DIPLOMARBEIT

Titel der Diplomarbeit

„Influence of Covalently Conjugated Monodisperse Polyethylene Glycol Chains on in Vitro Properties of siRNA Oligonucleotides"

Verfasserin

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Abstract

RNA interference (RNAi) is a recently discovered technique of gene silencing. It is based on a specific interference of a RNA sequence of approximately 21 nucleotides, called short interfering RNA (siRNA), which is complementary to a messenger RNA (mRNA) of interest. After hybridisation, the target mRNA is degraded and its expression inhibited.

The delivery of siRNA into cells has been a great challenge. While various modifications of the siRNA sequence and several delivery systems have been examined, so far none of them could fully meet the requirements for safe and efficient therapeutic application.

During this diploma thesis, I devised a synthetic procedure for the preparation of PEG-siRNA oligonucleotides and investigated the effect on their uptake and down-regulation of the targeted Bcl-2 gene in mammalian cells.

At first, anti-Bcl-2 siRNAs were synthesised and purified. Subsequently PEG chains of defined length were attached at the 3'- and 5'-ends of sense and antisense strands of the siRNA oligonucleotides. Conjugation of PEG at the 5'-OH group was carried out with a PEG-phosphoramidite building block added at the end of the RNA synthesis, but resulted in low yields. PEG chains were attached at the 3'-end post-synthetically by reaction of a NHS-ester activated polyethylene glycol to an aminohexyl modified oligonucleotide. The conjugation reaction showed approximately 75% conjugation yields.

The molecular weight of siRNA and its conjugates was analysed with ESI-MS. PAGE enabled analysis of purity and efficiency of the conjugation reaction. Quantification of the conjugation reaction and purification of the components was carried out with HPLC.

The influence of the PEG conjugates on the knockdown of the corresponding Bcl-2 mRNA was examined in a mammalian cell culture model (MCF-7 cells) by making use of a Dual-Luciferase Reporter Assay and by quantification of endogenous mRNA levels by RT-qPCR. After Lipofectamine[TM]2000 transfection, PEGylated siRNA resulted in strong knockdown, indicating no loss of activity through the 3'-end conjugation of both strands. Naked application showed no significant down-regulation of the target gene.

Although the polyethylene glycol chains could not effectively enhance unassisted siRNA uptake into mammalian cells, my work demonstrates the tolerance of the molecular effectors for 3'-PEGylation. These conjugates can be used for lipid and polymeric delivery

systems, and this and similar modifications are viable strategies for increasing the bioavailability of oligonucleotides, prolonging circulation times and increasing stability.

Zusammenfassung

RNA-Interferenz (RNAi) ist eine kürzlich entdeckte Technik des Gen-Silencing. Dieses Verfahren basiert auf einer spezifischen Interferenz einer RNA-Sequenz von ungefähr 21 Nukleotiden, auch short interfering RNA (siRNA) genannt. Diese sind komplementär in der Sequenz zur entsprechenden Boten-RNA (mRNA). Die Erkennung fördert den Abbau und hemmt die Expression der Ziel-Boten-RNA.

Die effiziente Zellaufnahme von siRNA ist zurzeit eine große Herausforderung. Während verschiedene Modifikationen der siRNA-Sequenz und mehrere Formulierungen untersucht worden sind, konnte bisher keine von ihnen die gewünschten Anforderungen vollständig erfüllen und ausreichende Sicherheit und Effizienz für eine therapeutische Anwendung gewährleisten.

Während dieser Diplomarbeit habe ich die Synthese und Darstellung von PEG-siRNA Konjugaten gestaltet und ihre Wirkung in Bezug auf die Aufnahme und Reduktion des Bcl-2 Gens in Säugerzellen untersucht.

Im Rahmen der Diplomarbeit wurden zunächst anti-Bcl-2 siRNA synthetisiert und gereinigt. Anschließend wurden PEG-Ketten definierter Länge an die 3'-und 5'-Enden der Sense- und Antisense-Stränge der siRNA-Oligonukleotide angehängt. Eine Modifikation mit PEG an der 5'-OH-Gruppe wurde mithilfe eines PEG-Phosphoramidit-Bausteins am Ende der RNA-Synthese versucht, was jedoch nur zu geringen Ausbeuten des Konjugats führte. PEG-Ketten wurden an das 3'-Ende der siRNA nach der Reaktion eines Aminohexyl-Linkers mit NHS-Ester aktiviertem PEG eingeführt. Die Konjugationsreaktion war mit ungefähr 75% der siRNA-Stränge mit einer Aminohexyl-Gruppe erfolgreich.

Die HPLC-Analyse ermöglichte Quantifizierung der Konjugationsreaktion und semipräparative Reinigung der Produkte; ESI-MS lieferte Daten über das Molekulargewicht der siRNA und der Reaktionsprodukte; Gelelektrophorese wurde verwendet, um die Reinheit von siRNA zu analysieren und die Umsetzung der Reaktion mit PEG zu verfolgen.

Der Einfluss der PEG-Konjugate auf das Knockdown der entsprechenden Bcl-2-mRNA in einem Säugerzellkultur-Modell (MCF-7-Zellen) wurde unter Verwendung eines Luciferase-Reporter-Assays und durch Quantifizierung der endogenen mRNA durch RT-qPCR untersucht. Nach einer Transfektion mit Lipofectamine™2000, PEGylierte siRNA erzielten

eine starke Herunterregulierung. Dies weist darauf hin, dass eine PEGylierung am 3'-Ende beider Stränge keinesfalls zum Wirkungsverlust führt. Auftragen von nackten siRNAs zeigte keine signifikante Herunterregulierung des gezielten Gens.

Obwohl die Polyethylenglykolketten, am 3'-Ende gebunden, die Transfektion von siRNA in Säugetierzellen nicht effektiv verbessern, beweist meine Arbeit die Toleranz der Effektormoleküle für solche Modifikation. Diese Konjugate können daher für Lipid- und Polymersysteme verwendet werden, welche als realisierbare Strategien zur Erhöhung der Bioverfügbarkeit, Verlängerung der Plasmahalbwertszeiten und Verbesserung der Stabilität gelten.
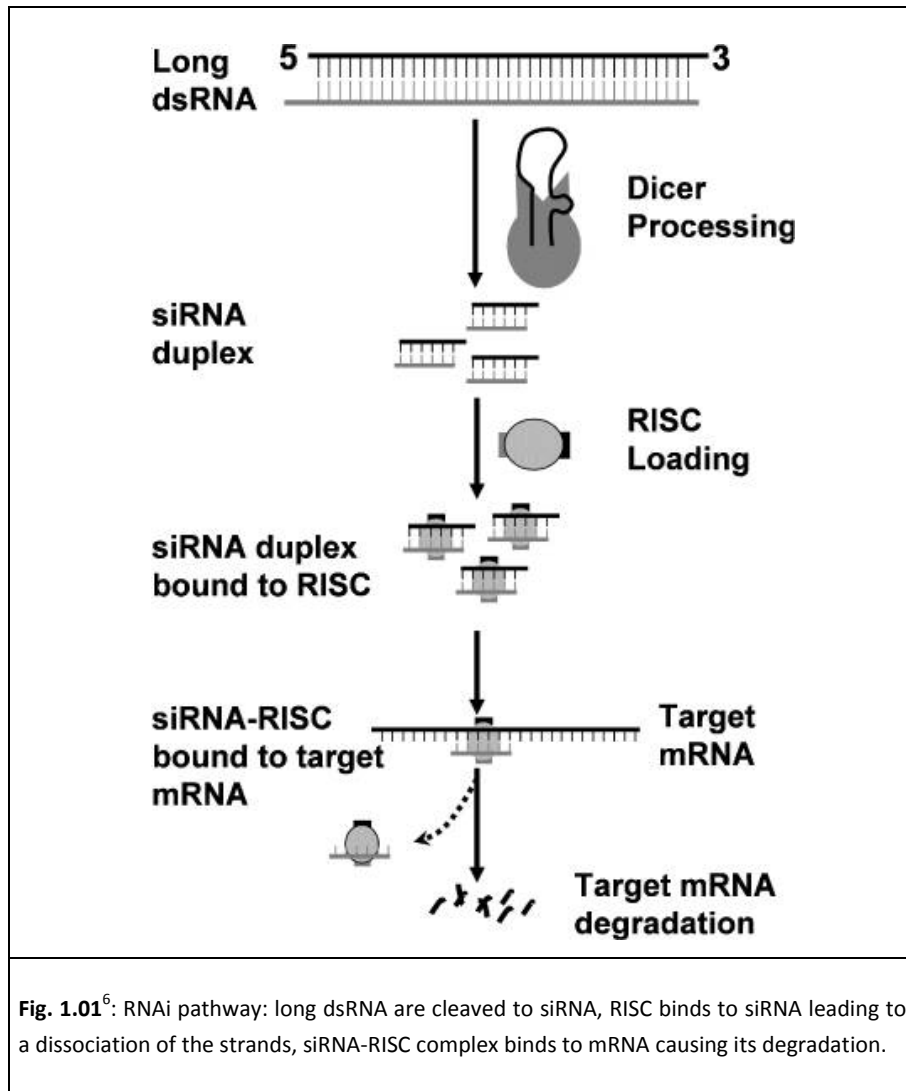
1 Introduction

1.1 RNA interference and gene silencing

RNA interference (RNAi) is a natural cellular process that occurs in many eukaryotic cells as part of an endogenous defence mechanism[1]. This way of post-transcriptional gene silencing is known to mediate resistance to both endogenous parasitic and exogenous pathogenic nucleic acids and regulates the expression of protein-coding genes[2].

The initial description of RNAi as a biological response to double-stranded RNA (dsRNA) came in 1998 by Fire A. et al after introducing long dsRNA into the nematode worm *Caenorhabditis elegans*. It was shown that gene silencing occurred after introduction of a dsRNA homologous in sequence to the gene of interest[3]. The effector molecules of sequence-specific gene silencing were later found to be the small interfering RNAs (siRNAs) that are dsRNA of approximately 21 nucleotides in length[4]. The first time siRNA-mediated gene silencing in mammalian cells was proved in 2001 after a succeeded transfection of siRNA into HEK293 and HeLa cells[5]. Longer dsRNAs induce an innate immune response in mammals, which is greatly overshadowing sequence-specific gene silencing effects. This was a crucial step towards the possibility of silencing of any specific gene of interest, much like the antisense technology, but with higher efficacy and specifity[8].

The pathway of the RNA interference is shown in Figure 1.01[6]. Long dsRNAs are cleaved in the cytoplasm by the endoribonuclease Dicer into shorter (20-25 bp) double-stranded RNA duplexes, the siRNAs. RNA-induced silencing complex (RISC), a protein complex found in all higher species, attaches itself to the siRNA. A component of RISC, the Argonaut-protein (Ago-2), has the ability to cleave and release the passenger strand from the dsRNA, resulting in an activated form of RISC with a single-strand RNA, the guide (or antisense) strand. The sequence of the guide strand is complementary to the target mRNA. It can bind through matching base pairing to the mRNA which enables Ago-2 to cleave the mRNA, thus causing degradation and gene down-regulation.

Fig. 1.01[6]: RNAi pathway: long dsRNA are cleaved to siRNA, RISC binds to siRNA leading to a dissociation of the strands, siRNA-RISC complex binds to mRNA causing its degradation.

Further gene silencing RNAs are short hairpin RNA (shRNA) and micro-RNA (miRNA). The expression of shRNAs in cells is enhanced by incorporating a plasmid vector into the cells that contain specific promoters. shRNA is a single stranded precursor structure of siRNA with a tight hairpin turn. miRNA are non-coding single stranded RNA that are not exactly complementary to the mRNA allowing each miRNA to potentially interact with many similar sets of the target mRNA[6]. All of the three types use the RISC complex to induce mRNA degradation and gene silencing but differ in specificity and efficiency[6,8].

1.2 RNAi-based therapy

The mechanism of RNAi is currently the source of new therapeutic approaches of several diseases with unmet medical need. The key therapeutic interest of applying RNAi lies in its ability to knock down the expression of disease-causing genes of known sequence[4]. First clinical applications of RNAi have been targeted at the treatment of wet, age-related macular degeneration (AMD) and respiratory syncytial virus (RSV) infection. In these cases, the local administration circumvents much of the problems associated with siRNAs poor pharmacokinetic parameters. Other promising RNAi-based therapies have been aimed for the treatment of neurodegenerative disorders, cancers or other viral diseases, such as hepatitis C virus (HCV) and human immunodeficiency syndrome (HIV). There are, however, concerns about therapeutic application of RNAi-based therapies, including delivery challenges and uncertainty about potential toxicity[4,7].

The first infectious agent targeted by RNAi was the HIV, possibly because of the well understood pattern of gene expression. Nevertheless, the high viral mutation rate and difficult delivery into immune cells made it a great challenge to attain a clinical application against the virus. Treatment of hepatitis caused by HCV and HBC (Hepatitis B virus) has been attempted *in vivo* in mice resulting in a high potency of knockdown of the hepatitis core antigens. Like with HIV therapeutics, the barrier of applying RNAi-based therapy for hepatitis viruses is their efficient delivery in hepatocytes[7].

Applications of RNAi in the treatment of cancer have been targeting dominant oncogenes or viral oncogenes in order to inhibit their function. In various studies siRNAs combined with cationic lipid complexes have been transfected into various cancer cell lines such as HeLa, lung adenocarcinoma, ovarian carcinoma or melanoma cells resulting a siRNA-dependent down-regulation of tumour targets such as Bcl-2, CDK-2, H-RAS and VEGF, which play an important role in regulation of cell proliferation and apoptosis. Cancer cells have been characterized with several defects, such as mutations, down-regulation, over-expression and deletion of the previously mentioned oncogenes and tumour suppressor genes. Another application of RNAi in oncology is the understanding of functions of various genes in tumours by knockdown studies and siRNA screens, which makes the search for new drug targets easier[8].

1.3 B-Cell Lymphoma 2 (Bcl-2) protein and its role in cancer

Apoptosis is a form of programmed cell death that, when inhibited through deregulation, contributes to a number of human diseases, such as cancer, autoimmune disorders and viral infections. Bcl-2 and related proteins play an important role in the regulation of the apoptotic pathway[9].

The B-Cell Lymphoma 2 gene was discovered, as the name says, due to a high rate of expression in B-Cell malignomes[10]. Vaux et al were the first to report that the Bcl-2 gene can extend life of cells[11]. Therefore Bcl-2 is seen as an important factor in cancer and resistance of cancer to conventional therapies. It has been reported that, when expressed extensively, Bcl-2 enhances the resistance of cancers to a wide spectrum of cytotoxic drugs and gamma irradiation which both are major therapeutic approaches in the cure of cancer[10,12]. Down-regulation of Bcl-2 showed to significantly increase sensitivity of cancer cells to cytotoxic drugs, such as etoposid and doxorubicin, thus reducing the number of viable cells[12].

