the TCEP assay also for single cell studies, the flow cytometric assay was evaluated for viable, fixed and fixed/permeabilized 5637 cells at 4°C and 37°C. With regard to the sensitivity of mammalian cells, two different buffers were used, as well as different TCEP concentrations; 3 mM iso HEPES/NaOH pH = 8,13 and 50 mM TCEP for viable cells and 0,4 M TRIS/HCl pH = 9,0 and 100 mM TCEP for immobilized cells, respectively.

The fluorescence signals detected showed a marked quenching effect for all three differently treated types of cells with a decrease of the TCEP quenching ability for viable and fixed cells incubated at 37°C (which should result in higher amounts of intracellularly accumulated a647-WGA) (Figure 38). As expected, fixed/permeabilized cells were penetrable for TCEP and resulted in a strong quenching effect irrespective of the incubation temperature. However, viable cells seemed to be less sensitive to the dequench via further washing. These results were inhomogeneous with either no marked dequench at all or only a slight one, with a

much lower increase of the signal as compared to fixed cells. For fixed and fixed/permeabilized cells, the dequench worked well with signal intensities of 70 % to 90 % as compared to the original ones. For permeabilized cells an agitation period of 5 minutes between every washing step was applied to guarantee a maximum dequench.

In summary, the results previously established on cell monolayers could be confirmed also for single cells.

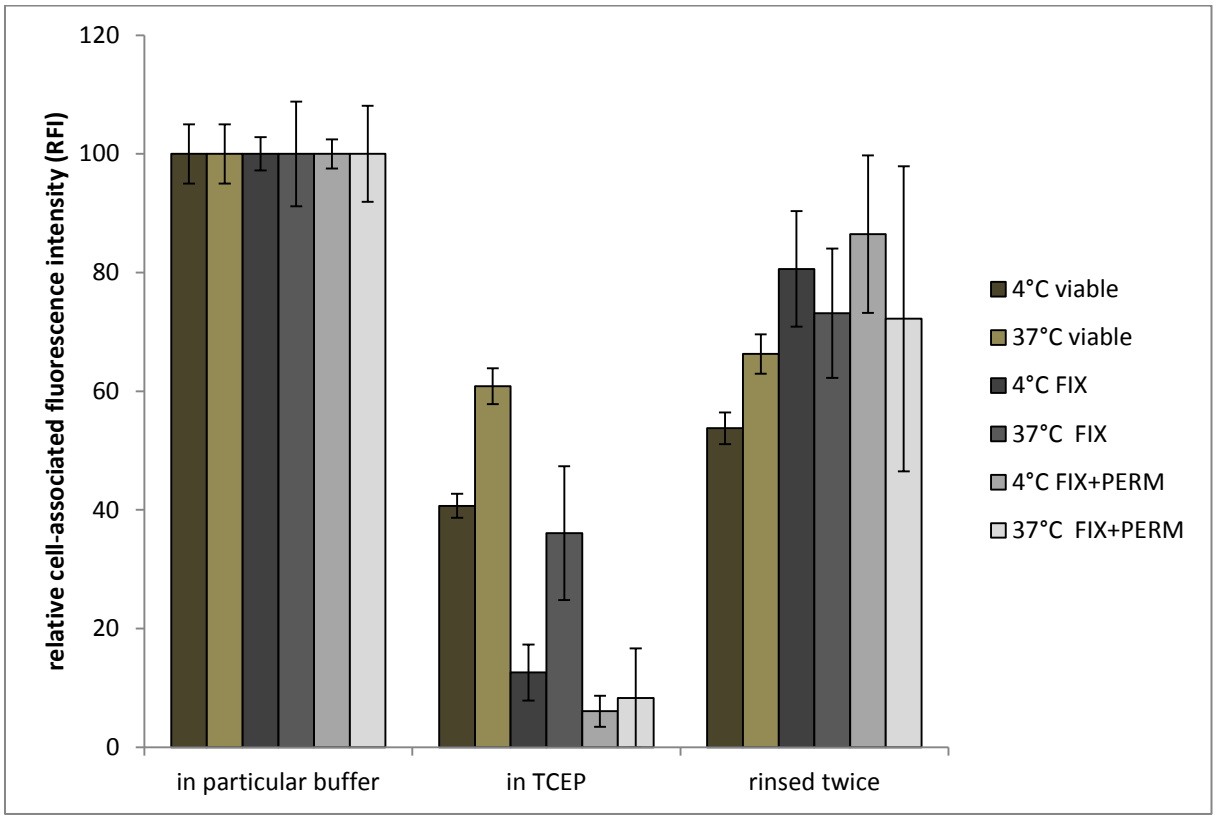


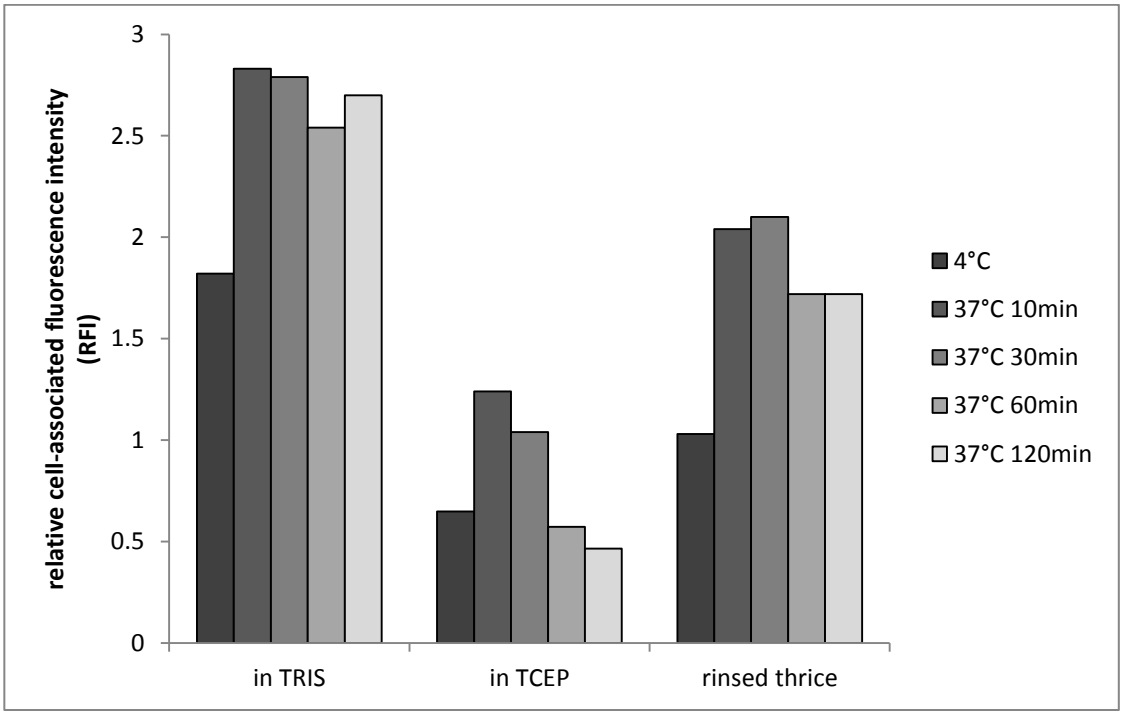
Figure 38: Comparison of the TCEP assay for viable, fixed and fixed/permeabilized cells via single cell detection. Single 5637 cells are incubated at 4°C and 37°C, analysed directly or fixed and fixed/permeabilized before quenchning. Both viable and fixed cells show a marked signal for internalized cargo, whereas fixed/permeabilized cells result in almost equal signals irrespective of WGA being outside or inside the cells. Dequench seems to be more efficient for fixed and fixed/permeabilized cells than viable ones. The values represent the mean RFI of a triplicate converted in percent, with taking the RFI directly before quench as the 100 % reference.

Internalization time course

Similar to the TCEP assay for cells in a closed tissue structure, an internalization time course was monitored with pulse/chase-incubation (30 min at 4°C, 0, 10, 30, 60 and 120 minutes at 37°C), with subsequent fixation using 4 % PFA. The quench was performed by adding 200 µl of 200 mM TCEP in 0,4 M TRIS/HCl pH = 9,0 to 200 µl of cell-suspension as described above.

Unfortunately, there was no clear difference in signals after TCEP treatment between the individual internalization periods (Figure 39). An increase of the fluorescence signal was noted only between the 0 and the 10 minutes samples, followed by a signal decrease for the 30, the 60 and the 120 minutes samples. Probably, incomplete removal (only one rinsing step) of free a647-WGA after incubation at 4°C led to additional surface loading during the 37°C chase period. Dequench of the fluorescence signal could be achieved but was far below its normal level of about 80 – 90 % of the original RFI. It should also be noted that a new TCEP charge was used and that the location of the cells in the scatter plot was shifted, which is possibly related to adverse effects of the new TCEP.

