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DIPLOMARBEIT

Titel der Diplomarbeit

„Influence of Covalently Conjugated Monodisperse Polyethylene Glycol Chains on in Vitro Properties of Antisense Oligonucleotides and Antisense Phosphorothioates“

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Contents

Kurzfassung

Gene silencing ist ein wichtiger Prozess in der Unterdrückung unerwünschter Genexpression durch die Blockierung der mRNA-Aktivität. Antisense Oligonukleotide wurden entwickelt, um an Ziel-RNA durch Watson-Crick-Basenpaarung zu binden. Sie verändern die Funktion der Ziel-RNA durch verschiedene Mechanismen, wie transkriptionalen Arrest.

Phosphorothioate sind eine der ersten Generationen von chemisch modifizierten Antisensemolekülen und werden als Rückgrat-modifizierte Arzneimittel bezeichnet. Dabei ist ein nicht-bindendes Schwefelatom im Phosphat-Rückgrat durch ein Sauerstoffatom ersetzt. Phosphorothioat-Substitutionen erhöhen die Resistenz gegen Nukleaseabbau und zeigen verbesserte pharmakokinetische Eigenschaften. Dennoch ist die Zellaufnahme nach wie vor eine große Barriere für die therapeutische Verwendung von Antisense Oligonukleotiden. Antisense Oligonukleotide mit kurzen Polyethylenglykolketten (PEG_{12}) wurden synthetisiert und deren Auswirkungen auf die Zellaufnahme in Säugetierzellen und ebenso deren Herunter-Regulationsaktivität auf die Zellen untersucht. Anti-Bcl-2- und anti-Galectin-oligodesoxyribonukleotide wurden für diese Experiment eingesetzt. Zunächst wurden anti-bcl-2- und anti-Galectin Phosphordiester und Phosphorothioate synthetisiert und gereinigt. Danach wurden PEG-Ketten definierter Länge mittels einem einfachen Biokonjugationsprozess am 3'-Ende und 5'-Ende der Oligonukleotide verknüpft. Die Analyse der Reinheit der Konjugate erfolgte mit Gelektrophorese und zeigte circa 50% Ausbeute, während mit Phosphorothioaten eine bessere Produktbildung am 3‘-Ende erzielt wurde. Die Analyse und Reinigung von den konjugierten Oligonukleotiden wurde mit HPLC und PAGE durchgeführt. CD-spektroskopische Analysen zeigten keinen signifikanten Unterschied zwischen Sekundärstrukturen von modifizierten und unmodifizierten Oligonukleotiden. Determinierung der Schmelztemperatur hat ebenfalls bewiesen, dass die PEGylation keinen Effekt auf die Affinität zum Gegenstrang ausgeübt haben.

Die Herunter-Regulation des Targetgens durch die modifizierten Oligonukleotide wurde mit einem Dual-Luciferase Reporter Assay in der Säugetierzelllinie MCF-7 untersucht. Im Vergleich zu den unkonjugierten, waren die konjugierten Phosphordiester Antisense Oligonukleotide gleich wirksam. Modifizierte Phosphorothioate führten zu einer effizienteren

herunterregulierung, wenn sie in hohen Konzentrationen wie 10 pMol eingesetzt wurden. Anschließend wurde der Einfluss von den Konjugaten auf die Zellproliferation und Zelltoxizität mittels MTT-Test untersucht. Es wurde herausgefunden, dass die PEGylierung keine zusätzliche Toxizität hervorgerufen hat, und in einigen Fällen wurde sogar eine geringe schützende Wirkung nachgewiesen. Die Daten zeigten, dass die Bindung von kurzen PEG-Ketten mit der pharmakologischen Wirkung von Antisense Oligonukleotiden nicht interferiert. Es könnte eine einfache Methode sein, um die pharmakokinetischen Eigenschaften zu verändern und möglicherweise die Toxizität von Phosphorothioat Oligonukleotiden zu vermindern.

Abstract:

Gene silencing is an important process in the suppression of unwanted gene expression by the blockage of mRNA activity. Antisense oligonucleotides were designed to bind to the

target RNA through Watson-Crick base pairing interaction and alter the function of target RNA by various mechanisms such as transcriptional arrest.

Phosphorothioates are one of the earliest generations of antisense chemical modifications and are referred as backbone-modified drugs, in which non-binding phosphate-backbone contains sulphur atoms instead of oxygen atoms. Phosphorothioate substitution greatly increases resistance against nuclease degradation and shows improved pharmacokinetic properties. Nevertheless, the cell uptake is still a major barrier in the therapeutic use of antisense oligonucleotides.

I designed antisense oligonucleotides with short polyethylene glycol (PEG_{12}) substitution and examined their effect on cell uptake into mammalian cells and their down-regulation activity. Antisense agents targeted at bcl-2 and galectin were used for this work.

At first, anti-bcl-2 and anti-galectin phosphordiesters and phosphorothioates were synthesized and purified. Afterwards PEG chains with defined lengths were linked at the 3'-end and 5'-end of oligonucleotides by a simple bioconjugation process.

The analysis of purity of the conjugates using PAGE demonstrated circa 50% conversion with phosphorothioates showing better reaction yields than phosphordiesters. The analysis and purification of conjugated oligonucleotide was performed with HPLC and PAGE. CD spectroscopic analysis showed no significant difference between secondary structures of modified and unmodified oligonucleotides. Determination of melting temperature likewise proved that PEGylation did not have an effect on counter strand affinity.

The down regulation of the targeted gene by the modified oligonucleotides was examined with a Dual-Luciferase reporter assay in the mammalian MCF-7 cell line. Compared to their unmodified counterparts, PEGylated phosphordiester antisense oligonucleotides were equally effective. PEGylated phosphorothioates resulted in more efficient target down regulation when applied in high concentrations. Subsequently, the influence of conjugates on

cell proliferations and cell toxicity was examined by MTT-test which, showed that no additional toxicity was caused by PEG. In some, even a small protective effect was detected.

The data demonstrates that attachment of short PEGyl chains does not interfere with the pharmacological effect of antisense oligonucleotides, and may be an easy method to alter pharmacokinetic properties and possibly mitigate the toxicity of phosphorothioate oligonucleotides.

1 Introduction

1.1 Antisense Oligonucleotide and Antisense Phosphorothioate

Antisense oligonucleotides are short single-stranded, usually chemically modified nucleic acids with around 10 to 30 nucleotides in length [1]. The potential of oligonucleotides for therapy has first been shown by Zamecnik and Stephenson [2,3,4]. They observed antiviral activity of 3' and 5'ends modified oligonucleotides, which was complementary to a target sequence of Rous sarcoma virus [2,4]. Antisense oligonucleotides affect protein synthesis at the translational level and lead to transcriptional arrest [1,2].

The molecular target for antisense drugs is RNA; this binds to the targeted RNA through Watson Crick base pairing [1].

The repression of mRNA takes place by various mechanisms, in figure 1-01 [5].

The more important mechanism is the activation of ribonuclease.

In order to activate RNase H, antisense oligonucleotides need at least a stretch of five to seven 2'- unmodified nucleotides [5]. RNase H is a ubiquitous enzyme that binds to DNA-RNA hetero-duplexes and causes degradation of the RNA part [5]. It thus enables the binding of DNA-oligonucleotide to other mRNA molecules [5]. Activation of RNAse H is highly sequence specific; an ideal ASO should be a perfect match of the base pairing to the target and more than 3 base pairings that do not match to all other genes [5].

Non-depleting antisense oligonucleotides, with fully modified 2' positions of the ribose do not lead to destruction of the target mRNA, but they exercise their effectiveness by tight binding to target mRNA and influence translation, splicing and can hybridize to microRNAs, preventing their effects [5]. Translations inhibitors can also prevent the binding of the ribosome and hence to lead translational arrest [5].

Antisense oligonucleotides that do not degrade mRNA can be used to influence mRNA processing after transcription, including the splicing process in which the non-coding parts of the so-called introns are excised by binding to splice injection. Additionally, they can prevent the formation of mature mRNA, inhibition of 5'-capping and polyadenylation, relevant components of the RNA-processing, which can also be inhibited by hybridization of an

oligonucleotide to the untranslated (UTR) region [5]. The utilization of oligonucleotides as therapeutic drugs for gene silencing of targeted gene sequences is an important approach for the treatment of various diseases as, for example, cancer [12].

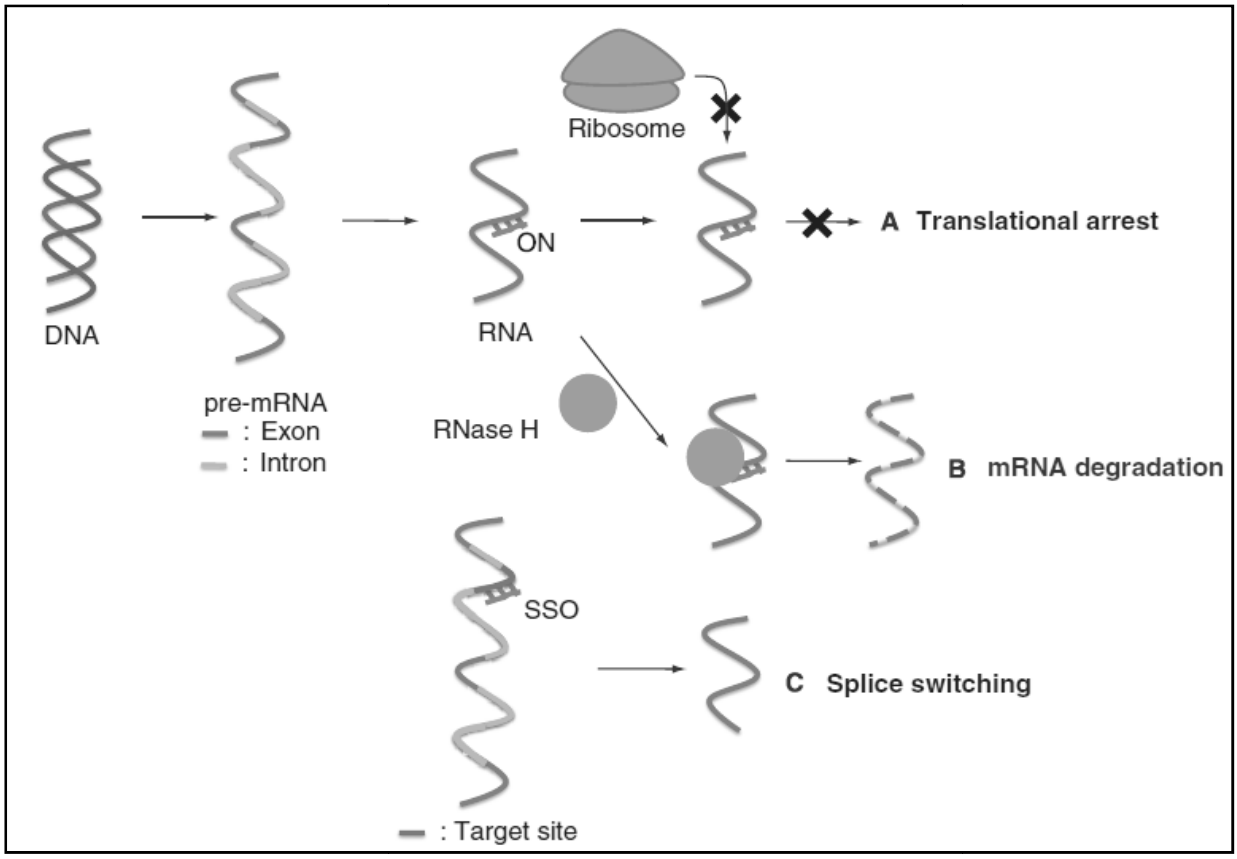

Figure 1-01[2]: Scheme representation of the antisense mechanism: Prevention of the binding ribosome, destruction of the mRNA by activation of ribonuclease, inhibition of the Formation of mature mRNA due to interfering with splicing.

Wild-type oligonucleotides are readily degraded by exonucleases and endonucleases. It was also found that oligonucleotides possess weak intrinsic binding affinity, and are rapidly eliminated through the kidney, which effectively prevents their therapeutic application. Therefore, a large number of different chemical modifications were developed to enable the therapeutic application of antisense oligonucleotides, among others, phosphorothioates [1,4,6,9].

General chemical modifications have been classified into three groups: backbone-modified oligonucleotides, sugar-modified oligonucleotides and nucleobase-modified oligonucleotides [2].

Phosphorothioate oligonucleotides belong to backbone-modified oligonucleotides in which an oxygen atom of phosphorus is substituted by a sulphur atom. There are regarded as a first generation of oligonucleotide-modifications and are able to activate of RNase H [1,2].