Various inhibitors of Bcl-2 proteins are in preclinical and clinical development. Oblimersen has at one stage been promising in the treatment of genitourinary tumors [13], chronic lymphocytic leukemia[13a] and advanced melanoma[13b]. It is an antisense oligonucleotide drug whose sequence is complementary to the sequence of the Bcl-2 mRNA[13].
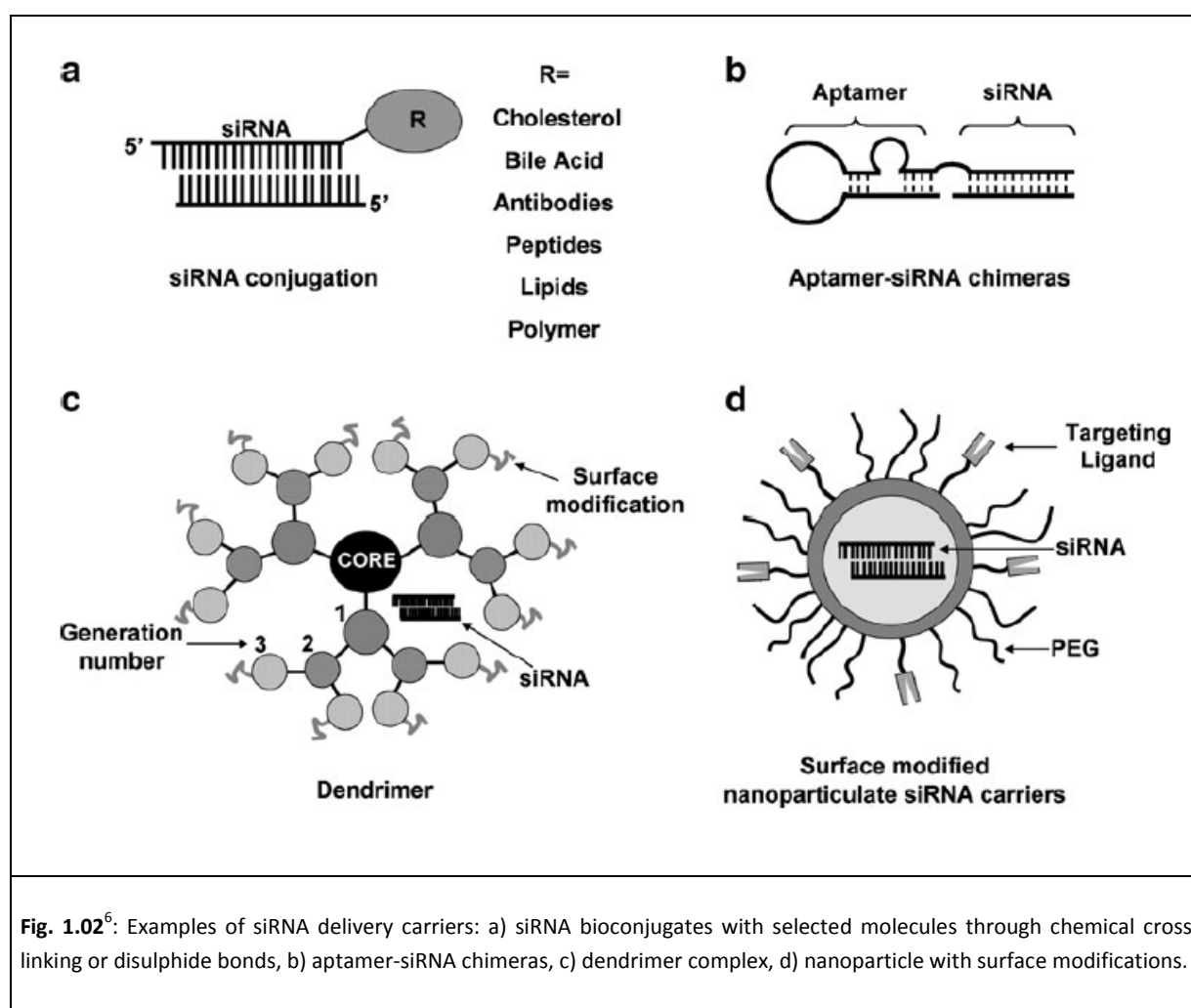
1.4 <u>siRNA therapeutics and barriers to delivery</u>

Several factors limit the utility of siRNA as therapeutics. The most important factor is the delivery of siRNA to its intracellular target site due to their unfavourable physicochemical properties and instability. siRNA are hydrophilic substances with multiple net charges and relatively large molecular weight and their wild-type form is very instable with plasma half-lives of only about 10 minutes. They are extremely poor active pharmaceutical ingredients[6,14].

Depending on the administration route and the targeted organ, there are multiple barriers a siRNA has to overcome after the application. After an intravenous injection, siRNA is distributed to organs and at the same time undergoes degradation through endonucleases and elimination. The transport of large molecules from the blood vessel to the interstitial space is mainly by convection. Absence of the lymphatic system in solid tumours increases interstitial fluid pressure which leads to inhibiting the convective transport, thus complicating the delivery of siRNA. With the negatively charged nature of siRNA that prevent it from having association with cell membrane components and the obvious lack of an active uptake system, simple internalisation of siRNA through cells is improbable[17]. A possible way of siRNA uptake is endocytosis, enabled by carrier structures that interact with anionic proteoglycans on the cell surface. However fusion of the endosomes with lysosomes causes a degradation of siRNA. The endosomal entrapment and lysosomal degradation are the major barriers for delivering the siRNA to its site of action[6].

1.5 Modifications of siRNA

Chemical modifications of the lead structure are an essential step to increase resistance against degradation and ameliorate the delivery of siRNA to its site of action in the cytosol. However these modifications are limited as the complex responsible for cleaving the targeted mRNA, the RISC, only tolerates minor alterations of the chemical structure[14]. Initial strategies involved substituting the 2'-OH-group on the ribose ring and modifications of the oligonucleotide backbone. It has been observed that these modifications increase the stability against enzymatic degradation[14,17]. Conjugation of siRNA with positively charged vectors, small molecules, lipids, polymers, antibodies or encapsulation of siRNA in nanoparticle formulations are popular alternatives of currently investigated delivery systems. Examples of such modifications are shown in Figure 1.02[6].

Fig. 1.02[6]: Examples of siRNA delivery carriers: a) siRNA bioconjugates with selected molecules through chemical cross linking or disulphide bonds, b) aptamer-siRNA chimeras, c) dendrimer complex, d) nanoparticle with surface modifications.

It has been reported that cells preferably internalise positively charged microparticles. This is why transfection with cationic reagents such as cationic lipids (Lipofectamine[TM]2000) or polymers (PEI) have a positive effect on increasing the uptake efficiency. Through interaction with negatively charged oligonucleotides cationic delivery systems enhance their incorporation into mammalian cells[16]. Cationic lipid formulations, like the commercially available Lipofectamine[TM]2000 (Invitrogen), have been the key transfection reagents for siRNA delivery in vitro.

Despite their efficiency, these delivery systems showed to have a cytotoxic effect on the host cells which prevents their therapeutic application[16,18]. Alternative ways to facilitate the delivery of siRNA are constantly developed aiming a lower cytotoxicity without losing sufficient uptake efficiency. Optimization of lipid formulations, known as SNALPs (stable lipid nucleid acid particles), have increased delivery efficiency. However these siRNA complexes are passively targeted to liver, thus being delivered to nontarget cells in the liver which contributes to toxicity[18]. Chemically stabilised bioconjugates of siRNA with cholesterol showed to have markedly improved pharmacological properties *in vitro* and *in vivo*. As a result, siRNA targeting the apoB mRNA could significantly decrease the levels of apoB protein in plasma and accordingly reduce total cholesterol. The degradation of apoB mRNA occurred specifically at the predicted site, in liver and jejunum. However, high doses are necessary, and the largest part ends up in the liver, which restricts this approach of hepatologic diseases[15]. Effective down regulation of the apoB gene and the PPAR-α gene *in vivo* was achieved also with siRNA dynamic polyconjugates (DPC) through targeted delivery into hepatocytes. Key features of the DPC technology include a membrane-active polymer with N-acetylgalactosamine (NAG) and polyethylene glycol (PEG) chains attached to the siRNA. PEG chains prevent the applied siRNA from non-specific interactions with other cells, hence reduce the cytotoxicity. NAG is important for targeted delivery into liver cells[18].

1.6 **PEG conjugates**

In 1977, Abuchowski et al. first described prolonged blood circulation life and non-immunogenicity of intravenously applied enzymes by their attachment to polyethylene glycol[22]. Since then, importance of PEG in drug delivery has been investigated more closely. Currently, PEG is considered to be a non-toxic polymer whose conjugates show a prolonged residence in body and decreased degradation by metabolic enzymes[23]. It has been observed that by inhibiting of activation of the complement, PEG modified substances do not show an immunogenic response in vivo[24]. The first PEG conjugate approved to the market was PEG-asparaginase used since 1994 for the treatment of acute lymphoblastic leukaemia. Ever since, PEG has found a wide range of applications in drug delivery[23], including liposome and nanoparticle formulations.

PEGylation of nucleic acids was commonly performed at the OH-group, directly or by introduction of a spacer. It was discovered that this modification extended plasma permanence and improved cellular uptake by covering the negative charges of oligonucleotides[23]. However, PEG has membrane stabilising properties which results in lower release of oligonucleotides from endosomal compartments which could decrease the silencing efficiency[14]. A PEGylated aptamer, aptanib, was approved by FDA in 2004 for treatment of AMD[23].

2 Aim

Specific cellular uptake of siRNA is an essential step to enhance silencing of a gene of interest. Developed siRNA carriers have already attained promising increase of internalisation of siRNA into cells. However the use of currently developed cationic polymers *in vivo* is avoided due to their cytotoxicity. The demand for new possibilities to delivery of siRNA is growing gradually.

The purpose of this diploma thesis was to investigate the effect of polyethylene glycol (PEG) chains covalently linked to siRNA on their uptake and knockdown effect of a targeted gene in mammalian cells. Due to its chemical, pharmacokinetic and pharmacodynamic properties, polyethylene glycol promises effective modification of siRNA with favourable characteristics.

PEG chains of defined length were aimed to be attached at the 3'- and 5'-OH-groups of sense and antisense strands of siRNA oligonucleotides. The conjugation was to be effected either by direct attachment of a PEG-phosphoramidite building block, which is compatible for automated RNA synthesis, or by using NHS-activated modifiers that catalyse the attachment in solution-phase after solid-supported assembly of the strands. In order to enable analysis and quantification of the reactions, suitable methods need to be applied. Accordingly an HPLC method for reliable separation and analysis of unreacted and reacted oligonucleotides was to be established.

The influence of position of the PEG ligands on the knockdown of the corresponding mRNA target was to be examined in a cell culture tumour model by making use of a luciferase reporter assay and by quantification of endogenous mRNA levels by RT-qPCR. The ability of the amphiphilic ligands to mediate unassisted cellular uptake was aimed to be investigated by applying the naked conjugates directly onto cells. A standard transfection reagent for specific cellular uptake was to be employed to allow a comparison in efficiency.

3   Materials and Methods

All reagents were obtained from Sigma-Aldrich or Serva in standard research grade, except where stated otherwise. Solvents were supplied from Fisher Scientific. Cell culture media and supplements were procured from Gibco, FCS from PAA. Tissue culture flasks and plates were obtained from Greiner Bio-One.

3.1 siRNA synthesis and purification

3.1.1 siRNA synthesis

Synthesis of the anti-Bcl-2-siRNA was performed on the PolyGen 10 column DNA/RNA synthesiser (Polygen), which enables full automation. Controlled Pore Glass (CPG) was used as solid phase having already the first nucleotide (the nucleotide at the 3'-end) attached. The nucleotides used for the synthesis had following protective groups: a silylether protecting the 2'-OH-group; a phosphoramidite group connected to the 3'-OH-end, a dimethoxytrityl-group attached to the 5'-end; acetylated free reactive groups on the bases (Fig. 3.01).
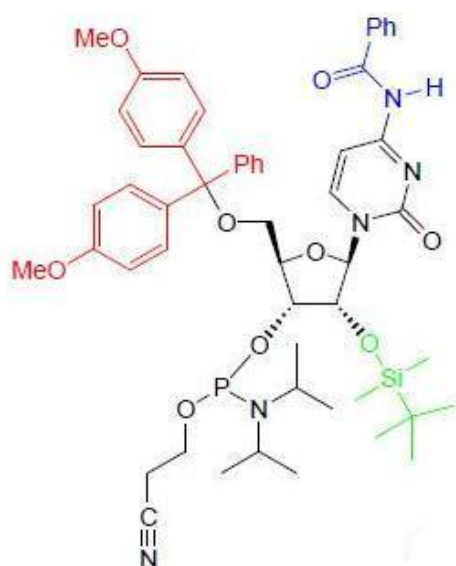
Fig. 3.01: Cytosine nucleotide used for synthesis with following modifications: the 2'-end OH-group is protected by a silylether, the 3'-end is modified with a phorsporamidite, the 5'-end carries a dimethoxytrityl, acetyl protective group on the pyrimidine ring of the cytosine base.

The oligonucleotide synthesis was done with standard protocols for RNA (Fig. 3.02), including 15 min coupling time, and dicyanoimidazol (DCI) as activating agent. For attaching an amino linker at the 3' terminus, amino-on-CPG (SAFC) was used, necessitating the chemical attachment of the first nucleotide. For 5'-aminohexyl-modified oligonucleoides, we used the corresponding PEG-phosphoramidite building block.

Fig. 3.02: Graphical representation of the siRNA synthesis on a solid phase: Detritylation, coupling, capping and oxidation are steps to extend the oligonucleotide chain by one nucleotide.
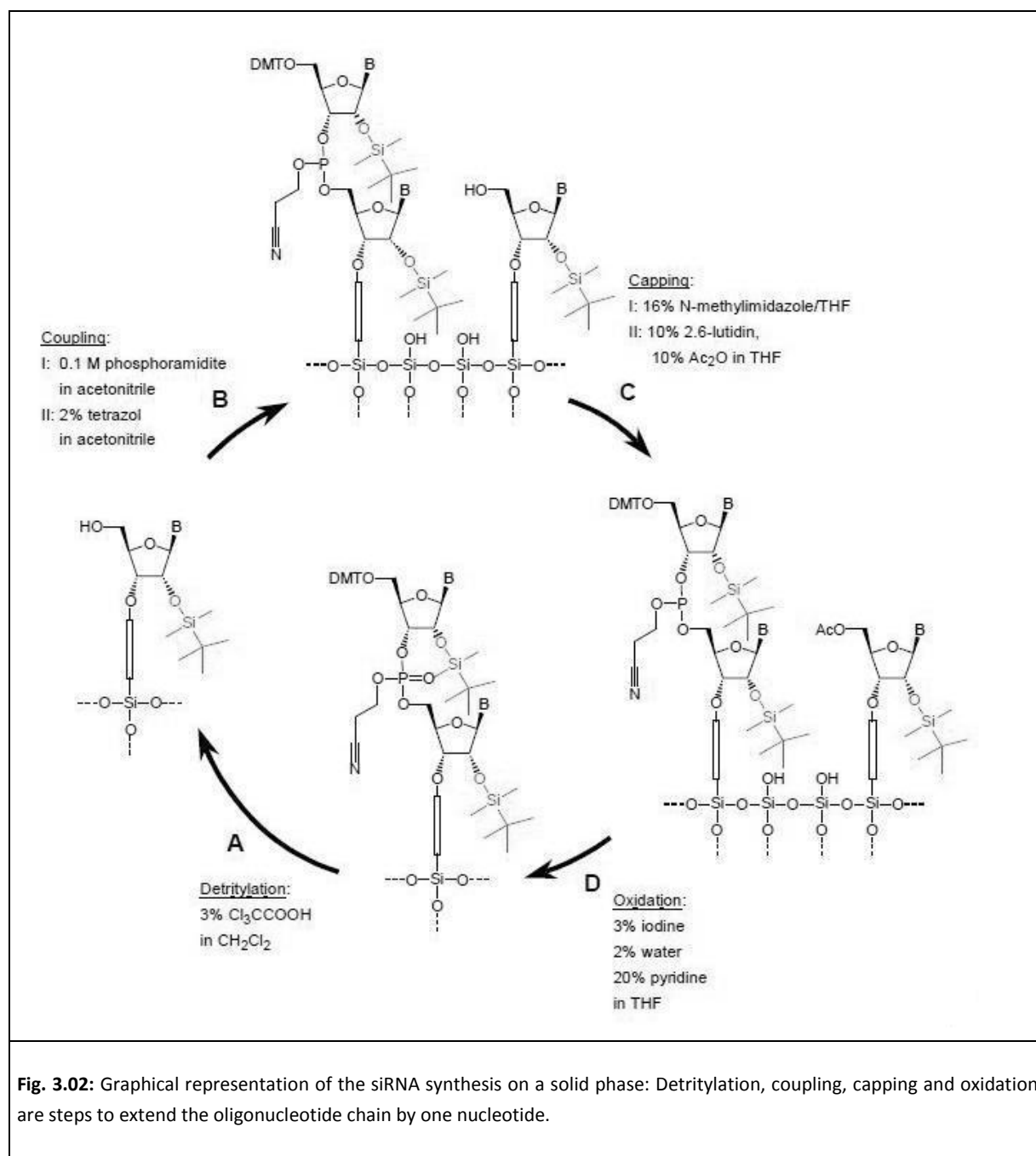
After the whole sequence was synthesised it was cleaved of the resin and purified.