a



b

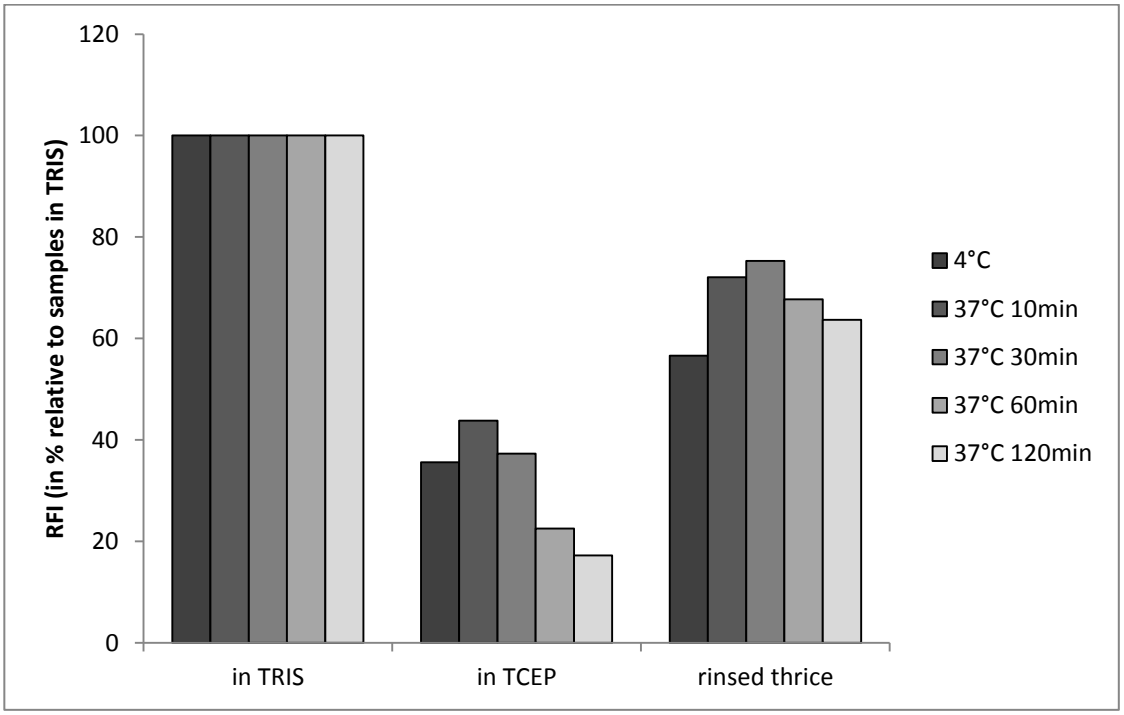


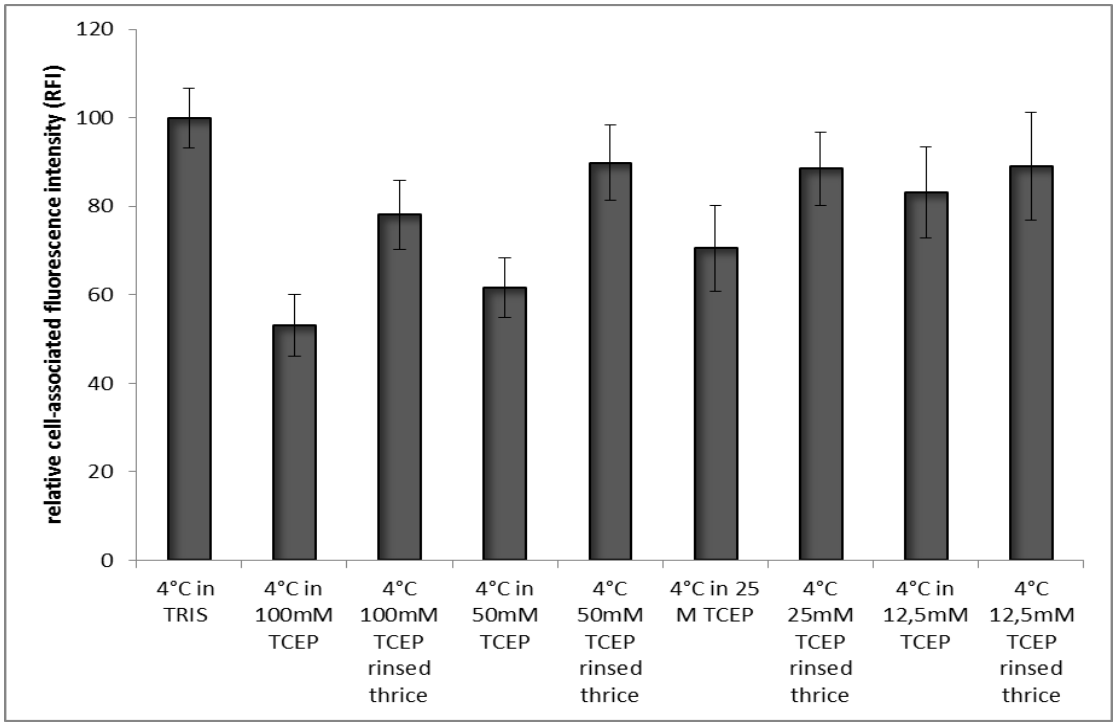
Figure 39: Internalization time course for a647-WGA in single 5637 cells detected via TCEP assay. Single 5637 cells are first incubated with a647-WGA at 4°C, followed by an incubation period at 37°C lasting for 0, 10, 30, 60 or 120 minutes. Prior to TCEP treatment the cells are fixed with 4 % PFA. An increase of the RFI during the quench is obvious between 0 and 10 minutes of incubation at 37°C, followed by a decrease from 30 to 60 minutes incubation. Therefore, the results of the time course achieved with cells in united cell structure cannot be corroborated with single cells. The values represent **(a)** the RFI or **(b)** the RFI in percent, taking the value before TCEP treatment as 100 % reference.

Evaluation of the ideal TCEP concentration

This assay was performed with incubation at 4°C or pulse/chase incubation (30 min at 4°C, 60 min at 37°C) and subsequent fixation. The final TCEP concentrations during the quench were 100 mM, 50 mM, 25 mM or 12,5 mM in 0,4 M TRIS/HCl pH = 9,0.

The signals recorded suggested that a concentration of 100 or 50 mM is to be preferred to achieve a satisfying quench and that these TCEP concentrations had no marked influence on the dequench via centrifugation/resuspension (Figure 40). Unfortunately, no difference in 4°C and 37°C samples could be found. An additional microscopic analysis of the single cells showed that some a647-WGA internalization occurred also in the 4°C samples.

a



b

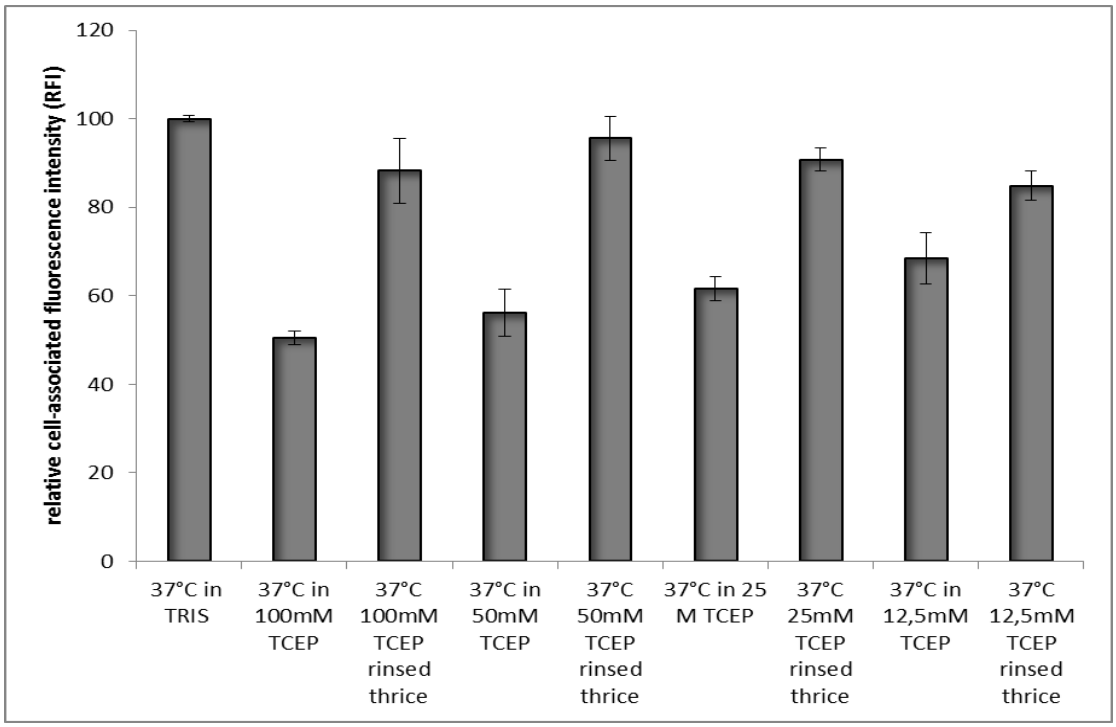


Figure 40: Evaluation of the quenching ability of various TCEP concentrations for cytoadhesive and cytoinvasive a647-WGA. 5637 single cells were incubated with a647-WGA **(a)** at 4°C or **(b)** at 4°C and 37°C and subsequently fixed with 4 % PFA. The quench is performed with 100 mM, 50 mM, 25 mM and 12,5 mM TCEP. The resulting signals suggest that a concentration of at least 50 mM should be used to achieve a pronounced quench. However, the various concentrations have no effect on the dequenching ability. Unfortunately, there is no marked difference between 4°C and 37°C samples, due to high outside temperatures during the assay performance. The values represent the mean RFI (n=2) converted in percent, with taking the RFI directly before quench as the 100 % reference.

4.6. Inhibition of endocytosis

The successful internalization of a647-WGA, suggested by the results generated so far, was to be substantiated by pharmacological endocytosis inhibition. In order to cover most of the possible uptake pathways, five different endocytosis inhibitors were evaluated as shown in Table 4.

inhibition agent	abbreviation	mechanism of action	recommended conc	stock solution
Dynasore	Dyn	inhibits GTPase activity of dynamin and with it clathrin-dependent endocytosis, but also caveolae pathways and micropinocytosis [20, 21]	80 µM	10 mg/ml in DMSO
Chlorpromazine	CPZ	inhibits clathrin mediated endocytosis [22]	10 µg/ml	1 mg/ml in aqua bidest.
Methyl-β-cyclodextrin	MβCD	inhibits cholesterol-dependent endocytic processes by reversibly extracting the steroid out of the plasma membrane [23-25]	10 mM	50 mg/ml in aqua bidest.
Nocodazole	Noco	disrupts microtubules and the mitotic spindle function and therefore arrests the cell cycle at G_2/M phase [26]	20 µM	1 mg/ml in DMSO
5-(N-Ethyl-N-isopropyl)amiloride	EIPA	inhibits macropinocytosis by lowering submembranous pH (through Na/H exchange inhibition) [27]	100 µM	10 mg/ml in MeOH

Table 4: Selected endocytosis inhibitors and their mechanism of action for further corroboration of successful internalization of lectins or lectin-coated particles using the TCEP assay.

4.6.1. Evaluation of the compatibility of the inhibitors with mammalian cells

In order to gain robust data with the TCEP assay, it is essentially required to have knowledge on putative detrimental effects of the different endocytosis inhibitors on the integrity of the cell membrane. Some endocytosis inhibitors bear the possibility of permeabilizing the cell membrane, which would lead to false conclusions concerning successful endocytosis and its inhibition. Therefore, cell viability was evaluated using Propidiumiodid (PI) as fluorescence agent and detection via flow cytometry and microscopic analysis. PI is an intercalating agent and a fluorescent molecule which is membrane-impermeant and generally excluded from viable cells. Thus, if the cells were permeabilized by an endocytosis inhibitor, the nuclear staining with PI should be visible. As a positive control,

5637 cells without contact to any inhibitor were incubated with Methanol (MeOH) for 25 minutes at -20°C to allow for fixation and complete permeabilization of the cells.

The evaluation was carried out with 100 µl of a 5637 cell-suspension, which was incubated with 100 µl of each of the inhibitors (see Table 5 for specific concentrations) for 30 minutes at RT and subsequently (without previous washing of the cells) treated with 0,4 µl of 1 mg/ml PI for another 30 minutes at RT. Controls were implemented by replacing the inhibitor with PBS ($+Ca^{2+}/Mg^{2+}$) or PBS ($+Ca^{2+}/Mg^{2+}$) with additionally 1 % DMSO and with MeOH-treated cells.

The results obtained via flow cytometry suggested a possible destruction of the cell membrane integrity by Dynasore and Chlorpromazine, whereas Nocodazole, EIPA and MβCD seemed to have no marked influence on the cell viability, irrespective of the inhibitor concentration tested (Table 5).

Microscopic analysis showed no obvious interference with cell viability upon treatment with the inhibitors, except for the cell-suspensions incubated with Dynasore and Nocodazole, which exhibited some indication of destructive potential as some apoptotic cells could be identified.

sample	**events in gate A**	**cells in gate A (in %)**	**RFI mean (PI)**
cells in PBS ($+Ca^{2+}/Mg^{2+}$)	4222	56,43	0,43
cells in PBS ($+Ca^{2+}/Mg^{2+}$) + 1% DMSO	9565	73,86	44,80
methanolized cells	3350	91,43	34,60
Dynasore 160µM	8782	68,28	35,10
Dynasore 200µM	8537	67,52	63,50
Chlorpromazine 10µg/ml	8693	73,32	32,80
Chlorpromazine 20µg/ml	7381	73,98	30,50
Chlorpromazine 30µg/ml	8348	74,53	0,73
MβCD 10mM	10697	69,26	0,76
MβCD 20mM	9206	68,10	1,89
Nocodazole 20µM	8687	70,66	0,34
Nocodazole 40µM	8816	71,20	0,34
Nocodazole 80µM	10077	73,12	0,55
EIPA 100µM	2695	58,11	1,61
EIPA 200µM	2075	51,10	4,19

Table 5: Evaluation of the influence of various inhibitors in different concentrations on cell viability, i.e. integrity of the cell membrane. Single 5637 cells are treated with various inhibitors and PI, which is only able to penetrate

damaged cell barriers. Therefore high RFI resemble cells which are destructed by endocytosis inhibitors and low RFI suggest that the cells are still viable. Negative controls are implemented by cells treated with PBS ($+Ca^{2+}/Mg^{2+}$), PBS ($+Ca^{2+}/Mg^{2+}$) and 1 % DMSO and a positive control is implemented by methanolized cells. Gate A includes all cells except smaller debris.