Phosphorothioates show distinct stability against nuclease degradation. Phosphorothioates are easy to synthesize and possess moderate systemic toxicity. In addition, they possess good pharmacokinetics features [1,2,7,9]. On the downside, it was recently demonstrated that phosphorothioates in high concentrations display toxic effects [3]. This effect is dependent on the dose and inhibits target mRNA sequence specifically and thus leads to a lesser secondary effect [3].

High plasma protein binding of phosphorothioates leads to prolonged plasma clearance, thus rapid elimination is impeded through slow metabolism and all of these facilitate receptor-binding and cellular uptake, and this result in *in vivo* pharmacological effects [1]. Antisense phosphorothioates cannot overcome the blood-brain barrier, but are distributed after parenteral applications in most peripheral tissues [11].

1.2 Challenges of Antisense Phosphorothioate Therapeutics

Several antisense oligonucleotides containing phosphorothioate modifications are at present in clinical trials for the treatment of a series of diseases such as viral infections (preventing the viral replication of infected cells without an influence of the cell metabolism), inflammatory disorders, cancer, neurodegenerative diseases and cardiovascular disorders [8,9,11]. Antisense phosphorothioates are negatively-charged and cannot penetrate the cell membrane by passive diffusion, whereas positively-charged particles can cross cell membranes by endocytosis [9,10]. Positively charged particles can serve as cationic carriers and are an important strategy to enable *in vitro* cell uptake which circumvent the intrinsic problems [9]. The identification of the pharmacokinetic and pharmacodynamic properties of phosphorothioates is pivotal for optimization of their utilization in therapy [8]. It was demonstrated that the liver possesses a relevant role in metabolism and disposition of PS-ODNs, similarly, the kidney, spleen, bone marrow, and lymph nodes are organs in which a high proportion of the does ends up [8]. To appraise phosphorothioate oligonucleotides, *in vivo* clearance from their *in vitro* metabolism the identification of hepatic metabolism contribution is of crucial importance [8].

To date, there are two phosphorothioate drugs, approved by the FDA (Food and Drug Administration): Vitravene™ (Formivirsen) was approved in 1998 for the treatment of cytomegalovirus retinitis, an AIDS-associated-disease, and recently mipomersen against familial hypercholesterolemia [11,12].

Phosphorothioates show a lower binding affinity toward complementary strand weak duplex binding affinity against ssDNA or ssRNA and also a lower melting temperature (Tm) compared with their phosphordiester counter parts [2,7].

Backbone-modified oligonucleotides have a sequence-independent toxicity profile [6]. The effects include increased coagulation time, pro-inflammatory effects and activation of the complementary system [6]. Oligonucleotides with certain sequences lead to stimulation of the immune system by interaction with Toll-like receptors or cause an unwanted side effect through direct binding to protein [6].

There are also two modified phophorothioate antisense drugs that were successful in clinical attempts; mipomersen and custirsen are so-called gampers, which possess centrally located 10 nucleotides and 2'-modified flanking regions [12]. The central nucleotides allow RNase H cleavage of mRNA and the modified flanks improve stability and reduce adverse side effects [12].

Mipomersen is a 2'-MOE phosphorothioate antisense gamper, targeted at apolipoprotein B 100, a protein that is responsible for the production of low-density lipoprotein [12]. Custirsen is an anti-apoptotic chaperone protein [12].

Duchenne muscular dystrophy is a hereditary muscle disease that is characterized by the disturbance of the reading frame in the dystrophin gene [13]. This leads to rapid, progressive muscle tissue degradation [13,14].

Transfection of an antisense oligonucleotide into cultured myocytes induced exon 19 skipping and leads to the expression of a functional dystrophin. A clinical delivery method into the skeletal muscles of DMD patients was not introduced [13,14], but it is suggested that the tissue damage found in DMD muscle cells allows for a certain extent of cellular uptake [13].

ISIS 14803 is a phosphorothioate oligonucleotide in the traetment of Hepatitis C virus infection [15].

1.3 B-Cell Lymphoma 2 (Bcl-2) Protein:

Apoptosis is an important biochemical process characterized by the activity of proteolytic effector caspases [16]. Effector caspases split specific aspartate residues and lead to the loss of housekeeping proteins that are required for cellular viability [16].

Bcl-2 is an anti-apoptotic representative belonging to the family of anti-apoptotic and pro-apoptotic proteins that regulate apoptosis and are involved in different human diseases [16]. Bcl-2 is over expressed in a variety of cancers [17]. Bcl-2 leads to the decrease of apoptosis, thereby improving resistance of tumor cells to cytotoxic chemotherapy drugs and gamma irradiation [16,17].

A strategy to overcome apoptotic resistance is the inhibition of Bcl-2 [17]. This leads to the programmed cell death operating again in a normal state and the tumor cells regaining sensitivity to chemotherapy and radiotherapy [17].

Currently, bcl-2 inhibitors are found in preclinical and clinical trials and are promising approaches to suppression of cancers [17,18]. Oblimersen is a synthetic oligodesoxyribonucleotide consisting of 18 bases and leads in diseases such as lymphoma, melanoma, breast, colon, ovarian, and prostate carcinoma cell lines to inhibition of bcl-2 mRNA [16,18].

The antitumor activity of docetaxel was improved by oblimersen through a dose-dependent inhibition of bcl-2 protein expression [16].

1.4 The roles of Galectin in various diseases:

Galectin belongs to Ca-independent soluble lectins. A common characteristic of these lectins is that they all contain carbohydrate recognition domains (CRDs) [19,23]. They have high affinity to ß-galactosidase and they play an important defensive role in the innate and required immune system [19]. Galectin-1 is involved in regulation and pathogenesis of autoimmune diseases and leads to dysregulation of B-cells and or T-cells [20].

Galectin exerts its effects either extracellularly or intracellularly [21,23]:

Extracellularly, it interacts with the cell surface and triggers signals, which ultimately lead to the control of immunity, migration and angiogenesis [22,23].

Intracellular galectin may exercise its effect through control of tumour transformation, proliferation and survival [23].

It has been shown, that galectin is part of an innate immune defence mechanism in mammalian cells against invasion of bacteria, fungi and viruses plays an important role [19]. For instance, galectin-1 shows anti-inflammatory effects through inhibition of leukocyte infiltration [19]. It was also shown that the pathogenesis of the influenza virus relies on binding of galectin-1 [19].

Nevertheless, galectin leads to resistance of cancer cells against apoptosis and causes tumorgenicity [21].

Galectin-1 is over expressed in various cancers such as colon cancer, breast cancer, hepatocellular cancer, prostate cancer, pancreatic ductal adenocarcinoma and galectin-1 over-expression is implicated in lung cancer [21,22,24,].

Thus suppression of overexpression of galectin-1 is an important therapeutic approach of to various cancers [24].

1.5 PEG conjugation

PEGylation is an important strategy for drug delivery and shows potential for therapeutic use [27]. PEGylation was first defined in the 1970s by Davies and Abuchowsky [25]. PEGylation is the non-toxic, constitute covalent binding of a polyethylene glycol polymer to proteins and oligonucleotides, thereby improving its pharmacokinetic properties [26].

Polyethylene glycol polymers shield the surface charges of protein and oligonucleotides from interaction and subsequent degradation by enzymes [26,27]. This results in prevention of interaction between particles, which, in turn, leads to rapid clearance in liver and lung. Subsequently, desired extended circulation lifetime is reached [27].

Through attachment of polyethylene glycol polymers improved absorption, bioavailability, and biodistribution of biological macromolecules results, whereas toxicity and immunological effects are generally reduced [26].

The covalent bond of PEG to the hydroxyl groups of nucleic acid was accomplished either directly or through a spacer link, which leads to stability against exonuclease and endonuclease [25].

Nevertheless, the utilization of PEG, by interaction between opposing charges of oligonucleotides with PEG, has resulted in less endosomal release of oligonucleotides, meaning that there is a decrease of transfection efficiency [27].

A PEGylated oligonucleotide, pegaptanib, which was approved by the FDA, consists of the 28mer oligomer aptanib for treatment of age-related macular degeneration of the retina, and PEG attachment which was afforded through a branched pentamino linker [25].

2 Aim

Gene knockdown is an essential approach for the potential treatment of wide range of disorders such as cancers. For this purpose, antisense oligonucleotides have proven to be successful for suppression of disregulated genes and thus treatment of diseases. As has been shown in various studies and clinical development, one of the major challenges of the therapeutic use of antisense oligonucleotides is to overcome their insufficient bioavailability.

In this diploma thesis, several short polyethylene glycol polymers with defined length were to be covalently linked at the 3’-and 5'-OH groups of antisense phosphorothioate oligonucleotides. An efficient protocol for the reaction of NHS-activated PEGyl chains to aminohexyl functionalized oligonucleotides was aimed to be developed. Successful PEG attachment should be analyzed by polyacrylamide electrophoresis (PAGE), to enable a proper assessment of the conjugation reaction.

A HPLC method should be established to be used for analyzing PEGylation yields.

CD spectroscopy was to be performed in order to examine the secondary structure of nucleic acid and for the measurement of melting temperature for the assessment of the affinity of PEGylated nucleic acid to the complementary strand.

The influence of amphiphilic PEG on *in vitro* cellular uptake should be investigated.

Using cell culture models, the effect of the PEGylation on the gene silencing activity should be assessed by comparison to isosequential unconjugated oligonucleotides.

Subsequently, an MTT-test, in order to examine the toxicity profile of modified nucleic acids was aimed to be conducted.

3 Materials and Methods

All reagents were supplied by Sigma-Aldrich, Serva, and Fisher Scientific in standard research grade. Cell culture media and supplements were purchased from, Gibco,FCS from PAA. Tissue culture flasks and plates were obtained from Greiner Bio-One.

3.1 ASO and PS synthesis and Purification

3.1.1 ASO and PS synthesis

ASO and PS Synthesis were carried out on a fully automatic Polygen 10 column DNA/RNA synthesizer. The synthesis of anti-Bcl-2-ASO, anti-galectin-ASO and anti-Bcl-2-PS, anti-galectin-PS was performed using standard DNA synthesis protocols. As a solid phase, controlled pore glass was utilized, in which the first nukleotide was already attached to the 3'-end. The nucleotides contained protective groups: a phosphoroamidite group was already attached to the 3'-OH-end, a dimethoxytritylgroup was also bound to the 5'-end and acetylated free reactive groups were on the bases.

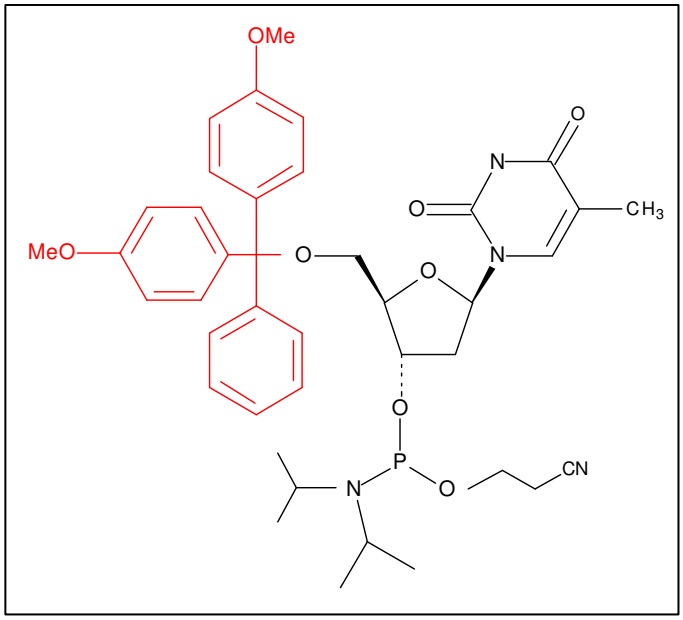

Figure 3-01: DMT-dT phosphoramidite; thymidin nucleotide for synthesis,at the 3'- end protected with 2-cyanoethyl-N,N'-diisopropylphosphoramidite, at the 5'-end dimethoxytrityl as protected groupe.

The synthesis of oligonucleotides was carried out according to the phosphoroamidite method. The synthesis was done from 3'- to 5'-end and consisted of four steps.

In the synthesis, dicyanoimidazole was used as an activating agent. For the binding of an amino linker at the 3' terminus, amino-on-CPG (SAFC) was utilized, which enabled the attachment of the first nucleotide. The synthesis of phosphorothioate was performed in a similar manner as DNA, with TETD (tetraethylthiuramdisulfide) as sulfurization reagent.