3.1.2 RNA cleavage and purification

The solid support was transferred from the PolyGen Synthesizer slider into a vial with 1 ml of AMA (methanolic methylamine/NH4OH; 1:1) and heated for 10 minutes at 65°C to eliminate protective groups of the bases. The supernatant containing the oligonucleotides was collected in sterile tubes with a pipette using a glass frit as a filter to separate the resin from the supernatant. The support was rinsed twice more with 100 µl of RNase free water and added to the supernatant. To check if the cleavage was complete, the absorbance was determined with a Nanodrop spectrophotometer at 260 nm.

Subsequently the tubes were opened and heated to 40 °C for 10 minutes to evaporate the majority of ammonia. Afterwards they were dried under vacuum using the Christ Speed-Vac RVC-2-18. The dried oligonucleotides were dissolved in 50 µl anhydrous DMSO (dimethylsulfoxide), 50 µl of TEA*3 HF were added and mixed well to initiate deprotection of the silylether from the 2'-OH-group. The tubes were heated to 65 °C for 2.5 hours.

Purification of oligonucleotides was performed by adding 200 µl of sodium acetate and 1 ml butanol. For complete precipitation, the tubes were incubated for 20 minutes or overnight at -70 °C. The samples were centrifuged for 10 minutes at 12,500 rpm after which the butanol was decanted and the pellet was washed in 750 µl cold 75 % ethanol and centrifuged. This washing step was performed twice after which the ethanol was always entirely decanted. The well-dried pellet was dissolved in 50 µl RNase free water and the absorbance was again determined with the Nanodrop spectrophotometer. The concentration of siRNA was calculated according to the Lambert-Beer Law, extinction coefficients were calculated using nearest-neighbor values[19,20]. The oligonucleotides were diluted to a concentration of 1 mM and stored at -80 °C.

siRNA duplexes were obtained by heating equivalent amounts of antisense and sense strands at 65 °C for 3 minutes. The high temperature unfolds the strands and the connection of complementary strands is more effective.

3.1.3 Desalting of oligonucleotides

- Purification with Sephadex G-50, a cross-linked dextran gel used for gel filtration: This method is often used to separate low and high molecular weight molecules. A column was filled with the Sephadex G-50 gel (3 ml, previously swelled with water for at least 2 hours) and washed with RNase free water. 100 µl of the sample was pipetted on the gel and eluted with RNase free water. Each fraction contained 500 µl of the eluent. Accordingly the absorbance of each fraction was determined at 260 nm and the fractions containing the oligonucleotides were lyophilised, diluted with a specific amount of RNase free water and analysed with HPLC.

- Precipitation with ethanol for analysis with ESI-MS was done according to the protocol from Samit Shah and Simon H. Friedman[21]: 30 µl of the 25 µM siRNA sample were mixed with 15 µl of 7.5 M ammonium acetate solution and left at room temperature for 1 hour. To help visualize the pellet after the centrifugation, 2 µl of PEG 4000 were added. After adding 115 µl of ethanol (this amount was duplicated with a higher concentration of the oligonucleotide solution) the tubes were left overnight at -80 °C. The next day the samples were centrifuged for 30 minutes at 17,400 x g and 0 °C to collect the precipitated oligonucleotides. The ethanol was decanted immediately after centrifugation to avoid resolubilisation of the siRNA. The remaining pellet was washed with 100 µl of ice cold 75 % ethanol and centrifuged for 20 minutes. The supernatant was again decanted instantly, the pellet was air-dried and dissolved in 32 µl of RNase free water. The absorbance was determined with Nanodrop Software and the purity of the samples was analysed.

3.2   Conjugation of siRNA with polyethylene glycol

The polyethylene glycol (PEG) chains were aimed to bind to the siRNA at the 3'-end at the aminohexyl-linker. Aminolinker modifications allow other modifications to be added to oligonucleotides post-synthetically.

The reaction of the aminolinker with NHS-ester activated polyethylene glycol was carried out at slightly alkaline conditions (pH 7-9). The target pH of a siRNA dilution was adjusted with buffer (PBS or Na-borate buffer). Accordingly a 250 mM $Me(PEG)_{12}$ (Thermo Scientific) stock solution in DMF was prepared and was added either in 5 or 50 equivalent amount of the siRNA. The tubes containing all reagents were stirred for 1 hour at room temperature and the reaction was monitored either with HPLC or PAGE.

3.3 Analytics of the siRNA and conjugates

3.3.1 Polyacrylamide Gel Electrophoresis

Preparation of a 20% acrylamide gel

7.2 g urea was dissolved in 1,5 ml 10xTBE (tris-borate-EDTA-buffer) and 7.5 ml acrylamide gel stock solution (40%, acrylamide/bis-acrylamide, 29:1, Serva) was added and the mixture was heated in the microwave for several seconds until the viscous liquid became clear. After cooling down, 7.5 μl TEMED and 75 μl APS (both from Serva) were added to induce polymerisation and the mixture was immediately poured into a gel forming sandwich. Ten or fifteen slots were formed with a comb. 1 x TBE was used as buffer for the run. The gel was pre-run for 30 minutes at 150 V.

Preparation of the samples

One nanomol of each sample was diluted with 10 μl formamide loading buffer. The samples were heated for 3 minutes at 95 °C and put on ice to cool down immediately after before being loaded onto the gel. As a reference a mixture of bromo phenol blue and xylene cyanol was used to allow a prediction of current position of the samples while the gel was running. The gel was run for 1.5 hours at 150 V.

Methylene blue dyeing and detection

After the run the gel was agitated in a 2% solution of methylene blue for 30 minutes. Consequently the oligonucleotide bands were clearly visible on the gel. Hence the gel was destained with water leaving the oligonucleotide bands coloured. The bands were detected with the Calibrated Imaging Densitometer GS-710 (BioRad) and the pictures were adjusted with Quantity One 4.6.3, 1-D Analysis Software (BioRad).

Purification of samples with gel electrophoresis

Purification of samples was performed with the gel electrophoresis as well. After the run the bands were detected with fluorescence to avoid methylene blue staining. Individual bands were cut out of the gel and the gel slices were put into tubes with 300 μl of 50 mM

TEAA. The tubes were left overnight at 35 °C allowing the oligonucleotides being saturated in the solution.

3.3.2 High performance liquid chromatography (HPLC)

All HPLC runs were performed with a LaChrom L-7100 (Merck Hitachi) system and analysed using the EZ Chrome Elite software. The HPLC was used mostly for analytics of the samples as well as for semi-preparative purification of individual components.

The HPLC runs were carried out in following conditions:
- mobile phase A: 50 mM triethyalmmonium acetate buffer (TEAA), TEAA was pepared with DEPC (diethylpyrocarbonate, 2%) treated water;
- mobile phase B: acetonitrile;
- gradient elution was altering depending on the sample properties, most commonly from 5% B to 30% B in 30 minutes at a flow rate of 1 ml/minute;
- column: Clarity 5 micron Oligo-RT 250 x 4.6 mm (Phenomenex);
- temperature of the column: room temperature.
- The HPLC was equipped with a UV detector at 260 nm.

Purification of oligonucleotides with HPLC

An autosampler (Pharmacia LKB Radi-Frac) was used to collect the chromatographically separated compounds of a sample. Absorbance of each fraction was measured and the fractions containing oligonucleotides were lyophilised (Christ Speed-Vac RVC-2-18), diluted in RNase free water and analysed with HPLC or PAGE.

3.3.3 Electrospray ionization – Mass spectrometry (ESI-MS)

Desalted samples (see above) dissolved in RNase free water were diluted in acetonitrile and triethylamine to obtain a final ratio of 50:50:1 (water:ACN:TEA).

Analysis was performed with a micrOTOF-Q II 10240 (Bruker). Calibration of the system was carried out with lithium-formiate. The samples were measured in negative ion mode, which means that the analysis depended on a deprotonation of the molecules. For the analysis 10 µl of a 0.3 µM (≈ 3 pmol) oligonucleotide dilution was commonly needed.

3.4 Cell culture assays

3.4.1 Cell cultivation and seeding

Cells were cultivated in DMEM (1x) + GlutaMAX^{TM} (Dulbecco's Modified Eagle Medium from Gibco) with 10% FBS (PAA) at 37 °C with 5 % CO_2. Nearly confluent cell layers in 25 cm^2 flasks contained approximately 5 million cells, 75 cm^2 delivered about three times as many cells. The cells were split depending on the growth of the cells about twice a week.

The cell line MCF-7 (Michigan Cancer Foundation – 7), obtained from the European cell culture collection (EACC) was used for all cell culture experiments. Only cells in their exponential growth phase were used for the experiments. At first the medium was removed and the cells were washed with 5 ml preheated 1 x PBS. The PBS was removed and 0.5 ml of 1 x trypsine solution was added avoiding instant contact with the cells. The trypsine solution was dispersed to cover the whole flask bottom and left incubating at 37 °C for 5 minutes. After this time the cells detached from the flask surface which was checked by tilting the flask. The cells were resuspended in an appropriate volume of preheated culture medium (to obtain approximately 1 million cells/ml). 10 µl of the suspension were pipetted to each side of a counting chamber and the cells were counted. The average count of cells in the squares was multiplied by 10 000 obtaining the number of cells per ml. The cell suspension was diluted depending on the concentration needed to perform either the Dual-Luciferase Reporter Assay or the RT-qPCR.

3.4.2 Multiplication and expression of psiCHECK-2 Bcl-2 plasmid

Transformation of E.coli *XL-1 Blue*

The propagation of psiCHECK-2 plasmid carrying the human Bcl-2 cDNA was carried out in *E. coli* XL-1 Blue. The psiCHECK-2 vector contains also genes for the Renilla luciferase and the firefly luciferase. The Renilla luciferase is fused to the Bcl-2 cDNA, therefore a quantification of the Bcl-2 gene expression is enabled. Firefly luciferase allows normalisation for the transfection.

200 μl of an *E.coli* XL-1 Blue stock were transformed with 100 ng psiCHECK-2 Bcl-2 plasmid and the tube was left on ice for 1 hour. The bacteria were heat shocked by transferring the content of the tube onto a preheated agar-plate with ampicillin.

The medium used for petri-plates was prepared out of 10 g LB Agar and 250 ml ddH$_2$O in an autoclaved Erlenmeyer flask. The mixture was heated in a microwave until the medium was fully dissolved. After the medium cooled down to room temperature, ampicillin was added and the flask was mixed well. The medium was poured into sterile petri-plates, left to solidify and the petri-plates were put into the incubator upside down.

Multiplication of the transformed E.coli *XL-1 Blue:*

After one day of cultivation in the incubator at 37 °C, several white shining colonies were visible on top of the medium. One of the colonies was picked and put into preheated liquid LB Broth medium. The bacteria were grown for 16 hours at 37 °C and 180 rpm.

Extraction of psiCHECK-2 Bcl-2 plasmid:

The plasmid was extracted using the GeneJET Plasmid Midiprep Kit from Thermo Scientific. At first the OD$_{600}$ (optical density – absorbance at a wavelength of 600 nm) of the medium with harvested bacteria was determined to obtain the maximum culture volume for the extraction. Plasmid isolation was done according to the manufacturer's instructions.

Concentration of the plasmid was obtained by measuring the absorbance at 260 nm. The purified plasmid was diluted to a final concentration of 100 ng/μl.

3.4.3 Dual-Luciferase Reporter Assay

The realisation of the assay consists of several steps: At first a specific number of MCF-7 cells was transfected with a plasmid containing the Bcl-2 cDNA. Subsequently anti-Bcl-2 siRNA was added at different concentrations with or without Lipofectamine^{TM}2000. After transfection the cells were incubated for 48 hours, lysed and the Dual-Luciferase Reporter Assay was performed.

Cell count

A 96 well plate was applied in this experiment. To obtain a confluency of 60% 30 000 cells were plated into each well. Hence the cells were diluted to 600 000 cells/ml and 50 µl of the suspension were transferred into each well.

Reverse co-transfection of plasmid and siRNA

The transfection mix of 50 µl/well included a psiCHECK plasmid carrying the Bcl-2 gene, siRNA and Lipofectamine^{TM}2000 diluted in Opti-MEM. The psiCHECK-2 Bcl-2 plasmid and siRNA duplexes were diluted with Opti-MEM depending on the final desired concentration for transfection. Generally 100 ng of plasmid was used for transfection for each well. The siRNA was diluted to three different concentrations: 0.1 pmol/well, 1 pmol /well and 10 pmol/well. The amount of 0.25 µl of Lipofectamine^{TM}2000 per well was diluted in Opti-MEM separately and left for incubation for 5 minutes. The components were poured into one flask and the whole transfection mix was incubated at room temperature for 20 minutes ensuring a favorable adsorbtion of LF at plasmid and siRNA.

Transfection of siRNA without a transfection reagent

Each well was set up with 100ng plasmid and 30 000 MCF-7 cells. Plasmid transfection was accomplished using 0.25 µl of Lipofectamine^{TM}2000. After 24 hours of incubation the wells were washed with 1 x PBS to remove residual LF, fresh medium was added and 1 or 10 pmol siRNA in 100 µl DMEM with 10 % was transferred to each well.

Lysis of Cells and Luminescence readout

The reagents were obtained from Promega. After 48 hours of incubation the medium was removed and the cells were lysed with 20 µl Lysis Buffer per well. Subsequently the well plate was shuffled for 20 minutes at room temperature. The Dual-Luminescence Reporter Assay was performed by the Tecan infinite M200Pro device and analysed with the Tecan i-control 1.7 software.