4.6.2. Quantitative evaluation of the inhibition of cellular uptake of a647-WGA

In order to determine the efficacy of the pharmacological endocytosis inhibitors, two different incubation methods for initiating energy-dependent uptake were investigated: the cells were either directly incubated at 37°C or via a pulse/chase incubation (with prior loading at 4°C).

In every case, a confluent 5637 monolayer was washed thoroughly and then either pretreated with 50 µl of each of the endocytosis inhibitors (see Table 6 for details) for 30 minutes at 37°C (precincubation period, as used conventionally in pharmacological inhibition assays, see [20-27]), followed by an incubation-period of 30 minutes at 37°C after adding 50 µl a647-WGA (see Table 6, as well as the following text), or analysed via a pulse/chase incubation protocol. For pulse/chase incubation, the monolayer was treated with 50 µl a647-WGA (see Table 6, as well as the following text) for 30 minutes at 4°C and subsequently rinsed three times with 150 µl PBS ($+Ca^{2+}/Mg^{2+}$). The volume/ well was adjusted to 50 µl with each of the endocytosis inhibitors (see Table 6 for details) prior to another 30 minutes` incubation-period at 4°C (precincubation period, as used conventionally in pharmacological inhibition assays, see [20-27]) followed by 60 minutes at 37°C. Irrespective of the incubation method used, the cells were rinsed with 150 µl of PBS ($+Ca^{2+}/Mg^{2+}$) after incubation at 37°C and treated with 150 µl of 4 % PFA for 20 minutes at RT to achieve fixation of the cells. Afterwards, the monolayer was rinsed again and the volume was adjusted to 100 µl/ well with 0,4 M TRIS/HCl pH = 9,0. Quenching was performed with 100 µl of 100 mM TCEP in 0,4 M TRIS/HCl pH = 9,0 and subsequent deqenching was initiated via three washing steps. The RFI was detected before and during the quench, as well as after the dequench via a Tecan Infinite® 200 Reader.

Assessment of different concentrations of each of the endocytosis inhibitors via direct incubation at 37°C

The assay was performed as described above by immediate incubation at 37°C using 100 pmol/ml a647-WGA and different concentrations of inhibitors as shown in Table 6.

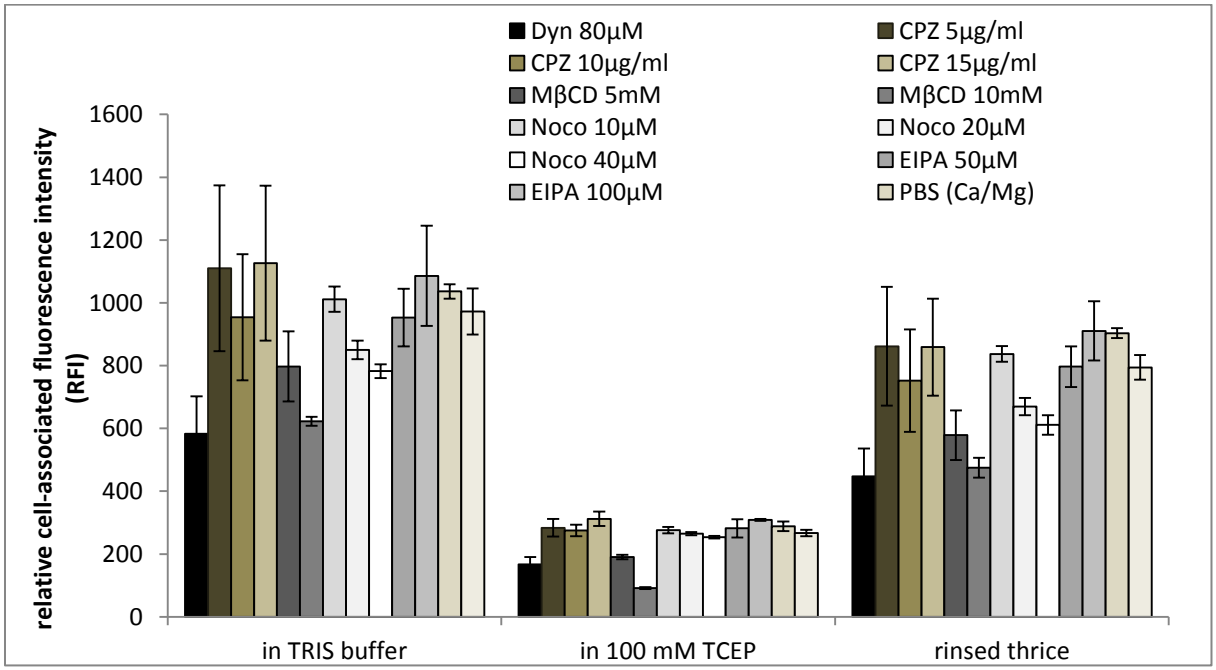
Controls were implemented using PBS (+Ca^{2+}/Mg^{2+}) at 4°C and 37°C and PBS (+Ca^{2+}/Mg^{2+}) containing the adequate amount of DMSO or MeOH.

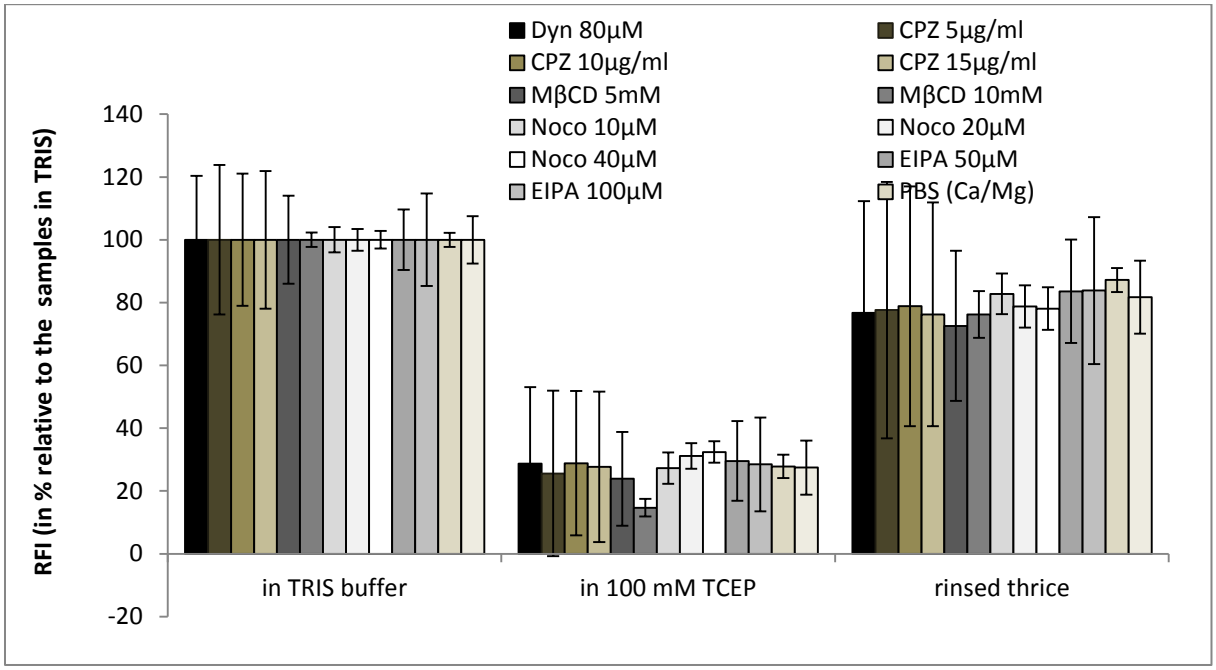
According to the quenching data determined, none of the inhibitors showed a clear effect on the endocytosis of a647-WGA (Figure 41). MβCD eventually displayed slight signs for inhibition, which could, however, also be due to cell permeabilization (an effect of MβCD on the cell membrane cannot be entirely excluded, despite promising results in the preliminary PI staining experiment; see also Table 4, *mechanism of action*). It should also be noted that the starting signals of a647-WGA differed strongly between the various inhibitors, which may be due to an interference with the specific interaction of WGA and the glycocalyx. Pulse/chase incubation could possibly reduce this effect because cell loading with a647-WGA at 4°C will take place in the absence of any inhibitor.

	immediate 37°C incubation (Figure 37a)			**immediate 37°C incubation (Figure 37b)**	**pulse/chase incubation (100pmol/ml a647-WGA)**	**pulse/chase incubation (50pmol/ml a647-WGA)**
inhibition agent	**final concentration**			**final concentration**	**final concentration**	**final concentration**
Dyn	40,0 µM			80,0 µM	100,0 µM	200,0 µM
CPZ	2,5 µg/ml	5,0 µg/ml	7,5 µg/ml	15,0 µg/ml	15,0 µg/ml	30,0 µg/ml
MβCD	2,5 mM	5,0 mM		5,0 mM	10,0 mM	20,0 mM
Noco	5,0 µM	10,0 µM	20,0 µM	20,0 µM	40,0 µM	80,0 µM
EIPA	25,0 µM	50,0 µM		100,0 µM	100,0 µM	200,0 µM

Table 6: Used concentrations of the selected endocytosis inhibitors in different assays. The results for the concentrations for the immediate 37°C incubation are shown in Figure 37. The results for the concentrations for the pulse/ chase incubation are shown in Figure 38.

a


Dyn 80µM
CPZ 5µg/ml
CPZ 10µg/ml
CPZ 15µg/ml
MβCD 5mM
MβCD 10mM
Noco 10µM
Noco 20µM
Noco 40µM
EIPA 50µM
EIPA 100µM
PBS (Ca/Mg)
relative cell-associated fluorescence intensity (RFI)
1600
1400
1200
1000
800
600
400
200
0
in TRIS buffer
in 100 mM TCEP
rinsed thrice


Dyn 80µM
CPZ 5µg/ml
CPZ 10µg/ml
CPZ 15µg/ml
MβCD 5mM
MβCD 10mM
Noco 10µM
Noco 20µM
Noco 40µM
EIPA 50µM
EIPA 100µM
PBS (Ca/Mg)
RFI (in % relative to the samples in TRIS)
140
120
100
80
60
40
20
0
-20
in TRIS buffer
in 100 mM TCEP
rinsed thrice