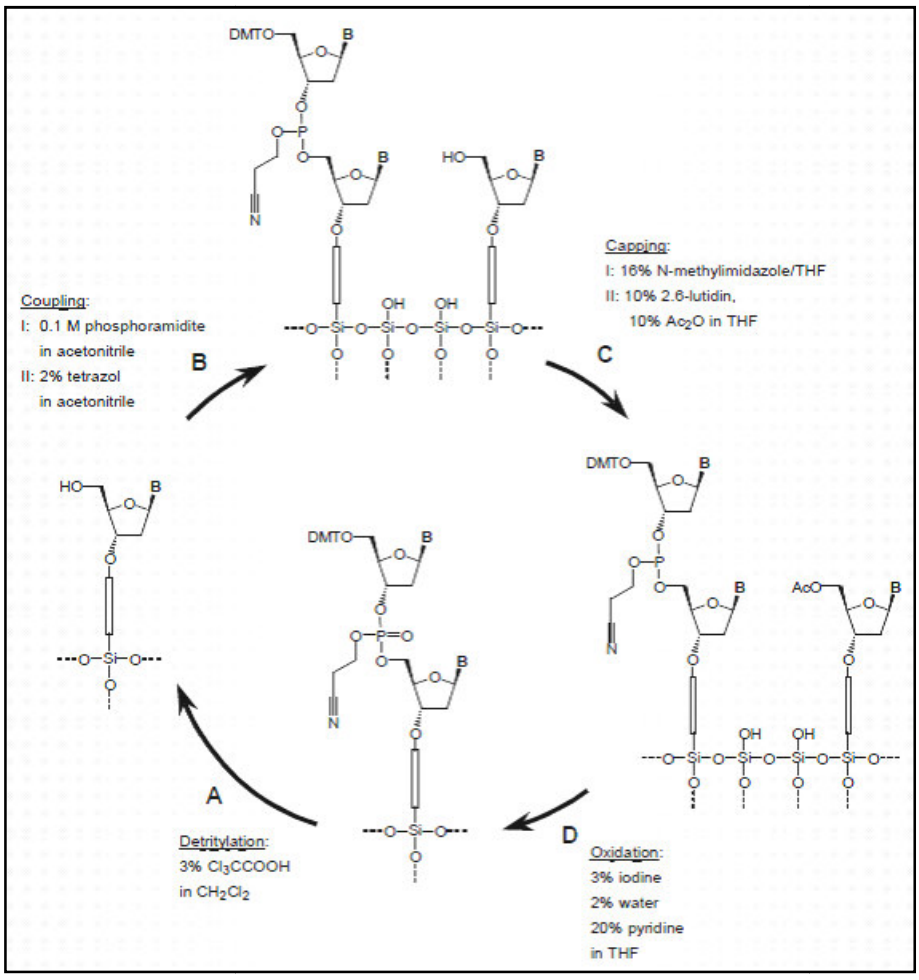

Figure 3-02: Graphical presentation of oligonucleotide synthesis consist of 4 steps for attachment of one nucleotide, Detritylation, Coupling, Capping, Oxidation.

3.1.2 DNA Cleavage, Reprocessing and Purification:

After the synthesis, the solid support was transferred from the Polygen synthesizer slider into a vial. The cleavage of oligonucleotides from the resin was afforded after half an hour by concentrated ammonia but for full deprotection of the bases the samples were heated in concentrated ammonia at 55°C for 16 hours. Initially, the supernatants were transferred to sterile reaction tubes. To verify complete cleavage the concentration was measured with a Nanodrop spectrophotometer (Thermo).

The major part of ammonia was removed by air flow, and then oligonucleotides were precipitated by addition of 5 µl ammonium acetate solution and isopropanol (to 1-5 ml) and cooled on ice for 30 minutes. After centrifugation (5 min, 12000g) the supernatants were removed and oligonucleotides pellets were washed with 75% ethanol. This process was repeated three times, the pellet was subsequently air dried and dissolved in 20-40 µl water. After concentration determination, the stock solution was diluted to 1mM.

3.1.3 Desalting of Oligonucleotides

- Purification with sephadex G-50

This method is used to separate low and high molecular weight molecules. Therefore, a cross-linked dextran gel is utilized. A column was filled with circa 3 ml sephadex G50, which was previously swelled in water for at least 2 hours, and then washed with water. 100 µl of the samples were pipetted into a column and eluted with water. Factions of 500 µl were collected in tubes, and then the oligonucleotide concentrations were determined at 260nm. Fractions containing oligonucleotides were concentrated and dissolved anew in water for further analysis.

3.2 Conjugation of ASO and PS with polyethylene glycol

MS (PEG) n reagents allow a simple and efficient modification of oligonucleotides. NHS was used as a reactive group for modification. The reaction of NHS activated polyethylene glycol with aminolinker was performed at PH 7-9 buffers, NHS-ester reagent react efficiently with the amino groups by nucleophilic attack, which leads to the forming of amid bound and the releasing of NHS. 5 nmol of the oligonucleotides (5 µl from a 1 mM solution) were dissolved in water (or PBS, Na-borate buffer). The pH were checked after the dissolving of the oligonucleotides and eventually adjusted with buffer. 250 mM stock solution of methyl-$(PEG)_{12}$-NHS reagents were used for attachment to oligonucleotides, 2, 5 or 50 equivalents of the linker were added 1:25 dilutions of stock were prepared with anhydrous DMF (table 3-1).

The reaction was carried out by stirring at room temperature for 2 hours; the samples were taken at certain time intervals of about 1 hour and analyzed by PAGE.

Samples (5 nMol)	Oligo-Stock (1mM)	Water µl	10X PBS µl	Linker (10 mM)
A+2 equiv.	5 µl	16.5	2.5	1 µl
B+5.equiv.	5 µl	15	2.5	2.5 µl
C+50.equiv.	5 µl	16.5	2.5	1 µl (from the 250 mM Stock)

Table 3-01: engaged amount of the each reagents to PEGylation. A,B,C: referred the samples, 2,5,50 equivalents of the inserted linker.

3.3 Analytics of the ASO, PS and conjugates

3.3.1 Polyacrylamide Gel Electrophoresis

- Preparation of a 20% acrylamide gel:

7.0 g urea was dissolved in 1.5 ml 10x TBE (Tris-borate-EDTA-Buffer), then 7.5 ml acrylamide stock solution (40%, 29:1 acrylamide:bis-acrylamide) was added. In order to dissolve urea, the mixture was heated in a microwave for a few seconds until the urea was completely dissolved and a clear solution was formed. After cooling down, 7.5 µl TEMED and 75 µl APS were added and which lead to polymerisation and the mixture was immediately applied into a gel forming sandwich, then it was shaped into ten slots using a 10-well comb. After polymerisation (circa 30 minutes) gel was pre-run at 150 V. As a buffer, 1x TBE was utilized.

- Preparation of the samples:

One nmol of the samples was mixed with 10 µl sample puffer (formamide buffer) and heated to 95°C for 3 minutes, immediately after; the samples were applied on a gel using a pipette or placed on ice. To follow the running position of the samples, a dyed sample buffer was used, which is a mixture of bromophenol blue and xylene cyanol. The gel was run at 150 V for circa 1.5 hours until the dyes indicated adequate sample migration.

- Methylene blue colouring and Detection:

After running, the gel was shaken in a 2% methylene blue solution for 30 minutes, consequently oligonucleoitdes bands were dyed, and then the gel was destained in water until the bands were clearly visible. The bands were digitalized with the calibrated Densitometer GS 710 scanner and analyzed using Quantity One software (BioRad).

- Separation and purification of samples from gel electrophoresis:

Purification of the samples was carried out by gel electrophoresis. Samples were loaded onto the gel. After the run was completed, oligonucleotide bands were visualized under UV light on a TLC plate with fluorescence indicator. Each band was cut out of the gel and the gel pieces were placed into tubes and cut into small pieces. For elution 1-3 ml TE was added and after shock-freezing the samples, they were shaken at 35 °C overnight.

3.3.2 Circular dichroismus

For CD experiments, a 200 µl or 150 µl oligonucleotide solutions in a 9µl or 4,5 µl concentration were prepared in 0.01 M Tris/ 0.15M NaCl buffer.(Table 3-02)

9 µM sample	4.5 µM sample
1.8 µl (1 mM) 20 µl buffer 178.2.µl $ddH_{12}O$	0.8 µl (1mM) 15 µl buffer 134.2µl $ddH_{12}O$

Table 3-02: preparation of samples, solution and buffer to CD experiments.

Melting temperature is determined as a measure of duplex stability and affinity for the counterstrand of oligonucleotides. The measurement can be done either by recording the UV absorption at 260 nm to 320 nm or the change in the CD value at a minimum or maximum. In both cases, a sigmoid curve arises.

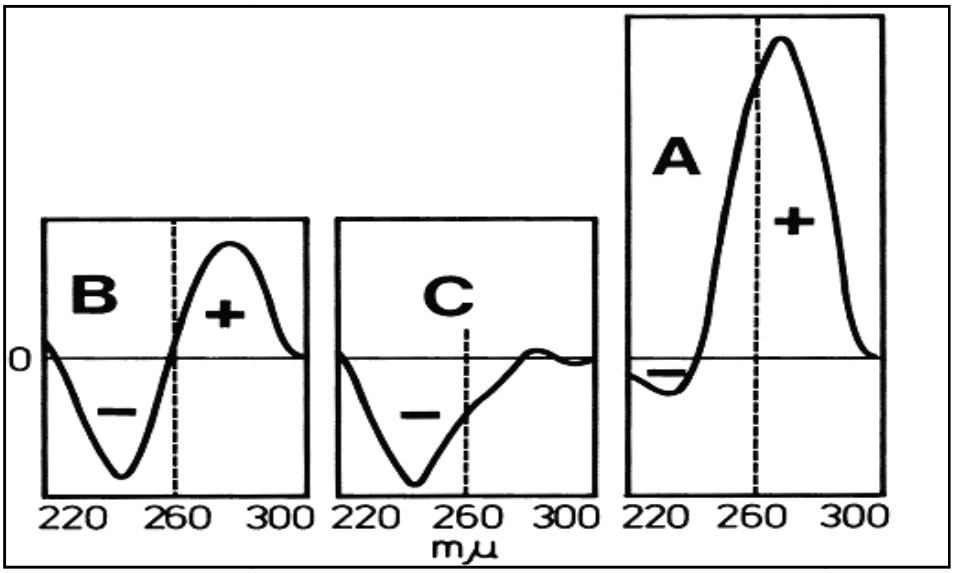

Figure 3-03: Scheme representatin of the three different conformations of Nucleic acids.

3.3.3 High performance liquid chromatography (HPLC)

The HPLC runs was carried out with a LaChrom L-7100 (Merck Hitachi) system and analyzed by EZ Chrome Elite software. The HPLC was utilized for samples analysis.

The HPLC was performed as described below:

Mobile phase A: 50 mM triethyalmmonium acetate buffer (TEAA), TEAA was prepared with RNase free, DEPC (diethylpyrocarbonate, 2%) treated water;

- Mobile phase B: methanol;
- Gradient elution was altered depending on the sample properties, usually 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.

3.4 Cell culture assays

3.4.1 Cultivation and cell seeding

The cells were cultivated in DMEM+GlutaMAX™ (Dulbecco's Modified Eagle Medium, Gibco) supplemented with 10 % FBS at 37 ˚C, 5 % CO_2 and an air humidity of 95 %. The cells were split at approximately 90 % confluence about twice a week.

For the cell experiments, the cell line MCF-7 was utilized (Michigan Cancer Foundation-7) obtained from the European Collection of Cell Cultures (ECACC). MCF-7 is a breast cancer cell line that was isolated in 1970 from a 69 year-old-Caucasian woman.

The medium was removed and the cells were washed with 3-5 ml of prewarmed PBS solution. After removing of PBS the cell layer was treated with 1 ml 1x trypsin solution. Thereby, avoiding direct contact with the cells, the trypsin solution was spread over the entire culture flasks area and the excess solution was removed. Subsequently, the culture flask was placed in an incubator at 37 ˚C for 5 minutes to disperse the cells. After incubation, the cells were detached from the surface, which was checked visually under a microscope.

After this, cells were filled up with medium to ca. 5 ml, from this suspension 10 µl were pipetted onto the Thoma counting chamber and the cells were counted.

Afterwards, the average was taken and multiplied with 40,000 to obtain the cell number per ml. The cell suspension was diluted to the required concentration.

3.4.2 Transfection process

White 96-well-plates were used for luminescence assays. In this case, 15 000

Cells were seeded into each well.

- Transfection mix:

Lipofectamine™ 2000 Opti-MEM®	In two concentrations 0.25 µl, 0.50 µl	Opti-MEM® Ad 75 µl
psiCHECK™-2 Opti-MEM®	 100 ng /well	Ad 75 µl per triplicate

Table 3-03: reagents for transfection

For the transfection, plasmid psiCHECK™-2 (Promega) with the cDNA of Galectin-1 or Bcl-2 cloned into the multiple cloning sites and Lipofectamine™ 2000 were mixed and diluted in Opti-MEM® according to the manufacturer's recommendation.