Preparation of the reagents was carried out as follows:

Reagents	µl/well	dilution	µl for a 96 well plate (incl. priming volume of 500 µl)
Passive Lysis Buffer 5x	20	1:5 in ddH2O	1920
Luciferase Assay Buffer II	50	--------------------------------	5300
Stop&Glo 50x	50	1:50 in Stop&Glo Buffer	5300

Table 3.01: reagents for execution of the Dual-Luminescence Reporter Assay

3.4.4 Reverse Transcriptase – quantitative Polymerase Chain Reaction (RT-qPCR)

The quantification of a gene of interest with the RT-qPCR implied several steps: at first the cells were transfected with siRNAs, after 48 hours of incubation the mRNA was extracted, transcribed to cDNA and measured.

Transfection of cells

The MCF-7 cells were transfected in a 24 well plate with siRNA targeting the Bcl-2 gene. Transfection of the siRNA was performed with or without the application of Lipofectamine™2000 as transfection reagent. In the first case, the in Opti-MEM diluted siRNA and LF were incubated for 20 minutes at room temperature and 100 µl of the transfection mixture was set up in each empty well. Amounts of 1 nM and 10 nM of siRNA per well with varying properties were used. Subsequently the MCF-7 cells were counted in a counting chamber, diluted and 400 µl of the cell suspension containing approximately 100 000 cells were put into each well. Transfection without a transfection reagent was carried out at higher amounts of siRNA. 10 or 100 nmol of siRNA were diluted with Opti-MEM, put into the wells and 400 µl of the prepared cell suspension were added.

RNA extraction and cDNA synthesis

After 48 hours of incubation the whole RNA was extracted. A column based RNA extraction was performed according to the Fermentas GeneJET RNA Purification Kit (K0731, K0732) manufacturer manual. Since a 24 well plate was used, the reagent volumes were adjusted: accordingly. Afterwards the concentration and purity of the extracted RNA was measured with the Nanodrop spectrophotometer. Five-hundred nanogram RNA of each sample were used for cDNA synthesis. The cDNA synthesis was performed the same day as freezing of RNA can cause its degradation. The Fermentas RevertAid First Strand cDNA Synthesis Kit (K1621, K1622) was used to prepare the reagents for the synthesis and add it to the extracted RNA.

The reagents were prepared as follows:

cDNA reaction component:	µl / sample
RNA (500 ng)	x
random hexamer primer	1
nuclease free water	to 12
5 x RT buffer	4
RiboLockRnase Inhi (40U/µl)	0,5
10 mM dNTP mix	2
Reverse Transcriptase (200U/µl)	1

Table 3.02: Reagents for the cDNA synthesis

The synthesis was performed with an Eppendorf Thermo Cycler. The tubes were heated to denaturise the secondary structure of the RNA to 65 °C and then cooled down to room temperature to enable annealing of the primers. The cDNA synthesis was effectuated at 42 °C, as the Reverse Transcriptase is active at this temperature. After 60 minutes the tubes were heated to 70 °C for 5 minutes to deactivate the polymerase. Subsequently the tubes were cooled down.

Preparation of the RT-qPCR and evaluation with REST 2009 software

The cDNA was diluted to 1:10 to avoid possible inhibition of the Taq Polymerase due to substances from the cDNA synthesis reaction. 2.0 µl of 1:10 diluted cDNA (equals 5.0 ng of template RNA) were used for each qPCR reaction. The master mix was prepared with reagents from Solis Biodyne and the primers were ordered from Microsynth.

RT-qPCR reaction component:	µl / sample
Nuclease free water	9.7
25 mM $MgCl_2$	2.4
10 x Buffer B2	2
dNTP mix 2 mM	2
Forward primer (10 µM)	0.5
Reverse primer (10 µM)	0.5
20 x EvaGreen dye	0.8
HotFirePol (5 U/µL)	0.2
Template cDNA	2
End volume	20 µl

Table 3.03: Master mix for the RT-qPCR

The RNA Polymerase II gene was used as reference gene, which allows a relative expression and quantification, as the transcription of this gene is not affected by the transfection of siRNA. A standard curve for determination of the reaction efficiency was prepared by making serial dilutions of a reference cDNA.

The cycle at which the dye intensity increases is the treshold cycle, named also Crossing point: Cp. The treshold depends on the initial amount of DNA copies in the sample. By normalising the Cp values of the samples to the Cp of the reference gene the rate of gene silencing was obtained (ΔΔCp method). After the RT-qPCR run the relative expression of the gene of interest is calculated with the REST 2009 software. The software needs the mean values of duplicated samples and the reaction efficiency of each gene obtained from the standard curve.

4 Results

4.1 Oligonucleotide synthesis

siRNA sense and antisense strands were prepared with and without attachment sites for ligand coupling. Oligonucleotide sequences are listed in table X. For 3'-PEG-tethering, commercially available amino-on CPG (SAFC) was used instead of the standard CPG resin. For 5'-PEGylation, I used 17-O-(4,4'-dimethoxytrityl)-hexaethyleneglycol-1-[(2-cyanoethyl)-(N,N-diisopropyl)]-phosphoramidite (Linktech, Strathclyde, UK).
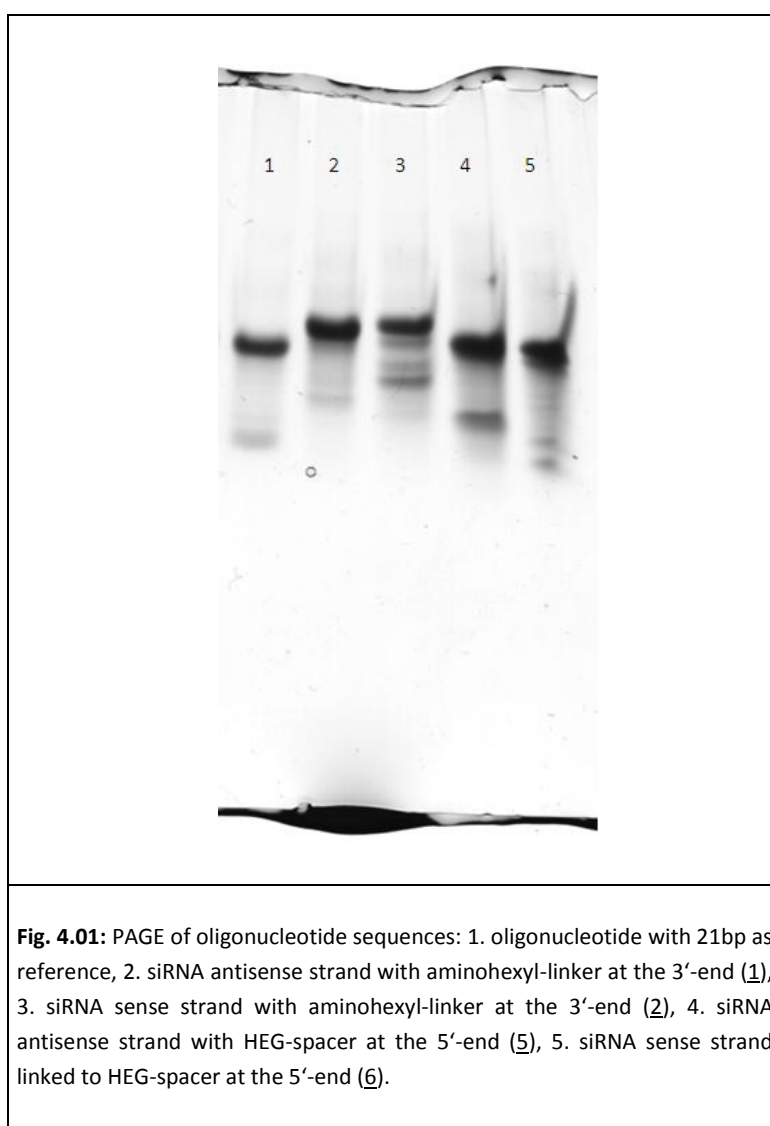
Compound number	Oligonucleotide sequence	Strand	Modification
<u>1</u>	UCA-GGU-ACU-CAG-UCA-UCC-ACA-T-amino-on	AS	-
<u>2</u>	UGU-GGA-UGA-CUG-AGU-ACC-UGA-T-amino-on	S	-
<u>3</u>	UCA-GGU-ACU-CAG-UCA-UCC-ACA-T-amino-on	AS	3'-PEG
<u>4</u>	UGU-GGA-UGA-CUG-AGU-ACC-UGA-T-amino-on	S	3'-PEG
<u>5</u>	UCA-GGU-ACU-CAG-UCA-UCC-ACA-TT	AS	5'-PEG
<u>6</u>	UGU-GGA-UGA-CUG-AGU-ACC-UGA-TT	S	5'-PEG

Table 4.01: synthesized anti-Bcl-2 siRNA strands: oligonucleotide sequences and modifications; A: adenosine, C: cytidine, G: guanosine, U: uridine, T: thymidine; AS: antisense strand, S: sense strand

Compound numbers (Table 4.01) are used in the text below for better understanding of the siRNA properties.

4.2 Polyacrylamide gel electrophoresis (PAGE)

The analysis of siRNA on the polyacrylamide gel was performed to determine identity and purity of the compounds. By comparing the siRNA bands to a variable oligonucleotide with 21 bp it is possible to roughly estimate the length of the synthetised siRNA strands, but because gel migration is sequence-dependent to a certain extent, no absolute length determination is possible.

Fig. 4.01: PAGE of oligonucleotide sequences: 1. oligonucleotide with 21bp as reference, 2. siRNA antisense strand with aminohexyl-linker at the 3'-end (1), 3. siRNA sense strand with aminohexyl-linker at the 3'-end (2), 4. siRNA antisense strand with HEG-spacer at the 5'-end (5), 5. siRNA sense strand linked to HEG-spacer at the 5'-end (6).

In Figure 4.01 the antisense strand (2) and the sense strand (3) of the siRNA migrate slightly slower than the 21bp oligonucleotide (1) as a consequence of the higher moleculer weight caused by attachment of the aminohexyl-linker at the 3'-end. Small impurities of shorter sequences with fast migration rates are visible in some cases. The siRNA strands that were linked to a HEG-spacer at the 5'-end, the antisense (4) and the sense (5) strand, are on the same level as the reference 21bp oligonucleotide. This possibly means that the HEG-spacer failed to be attached to the 5'-end or that the sample contained degraded thus shorter siRNA sequences. Accordingly the ESI-MS analysis of these sequences proved that the HEG-spacer failed to be attached effectively to the oligonucleotides.

The gel electrophoresis was also used to monitor the conjugation of siRNA to polyethylene glycol chains. NHS ester-activated polyethylene glycol chains react with the aminohexyl-linker at the 3'-end of the oligonucleotide strands to yield stable amide bonds (Fig. 4.02). The reaction releases N-hydroxysuccinimide (NHS).

Fig. 4.02: Reaction of a NHS ester-activated Me(PEG)$_{12}$ chain with the aminohexyl-linker at the 3'-end-phosphate group of the siRNA (compounds 3 and 4).
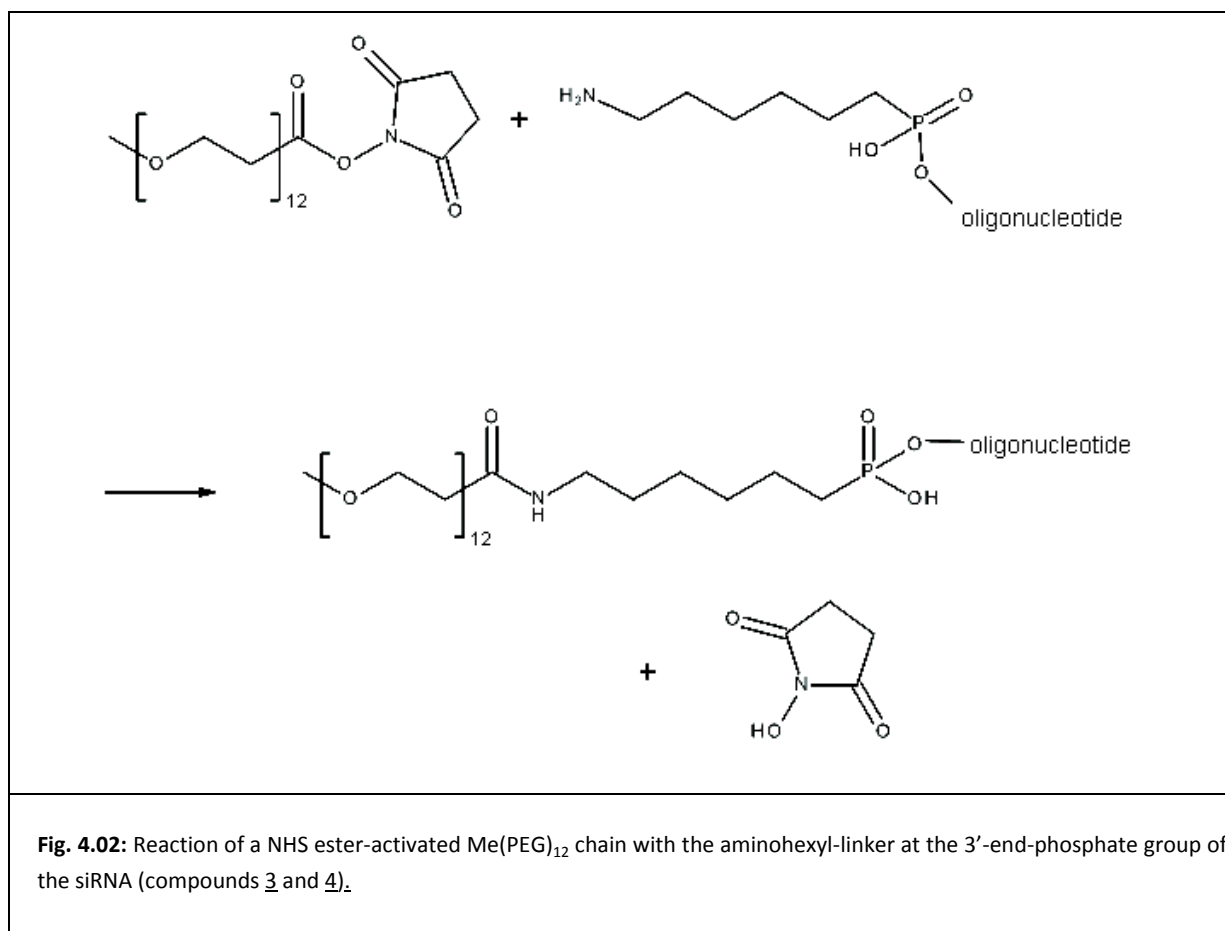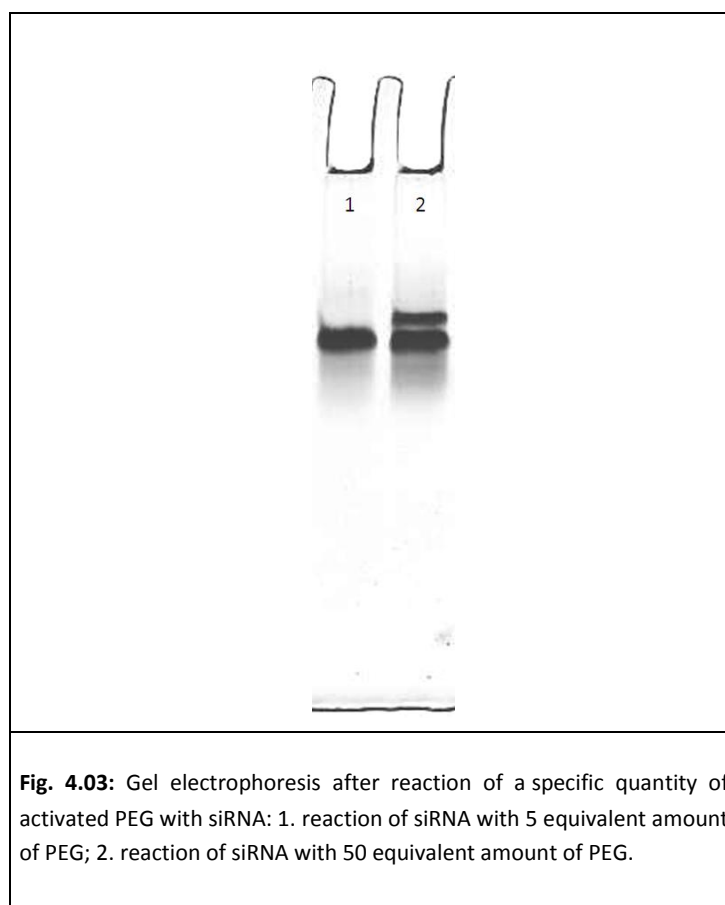
Figure 4.03 shows bands on a gel after reaction of siRNA with 5 (1) and 50 (2) equivalent amounts of activated polyethylene glycol, respectively. The product of reaction of siRNA with 5 equivalent amount of PEG shows one band on the gel. A comparison among the samples shows that the product of the reaction of siRNA with a higher amount of PEG appears in two separate bands, thus exhibiting an additional band of a product with greater molecular weight. The additional band is the product of the reaction itself, representing the conjugate of siRNA with polyethylene glycol. Given that a part of the siRNA was not PEGylated, the second band indicates the original unmodified siRNA. Additionally, because amino deprotection was incomplete, the respective nitrophenyl adduct (see below), unreactive towards NHS reactions, is contained in the lower band.