b

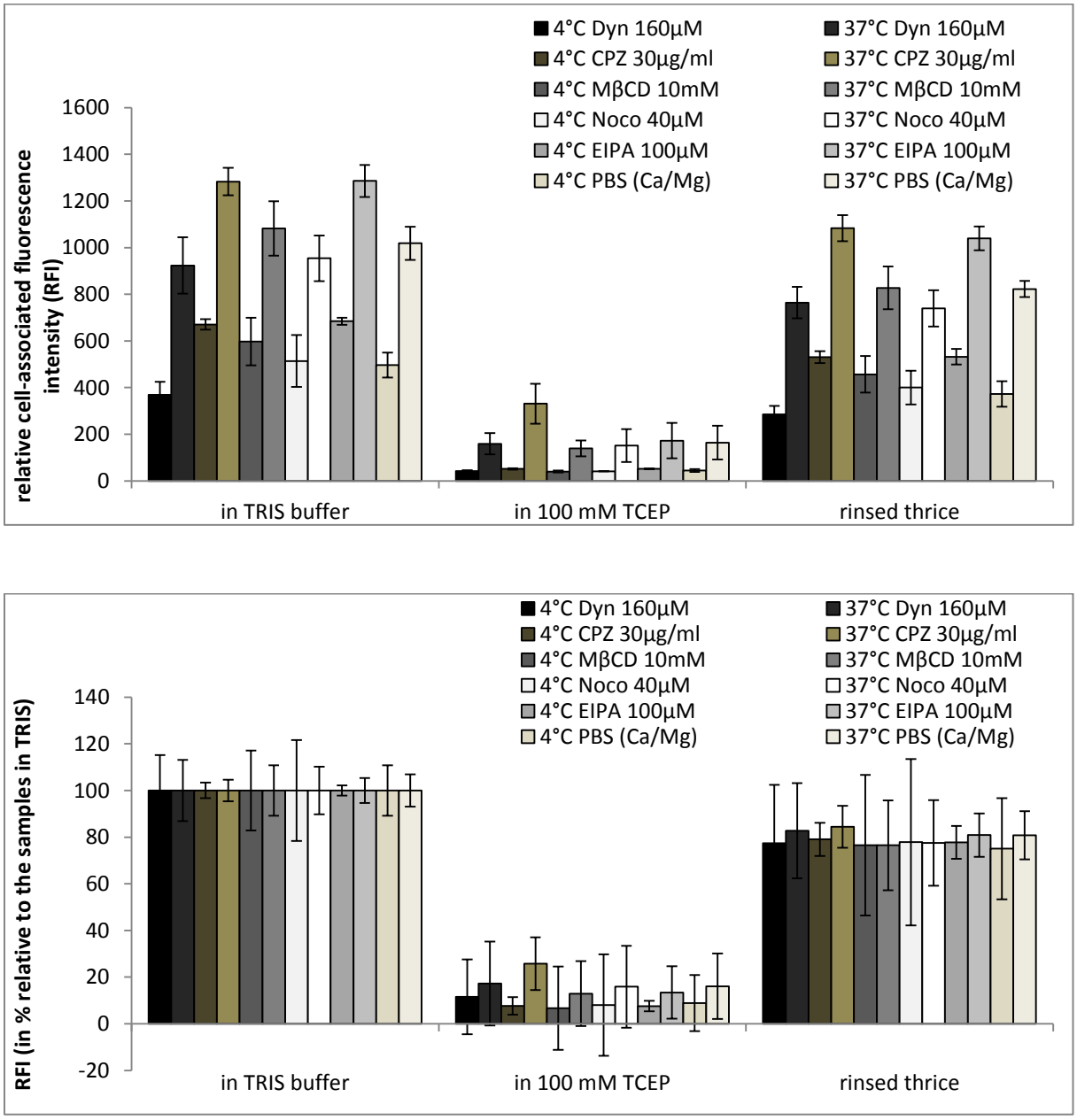


Figure 41: Evaluation of the inhibitory ability of different agents in various concentrations performed via TCEP assay. **(a)** 5637 monolayers are pretreated with the various inhibitors at 37°C and then incubated with 100 pmol/ml a647-WGA at 37°C. Eventually, MβCD seems to have a slight inhibitory effect on the internalization of WGA. **(b)** Confluent cells are treated analogous but a 4°C control for every inhibitor is implemented to allow better differentiation of the inhibitory effects. Again, only MβCD could possibly have inhibitory potential. All values represent the mean RFI ± SEM of a triplicate. Both Figure **(a)** and **(b)** are also shown in RFI (%), i.e. the mean RFI is converted in percent, with the RFI directly before quench as the 100 % reference.

Assessment of different concentrations of each of the endocytosis inhibitors via pulse/chase incubation

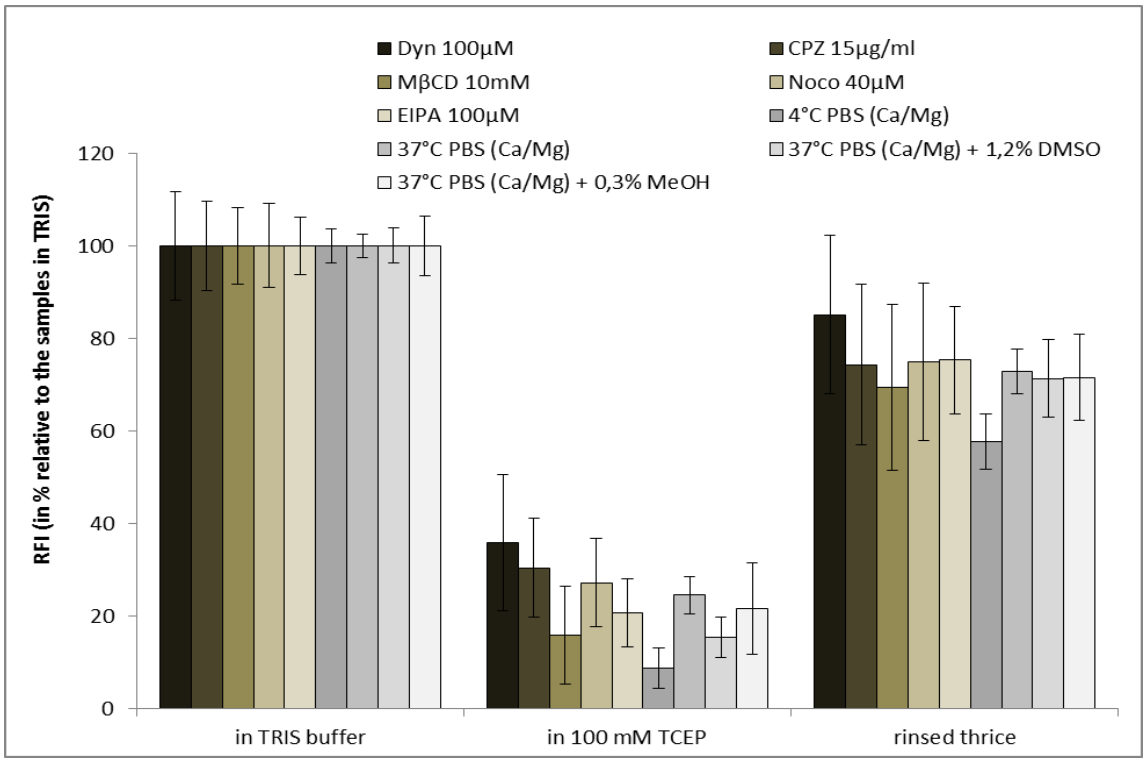
Pulse/chase incubation was performed with 100 or 50 pmol/ml a647-WGA as described above and with different concentrations of the selected inhibitors (Table 6). Again, controls

were implemented using PBS (+Ca^{2+}/Mg^{2+}) at 4°C and 37°C and PBS (+Ca^{2+}/Mg^{2+}) containing the adequate amount of DMSO or MeOH. The 37°C control with PBS (+Ca^{2+}/Mg^{2+}) was also performed with permeabilized cells to allow for an estimation of the integrity of the cell membrane after treatment with the inhibitors.

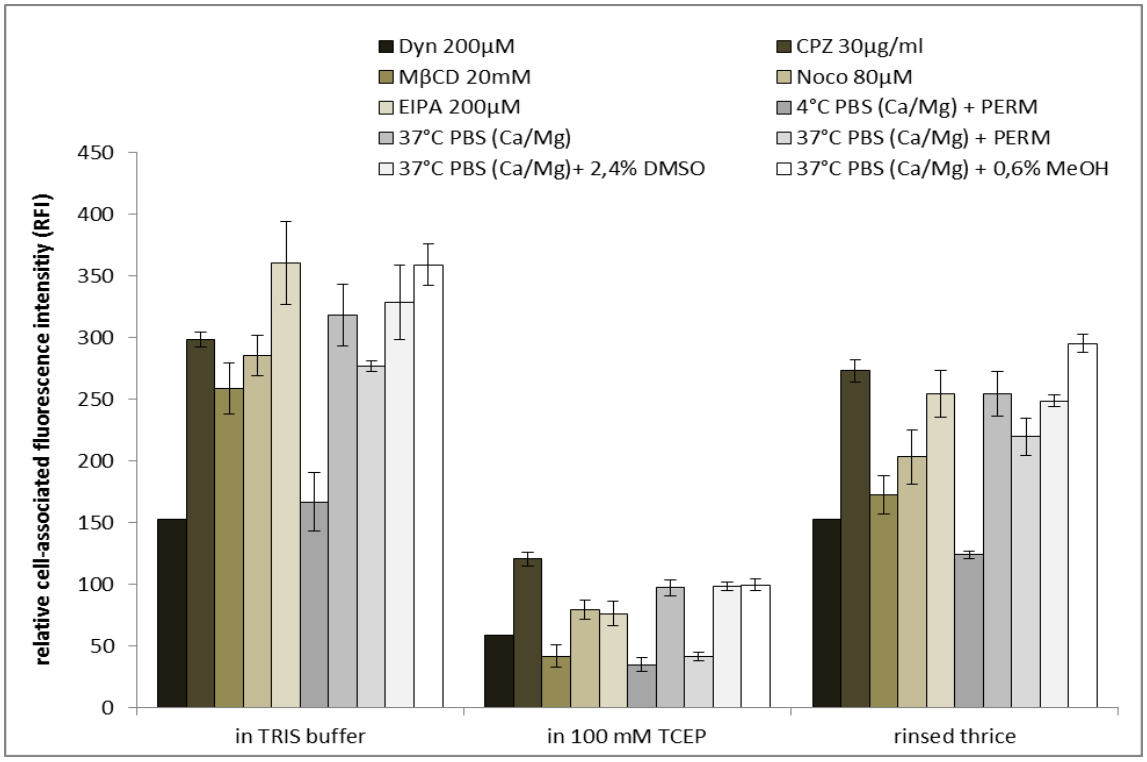
The assay using 100 pmol/ml a647-WGA showed no inhibition of cargo uptake, except for MβCD. Here, the signal intensity was between that of the 4°C and the 37°C controls (Figure 42a). A possible reason for this could be an overloading of the cell with a647-WGA, which may obscure the effect of a slight inhibition.

As a consequence, the assay was repeated with 50 pmol/ml a647-WGA, again with unclear result (Figure 42b). A further examination by microscope revealed a partially desintegrated monolayer for EIPA and MβCD.