- Co-transfection mix:

For simultaneous transfection of reporter plasmid and oligonucleotides,

siRNA targeted against galectin-1 or Bcl-2 and PEGylated DNA (phosphorodiester and phashorothiate) in two different amount per well were used. All dilutions were prepared in PBS solution. As a control, unmodified DNA oligonucleotides were applied as well.

Samples (siRNA,P=O,P=S)	Sample concentration	OptiMem+psiCHECK	OptiMem+LP
1 pMol	0.3 µl (10 µM) per triplicate	0.1.µl.in.25.µl mixture	addition of 25 µl mixture
10 pMol	3 µl (10 µM) Per triplicate	1.µl.in.25.µl mixture	addition of 25 µl mixture

Table 3-04: reagents for co-transfection mix.

First, the samples were transferred into each well and followed by the pasmid-lipofectamine. After dilution, lipofectamine was allowed to stand for 5 minutes before being added to the samples. The transfection mix was incubated for 20 minutes before 50 µl of the cell suspension was pipetted into each well.

As a reference, cells were transfected with plasmid only. The plate was placed in an incubator for 24 hours.

- Treatment without a transfection reagent:

15 000 cells were seeded per well and plasmid complexed to the indicated amounts of lipofectamine was added. On the following day, the medium was removed and fresh culture medium was added. For examination of uptake in the absence of lipofectamine 20 µl of the samples with the indicated amounts of oligonucleotides (10 pMol, and 100 pMol) were added.

3.4.3 Cell lysis and Luminescence Readout

The luminescence readout was performed according to the instructions of the manufacturer. After two days of incubation the medium was removed and cells were washed with 1x 100 µl PBS, before being lysed with 20 µl lysis buffer per well. The culture flask was put on the shaker for 15 minutes at room temperature. Thereafter cells were examined for complete lysis monitor with a microscope.

Luciferase activity was measured by a TECAN Infinite M200Pro luminometer using TECAN i-control 1.7 software.

Required reagents for 96 wells for the Luminescence assay in the table below:

The reagents were obtained from Promega.

5x PLB	**LAR II**	**50X Stop & Glo® reagent**
-20 µl / well	-50 µl /well	-50 µl /well
-1:5 diluted in ddH2O	---------- ----------	-1:50 in Stop & Glo® buffer
-1920 µl for 96 well	-5300 µl for 96 well	-5300 µl for 96 well

Table 3-05: reagents for conducting Dual-Luciferase Reporter Assay

With the Dual-Luciferase® Reporter Assay System two measurements of a sample are carried out. psiCHECK-2 vector contains genes for the Renilla luciferase and the firefly luciferase. The first Luminescence signal is generated by firefly luciferase reporter by adding luciferase assay reagent II. Firefly enables the quantification of the measurement and also normalization of the transfection. Thereafter, Renilla luciferase reporter, which is indicative of knockdown of the gene of interest similarly, is measured by adding Stop & Glo® reagent.

3.5 Cell proliferation and Cytotoxicity assay

The MTT- test (EZ4U, Biomedical) is based on the reduction of the yellow water-soluble dye 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) into a blue-violet water-insoluble formazan. This method allows a distinction between living and dead cells, living cells convert the colourless tetrazolium in coloured formazan. For this method, intact mitochondria are required, which are inactive a few minutes after cell death.

The test was performed using the TECAN plate reader and i-control 1.7 software.

Day 1: MCF-7 cells were trypsinized, an antibiotic-free medium added, and according to 20, 000 cells/well diluted and 100 µl were pipetted into each well. As a control, 3 wells were left empty. The microtiter plate was placed for in the incubator 24 hours.

Day 2: The medium was removed and 80 µl of prewarmed fresh medium added. Therefore, 20 µl of the 1 pMol or 10 pMol oligonucleotides diluted in medium were pipetted into wells and incubated for another 24 hours.

Day 3: 100 µl fresh medium was pipetted into each well and the MTT-test performed according to the manufacturer's instructions.

- Reagents preparation:

Commonly, 20 µl of dye solution was added to 200 µl cell culture. SUB (substrate, contains corresponding quantity for 96 well) was dissolved (substrate) 2.5 ml ACT (activator) solution.

The absorbance was measured in the microplate-reader at 470 nm the 650 nm as reference. The reference absorbance serves for correction of signals caused by nonspecific and turbidity, for example, fingerprints and cell debris.

4 Results

4.1 Oligonucleotide synthesis

Sense and antisense strands of phosphordiester and phosphorothioate oligonucleotides were synthesized, some of these with ligand binding sites for coupling and others without ligand binding sites as for control experiments. For 3'-PEGylation, ready bound amino-on CPG instead of conventional CPG resin was utilized. For 5' PEGylation, MMT-hexylaminolinkerphosphoroamidite was utilized

Code number	Oligonucleotide sequences	Strand	Modification
1	TTC-GTA-TCC-ATC-TGG-CAG-C	AS	-
2	Aminohexyl-TT-CGT-ATC-CAT-CTG-GCA-G-C	AS	5'-Aminohexyl
3	PEG-TT-CGT-ATC-CAT-CTG-GCA-GC	AS	5'-PEG
4	TTC-GTA-TCC-ATC-TGG-CAG-C-Aminohexyl	AS	3'-Aminohexyl
5	TT-CGT-ATC-CAT-CTG-GCA-GC-PEG	AS	3'-PEG
6	GCT-GCC-AGA-TGG-ATA-CGA-A	S	-

Table 4-01: Synthesized anti-Galectin-1 sequences (18 bp), A: adenosine, C: cytidine, G: guanosine, T: thymidine, U: uridine, AS: antisense strand, S: sense strand.

Code number	Oligonucleotide sequences	Strand	Modification
7	TCT-CCC-AGC-GTG-CGC-CAT	AS	
8	Aminohexyl-TC-TCC-CAG-CGT-GCG-CCA-T	AS	5'-Aminohexyl
9	PEG-TC-TCC-CAG-CGT-GCG-CCA-T	AS	5'-PEG
10	TCT-CCC-AGC-GTG-CGC-CA-T-Aminohexyl	AS	3'-Aminohexyl
11	TCT-CCC-AGC-GTG-CGC-CA-T-PEG	AS	3'-PEG
12	ATG-GCG-CAC-GCT-GGG-AG-(A)	S	-

Table 4-02: Synthesized anti Bcl-2 sequences (18 bp), A: adenosine, C: cytidine, G: guanosine, T: thymidine, U: uridine AS: antisense strand, S: sense strand.

4.2 Polyacrylamide gel electrophoresis

In order to examine the purity of synthesized oligonucleotides polyacrylamide gel electrophoresis was carried out. By attachment of an amino linker, the reaction product has a slightly higher molecular weight. The mobility of nucleic acids during the run depends on molecular size and length. The analysis was performed by comparison of reacted and unreacted samples, which were applied to the gel.

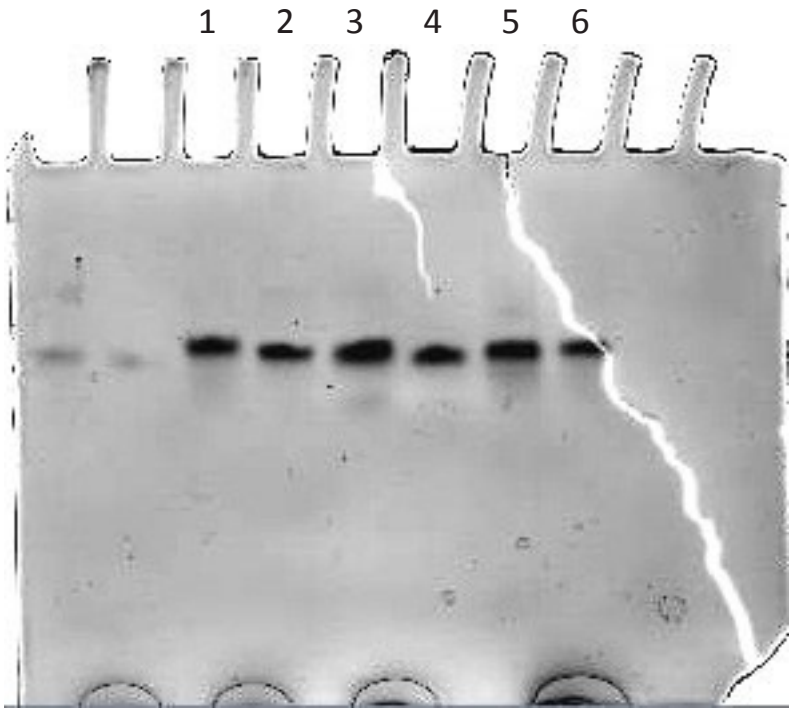

Figure 4-01: PAGE of oligonucleotides sequences.**1)** Galetin-1 antisense **2)** Galectin-1 **(1)** antisense with aminohexyl-linker at the 3'-end **(4)**, **3).**Bcl-2 antisense with MMT-Aminohexyllinker at the 5'end, **4)** Bcl-2 antisense without linker at the 5'-end **(9)**, 7) Bcl-2 antisense with aminohexyl-linker at the 3'-end **(10)**, 8) Bcl-2 antisense **(7).**

Only minimal differences in migration in the gel was detected for aminohexyl-modified phosphodiester oligonucleotides compared to their counterparts without the linker. The increase in molecular weight accounts to only about 180 Da, compared to the molecular weight of the oligonucleotides of around 5800 Da. Consequently, it was concluded that the differences are too small for unequivocal analysis of successful linker attachment.

the synthesis of phosphorothioate oligonucleotides was also performed and equally after conjugation, in order to observe the process of conjugation, gel electrophoresis was carried out. As PEGylation reagents, the corresponding N-hydroxysuccinimide (NHS) esters were employed. The chemical reaction is presented in the figure below.

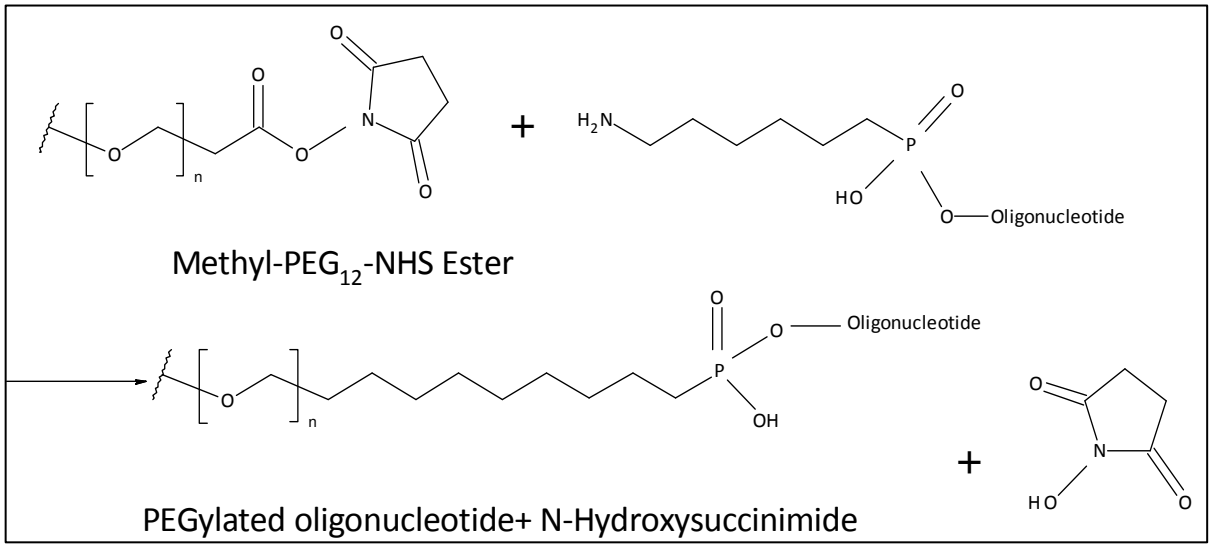

Figure 4-02: Scheme of oligonucleotide PEGylation at the 3'-end with aminohexyllinker with MS (PEG) $_{12}$; Methyl-PEG$_{12}$-NHS Ester

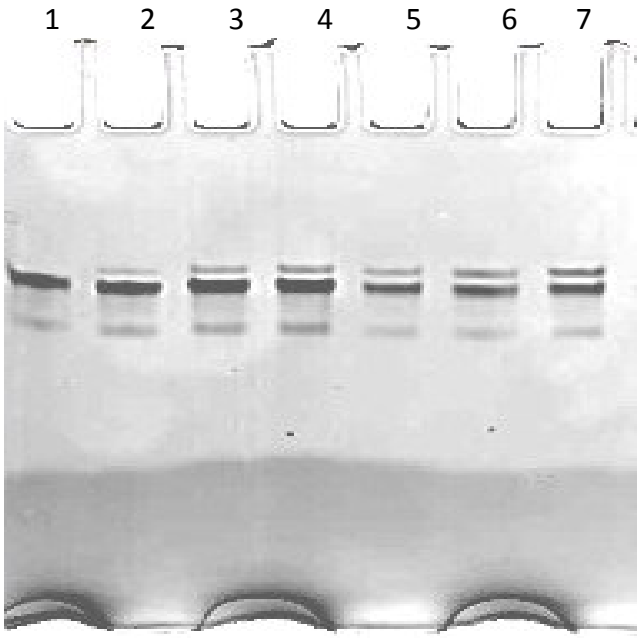

Figure 4-03: Gel electrophoresis of conjugated oligonucleotides at the 5'-end after elution with Sephadex gel: **1)** antisense as a reference **(7)**, **2,3,4)** PEGylation with 5 equivalent **(10)**, **5,6,7)** PEGylation with 50 equivalent with sampling-taking after 10,60 120 minutes **(10)**.