Fig. 4.03: Gel electrophoresis after reaction of a specific quantity of activated PEG with siRNA: 1. reaction of siRNA with 5 equivalent amount of PEG; 2. reaction of siRNA with 50 equivalent amount of PEG.

Purification of samples was performed with gel electrophoresis as well. Bands containing the PEGylated siRNAs were cut out and gel slices were incubated with extraction buffer. Analysis of the extracted samples was performed by HPLC (see section 4.3).
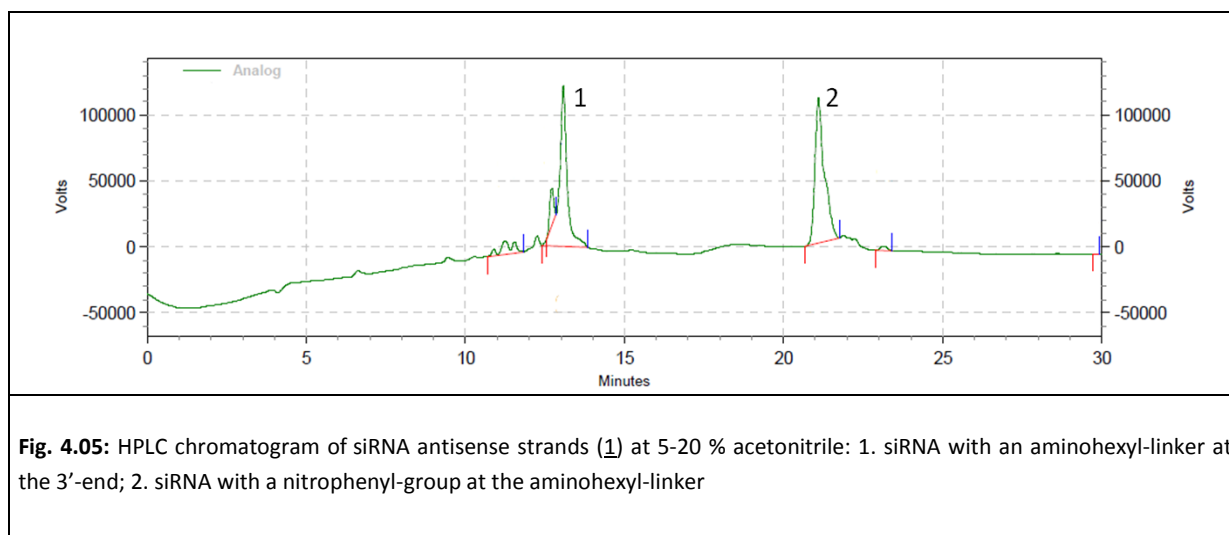
4.3 HPLC

HPLC enabled analysis and semi-preparative purification of the samples. The adequate methods were optimised according to the properties of the samples.

The synthesised siRNAs were analysed at a gradient elution of acetonitrile at 5 to 20 %. siRNA strands having an aminohexyl-group linked to the 3'-end appeared in two separate peaks. Mass spectrometry showed a mass difference of 179 Da between the two products. Review of the amino-on structure indicated that a nitrophenyl group remained connected to the aminohexyl linker due to incomplete deprotection after cleavage of the siRNA (Fig. 4.04). Basic hydrolysis with a mixture of ammonia and methanolic hydroxide cleaves both esters and amides, the former having faster hydrolysis rates. Although we used the protocol provided by the manufacture for resin cleavage, it proved to be insufficient for complete deprotection of the aminohexyl linker. To accomplish complete deprotection of the amino hexyl linker, the mixture was incubated for 2 hours with concentrated ammonia at 55 °C, providing a homogenous sample[25].

Fig. 4.04: Incomplete deprotection of the amino-on structure: fully deprotected amino hexyl linker and a nitrophenyl-protected amino hexyl linker appear in the sample
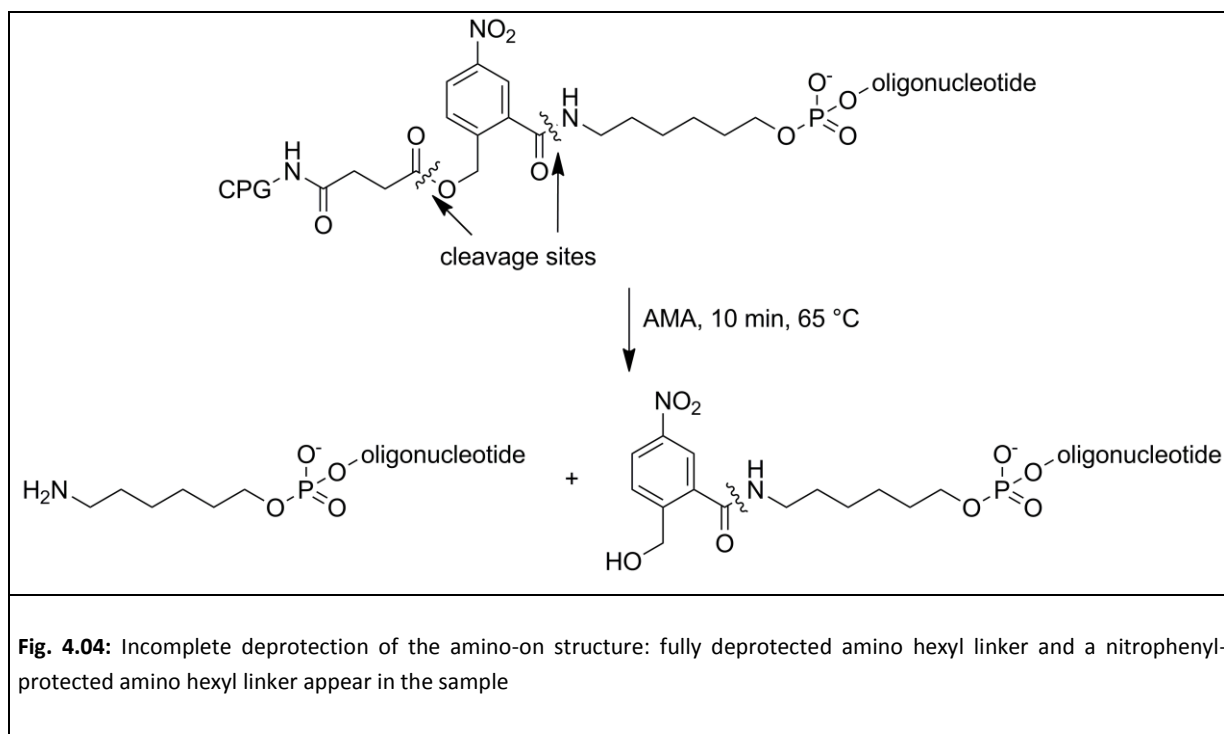
The first peak of the sample representing the siRNA without a nitrophenyl group appears at approximately 12 % of acetonitrile in the mobile phase and the second peak at approximately 16 % of acetonitrile. Comparison of the area percentages of the peaks shows that the nitrophenyl-protected siRNA initially represented approximately 50 % of the siRNA sample.

Fig. 4.05: HPLC chromatogram of siRNA antisense strands (1) at 5-20 % acetonitrile: 1. siRNA with an aminohexyl-linker at the 3'-end; 2. siRNA with a nitrophenyl-group at the aminohexyl-linker

After PEGylation of the siRNA strands, analysis was performed at a gradient elution of acetonitrile at 8 to 30 %. The chromatogram shows two additional peaks after the reaction: a peak at the beginning representing the NHS hydrolysis product and an additional peak representing the siRNAs connected to polyethylene glycol chains (Fig. 4.06). Like with the unmodified sample, the siRNA appears at 12 % of acetonitrile and the nitrophenyl-protected siRNA appears at 16 % of acetonitrile. Being most apolar the PEGylated siRNA occurs in the chromatogram at 18 % of acetonitrile. Calculation of the peaks shows that PEGylation of 75 % aminohexyl-linked siRNA was accomplished. PEGylation of nitrophenyl-protected siRNA failed, as the amino group was blocked.
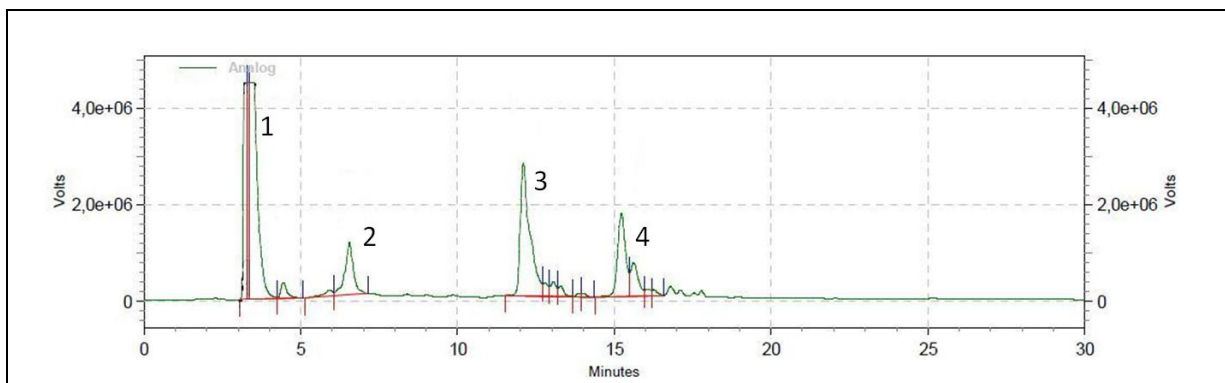
Fig. 4.06: HPLC chromatogram of siRNA antisense strands after reaction with activated polyethylene glycol (3) at 8-30 % acetonitrile: 1. N-hydroxysuccinimide; 2. siRNA with aminohexyl-linker at the 3'-end; 3. siRNA with a nitrophenyl group linked at the aminohexyl-linker; 4. siRNA with PEG chains attached to the aminohexyl-linker.

Semi-preparative purification of the PEGylated siRNA strands with HPLC showed to be successful (Fig. 4.07). Analysis of the purification product was performed at a gradient of 8 to 30 % of acetonitrile. The siRNA strands linked to polyethylene glycol chains appear in one peak at approximately 18 % of acetonitrile.
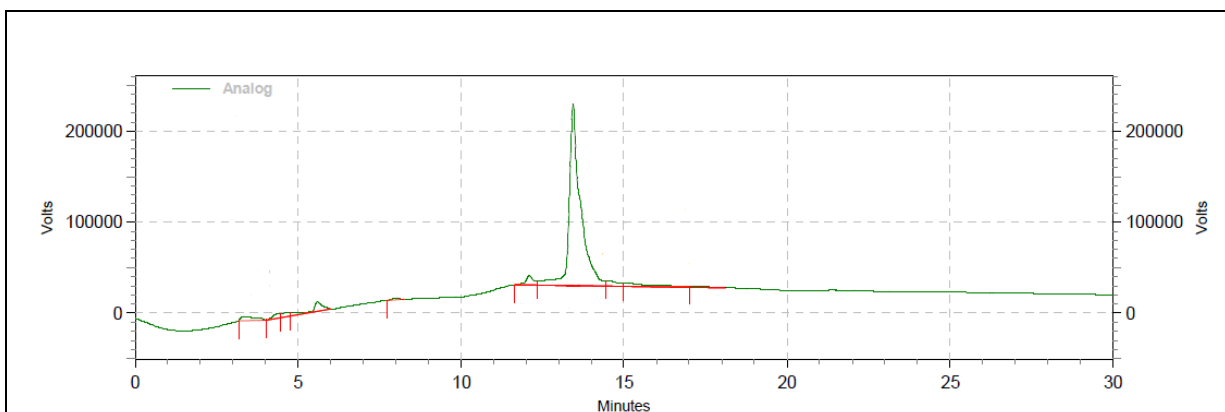
Fig. 4.07: siRNA linked to PEG chains after purification with HPLC, analysis at 8-30 % acetonitrile

Analysis of samples separated with the gel electrophoresis was performed at mobile phase composition of 8 to 30 % of acetonitrile. Extraction yields from the gel were low. In addition, analysis with HPLC showed that the sample was contaminated with impurities. Purification of PEG-siRNA oligonucleotides with HPLC was therefore the superior procedure.

4.4 ESI-MS

The obtained mass spectra showed multiply-charged ions of the form $[M+nH]^{n+}$. The estimated mass of each sample was compared to their respectively multiplied peaks. Negative ion adducts with sodium $[M-H+Na]^-$ and with potassium $[M-H+K]^-$ were partially observed as well, so the mass had to be adjusted accordingly. This phenomenon was more common with oligonucleotides connected to polyethylene glycol chains (Table 4.02).

At first the molecular weight of the siRNA strands was calculated, representing the expected mass of the components in the samples. After the measurements the main peaks were selected and their masses were copied. Multiplied masses of the peaks were compared to the expected masses.

Masses of the samples were calculated as follows:

(MW of peak+1,01) * $[M-x]^{x-}$ = calculated mass

Molecular weight of peak	$[M-x]^{x-}$	calculated mass	expected mass
946,77	8	7582,2 (+ nitrophenyl)	7403,21
1056,47	7	7402,3	
1233,02	6	7404,2	

Table 4.02: ESI-MS of a Bcl-2 siRNA antisense strand (1): the expected mass of the antisense strand corresponds to the calculated mass of the sample. The first peak shows an incompletely deprotected product, where the amino-on linker binds to a nitrophenyl group.