a



b



c

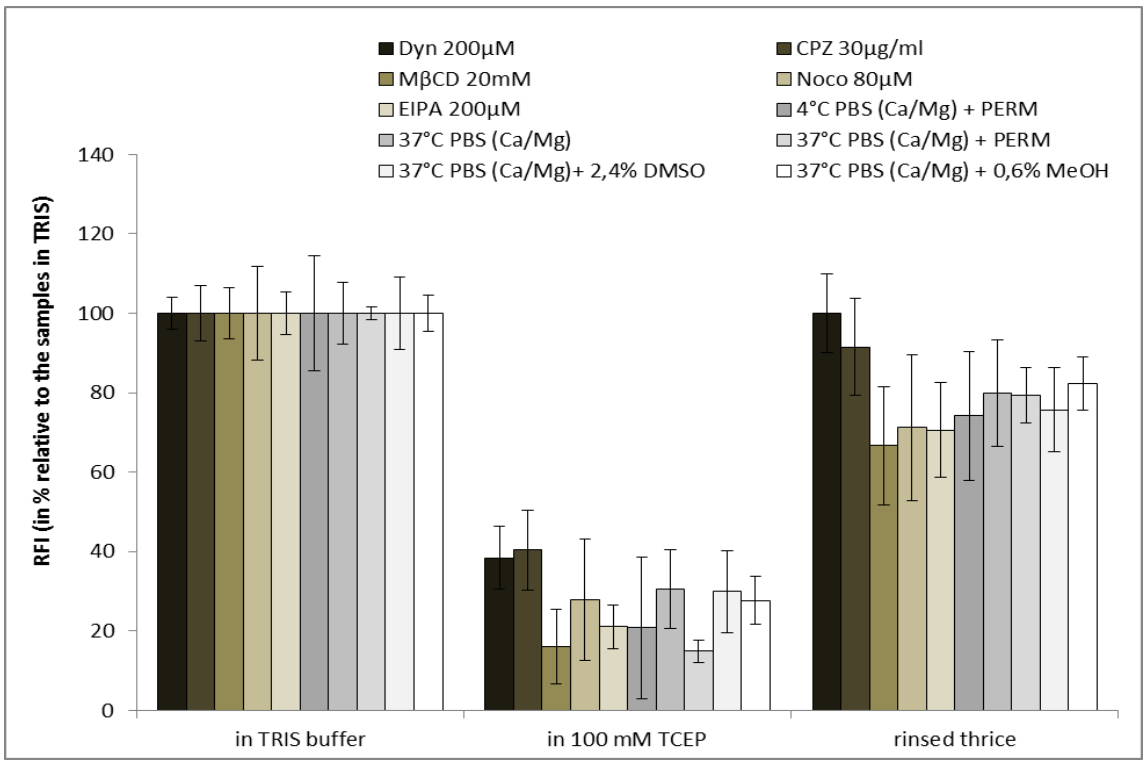


Figure 42: Evaluation of the inhibitory potential of different agents in various concentrations performed via TCEP assay. 5637 monolayers are pulse/chase incubated with **(a)** 100 pmol/ml or **(b) (c)** 50 pmol/ml a647-WGA and various endocytosis inhibitors (which are added after cell loading as described above). **(a)** Possibly due to an overload of cargo using 100 pmol/ml a647-WGA, only MβCD wells show a probable inhibition of WGA internalization. **(b) (c)** Cells loaded with 50 pmol/ml a647-WGA indicate that three agents may have inhibitory ability: EIPA, MβCD and Nocodazole. All values represent the mean RFI ± SEM of a triplicate performance. In figure **(a)** the mean RFI is represented in percent, taking the RFI before TCEP treatment as 100 % reference. In

figure **(b)** the result are presented as the recorded RFI and in figure **(c)** also represented in percent, calculated analogously to figure **(a)**.

4.6.3. Qualitative evaluation of the inhibition of cellular uptake of a647-WGA

This assay was carried out on confluent 5637 monolayers on glass coverslips that were mounted in FlexiPERM®s. Cells were loaded with 50 µl of 12,5 pmol/ml a647-WGA or a mixture of 12,5 pmol/ml a647-WGA and 12,5 pml/ml f-WGA (as control) at 4°C for 30 minutes and rinsed three times with 150 µl PBS ($+Ca^{2+}/Mg^{2+}$). To each well 50 µl of either 200 µM Dyn, 30 µg/ml CPZ, 15 mM MβCD, 80 µM Noco or 200 µM EIPA were added, cells were kept at 4°C for 30 minutes (precincubation period, as used conventionally in pharmacological inhibition assays, see [20-27]) and subsequently incubated for 60 minutes at 37°C to initialize endocytosis. After another three rinsing steps, fixation was performed with 4 % PFA for 20 minutes at RT. The monolayer was rinsed again and the volume adjusted to 100 µl of 0,4 M TRIS/HCl pH = 9,0. The fluorescence quenching was initiated by replacing the buffer with 100 µl of 100 mM TCEP in 0,4 M TRIS/HCl pH = 9,0. Dequenching was performed via washing the monolayer thoroughly with 0,4 M TRIS/HCl pH = 9,0. Fluorescence images were captured before and during the quench, as well as after dequenching of the cargo. Controls were carried out with PBS ($+Ca^{2+}/Mg^{2+}$) at 4°C and 37°C (either fixed or fixed/permeabilized) and PBS ($+Ca^{2+}/Mg^{2+}$) + 2,4 % DMSO or PBS ($+Ca^{2+}/Mg^{2+}$) + 0,6 % MeOH.

For all controls the expected results could be substantiated, i.e. 4°C experiments and fixed/permeabilized cells showed a nearly complete quench of a647-WGA, whereas intracellular WGA in cells incubated at 37°C could not be quenched, irrespective of the buffer used (see Figure 31). A widespread colocalization of a647-WGA and f-WGA was always visible, with f-WGA being unaffected by the quenching agent. Overall, the fluorescence images suggested that the endocytosis inhibitors had no effect on the internalization or on cell membrane integrity. Cells treated with Dyn or MβCD showed a little less intracellular staining, which may probably be due to an inhibitory effect of these agents (Figure 43).

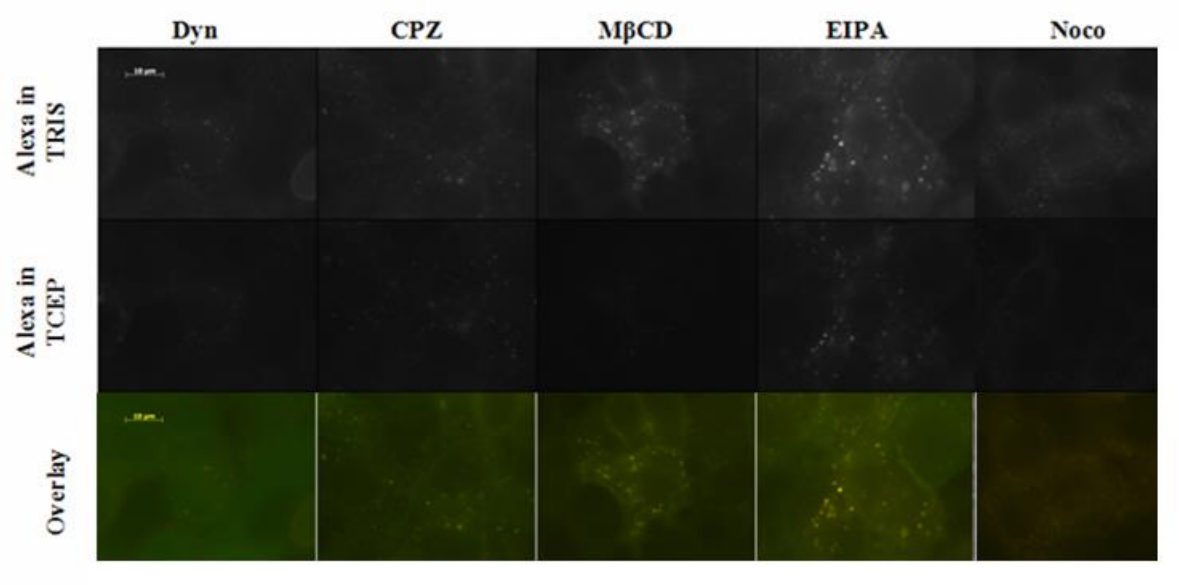


Figure 43: Microscopic analysis of the ability of endocytosis inhibition of different agents, verified through the TCEP assay. A confluent monolayer (5637) is treated with various endocytosis inhibitors at 4°C for 30 minutes, followed by pulse/chase incubation with 12,5 pmol/ml a647-WGA or a mixture of each 12,5 pmol/ml a647-WGA and f-WGA (see lower panel, i.e. overlay of the *Alexa* and *FITC* channel). Prior to TCEP treatment all cells are fixed with 4 % PFA. All cells show the typical intracellular staining pattern of a647-WGA and no obvious damage of the biological membrane irrespective of the used inhibitor. Only MβCD and Dyn may have an inhibitory effect on endocytosis because the amount of intracellular cargo seems diminished. All images were exposed to identical imaging conditions. (green channel, *FITC* 470nm, 100%, 300 ms; red channel, *Alexa*, 590nm, 100%, 1500 ms)

4.6.4. Discussion

Unfortunately, the results of the PI assay that should screen for inhibitors with permeabilizing potential showed quite different results than those acquired via microscopic analysis. An inhibitory effect could be found for 80 µM Noco and 10 – 20 mM MβCD, but a potential disturbance of cell membrane integrity could not been proven or ruled out for these two agents, although the results using PI were promising.

Summarizing, no clear conclusion of the potency of the inhibitory effect of any of the inhibitors can be drawn, nor can there be made a statement about their effect on cell viability and cell membrane integrity respectively.

4.7. Differentiation of intra- and extracellular localisation of NP

So far, it is unclear whether or not PLGA NP can be taken up by bladder urothelial cells. Hence, a robust detection method to determine internalization is necessary, which may be

found in the TCEP assay. The assay had already been evaluated using bioconjugates (see Chapter 4.4) and as such was predisposed to be tested with surface-modified NP.

4.7.1. Evaluation of the NP surface modification

PLGA NP were prepared via sonication as detailed in *6.2 Preparation of PLGA nano- to microparticles* containing Bodipy® to allow for calculation of the particle amount during experiments. For covalent surface modification, accessible carboxyl groups on the particle surface were activated by incubating 20 ml of the particle suspension (as obtained from standardized production, see *6.2 Preparation of PLGA nano- to microparticles*) with 750 µl of 240,36 mg EDAC and 500 µl of 10,28 mg NHS, both in 20 mM HEPES/NaOH pH = 7,0 with 0,1% Pluronic® F-68, for 30 min at RT. In order to remove excessive activating agent, the particles were washed twice with 80 ml of 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68 via a Vivaflow® 50 cassette. Afterwards, 20 ml of the particle suspension were treated either with 670 µl of a 1 mg/ml WGA-solution, containing $^{1}/_{10}$ a647-WGA and $^{9}/_{10}$ WGA (a647-WGA/WGA NP), or 1240 µl of a 1 mg/ml HSA-solution (HSA NP) or 1240 µl of a 1 mg/ml BSA-solution, containing $^{1}/_{10}$ a647-BSA and $^{9}/_{10}$ BSA (a647-BSA/BSA NP), for 15 hours during constant agitation. After 2 hours of saturation of non-reacted active ester groups with 6 ml of a 100 mg/ml glycine solution in 20 mM HEPES/NaOH pH = 8,0 the particles were washed twice with 40 ml of 20mM HEPES/NaOH pH=7,4 with 0,1% Pluronic® F-68 by the Vivaflow® 50 cassette.

In order to avoid any agglomeration of the NP, samples were stored in aliquots of 1 ml, kept at 4°C and sonicated for 10 minutes before usage. Additionally, the mean diameter and particle size distribution were always cross-checked by Zetasizer (Nano series-ZS, Malvern instruments, Worcestershire, UK) before usage.

For evaluation of the surface modification, NP produced as described above were diluted 1:10, 1:50 or 1:100 with 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68 and subsequently the surface-associated fluorescence label was detected in unquenched or quenched state. For this, 10 µl of each of the various particle suspensions were mixed either with 90 µl of 100 mM TCEP in 0,4 M TRIS/HCl pH = 9,0 or with 90 µl of 0,4 M TRIS/HCl pH = 9,0 and immediately analysed via Tecan Infinite® 200 Reader (Alexa: ex/ em = 647/ 675 nm; Bodipy: ex/em = 485/ 525 nm). The recorded data suggested successful particle modification as well as a quench of the a647-WGA by TCEP, which was indicative of a relatively homogenous

particle suspension without major aggregation. It should be noted that a slight decrease of the Bodipy® fluorescence signal was also visible (Figure 44).