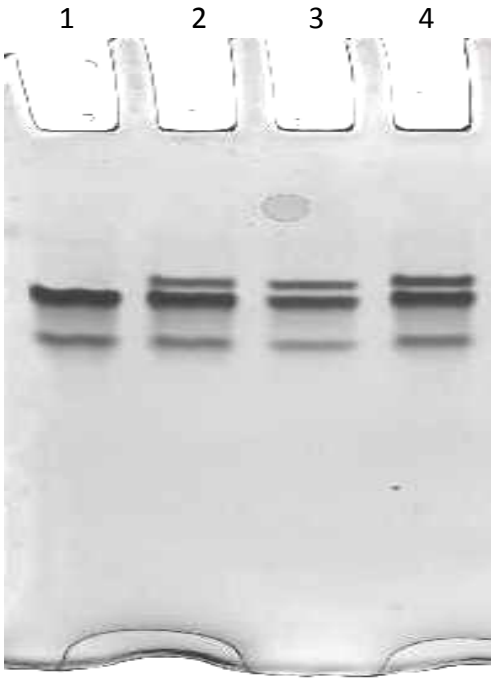

Figure 4-04: Gel.electrophoresis after conjugation of oligonucleotides with PEG, **1)** reference **(1)**, **2,3,4)** reaction with 50 equivalent PEG with sampling-taking after 10, 60, 120 minutes **(3)**.

In order to examine and optimized the conjugation, the reaction was studied in a time- and concentration-dependent manner.

The band in the 2 position in the electrophoresis gel (Fig.4.03) presents the reaction with 5 equivalents PEG. The more intense band is the educt (oligonucleotide with aminohexyl linker), and the band with slower migration (higher molecular weight) the PEGylated oligonucleotide. Although the product was successfully formed, we aimed to improve the reaction yields by increasing the molar surplus of the NHS-PEG reagent. On the second electrophoresis gel figure 4-04 one sees that the conjugation has worked much better with 50 equivalents PEG compared to only 5 equivalents. Comparing to yields after 10, 60, and 120 minutes showed that after 120 minutes reaction time, the intensity of the bands is approximately equal, indicative of a 50% reaction yield.

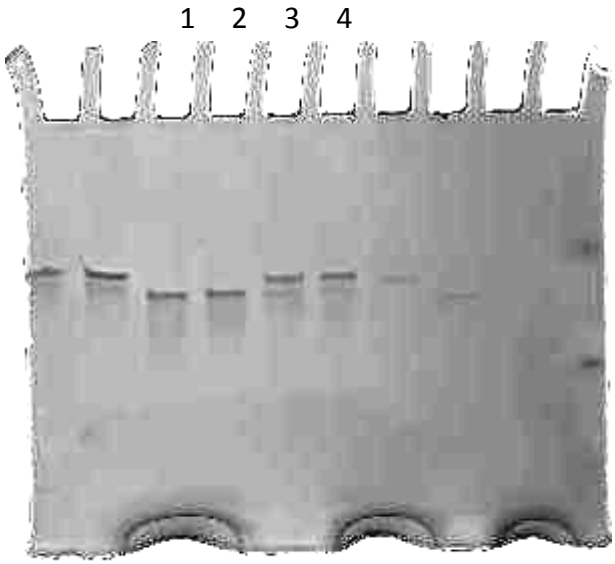

Figure 4-05: Phosphorothioate sequences, **1,2)** anti-Bcl-2 sequences PEGylated at the 5'-end **(8)**, **3,4)** anti-Bcl-2 sequences PEGylated at the 3'end with taking samples after 30 and 120 minutes **(10)**.

In figure 4-05, the gel electrophoresis of phosphorothioates shows that PEGylation has not worked at the 5'-end, whereas the 3- and 4- band, which have been PEGylated at the 3'-end the conjugation proceeded well. A comparison of the reaction with that of the phosphodiester conjugation showed higher conversion rate for phosphorothioates.

The incomplete reaction at the 5’-terminus may be due to poor attachment yields of the aminohexyl-phosphoroamidite.

Because the 3’-PEGylation proceeded well, no re-synthesis of the 5’-amino-oligonucleotides was undertaken. Purification of samples was carried out by preparative gel electrophoresis. PEGylated bands were cut out and gel pieces were incubated with TE-buffer and consequently analysed with CD and HPLC.

4.3 HPLC

Analytical HPLC was used for further confirmation of successful PEGylation. Therefore, a suitable method was developed. In my diploma thesis, I performed HPLC analysis of PEGylated and unPEGylated oligonucleotides with gradient elution of 23. 5-37% MeOH within 30 minutes.

HPLC analysis PEGylated anti-Galectin-1 at the 5’-end **(3)** and altogether appear the 2 peaks (Figure.4-06). The first peak (2.2 minutes) corresponds to the cleavage product of the N-hydroxysuccinimide ester, which exhibits high absorbance at 260 nm. The second peak was allocated to the unconjugated oligonucleotide and the third peak to the conjugated oligonucleotide through comparison to unreacted and purified compounds.

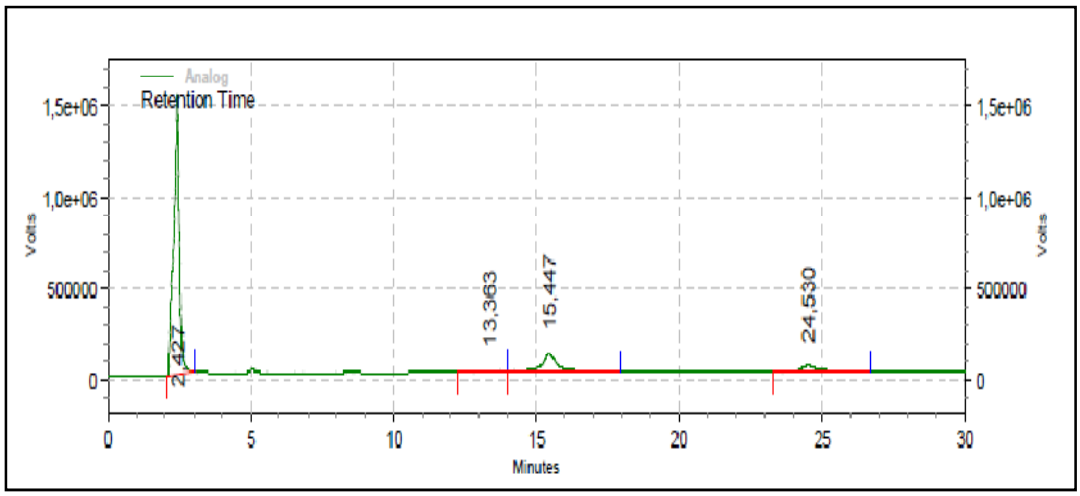

Figure 4-06: HPLC chromatogram of anti-galectin-1 antisense with PEG at the 5’-end **(3)**.

In Figure 4-07, HPLC was performed of unmodified Bcl-2. We can see that only one peak appear in the diagram, which indicates that the synthesis and purification was successful and no shorter oligonucleotide sequences were detected.

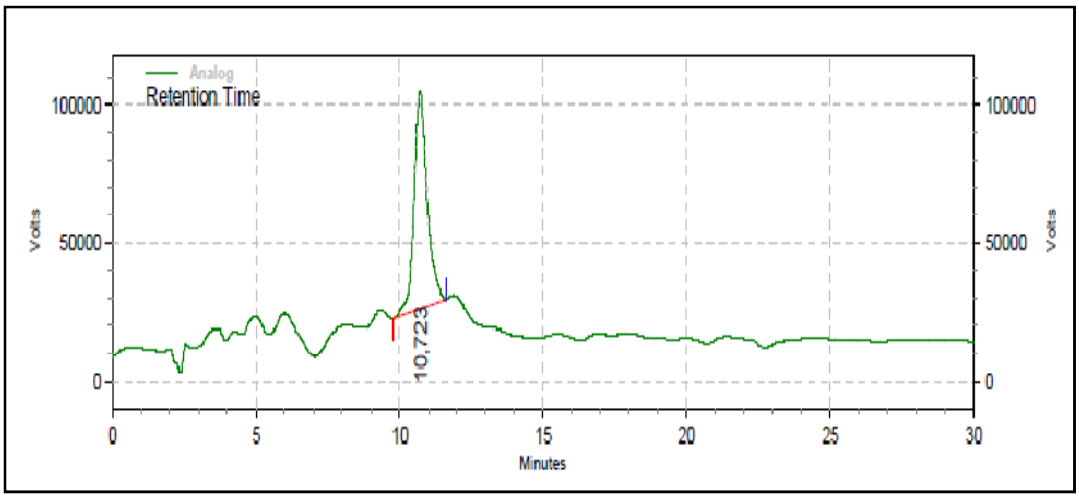

Figure 4-07: HPLC chromatogram of anti-Bcl-2 antisense oligonucleotide sequence without PEG **(7)**.

In Figure 4-08, PEGylated oligonucleotide was analysed after purification by gel electrophoresis. Only one peak, corresponding to the conjugated oligonucleotide was detected. The PEGylated oligonucleotide was eluted later from the Column because of the increased polarity caused by the PEG chain.

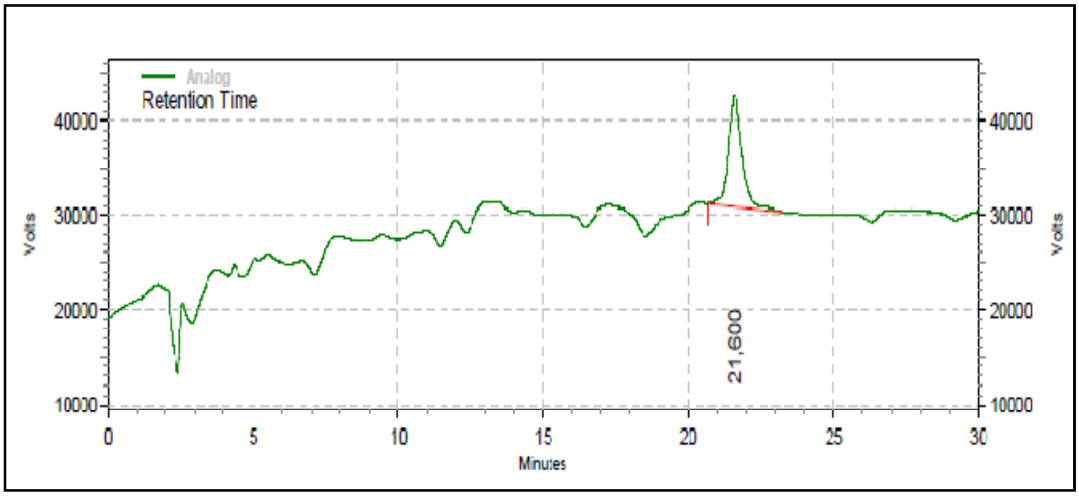

Figure 4-08: HPLC chromatogram of anti-Bcl-2 antisense oligonucleotide sequence with PEG at the 3'-end **(10)**.

4.4 CD Spectroscopy

CD spectroscopy was used to obtain information about the secondary structure of the nucleic acids. When the components in the samples exhibit the same absorption of both types of circularly polarized light, a linear amplitude perpendicular to the propagation direction results. On the other hand, if the components possess different absorbance strength, the amplitude of the strongly absorbed component becomes smaller, which results in the formation of an ellipse projection instead of a linear amplitude. CD spectroscopy provides information about melting temperature of samples and thereby enables analysis of thermodynamic parameter as well as information on folding and unfolding of secondary structures, altering the stability between conjugated and unconjugated oligonucleotide and affinity to the complementary structure.