Molecular weight of peak	$[M-x]^{x-}$	calculated mass	expected mass
800,14	10	8011,5 ($+K^+$)	7974,54
889,17	9	8011,6 ($+K^+$)	
1003,43	8	8035,5 ($+K^+$, Na^+)	
1146,76	7	8034,4 ($+K^+$, Na^+)	

Table 4.03: ESI-MS of a Bcl-2 siRNA antisense strand linked to PEG chains (3): the expected mass corresponds to the calculated mass, adducts with potassium and sodium are observed.

MW of peak	[M-x]$^{x-}$	calculated mass	expected mass (w/o HEG spacer)
737,42	10	7384,3 (+Na$^+$)	7361,22
819,43	9	7383,9 (+Na$^+$)	
922,00	8	7384,1 (+Na$^+$)	
1229,69	6	7384,2 (+Na$^+$)	

Table 4.04: ESI-MS of a Bcl-2 siRNA sense strand, connected to a HEG-spacer at the 5'-end (6): the calculated mass of the sample equals the sense strand of Bcl-2 siRNA without the HEG-spacer connected at the 5'-end as a sodium adduct.

A part of the siRNA having an aminohexyl-linker at the 3'-end showed a higher molecular weight by 179 Da (Table 4.02), which equals the molecular weight of a nitrophenyl group that was not detached after cleavage.
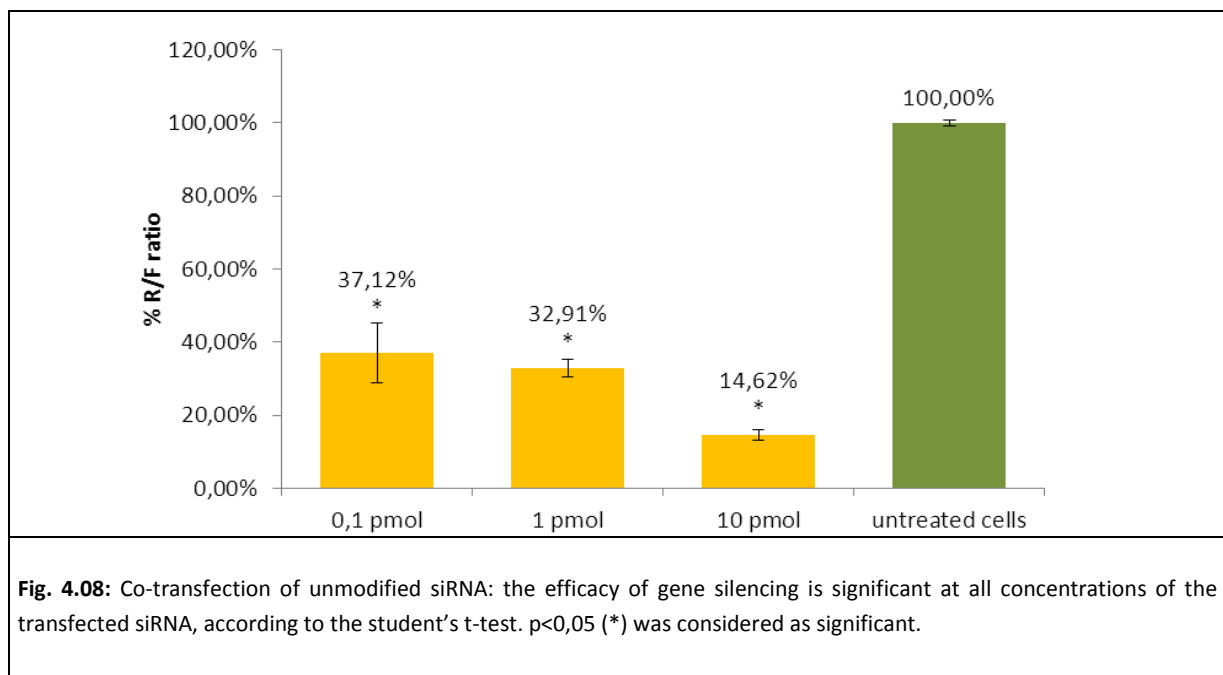
Expected masses of the synthesised siRNA strands corresponded to calculated masses of the samples. This leads to the conclusion that the oligonucleotides were synthesised as required. The ESI-MS proves that the siRNA having aminohexyl-linker at the 3'-end were successfully conjugated to polyethylene glycol chains (Table 4.03). However linking the HEG18-spacer at the 5'-end of our siRNA by the synthesizer failed (Table 4.04), possibly due to a too short coupling time. Relevant ESI-MS spectra are attached at the end of this thesis in the appendix. Since PEGylation at the 3'-end was successful, no attempts to optimize HEG-attachments by increasing coupling time or concentration of the building block, or by employing more active catalysts were undertaken.
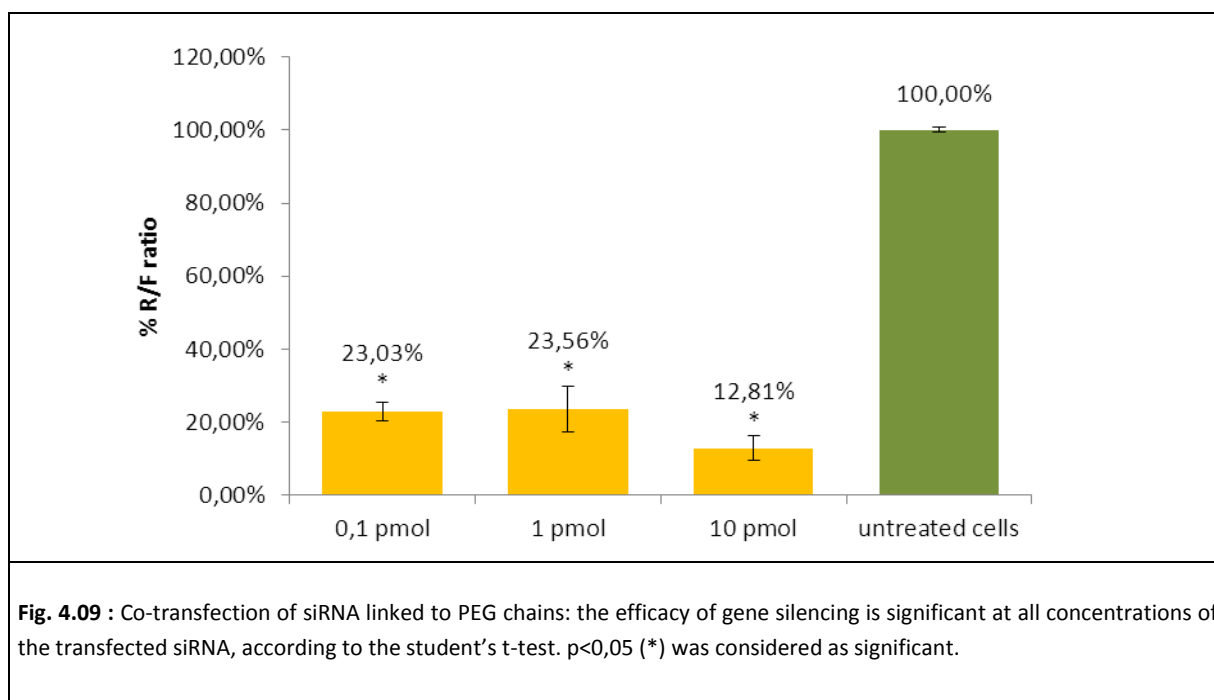
4.5 **Dual-Luciferase Reporter Assay**

A Dual-Luciferase Reporter Assay was used to compare the biological efficiency of PEGylated siRNA to its unmodified counterpart. Hence the psiCHECK 2 Bcl-2 plasmid was co-transfected with siRNA agents into MCF-7 cells. This specific plasmid comprises the Renilla gene, fused to human Bcl-2 cDNA and the Firefly gene, which allows normalisation. If the Bcl-2 mRNA is degraded by siRNA, no Renilla luciferase protein is produced. To determine the level of gene silencing, the down-regulation of the Renilla luciferase was compared to the unaffected, constitutively expressed Firefly luciferase. The detection was performed after approximately 48 hours of incubation with siRNA.

siRNA with different properties were transfected into the cells: siRNA without a PEG modification (1+2), siRNA where the sense strand was linked to PEG chains (1+4) and siRNA where both strands were linked to PEG chains (3+4). Comparing the differences in gene silencing in reference to the siRNA properties allowed a statement which level of PEGylation provides the most efficiency.

siRNA that were co-transfected along with the plasmid with LF had a significant effect on the Bcl-2 mRNA levels. Efficient down-regulation resulted with unmodified (Fig. 4.08) as

Fig. 4.08: Co-transfection of unmodified siRNA: the efficacy of gene silencing is significant at all concentrations of the transfected siRNA, according to the student's t-test. $p < 0.05$ (*) was considered as significant.

well as with modified siRNA (Fig. 4.09). The modified siRNA show slightly higher efficacy in lower quantities than unmodified siRNA, but according to the student's t-test, the differences in Bcl-2 gene silencing between unmodified and modified siRNA were not statistically significant.

Fig. 4.09 : Co-transfection of siRNA linked to PEG chains: the efficacy of gene silencing is significant at all concentrations of the transfected siRNA, according to the student's t-test. $p < 0.05$ (*) was considered as significant.

To examine a possible increase in uptake mediated by amphiphilic PEG chains, modified siRNAs were applied without a transfection reagent. In reference to the investigated cellular uptake of siRNA and its intrinsic activity where use of Lipofectamine[TM]2000 as transfection reagent was avoided, the efficacy of gene silencing was minimal (Fig. 4.10). In comparison to untreated cells or unmodified siRNA, the effect of PEGylated siRNA on the Bcl-2 expression was not significant.
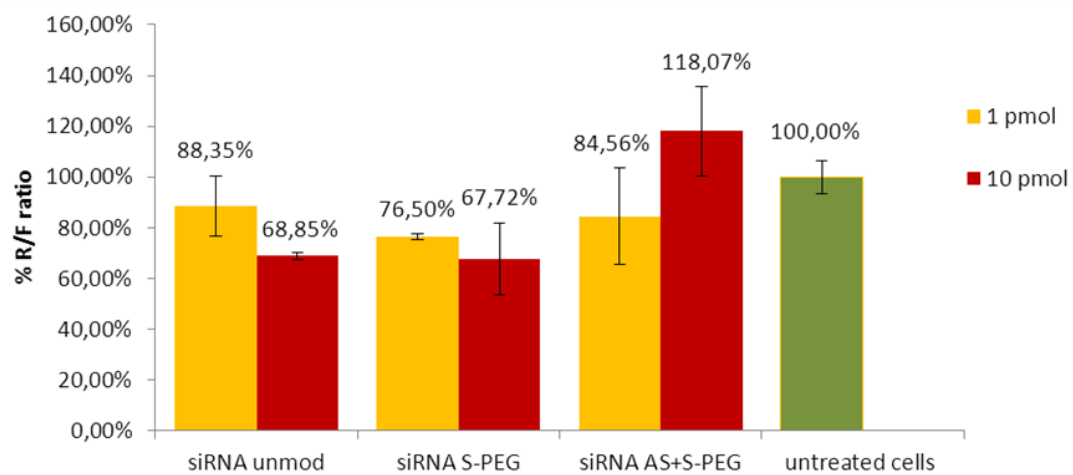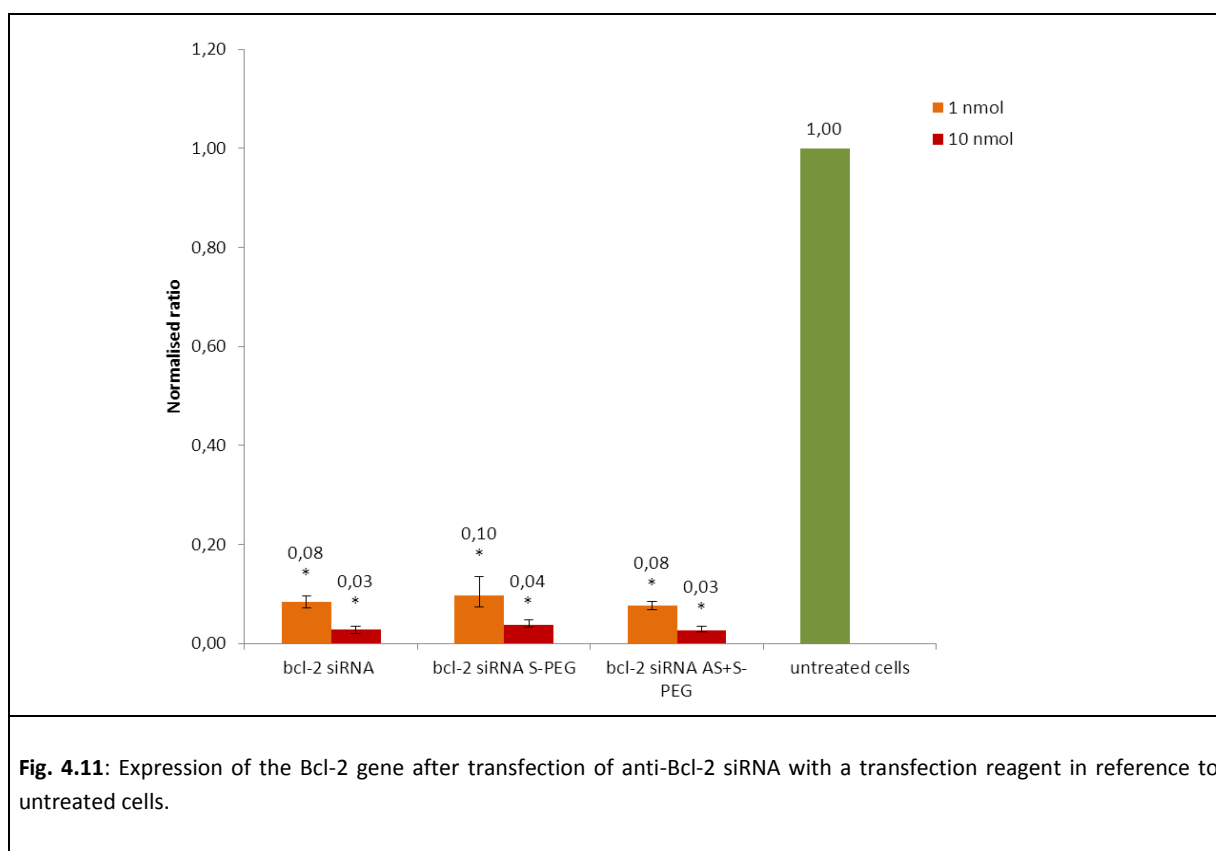
Fig. 4.10 : transfection of siRNA without a transfection reagent: the gene silencing of siRNA without the use of a standard transfection reagent was absent

4.6 RT-qPCR

To quantify the Bcl-2 mRNA levels in MCF-7 cells after transfection of anti-Bcl-2 siRNA, a RT-qPCR was performed. After extraction of the cellular RNA and its transcription to cDNA, the effect of the transfected siRNA on the Bcl-2 gene was measured. RNA Polymerase II was chosen as the reference gene as its expression is not affected by a transfection of anti-Bcl-2 siRNA.