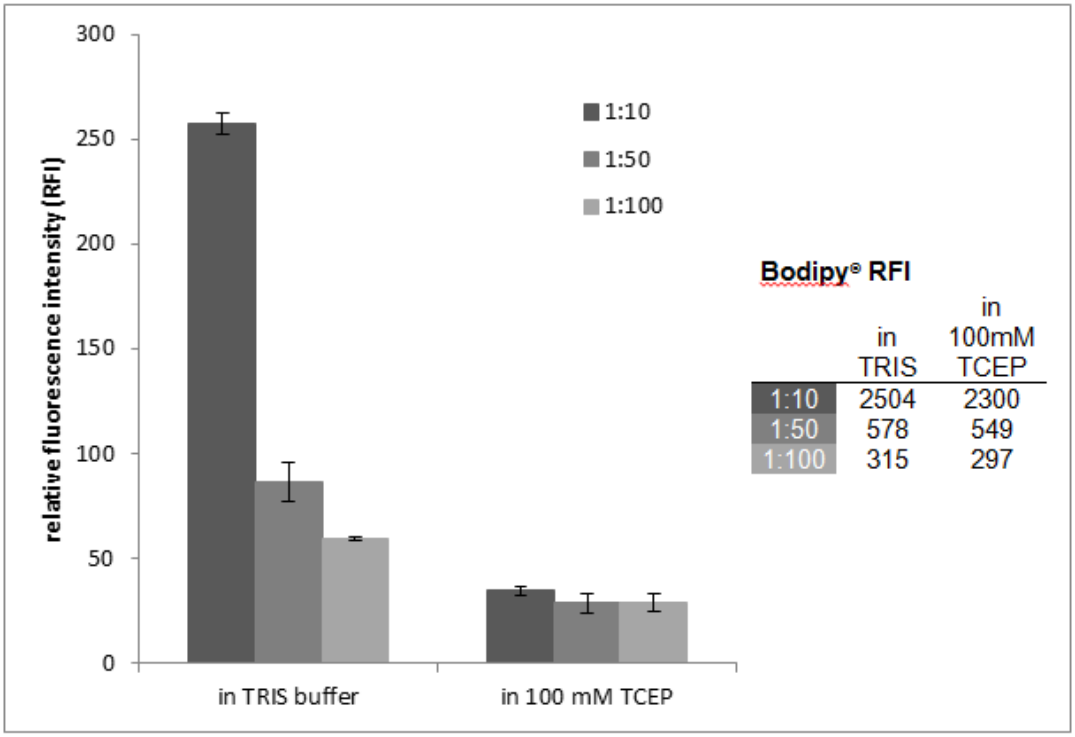


Figure 44: Confirmation of successful NP surface modification. NP with immobilized a647-WGA/WGA, are diluted 1:10, 1:50 or 1:100 with 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68 and analysed for fluorescence (ex/em: 647/675 nm) either in TRIS/HCl pH = 9,0 or in 100 mM TCEP in the same buffer. Irrespective of the previous dilution, the RFI of all quenched samples reaches nearly the same level, which indicates that the particle suspension is homogenous and no major agglomeration has occurred. In addition, the successful quench corroborates the accessibility of the fluorescence label at the particle surface. Furthermore, the Bodipy® signals verified comparable amounts of detectable particles/ well. The values represent the mean RFI ± SEM, calculated from triplicates.

In order to assess in a model system the theoretical capability of the TCEP assay to differentiate between NP inside and outside the cell, an assay was performed with NP embedded in gels. For this, a647-WGA/WGA NP and a647-BSA/BSA NP were adjusted to the same level concerning their Bodipy® fluorescence and analysed either encapsulated in the gel matrix or topped onto the surface of two different layers of gels: 1,5 % agarose gel in aqua dest or FluorSave™ Reagent (Merck Millipore, Vienna, AUT) . The wells of a 96 well microtiterplate were loaded either with a mixture of 40 µl of agarose solution and 10 µl of NP suspension or 40 µl of agarose solution only, followed by a resting time of 24 hours at RT to allow the gel to solidify. Afterwards, the wells were treated either with (i) 50 µl of 100 mM TCEP in TRIS/HCl, (ii) 50 µl of 0,4 M TRIS/HCl pH = 9,0, (iii) a mixture of 10 µl of NP suspension with 50 µl of 100 mM TCEP in TRIS/HCl, or (iv) a mixture of 10 µl NP suspension

with 50 µl of 0,4 M TRIS/HCl pH = 9,0, always resulting in a total volume of 100 µl per well. A control was implemented by mixing 10 µl of each of the NP suspensions and 90 µl of 100 mM TCEP in TRIS/HCl or 90 µl of 0,4 M TRIS/HCl pH = 9,0 as a reference. The fluorescence signals were detected via a Tecan Infinite® 200 Reader.

The recorded RFI signals showed that TCEP was not able to penetrate into the gel barrier, similar to biological membranes. This was demonstrated by higher remaining RFI signals of embedded NP in wells treated with the quenching agent. However, a difference between the two embedding agents could be observed, especially in samples where the NP solutions were only topped onto the gel surface (corresponding to extracellular cargo). NP on the 1,5 % agarose gel achieved similar quenching ratios as the control, whereas the RFI of the FluorSave™ samples could not be decreased by this amount, which may be due to an interference between components of the gel and the fluorescence label (Figure 45).

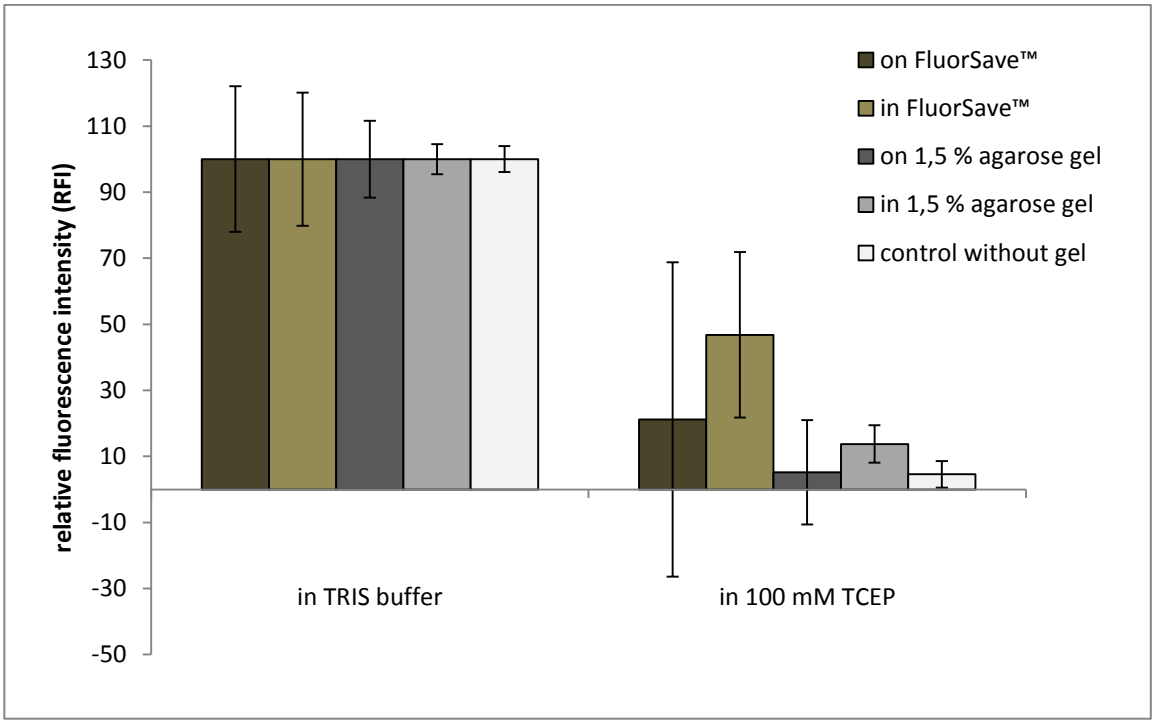


Figure 45: Demonstration of the ability of TCEP to differentiate between NP inside or outside the cells, mimicked by NP embedded either in the matrix (representing internalized NP) or on the surface (representing membrane-bound NP) of 1,5 % agarose gel and FluorSave™ respectively. A trend between the "internalized" and "membrane-bound" samples with lower RFI under quenching for the latter can be seen, corroborating the inability of TCEP to penetrate into the gel barrier. However, the RFI values of 1,5 % agarose gel are closer to the value of a control without gel, implying a possible interference of FluorSave™ and a647-WGA. The values represent the mean RFI of a triplicate performance converted in percent, with taking the RFI in TRIS/HCl buffer as 100 % reference.

4.7.2. Analysis of the NP-cell interaction via the TCEP assay

The quantitative assays were carried out on confluent 5637 monolayers, which were loaded with 50 µl of a suspension of a647-WGA/WGA NP or a647-BSA/BSA NP or HSA NP – as control – at 4°C for 30 minutes, rinsed three times with 150 µl PBS ($+Ca^{2+}/Mg^{2+}$) and subsequently incubated at 37°C to initialize endocytosis. Fixation was performed with 4 % PFA for 20 minutes at RT either directly after the 4°C incubation period or after the internalization period. The monolayer was rinsed again and the volume adjusted to 100 µl using 0,4 M TRIS/HCl pH = 9,0 per well. Quenching was performed by replacing the buffer with 100 µl of 100 mM TCEP in 0,4 M TRIS/HCl pH = 9,0, and afterwards dequenching was carried out via additional washing of the monolayer. RFI was detected via a Tecan Infinite® 200 Reader before and during the quench as well as after the dequench.

Internalization time course

In order to evaluate the ability of the cells for an intracellular uptake of NP, an internalization time course assay was performed, by incubating the cells at 37°C for 30, 60 and 120 minutes, respectively. HSA NP were treated analogously as a control.

In general, HSA NP were eluted nearly completely during the washing steps (Bodipy® signal, data not shown), in contrast to a647-WGA/WGA NP. However, the amount of remaining a647-WGA/WGA NP at or in the cells was too low to allow for a clear differentiation between cargo inside and outside the cell. It is possible that NP internalization took place beginning at 60 minutes of incubation, but no definitive statement can be given on that.

Internalization assays

Further internalization experiments suggested a possible internalization of the NP for incubation times of at least 60 minutes, which could be verified by a control with a647-WGA only (Figure 46). The NP were applied to the cells in 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68 to avoid particle agglomeration. In order to prove that this buffer has no influence on internalization, the quenching assay or the integrity of the cell membrane, two different controls with a647-WGA were implemented: cells were treated with 100 pmol/ml a647-WGA either in PBS ($+Ca^{2+}/Mg^{2+}$) as usual or in 20 mM HEPES/NaOH pH = 7 ,4 with 0,1% Pluronic® F-68. A tendency to higher values (under TCEP quenching) for 37°C samples

was visible, and probably also buffer type may have an influence on assay performance (Figure 46).

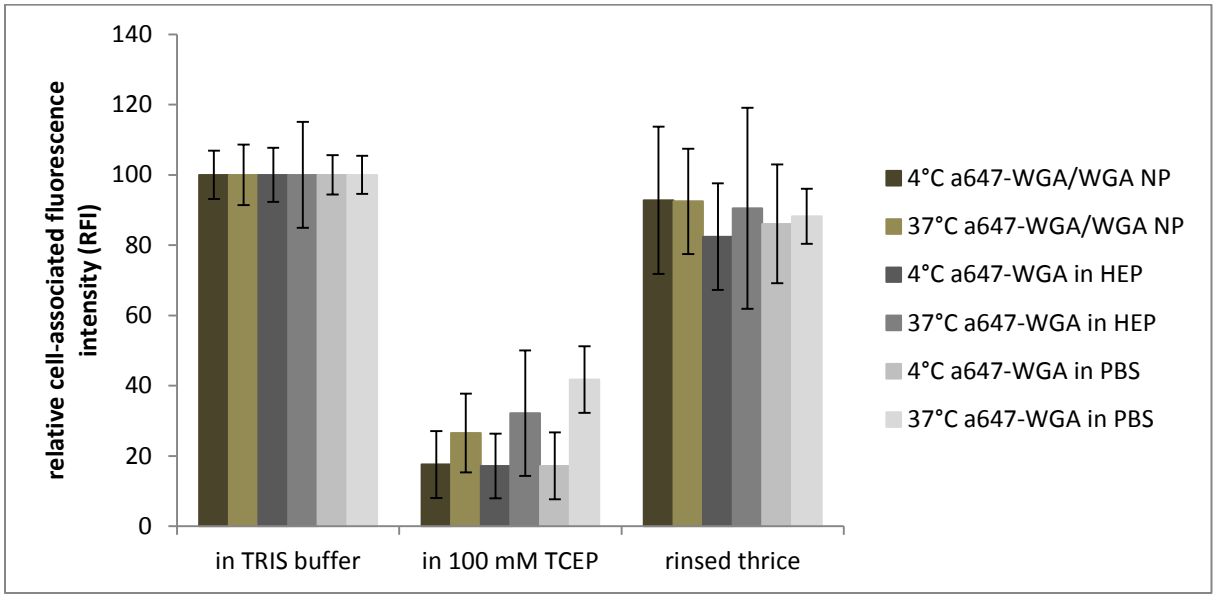


Figure 46: Internalization of a647-WGA/WGA NP and a647-WGA in PBS (+Ca^{2+}/Mg^{2+}) or in 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68 as control, detected via the TCEP assay. It is possible that cargo in PBS (+Ca^{2+}/Mg^{2+}) is internalized more easily by the cells (5637) than cargo in 20 mM HEPES/NaOH pH = 7,4 with 0,1% Pluronic® F-68. However, a difference in the RFI during quench between samples kept at 4°C and incubated at 37°C is notable for the various types of cargo. The values represent the mean RFI ± SEM in percent, relative to the value before TCEP treatment (set as 100 %).