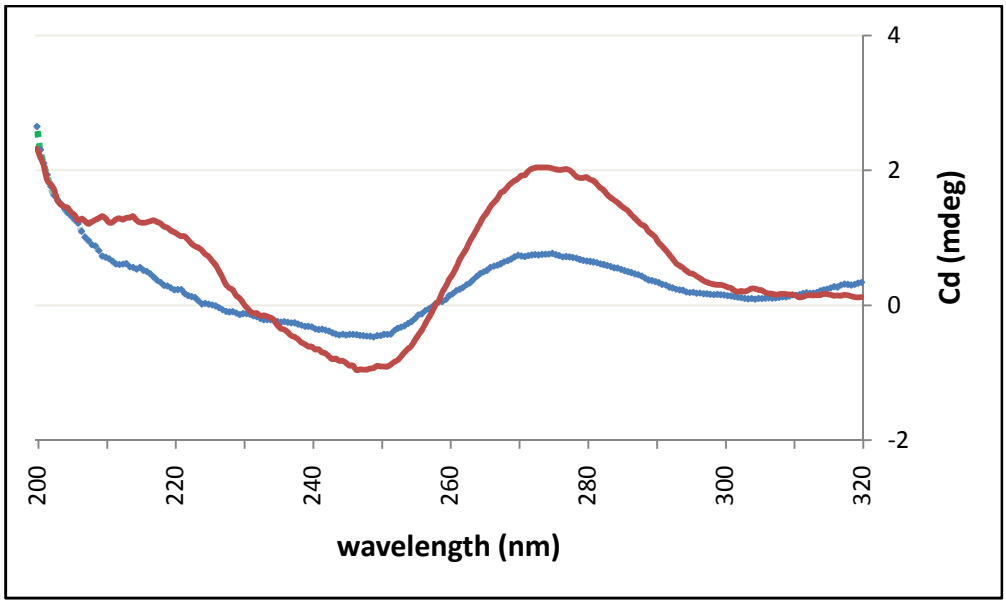

Diagram 4-01: red curve: PEGylated antisense ss, green curve: unPEGylated antisense ss.

In diagram 4-01, curve shapes of the PEGylated and unPEGylated single stranded oligonucleotides are equal. The intensity of the conjugated oligonucleotide is minimally stronger than that of the isosequential wild type oligonucleotide. Small differences of the secondary structure folding may have occurred.

In diagram 4-02, the curve shapes of double stranded oligonucleotides are very similar; indicating that, no substantial change in the secondary structure is triggered by PEGylation. Nevertheless, intensity of PEGylated oligonucleotide compared to unPEGylated oligonucleotide is slightly higher.

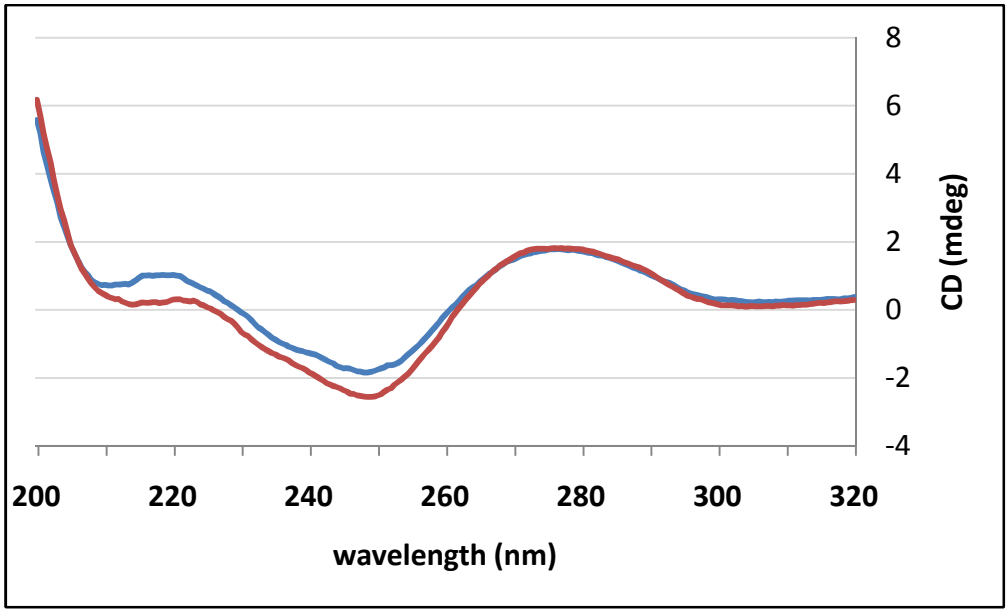

Diagram 4-02: red curve: PEGylated ds, blue curve: unPEGylated ds.

In diagram 4-03, the curve shapes of phosphorothioate double strands with and without PEG are similar only the intensity of PEGylated strand is slightly higher. Possibly due to the chirality generated by the phosphorothioate backbone modification. The PEGylated phosphorothioate CD intensity differs marginally compared to that of the phosphordiester.

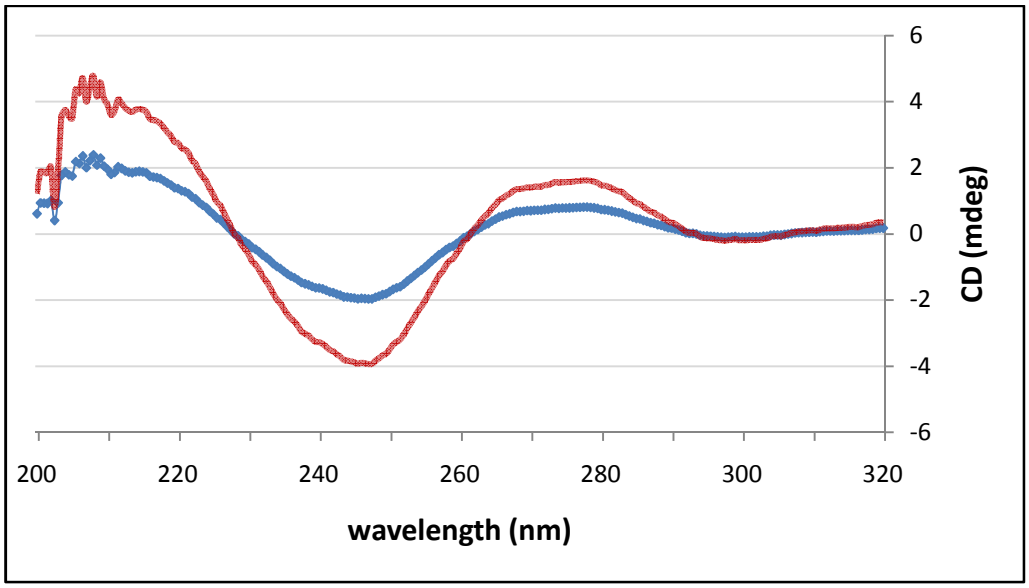

Diagram 4-03: red curve: PEGylated phosphorothioate, blue curve unPEGylated phosphorothioate.

In diagram 4-05, heating curves are presented. By heating up, denaturation of double strands occurs. The curve turning points are referred to as melting temperatures.

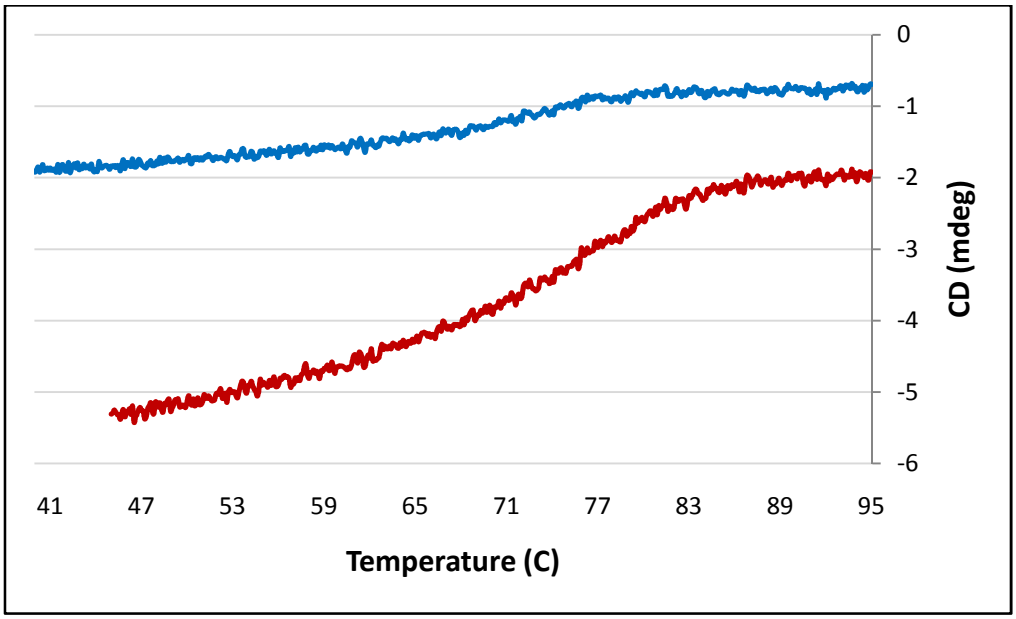

Diagram 4-04: measurement of melting temperature with CD: red curve: PEGylated phosphorodiester, blue curve: unPEGylated phosphordiester.

Samples	Medium	medium±SD(±0.14)
PEG P=O	78.2	78.06-78.34
AS.P=O	80.8	80.66-80.94
PEG P=O	86.3	86.16-86.44
AS.P=O	85.7	85.56-85.84
PEG P=S	57.7	57.56-57.84
AS P=S	52	51.86-52.14

Table 4-03: measured melting temperature of representative samples

In table 4-03, one sees that the melting temperatures of PEGylated and unPEGylated phosphordiester oligonucleotides are very similar. A stabilizing effect of the PEGylation was detected in the case of phsphorothioate.

4.5 Dual-Luciferase Reporter Assay

Dual Luciferase Reporter Assay serves as an assessment of the biological knockdown efficiency of the prepared oligonucleotides. The psiCHECK2-bcl-2 or -galectin-1 plasmid was co-transfected with the corresponding oligonucleotides into MCF-7 cells. This psiCHECK-2 vector contains renilla and firefly reporter genes, with renilla luciferase fused to the human Bcl-2 or galectin-1 cDNA. This set up enables the generation of renilla luciferase (dependent on bcl-2 or galectin-1 expression) and firefly luciferase (constitutively active, used for normalization of transfection) signals and the monitoring of alteration in gene expression and thus the assessment of gene silencing. When mRNA is degraded, the expression of renilla protein is reduced. siRNA, phosphordiester and phosphorothioate antisense oligonucleotides (with and without PEGylation) were applied in two amounts (1 and 10 pMol per well). After 48 hours incubation, the detection was carried out.

For analysis, normal and symmetrical distribution of variables using the of descriptive statistics as indicator of central tendency (mode, median, mean), and dispersion (, variance, standard deviation) and distribution (standard error of mean, skewness,kurtosis) and also kolmogorov-smirnov test were employed, in consideration of normal and symmetric distribution above can be used parametric statistical models.

For this study, the statistical model t was used; two independent groups (PEGylated compared to unPEGylated) and t-one sample (the comparison of empirical and theoretical mean). It is noted that, in the end, in case of significant different between groups, the potency and the rate effect of PEG on cells were evaluated.

One Sample t-Test $$t = \frac{\mu - \bar{x}}{S_{\bar{x}}} \qquad df = N - 1$$

Independent Samples t-Test $$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}} \qquad df = n_1 + n_2 - 2$$

Effect Size $$ES = \sqrt{\frac{t}{t+df}} \qquad df = n_1 + n_2 - 2$$

Central Tendency			Dispersion		
Mode	Median	Mean	Range	Variance	Std.Deviation
42.80	42.80	39.94	39.67	189.05	13.74

Table 4-04: Statistics indicator: related to the description of the variables (oligonucleotides)

Distribution		
Std.Error of Mean	Skewness	Kurtosis
3.96	-0.40	-1.21

Table 4-05: Statistics indicator: related to the description of the variables.

According to tables 4-04, 4-05 and with the emphasis on the minor difference that there is between the mode, median and mean and in consideration of the rate of skewness and kurtosis (lesser than 1.96), it can be mentioned, that the distribution above possesses the assumption of normality and thus the mean can be used as indicator of central tendency.

Variable	Kolmogorov-Smirnov Z	Sig
oligonucleotide	0.514	0.954

Table 4-06: Kolmogorov-smirnov Z: to evaluate for normality of variable.

Regarding to observed Z=0.514 in the table 4-06, that at the level of α=0.05 is not significant. We can say that the variable above is normality and thus we can use parametric statistical models.