Transfection of unmodified and modified siRNA with Lipofectamine[TM]2000 as transfection reagent resulted in significant down-regulation of the Bcl-2 expression with both concentrations (Fig. 4.11). All tested substances resulted in concentration-dependent target down-regulation. According to the student's t-test, efficiency differences between unmodified and modified siRNA were not significant. The results indicated that polyethylene glycol chains have no detrimental effect on uptake of the siRNA and gene silencing.

Fig. 4.11: Expression of the Bcl-2 gene after transfection of anti-Bcl-2 siRNA with a transfection reagent in reference to untreated cells.

To examine a possible membrane permeation enhancing effect of polyethylene glycol chains on the gene silencing more precisely, siRNA targeting the Bcl-2 gene were applied onto MCF-7 cells without a transfection reagent. Unmodified siRNA (1+2) transfected with Lipofectamine[TM]2000 served as positive control (Fig. 4.12). Transfection of siRNA with a transfection reagent resulted in a significant gene silencing. However, in reference to the expression of the RNA Polymerase II gene, transfection of anti-Bcl-2 siRNA without a transfection reagent did not have any effect on the mRNA levels of the Bcl-2 gene. Furthermore, PEGylation did not affect the uptake or gene silencing so that the effect of unmodified siRNA was comparable with modified siRNA. The observed apparent increase in Bcl-2 mRNA is present due to variations in RNA yield of treated and untreated samples. Because no significant down regulation was found, no attempts to optimize the qPCR analysis were undertaken.
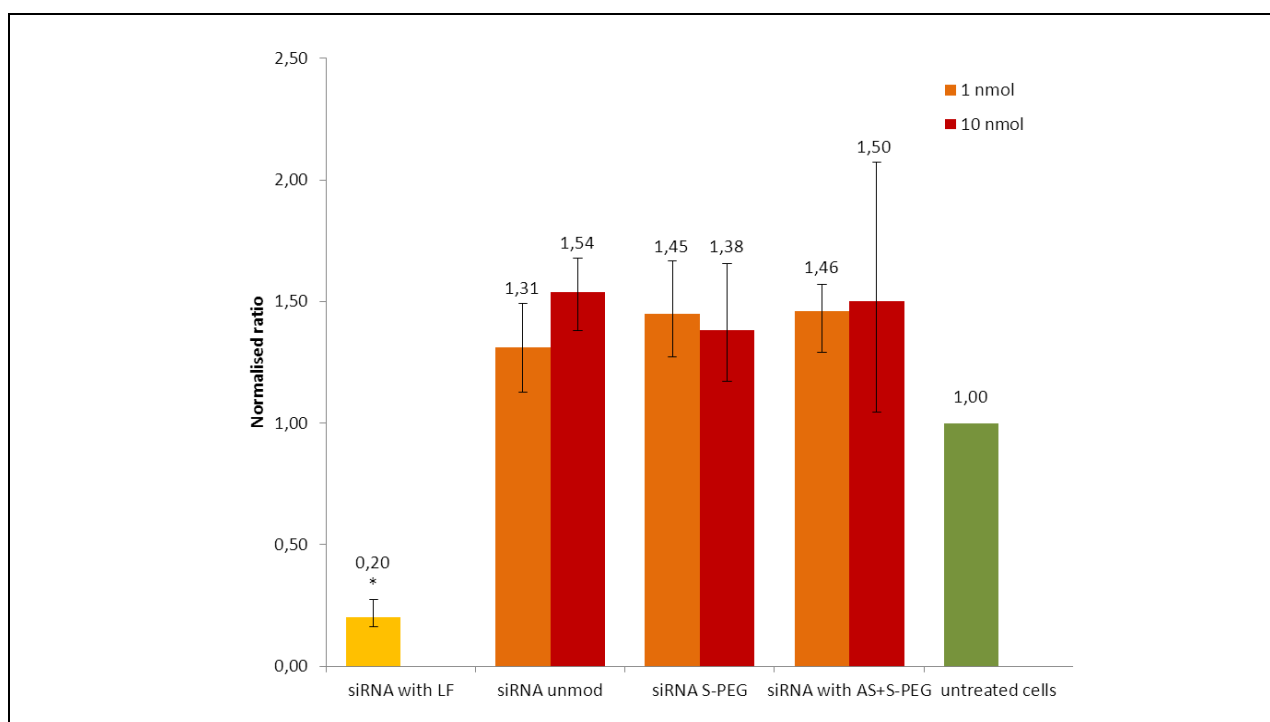
Fig. 4.12: Expression of the Bcl-2 gene after transfection of anti-Bcl-2 siRNA without a transfection reagent in reference to untreated cells.

5   Discussion

5.1   siRNA and conjugates

5.1.1   siRNA synthesis

The anti-Bcl-2 siRNA strands were synthesised according to the standard RNA synthesis methods. The synthesis generally consists of four steps: detritylation, coupling, capping and oxidation. These steps are essential to bind every single nucleotide unit to the previous one (Fig. 3.02). The synthesis proceeds from the 3' to the 5'-end.

The synthesis cycle starts with the dissociation of the DMT group from the nucleotide in acidic conditions (A). The leaving group, an orange cation, can be measured spectrophotometrically. This ability was used as an in process control of the synthesis. Coupling of the next phosphoramidite nucleotide takes about 15 minutes (B). The coupling of RNA lasts longer than that of DNA as there are sterical constraints due to a silylether protecting group at the 2'-OH position. During the coupling reaction the diisopropylamine dissociates from the phosporamidite, cyanoethyl groups stays as protecting group. Tetrazol or a related chemical is used as catalyst to facilitate this reaction. As a result coupling rate of 95-100% can be achieved. In the next step, capping, free 5'-end OH-groups are acetylated to avoid further reactions (C). Oxidation of P(III) to P(V) is effected by iodine/water/pyridine in THF (D). The phosphortriester is stable against hydrolysis which is important during detritylation. Graphical representation of the synthesis can be found in section 3.1.1, page 18.

5.1.2   Analytics of the siRNA

Identity and purity of the synthesised siRNA were analysed with gel electrophoresis, HPLC and ESI-MS. Gel electrophoresis enabled comparison of sample migration to sequences of known length. However as gel migration depends partly on the sequence, it is not possible to determine exactly the absolute length. Purity of the samples can be analysed and shorter sequences of the siRNA have been observed in some cases, which is common after an

automated synthesis on the synthesiser. Nevertheless, purification of the compounds with gel electrophoresis showed not to be effective due to low amount of gathered sample and insufficient purity after separation.

ESI-MS is a technique to obtain the exact mass of a molecule in a diluted sample. During the analysis, the sample is dispersed in a fine aerosol forming ions that are accelerated in an electric field and depending on their detection, the mass can be determined. This method enabled confirmation of identity of the synthesised siRNAs. It could be proved that the sequences were synthesised as desired with the right length, which was an important factor to affirming the gene silencing efficiency.

Purity of the samples was analysed with HPLC. After optimisation of the HPLC methods in terms of acetonitrile gradient, it was possible to separate the different compounds, purify them in semi-preparative scale and accordingly analyse either with ESI-MS to find out the molecular weight of the specific compound, or repeatedly with HPLC to confirm the identity. Purification of the compounds was very efficient and adequate amounts of the applied sample could be obtained. Optimisation of the HPLC was necessary depending on properties of the samples. Methanol was used at first as the apolar compound of the mobile phase, however a gradient elution of acetonitrile delivered better chromatograms and therefore has been used for all analysis.

5.1.3 Characterisation and analytics of the siRNA-PEG conjugates

Two distinct approaches for covalent conjugation of PEG to the siRNA were established. PEG chains were aimed to be attached either at the 3'- or at the 5'-end of the oligonucleotides. To modify the siRNA at the 5'-end we used a PEG-phosphoramidite building block (HEG18-spacer) which is appropriate for automated RNA synthesis. It reacts with the 5'-OH at the end of the synthesis at the synthesizer. These oligonucleotides showed one peak in HPL chromatograms and one band on the PAGE gel. The analysis with ESI-MS revealed that the HEG18-spacer failed to be connected to the oligonucleotide. Possible reason is a too short coupling time at the synthesizer. Improving of the attachment could be possibly done either by increasing the coupling time or concentration of the building block or by addition of more potent catalysts.

Reaction of NHS-activated esters of polyethylene glycol with aminolinker at the 3'-end showed to be successful. The reaction was commonly performed at pH 7-9, a pH at which primary amines of the aminohexyl-linker easily react with NHS esters to form stable amides. A high surplus of PEG-NHS ligand was shown to be necessary for achieving high yields. An additional band on the PAGE gel and an additional peak in the HPLC chromatogram appeared representing the product of the conjugation reaction. An efficient conjugation of siRNA with PEG was confirmed with ESI-MS by calculation of the molecular weight of the examined sample and comparison to expected molecular mass of the reaction product. The PEGylated siRNA strands were purified with HPLC. A PEGylation yield of approximately 75% of aminohexyl-linked siRNA was achieved. Understandably, no attachment of PEG was achieved with the incompletely attached synthesis side product, where a nitrophenyl-group stayed attached to the aminohexyl-linker.

Since conjugation with polyethylene glycol chains at the 3'-end proved to be efficient, optimization of HEG-attachments was not effectuated. The attachment site of the PEG is arguably of minor importance for functional assays.

5.2 Cell culture assays

Modified and unmodified siRNAs were transfected into mammalian cells and the knockdown of the target Bcl-2 mRNA was investigated with both Dual-Luciferase Reporter Assay and RT-qPCR.

The principle of the Dual-Luciferase Reporter Assay is based on the measurement of both Firefly and Renilla luciferases of each sample. A plasmid, psiCHECK-2, encoding the Bcl-2 gene along with the Firefly and the Renilla luciferases was transfected into MCF-7 breast cancer cells. The Renilla luciferase is used as the experimental construct; its gene is fused to the gene of interest. As a result, a change of expression of the target gene, in this case the Bcl-2 gene, changes the expression of the Renilla gene. Firefly luciferase is considered as internal standard. Accordingly the Renilla/Firefly ratio enables the analysis of gene silencing by transfected siRNA. It as well allows normalisation from effects such as variations in transfection efficiency and cytotoxicity. The Stop&Glo substrate is an important tool used to quench the Firefly luminescence to avoid an interference with Renilla luminescence. A dual

injector luminometer is necessary for the procedure due to short signal half life of the reagents. The assay was performed several times and the reported results are from a representative example.

The most important problem we encountered during the test was poor transfection of the psiCHECK-2 plasmid into the cells, which complicated interpretation of the results. As a result from the low luminescence levels measures, the standard deviation ended up being too high and the knockdown of the target gene was not significant when calculated with the student's t-test. Bcl-2 down-regulation was achieved only when a standard transfection reagent was used with siRNAs to enhance cellular uptake. Naked PEG conjugates showed only a low extent of down-regulation. No difference in efficiency between unmodified siRNA, siRNA with only sense-modified strand and siRNA which had both strands attached to PEG was found.

RT-qPCR enabled exact quantification of endogenous mRNA levels in cells with high sensitivity. Extracted mRNAs were transcribed into cDNA and amplified with PCR. The quantification of a gene of a cDNA of interest was enabled with a dye, Eva Green (Biotium), which binds only to dsDNA. The fluorescence intensity increases with each amplification of the DNA product. The treshold is specific for each sample depending on the original amount of the sample. This method does not imply the necessity for transfection of a plasmid, which was an advantage. The siRNA were added with or without a transfection reagent, hence the comparison of the effect was more precise with low standard deviation between samples. Endogenous expression of the RNA Polymerase II gene was used as reference, since it is not influenced by the transfection of anti-Bcl-2 siRNA. Evaluation of the efficiency with the REST 2009 software has shown that the modification of siRNA with PEG did not lead to significant Bcl-2 gene silencing in absence of lipofectamine.

In conclusion, both of the cell culture assays have yielded to a similar result. Without transfection reagent, cellular uptake was minimal and siRNA did not manifest any significant influence of down-regulation of the gene of interest. Although different concentrations of siRNA have been applied, no significant difference in efficiency has been observed between the samples. Transfection of siRNA by means of a standard transfection reagent leads to a significant down-regulation of the gene of interest. The grade of modification was unimportant in regard of the effect. Gene silencing was attained with higher concentrations of siRNA more sustainably. Since we applied only two different concentrations, no full dose-

response relationship regarding the knockdown of the target gene with siRNA could be established, but higher, 10 nM concentrations were more efficient that 1 nM, indicating a dose-dependency.

5.3 Conclusion

The RISC, which is responsible for siRNA mediated gene silencing, has a generally low tolerance for chemical modifications, especially of the antisense strand. Thus, it was somehow surprising that no significant differences resulted in efficiencies of the unmodified, sense-modified, and fully modified duplexes. The PEGylation at the 3'-end is obviously sufficiently tolerated at both strands, having obviously no harming effect on down-regulation of the gene of interest by the siRNA. It can be speculated that the PEGylation influences membrane permeation when used in conjunction with lipoplex delivery, thus enhancing endosomal escape which is either inefficiently done with the small PEG chains we used, or is on the other hand offset by lower RISC recruitment through the structural modification.

The PEG-siRNA conjugates can be combined with standard liposomal or nanoparticle delivery systems for shielding from nuclease degradation and enhancement of pharmacokinetic parameters. The conjugates possess the same charge and can be equally loaded into positively charged vesicles as unmodified siRNA. The conjugation is compatible with other oligonucleotide modifications such as 2'-methylation or phosphorothioates for increasing enzymatic stability. Longer PEG chains and PEGylation at multiple sites remain to be evaluated for a possible enhanced membrane permeabilitation effect, for RISC recruitment, and for pharmacokinetic properties. If longer chains can be used in siRNAs, the molecular size could be raised above the renal filtration limit, and, together with a targeting strategy, enable therapeutic application of siRNA without complex multicomponent delivery systems.