In a further internalization assay, which was performed with particle suspensions diluted 1:2 with PBS (+Ca^{2+}/Mg^{2+}) prior to cell loading, a similar trend to particle internalization was visible (Figure 47a). However, due to the previous dilution step, the particle amount in the cells or at the cell surface was quite low, which should be considered for data interpretation. In spite of the low amount of cell-adherent NP (Bodipy® signal), a slight difference in the absorptive behaviour between a647-WGA/WGA NP and a647-BSA/BSA NP could be found (Figure 47b). Furthermore, the Bodipy® signals showed that a minimal amount of NP is eluted, especially in case of the a647-WGA NP. However, the amount eluted seemed to be consistent over different samples, which may allow for comparison. For a647-BSA/BSA NP, the obtained fluorescence values may already be close to the endogenous cell autofluorescence. Unfortunately, additional experiments under the same conditions but with freshly prepared NPs (analogous to the preparation protocol above) showed that the reproducibility of the NP internalization assay is not sufficient to allow for definite conclusions.

a **b**

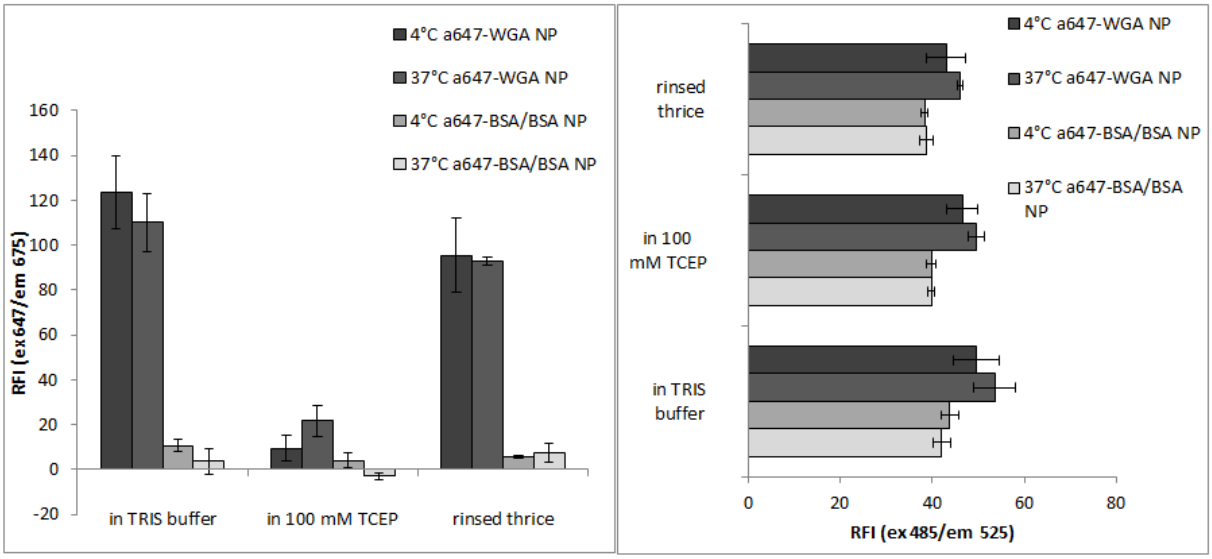


Figure 47: Internalization of a647-WGA/WGA NP and a647-BSA/BSA NP diluted 1:2 in PBS (+Ca^{2+}/Mg^{2+}) detected via the standard TCEP assay. The various NP formulations of this charge are diluted 1:2 with buffer and subsequently pulse/chase incubated with monolayers (5637) in FlexiPERM® chambers for 60 minutes at 4°C and (optionally) for another 60 minutes at 37°C. See 2.7.4 *Analysis of the NP-cell interaction via the TCEP assay* for a detailed description of the assay conditions. After fixation via 4 % PFA, a647-WGA and a647-BSA are quenched with 100 mM TCEP in TRIS/HCl buffer. **(a)** The signals, originating from a647-WGA or a647-BSA (immobilized at the NP surface, 4°C, or internalized, 37°C, respectively), would indicate a successful internalization of the NP. **(b)** However, due to the very low amount of surface-adherent or intracellular cargo (quantified via the entrapped bodipy® label, and assessed analogously to the other TCEP/NP experiments), an unambiguous statement concerning NP internalization cannot be made. The assay was performed in triplicate and the values symbolize the mean RFI ± SEM.

4.7.3. Discussion

As different charges of NP brought different results concerning a possible internalization, putative unintended differences in the particle modification should be taken under consideration. Also, free modification agent (like a647-WGA) that was not completely rinsed off in purification, might have led to false positive results. Therefore, it is still not absolutely clear whether bladder cells are able to internalize NP. However, the successful NP surface modification could be demonstrated and preliminary indication could be established for the ability of TCEP to differentiate between NP inside or outside of accessible areas (e.g. gels or cells). .

5. Conclusion

All in all, three different detection methods for the analysis of endocytosis phenomena were tested, which all showed different performance, especially regarding their applicability for the investigation of the interaction between micro- to nanoparticles and cells.

TMB assay

(i) Utilizing the interaction between Avidin and Biotin

With aim of improving and intensifying the interaction between Avidin, conjugated to HRP for further detection via the TMB assay, and Biotin, immobilized at the MP surface, a panel of MP variants with different amounts of particle-associated Biotin was evaluated. PLGA MP were either modified with b-WGA only, additionally biotinylated with BIO C5 NHS (bb-WGA) or hyperbiotinylated (bbb-WGA). Each time, the increase in the Avidin-accessible Biotin at the MP surface resulted in an improvement of the intensity of the recorded signals. Nevertheless, the overall signal level remained too low to allow for a clear disclosure of a possible endocytic uptake of the particles.

(ii) Immobilizing HRP directly at the MP surface

A successful modification of MP with HRP or HRP/WGA could be demonstrated, as well as the effect of the specific targeter WGA for stable cytoadhesion. Besides, for this variant the intensity of the signal had finally reached a level high enough for distinction of possible endocytosis. However, potential saturation phenomena or other disturbances of the enzyme reaction point to additional processes that may go on during TMB development. The mechanistic background is not yet characterized and requires further research.

TSA assay

The TSA assay is based on an enzymatic reaction between fluorescence-labelled tyramide and HRP, which becomes detectable through covalent binding of the tyramide to nearby accessible reactive groups at the cell/MP surface. In general, this detection method showed some promising characteristics when assessed for bioconjugates (hrp- and f-WGA): The tyramide dying agent evidently is not able to penetrate intact viable cell membranes, which facilitates discrimination between cytoadhesive and cytoinvasive cargo. For further

corroboration, fixed/ permeabilized cells were treated with the dye resulting in additional staining of intracellular hrp-WGA. Besides, blocking of the enzyme HRP with H_2O_2 demonstrated the specificity of the staining reaction.

Nevertheless, the method turned out to be limited in certain aspects, which exclude it as a reliable detection method for endocytosis in general. The TSA assay could be applied for a qualitative detection of bioconjugates. Successful detection of modified MP (HRP MP) could not be achieved, neither qualitatively nor quantitatively.

TCEP assay

The recently discovered quenching ability of TCEP for several fluorescent molecules turned out to be the most promising detection method assessed in this work. The assay could be applied and optimized for different detection devices, and for generating both qualitative and quantitative results (fluorescence microscopy, fluorimetry via Tecan Infinite® 200 Reader and flow cytometry). Moreover, applicability for bioconjugates (a647-WGA) as well as NP could be demonstrated. The discrimination between cytoadhesive and cytoinvasive cargo could be achieved due to the impenetrability of the cell membrane for TCEP, which was verified in control assays via cell permeabilization.

Because of difficulties in the surface modification of NP, a clear prove of successful internalization of particles in 5637 BCa cells could not be established. Nevertheless, the desired goal of establishing a simple and robust method to discriminate between cargo inside and outside the cells could be met to a certain extent.

6. Materials and methods

6.1. Chemicals and cell line

WGA, b-WGA, hrp-WGA, f-WGA, tr-Avidin and hrp-Avidin were supplied by Vector laboratories. a647-WGA, a647-BSA, HRP, HSA, BIO C5 NHS, TCEP, PFA and Pluronic® F-68 as well as all endocytosis inhibitors (Noco, MβCD, Dynasore, EIPA and CPZ) were obtained from Sigma-Aldrich®(St. Louis, USA). BSA was supplied by PAA Laboratories (Pasching, AUT) and the tyramide dye (including the solutions for dilution) by Invitrogen™ (Carlsbad, USA). Ethylacetate, H_2O_2, HEPES and H_2SO_4 were obtained from Roth® (Karlsruhe, DE) and DMSO from Fluka (St. Louis, USA). TMB was supplied by Sciotec® (Vienna, AUT) in form of a proprietary standard solution. For application in assays the TMB solution was diluted in PBS ($+Ca^{2+}/Mg^{2+}$) with 0,0015% hydrogen peroxide at a particular ratio, described in the various sections.

The 5637 cells, an urothelial carcinoma cell line, and the urinary bladder cell line Sv-Huc, isolated from a donor without history of malignant transformation, were obtained from ATCC (Middlesex, UK).

6.2. Preparation of PLGA nano- to microparticles

In order to prepare both, PLGA NP and MP a solvent evaporation protocol was carried out. For preparation of MP, 200 mg of poly(lactide-co-glycolide) (PLGA) were dissolved in a mixture of 1,215 ml ethylacetate (EtOAc) and 125 µl of a Bodipy® solution (1 mg/ml Bodipy® (493/503) dye in EtOAc) and cooled to 4 °C. This organic PLGA-phase was dispersed in 4 ml of an aqueous phase (aqua dest. + 1 % of Pluronic® F-68, acting as stabilizer) via 40 s of sonication (Bandelin, Sonopuls HD 70) at 20 % output power. Immediately this emulsion was diluted with 50 ml of an aqueous solution (aqua dest. + 0,25 % Pluronic® F-68). In order to evaporate the organic solvent, the emulsion was magnetically stirred for 1 hour and subsequently kept under reduced pressure for another hour on a rotary evaporator. Finally, the produced MP were washed thrice via centrifugation (4000 rpm, 10 minutes, 20°C) and resuspension in 20 mM HEPES/NaOH pH = 7,4 + 0,1% Pluronic® F-68 to remove free polymer and non-encapsulated components.