According to the table 4-07 and 4-08 with emphasis on observed t values (t=0.02 in 1 pMol conc. and t=1.11 in 10 pMol conc.), can be raised, that a significant difference (at level 0.05=α) between the means of the unmodified and modified oligonucleotides was not observed. Thus the effect of linking PEG did not result in significant change of the pharmacologic effect.

	Group	Std.Deviation	t.value	Sig
1 pMol conc.	P=O conjP=O	8.70 5.85	0.02	0.980

Table 4-07: P=O: phosphordiester in 1 pMol concentration, conj P=O: PEGylated phosphordiester

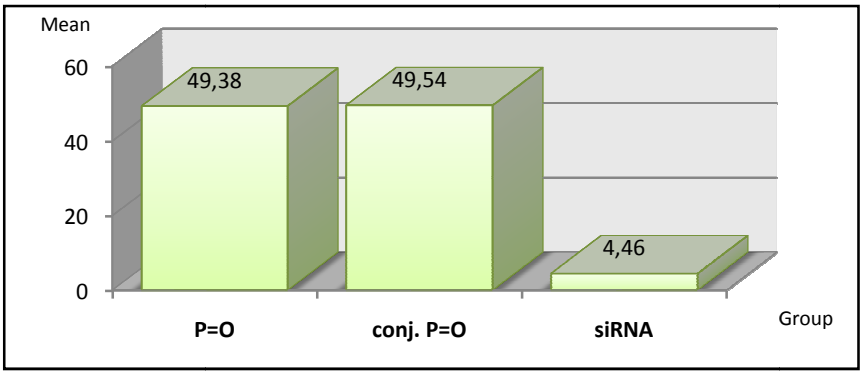

Diagram 4-06: scheme representation of Luciferase assay in 1 pMol concentration.

	Group	Std.Deviation	t.value	Sig
10 pMol conc.	P=O conjP=O	16.57 4.49	1.11	0.329

Table-4-08: P=O: phosphordiester, conj P=O: PEGylated phosphordiester

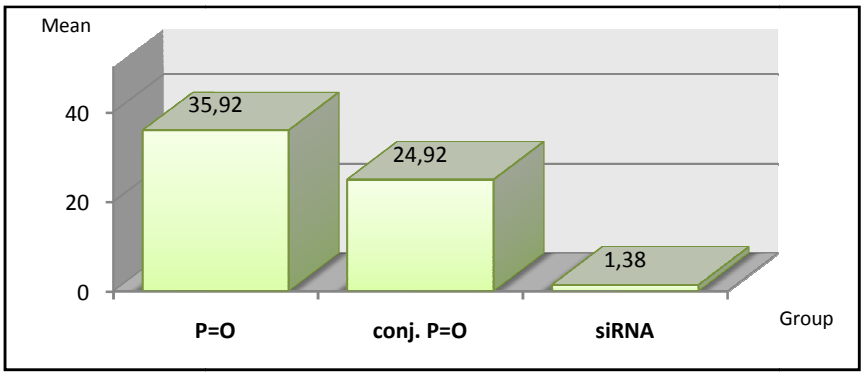

Diagram 4-07: scheme representation of Luciferase asay in 10 pMol concentration

	Group	Std.Deviation	t.value	Sig
10 pMol conc.	P=S conjP=S	2.31 2.70	1.24	0.280

Table 4-09: P=S: unPEGylated phosphorothioate, Conj. P=S: PEGylated phosphorothioate.

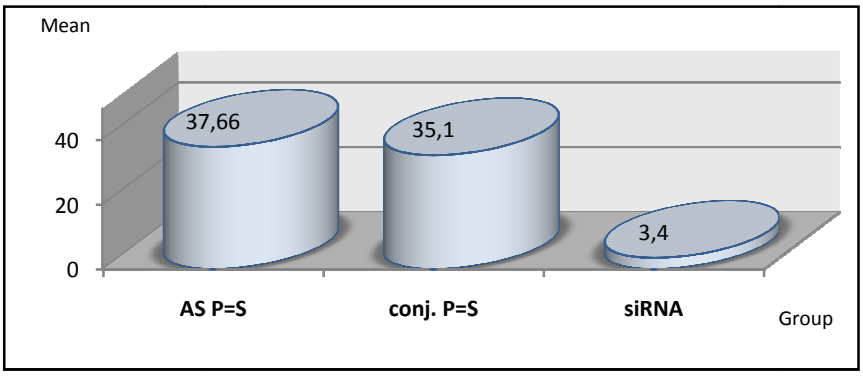

Diagram 4-08: schematic representation of Luciferase asay in 1 pMol concentration

According to the table 4-09 with the observed the t values of 1.24 no significant difference was observed (at level 0.05=α) between the unmodified and modified oligonucleotides.

	Group	Std.Deviation	t.value	Sig
10 pMol conc.	P=S conjP=S	2.03 1.83	5.80	0.004

Table 4-10: P=S: unPEGylated phosphorothioate, Conj. P=S: PEGylated phosphorothioate.

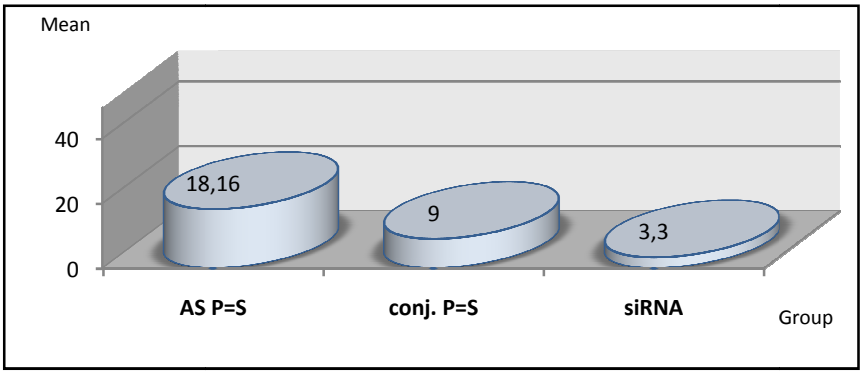

Diagram 4-09: scheme representation of Luciferase assay in 1 pMol concentration.

In table 4-10, regarding to the observed t-value of 5.80, there is significant difference between the effect of PEGylated and unPEGylated oligonucleotides , when applied in a 10pMol amount per well. The ES=0.77 effect size was also evaluated; this implied the effectiveness of PEGylated oligonucleotide in 10 pMol.

PEGylated and unmodified phosphordiester and phosphorothioate antisense oligonucleotides were also applied without any transfection reagent. Again the cells were incubated for 48 hours and the luciferase assay readout was performed as described above.

No knockdown of the target genes was detected after treatment with phosphodiester compounds (table 4.011-4.12). Obviously, the cellular uptake of those oligonucleotides is negligible, and their stability in culture medium is likely low.

	Group	Std.Deviation	t-value	Sig
1 pMol Conc.	AS P=O conj. P=O	10.22 22.71	2.14	0.099

Table 4-11: P=O: unPEGylated phosphordiester, conj P=O: PEGylated phosphordiester.

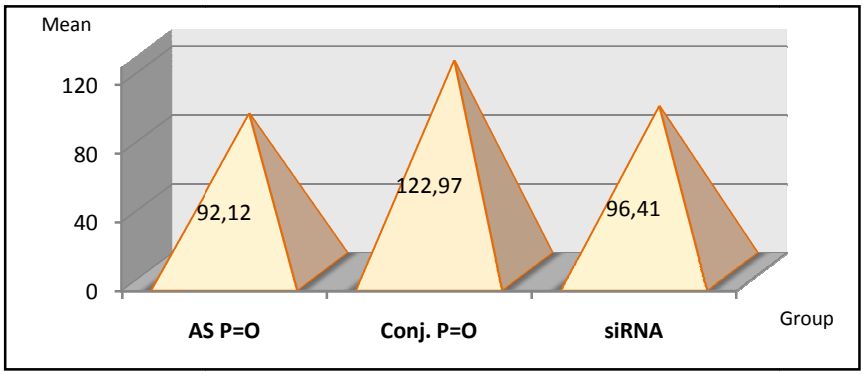

Diagram 4-10: scheme representation of Luciferase assay in 1 pMol concentration

	Group	Std.Deviation	t-value	Sig
10 pMol Conc.	AS P=O conj. P=O	40.53 23.26	-0.47	0.660

Table: 4-12: P=O: unPEGylated phosphordiester, conj P=O: PEGylated phosphordiester

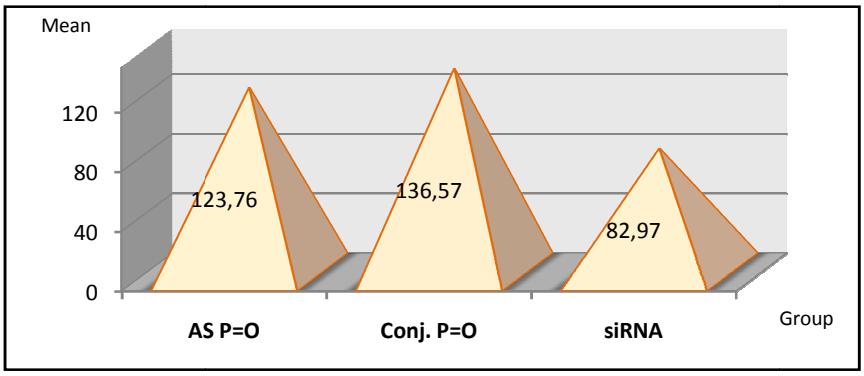

Diagram 4-11: schematic representation of Luciferase asay in 10 pMol concentration

Treatment with phosphorothioate derivatives (table 4.13-4.14) resulted in a certain extent of gene knockdown.

The effect was concentration-dependent, i.e. in 10 pMol per well a superior knockdown resulted. No statistically significant differences between PEGylated and non-PEGylated antisense agents were detected. A rather high variation of expression levels mirrored by a high standard deviation was detected after application of the oligonucleotides at 10 pMol/well.

	Group	Std.Deviation	t-value	Sig
10 pMol Conc.	AS P=S conj. P=S	3.28 6.85	2.38	0.076

Table-4-13: P=S: unPEGylated phosphorothioate, conj. P=S: PEGylated phosphorothioate.

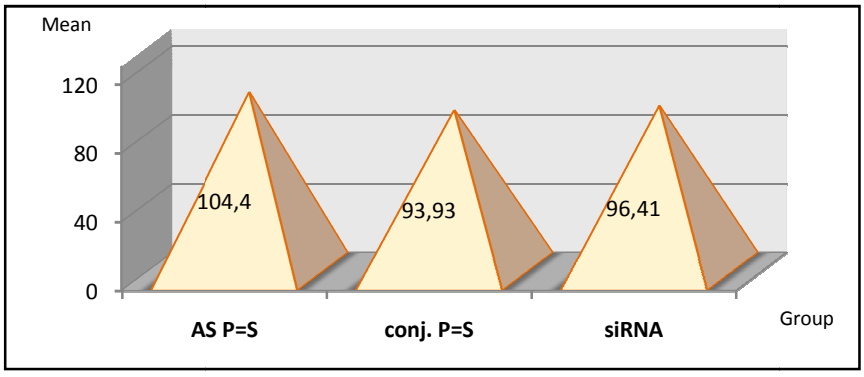

Diagram 4-12: scheme representation of Luciferase assay in 1 pMol concentration.

	Group	Std.Deviation	t-value	Sig
10 pMol Conc.	AS P=S	17.30	0.51	0.634
	conj. P=S	13.23		

Table 4-14: P=S: unPEGylated phosphorothioate, conj. P=S: PEGylated phosphorothioate.

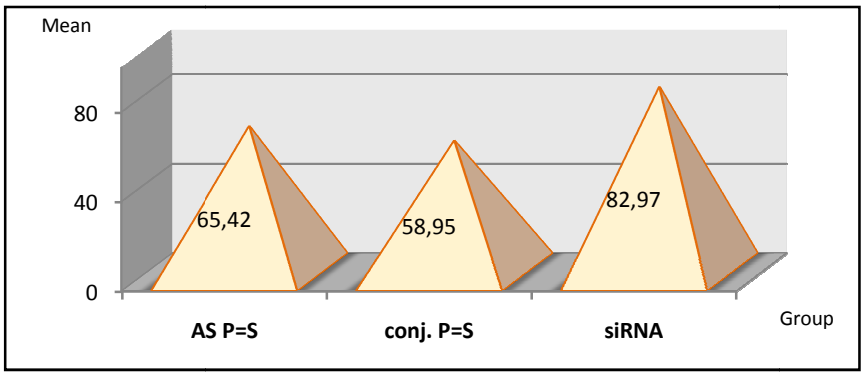

Diagram 4-14: scheme representation of Luciferase asay in 10 pMol concentration

4.6 Cell proliferation and Cytotoxicity assay

Using the MTT-test, the cell proliferation rate can be examined. MTT is metabolised by living cells in formazan. This reaction requires intact mitochondria. After cell lysis by the solubilisation reagent the formation of this complex is proportional to the extent of cell proliferation. The MTT assay enables quantification of cell proliferation and thus toxicity. High sensitivity to the test is among the advantages.