6   List of abbreviations

ACN	acetonitrile
Ago-2	Argonaut-protein 2
AMA	methanolic methylamine/NH4OH; 1:1
AMD	age-related macular degeneration
apoB	apolipoprotein B
APS	ammonium persulfate
Bcl–2	B-Cell Lymphoma – 2
bp	base pair
cDNA	copy DNA
Cp	Crossing point
CPG	Controlled Pore Glass
DCI	dicyanoimidazol
ddH_2O	double-distilled water
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modified Eagle Medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DMT	dimethoxytrityl
DNA	desoxyribonucleic acid
DPC	dynamic polyconjugates
dsRNA	double-stranded RNA
EDTA	ethylenediaminetetraacetic acid
ESI-MS	Electrospray ionisation-Mass spectrometry
HBV	hepatitis B virus
HCV	hepatitis C virus
HEG	heptaethylene glycol
HF	hydrogen fluoride
HIV	human immunodeficiency syndrome
HPLC	High Performance Liquid Chromatography
LB	Lysogeny broth

LF	Lipofectamine^{TM}2000
MCF-7	Michigan Cancer Foundation - 7
miRNA	micro RNA
mRNA	messenger RNA
NAG	N-acetylgalactosamine
NHS	N-hydroxysuccinimide
PAGE	Polyacrylamide Gel Electrophoresis
PBS	phosphate buffered saline
PEG	polyethylene glycol
PEI	polyethylene imine
PPAR-α	Peroxisome proliferator-activated receptor alpha
RISC	RNA-induced silencing complex
RNA	ribonucleic acid
RNAi	RNA interference
RSV	respiratory syncytial virus
RT-qPCR	Reverse Transcriptase – quantitative Polymerase Chain Reaction
shRNA	short hairpin RNA
siRNA	small interfering RNA
SNALP	stable lipid nucleid acid particles
TBE	Tris/Borate/EDTA
TEA	triethanolamine
TEAA	triethylammonium acetate
TEMED	N, N, N', N'-tetramethylethylenediamine
THF	tetrahydrofuran

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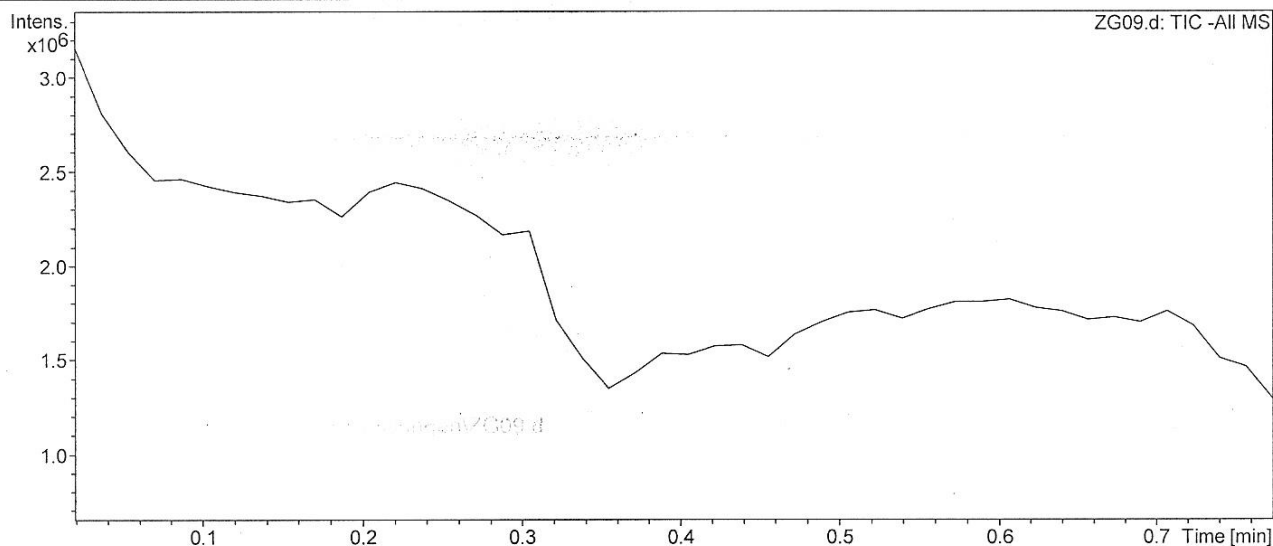
Appendix

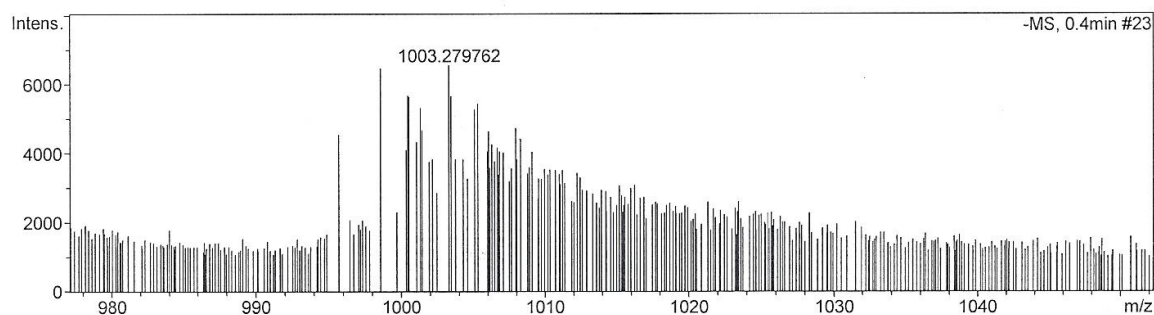I) ESI-MS of a bcl-2 siRNA antisense strand:

Acquisition Parameter

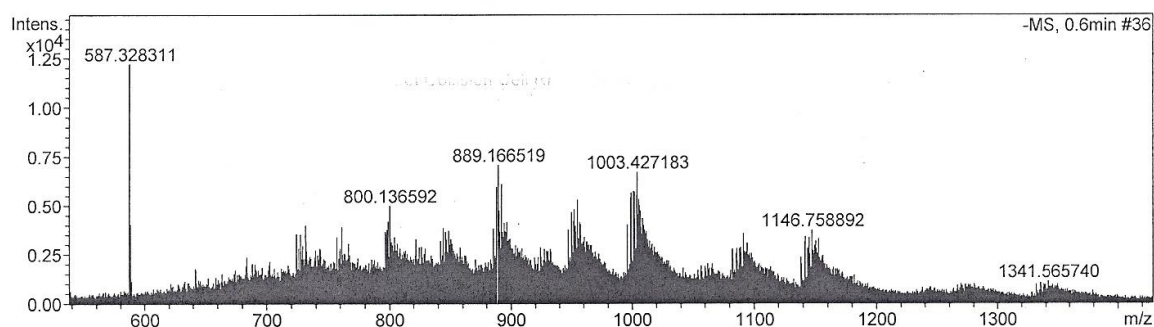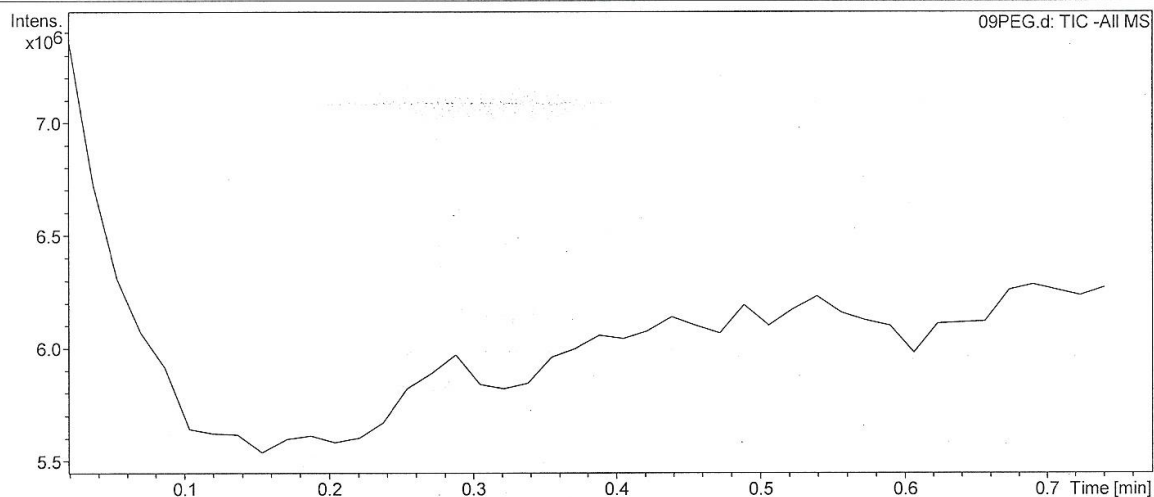|					
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Scan End	2000 m/z	Set Collision Cell RF	500.0 Vpp	Set Divert Valve	Waste
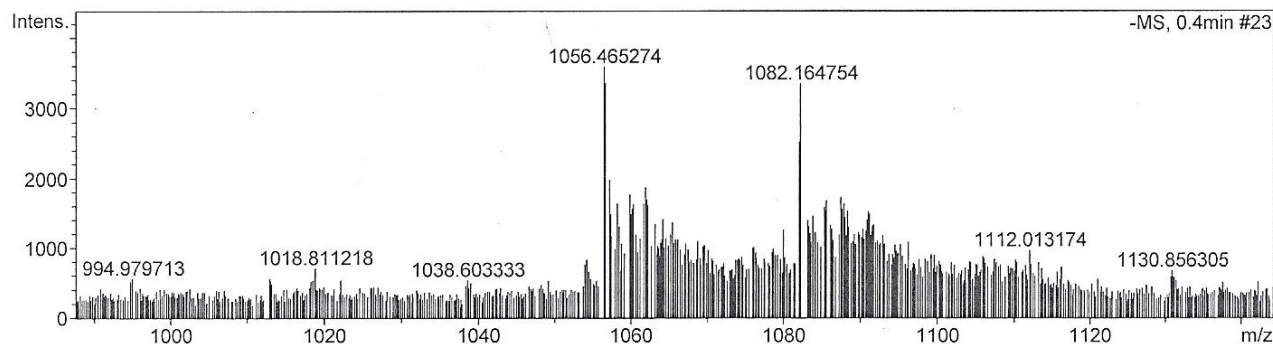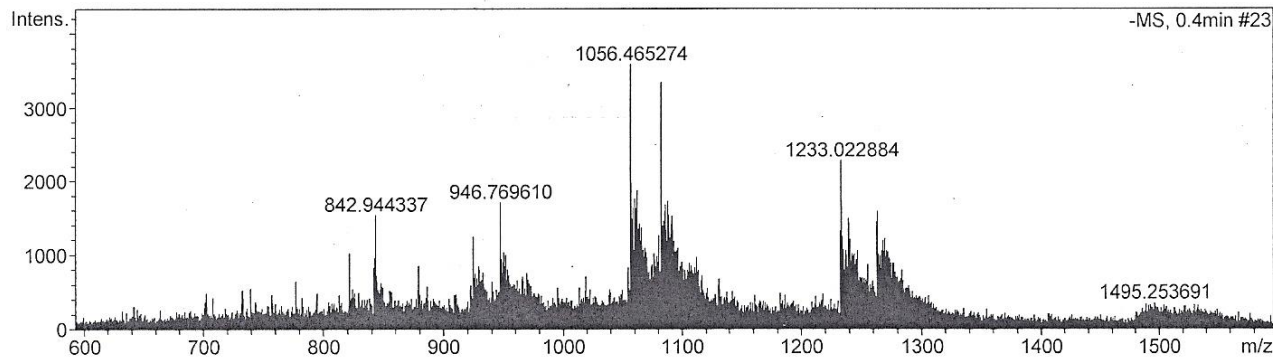
II) ESI-MS of a bcl-2 siRNA antisense strand linked to PEG chains:

Acquisition Parameter

Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.4 Bar
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Scan Begin	100 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	2000 m/z	Set Collision Cell RF	500.0 Vpp	Set Divert Valve	Waste

III) ESI-MS of a bcl-2 siRNA sense strand, connected to a HEG-spacer at the 5'-end:

Acquisition Parameter

Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.4 Bar
Focus	Active	Set Capillary	3200 V	Set Dry Heater	180 °C
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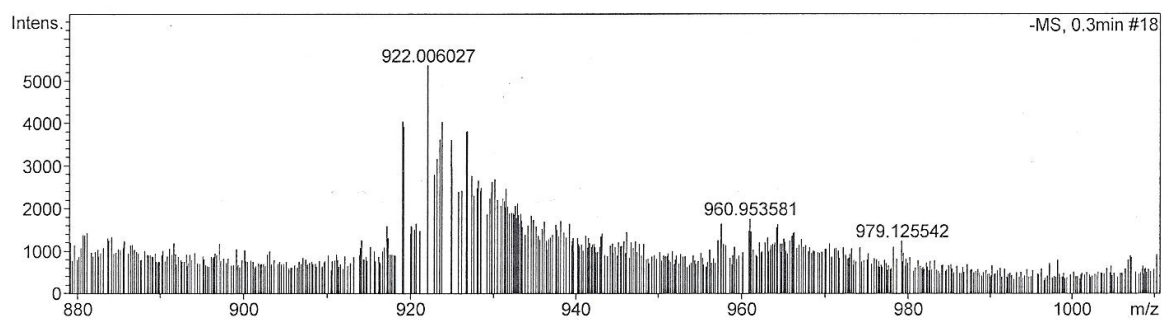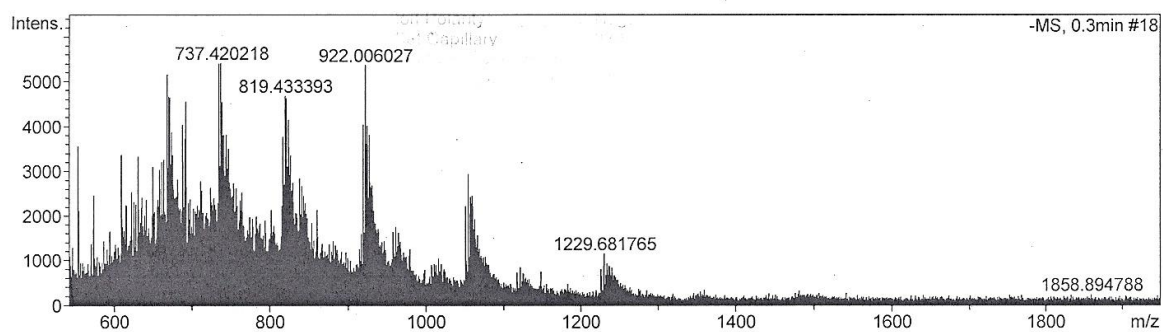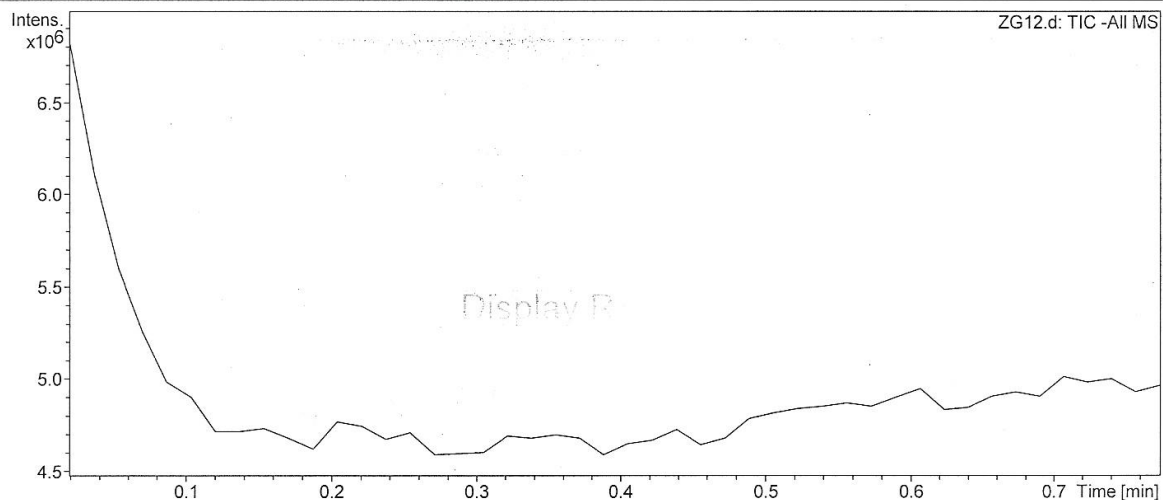
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Sprachen

Slowakisch:	Muttersprache
Deutsch:	Fließend in Wort und Schrift
Englisch:	Fließend in Wort und Schrift
Französisch:	Fließend in Wort und Schrift
Portugiesisch:	Gut
Japanisch:	Grundkenntnisse