NP were prepared by dissolving 400 mg of PLGA in a mixture of 2 g EtOAc and 125 µl of the Bodipy® solution (as above). After cooling the solution to 4 °C, it was dispersed in 6 g of aqua dest. + 3 % of Pluronic® F-68 via 40 s of sonication at 40 % output power. The resultant emulsion was diluted with 100 g of aqua dest. + 0,1 % Pluronic® F-68 and the organic solvent was subsequently evaporated using the same procedure as for MP. Finally, the NP suspension was filtered (1 µm pore size) and washed twice with 40 ml of 20 mM HEPES/NaOH pH = 7,0 + 0,1% Pluronic® F-68 via a Vivaflow® 50 cassette (Sartorius Stedim Biotech).

All particles were stored in aliquots of 1 ml either at 4°C for immediate use or -80°C for long-term storage.

6.3. Cell culture

All cells were cultivated in adherent culture in a humidified air atmosphere containing 5 % CO_2 at 37 °C in either RPMI (5637) or HAMS (Sv-Huc) medium. RPMI medium was supplied by life technologies and completed by addition of 10 % fetal bovine serum (FBS), 4 mM L-glutamine and 150 µg/ml gentamycin. HAMS-F12 medium was supplied by life technologies and 1000 ml thereof were further supplemented with 100 ml FCS, 10 ml Pen/Strep and 0,146 g L-glutamine. Adherent cultures were grown in 75 cm^2 tissue culture flasks and passaged with trypsin/EDTA. For the various assays 17000 cells/ well were seeded in 96-well microtiterplates or in flexiPERM® Micro12 chambers (Prod. No. 90011436, GreinerBioOne, Vienna, Austria). The resultant monolayers were used at 100 % confluency when grown in microtiterplates or at about 80 % confluency when cultured in flexiPERM® chambers.

6.4. Adjusting of PLGA MP/ NP concentrations

In order to allow for direct comparison between differently modified NP/MP, the amount of detectable NP (via TMB/TSA or TCEP) particles per well – either free or cytoadherent – had to be adjusted to the same level. All particles were thus prepared via entrapment of Bodipy®, a fluorescence dye which allows for estimation of the particle concentration. For particle quantification, 100 µl per well of the various particle suspensions, previously homogenized via vortexing (and sonication in case of NP), were settled in a 96 well microtiterplate and the RFI (ex/em: 485/525) was detected via a Tecan Infinite® 200 Reader. Subsequently the suspensions were diluted accordingly to attain a specified RFI level. 20 mM

HEPES/NaOH pH = 7,4 + 0,1% Pluronic® F-68 was used for this dilution step. Again, 100 µl of the now diluted particle suspensions were analysed to confirm successful adjustment of the particle amounts. This adjustment was performed in triplicate.

List of figures

List of tables

List of abbreviations

substance	abbreviation
Alexa Fluor® 647-labelled bovine serum albumin	a647-BSA
Alexa Fluor® 647-labelled wheat germ agglutinin	a647-WGA
Alexa Fluor® 594-labelled wheat germ agglutinin	a594-WGA
bovine serum albumin	BSA
Biotinamidocaproate-N-hydroxysuccinimideester	BIO C5 NHS
Biotin-labelled wheat germ agglutinin	b-WGA
Chlorpromazine	CPZ
dimethylsulfoxid	DMSO
Dynasore	Dyn
1-Ethyl-3-(3-dimethylaminopropyl)carbodiimid	EDAC
Ethylendiamin-tetraacetate	EDTA
5-(N-Ethyl-N-isopropyl)amiloride	EIPA
ethylacetate	EtOAc
Fluorescein-labelled wheat germ agglutinin	f-WGA
glutaraldehyde	GlutAld
N-acetyl-D-glucosamine	GlcNAc
hydrochloric acid	HCl
hydrogen peroxide	H_2O_2
sulphuric acid	H_2SO_4
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid	HEPES
horseradish peroxidase	HRP
horseradish peroxidase-labelled Avidin	hrp-Avidin
horseradish peroxidase-labelled wheat germ agglutinin	hrp-WGA
human serum albumin	HSA
Methyl-β-cyclodextrin	MβCD
Methanol	MeOH
natriumhydroxid	NaOH
N-hydroxysuccinimide	NHS
Nocodazole	Noco
phospate saline buffer	PBS
paraformaldehyd	PFA
poly(lactide-co-glycolide)	PLGA
Tris-2-carboxyethylphosphine hydrochloride	TCEP
3,3’,5,5’-Tetramethylbenzidine	TMB
Tris(hydroxymethyl)-aminomethan	TRIS
wheat germ agglutinin	WGA

List of references

1. Kaufman, D.S., W.U. Shipley, and A.S. Feldman, *Bladder cancer.* The Lancet, 2009. **374**(9685): p. 239 - 249.
2. Colgan, R. and M. Williams, *Diagnosis and Treatment of Acute Uncomplicated Cystitis.* American Family Physician, 2011. **84**(7): p. 771-776.
3. Salvatore, S., et al., *Urinary tract infections in women.* European Journal of Obstetrics & Gynecology and Reproductive Biology, 2011. **156**(2): p. 131-136.
4. van Rhijn, B.W., et al., *Recurrence and progression of disease in non-muscle-invasive bladder cancer: from epidemiology to treatment strategy.* Eur Urol, 2009. **56**(3): p. 430S-442.
5. Neutsch, L., et al., *Lectin bioconjugates trigger urothelial cytoinvasion – A glycotargeted approach for improved intravesical drug delivery.* Eur J Pharm Biopharm, 2012. **82**: p. 367–375.
6. Neutsch, L., et al., *Synergistic targeting/prodrug strategies for intravesical drug delivery — Lectin-modified PLGA microparticles enhance cytotoxicity of stearoyl gemcitabine by contact-dependent transfer.* J Control Release, 2013(169): p. 62-72.
7. Neutsch, L., et al., *UPEC biomimickry at the urothelial barrier: Lectin-functionalized PLGA microparticles for improved intravesical chemotherapy.* Int J Pharm, 2013. **450**: p. 163–176.
8. Pietzonka, P., et al., *Transfer of lipophilic markers from PLGA and polystyrene nanoparticles to caco-2 monolayers mimics particle uptake.* Pharm Res, 2002. **19**(5): p. 595-601.
9. Xu, P., et al., *Intracellular drug delivery by poly(lactic-co-glycolic acid) nanoparticles, revisited.* Mol Pharm, 2009. **6**(1): p. 190-201.
10. Kreft, M.E., et al., *Apical plasma membrane traffic in superficial cells of bladder urothelium.* Ann N Y Acad Sci, 2009. **1152**: p. 18-29.
11. Kreft, M.E., et al., *Endocytotic activity of bladder superficial urothelial cells is inversely related to their differentiation stage.* Differentiation, 2009. **77**(1): p. 48-59.
12. Dhakal, B.K., R.R. Kulesus, and M.A. Mulvey, *Mechanisms and consequences of bladder cell invasion by uropathogenic Escherichia coli.* Eur J Clin Invest, 2008. **38**: p. 2-11.
13. Martinez, J.J., et al., *Type 1 pilus-mediated bacterial invasion of bladder epithelial cells.* EMBO J, 2000. **19**(12): p. 2803-12.
14. Green, N.M., *Avidin. 3. The Nature of the Biotin-Binding Site.* Biochem J, 1963. **89**: p. 599-609.
15. Zhu, Y.C., J.J. Adamczyk, and S. West, *Avidin, a potential biopesticide and synergist to Bacillus thuringiensis toxins against field crop insects.* Econ Entomol, 2005. **98**(5): p. 1566 - 1571.
16. Kohanski, R.A. and M.D. Lane, *Monovalent avidin affinity columns.* Methods Enzymol, 1990. **184**: p. 194-200.
17. Invitrogen, *Tyramide Signal Amplification (TSA) Technology*, 2002. p. 152-159.
18. Invitrogen, *Tyramide Signal Amplification Kits*, 2011.
19. Vaughan, J.C., et al., *Phosphine quenching of cyanine dyes as a versatile tool for fluorescence microscopy.* J Am Chem Soc, 2013. **135**(4): p. 1197-200.
20. Macia, E., et al., *Dynasore, a cell-permeable inhibitor of dynamin.* Dev Cell, 2006. **10**(6): p. 839-50.
21. McNiven, M.A., et al., *The dynamin family of mechanoenzymes: pinching in new places.* Trends Biochem Sci, 2000. **25**(3): p. 115-20.

22. Khandelwal, P., W.G. Ruiz, and G. Apodaca, *Compensatory endocytosis in bladder umbrella cells occurs through an integrin-regulated and RhoA- and dynamin-dependent pathway.* EMBO J, 2010. **29**(12): p. 1961-75.
23. Grimmer, S., B. van Deurs, and K. Sandvig, *Membrane ruffling and macropinocytosis in A431 cells require cholesterol.* J Cell Sci, 2002. **115**(Pt 14): p. 2953-62.
24. Simons, K. and D. Toomre, *Lipid rafts and signal transduction.* Nat Rev Mol Cell Biol, 2000. **1**(1): p. 31-9.
25. Subtil, A., et al., *Acute cholesterol depletion inhibits clathrin-coated pit budding.* Proc Natl Acad Sci U S A, 1999. **96**(12): p. 6775-80.
26. dos Santos, T., et al., *Effects of transport inhibitors on the cellular uptake of carboxylated polystyrene nanoparticles in different cell lines.* PLoS One, 2011. **6**(9): p. e24438.
27. West, M.A., M.S. Bretscher, and C. Watts, *Distinct endocytotic pathways in epidermal growth factor-stimulated human carcinoma A431 cells.* J Cell Biol, 1989. **109**(6 Pt 1): p. 2731-9.

LEBENSLAUF

Bildungsweg

1994 - 1998 Volksschule Bischof Faber Platz in Wien

1998 - 2006 Neusprachliches Gymnasium Wien (mit ausgezeichnetem Erfolg maturiert)

Seit 2006 Studium der Pharmazie an der Universität Wien

Berufliche Tätigkeiten

2014 (SS) – Praktikum - TU Wien, Institut für Verfahrenstechnik
- Tätigkeitsbereich: DoE zur Optimierung der Produktion von Carotinoiden mittels halophiler Archaee

2013 (WS) – Tutorium – Industrielle Arzneimittelherstellung

2013 (SS) – Tutorium – Grundpraktikum aus pharmazeutischer Technologie

2011 bis 2013 (WS/SS/WS) – Tutorium – Identitäts- und Reinheitsprüfung biogener Arzneimittel (inklusive Arzneibuchanalytik)

2011 – Ferialpraktikum – Movianto Österreich, Geschäftsbereich der Sanova Pharma GesmbH
- Tätigkeitsbereich: Kommissionierung

2011 (SS) – Tutorium – Morphologie, Anatomie und Systematik arzneistofflieferner Organismen

2010 – Ferialpraktikum – ChEck iT! Verein Wiener Sozialprojekte
- Tätigkeitsbereich: HPLC – Analysen von Drogen und Methodenoptimierung

2008 – Ferialpraktikum - PFIZER Corporation Austria
- Tätigkeitsbereich: Literaturrecherche (PubMed) und Datenbankpflege

2007 – Ferialpraktikum - RAUCH Mühle Innsbruck (http://www.rauchmehl.at/)
- Tätigkeitsbereich: Qualitätsprüfung und Qualitätssicherung im Labor

2005 – Ferialpraktikum – SOWI Apotheke Innsbruck
- Tätigkeitsbereich: Rezepturenherstellung und Warenlogistik

2004 – Ferialpraktikum – SOWI Apotheke Innsbruck
- Tätigkeitsbereich: Rezepturenherstellung und Warenlogistik