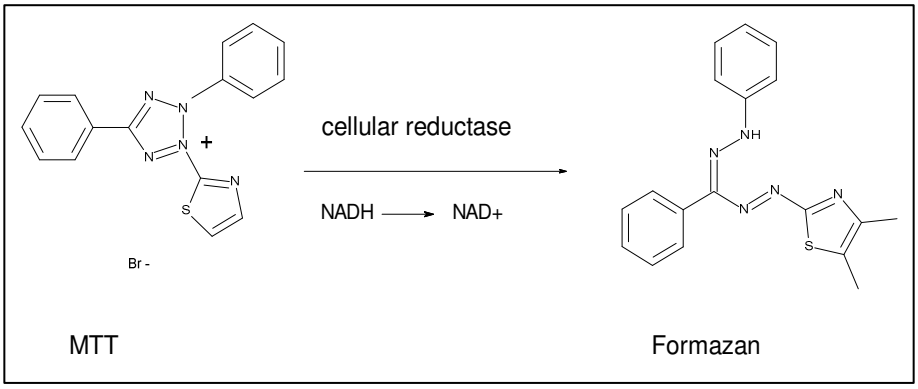

Figure 4-06: Conversion of MTT in Formazan by living cell

The samples were inserted into two concentrations and a proliferation test was performed.

Employing MTT, the absorbance of the solution is quantified. This means that higher absorbance is indicative to a higher number of living and proliferating cells. The metabolic activity of phosphordiester and phosphorthioate was only minimally reduced compared to siRNA. Phosphorothioates generally indicates lower metabolic activity compared to phosphodiester. The toxicity of phoshorothioates is well documented, and the effect found corresponds well with literature data [28].

	Samples Conc. (pMol)	Mean	t-value	Sig.	μ
1)	siRNA siRNA 10	94.92 86.06	-0.40 -78.80	0.723 0.001	100
2)	galectin 1 galectin 10	90.98 96.15	-2.26 -1.50	0.152 0.269	100
3)	galectin PEG at the 5'-end **1** galectin PEG at the 5'-end **10**	93.33 88.08	-2.15 -4.71	0.164 0.042	100
4)	unPEGylated **1** unPEGYlated **10**	91.88 85.81	-1.98 -10.22	0.186 0.009	100
5)	PEG at the 3'-end **1** PEG at the 3'-end **10**	88.46 90.02	-5.96 -4.36	0.027 0.049	100
6)	bcl-2 PEG at the 5'-end **1** bcl-2 PEG at the 5'-end **10**	85.01 90.7	-8.33 -1.74	0.014 0.223	100
7)	unPEGylated **1** unPEGylated **10**	84.29 94.43	-9.95 -2.21	0.010 0.157	100
8)	bcl-2 PEG at the 3'-end **1** bcl-2 PEG at the 3'-end **10**	94.89 83.37	-1.40 -18.96	0.295 0.003	100
	Phosphorothioate				
9)	galectin PEG at the 5'-end **1** galectin PEG at the 5'-end **10**	90.48 83.36	-0.91 -10.70	0.456 0.009	100
10)	unPEGylated **1** unPEGYlated **10**	90.80 93.53	-2.41 -4.26	0.137 0.051	100
11)	PEG at the 3'-end **1** PEG at the 3'-end **10**	85.88 94.29	-6.84 -0.78	0.0210 514	100
12)	bcl-2 PEG at the 5'-end **1** bcl-2 PEG at the 5'-end **10**	94.55 98.41	-4.30 -0.56	0460 0.177	100
13)	unPEGylated **1** unPEGylated **10**	90.46 94.13	-12.72 -3.01	0.006 0.095	100
14)	bcl-2 PEG at the 3'-end **1** bcl-2 PEG at the 3'-end **10**	93.90 94.54	-0.90 -2.04	0.460 0.177	100

Table 4-15: For assessment of cytotoxicity observed t-values of treated and untreated cells.

According to the table 4-15, a minimal difference was observed between the PEGylated- and unPEGylated groups compared to untreated groups. The comparison of the values of the PEGylated and unPEGylated components shows that the PEGylation have in some cases possibly exercised a positive effect on the proliferation and cytotoxicity. Moreover, this minimal difference there is also between galectin and siRNA compared to untreated.

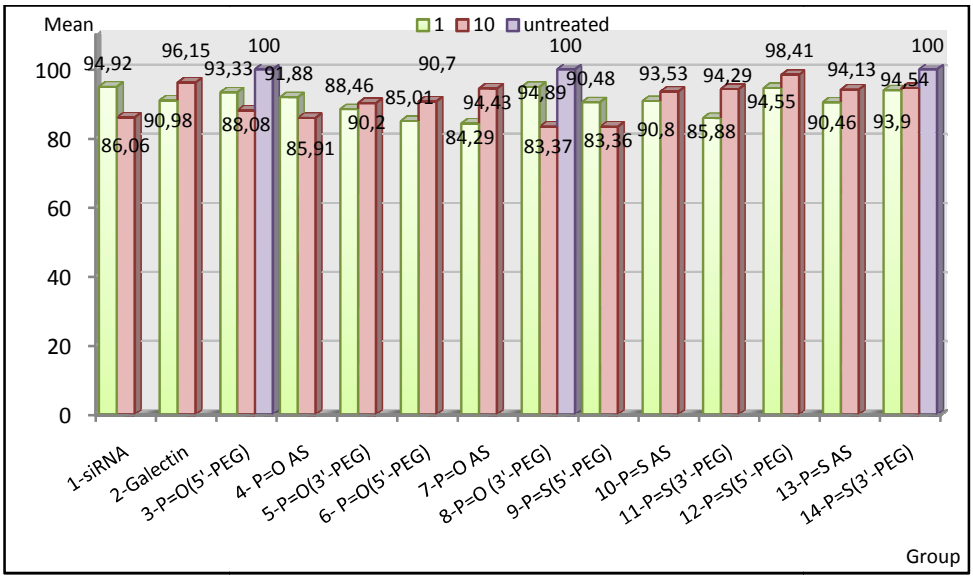

Diagram 4-15: Graphical presentation of performed MTT-test in two concentrations 1- and 10-pMol,PEG: polyethylene glycol,P=O: phosphordiester, P=S: phosphorthioate.

Samples	μ(siRNA)	t-Value	Sig
Galectin	**94/92**	**-.988**	**.427**
PEG at the 5'-end P=O	**94/92**	**-5.512**	**.031**
AS P=O	94/92	-6.737	.021
PEG at the 3'-end P=S	**94/92**	**-4.382**	**0.048**
AS P=S	94/92	-1.082	0.393

Table 4-16: observed t-value of samples with 1 pMOl concentration: galectin,PEG: polyethylene glycol P=O: phosphordiester, AS: unmodified, P=S:phosphorothioate.

Regarding to the table 4-16 and 4-17 with observed t-values, there is no significant difference between the values of samples. The comparison of PEGylated and unPEGylated phosphorothioates showed unmodified compound a significant of more than $\alpha > 0.05$ and thus a weak toxicity, whereas PEGylated compound $\alpha < 0.05$. It can be concluded, that the observed toxicity is caused rather by the phosphorothioate modification.

Diagram 4-16: Graphical presentation of performed MTT-test in concentration 1 pMol, galectin, PEG: polyethylene glycol, P=O: phosphordiester, AS: unmodified P=S: phosphorthioate.

Samples	μ(siRNA)	t-Value	Sig
galectin	**86**	**-50.082**	**0.000**
PEG at the 5'-end P=O	**86**	**-6.561**	**0.022**
AS P=O	86	-13.169	0.006
PEG at the 3'-end P=S	**86**	**-7.434**	**0.018**
AS P=S	**86**	-0.086	0.940

Table 4-17: observed t-value of samples with 10 pMOl concentration: PEG.P=O: PEGylated phosphordiester, AS P=O: antisense phosphordiester, PEG.P=S: PEGylated phosphorothioate, AS P=S: antisense phosphorothioate.

According to the table 4-17 with observed t-values, it can be lifted that no significant different were observed. However, it can be determined, when 10 pMol concentration are applied the toxicity of phosphorothioate will increase.

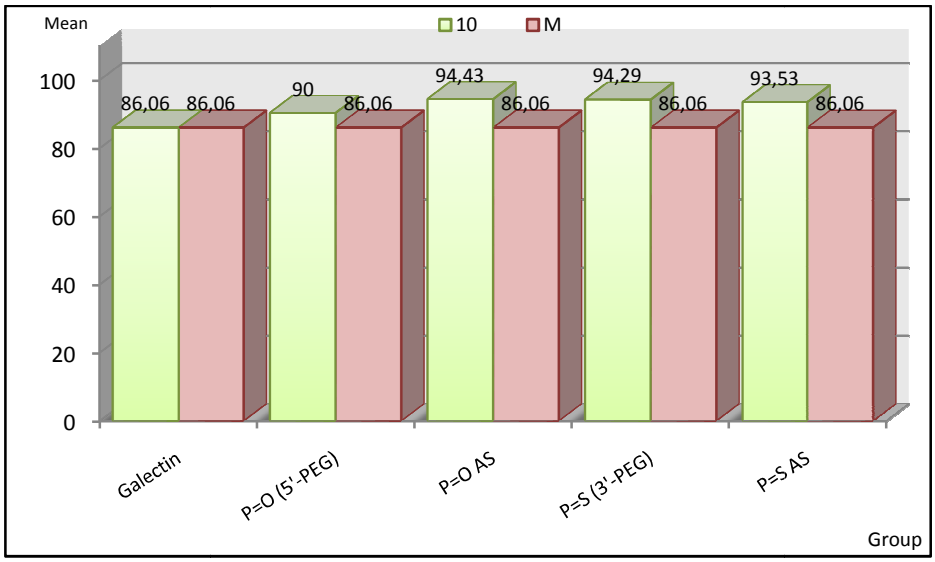

Diagram 4-17: Graphical presentation of performed MTT-test in concentration 10 pMol, galectin, PEG: polyethylene glycol,P=O: phosphordiester,P=S: phosphorthioate.

5 Discussion

5.1 ASO, PS synthesis and conjugation

5.1.1 ASO and PS synthesis

The anti-bcl-2 and anti-galectin phosphordiester and phosphorothioate antisense and sense strands were successfully assembled on the DNA synthesizer. The attachment of the each nucleotide is achieved by 4 steps: detritylation, coupling, capping and oxidation. The synthesis process takes place from the 3' to the 5'-end.

The synthesis cycle begins with the secession of protective groups DMT from the nucleotides in acidic conditions (A). The orange dyed DMT cation can visually be followed and spectrophotometrically measured. This property was used as an in process control of the synthesis. The coupling of the unmodified phosphoroamidite nucleotide lasts one minute (B). In the coupling phase ensues splitting of diisopropylamine from the phosphoroamidite, whereas cyanoethyl groups are retained as a protective group. For the coupling, the weak acid tetrazole is used as catalyst and thus facilitates the synthesis procedure. The coupling rate according to the synthesizer was routinely between 95-100%. In the capping step, the free 5'-end OH-groups were acetylated, in order to prevent further strand prolongation (C). In the 4^{th} step, the phosphorus is oxidized from P^{3+} in the P^{5+} (D). The resulting phosphortriester is more stable towards hydrolysis which is important during detritylation. Phosphorothioate can also be synthesized. The oxidation was performed with tetraethylthiuramdisulfide (TETD) for efficient substitution of sulphur for an oxygen atom.

5.1.2 Analytics of ASO and PS

The PEG-chains were attached at the 3’-end and 5’-end of the oligonucleotides. For the modification at the 5’-end, a MMT (monomethoxytrityl) linker was attached during solid-phase oligonucleotide synthesis. In principle, the modification results with polyethylene glycol (PEG) spacers with terminal methyl groups (Methyl-PEG12-NHS Ester). The PEGylation

at the 3’-end was also accomplished with MS-PEG12-NHS Ester, and the aminohexyl linker was introduced by use of a correspondingly modified resin. It was essential that the reaction was carried out in pH 7-9 buffers, because at this pH value the NHS-ester reacts efficiently with primary amino groups so that stable amide bonds accrues and NHS-ester is released.

Quality analysis and purity of synthesized ASO and PS were examined by gel electrophoresis and HPLC. Through gel electrophoresis, the migration of samples according to known sequence length can be compared but this statement is relative because the gel migration partly depends on the sequences. The exact examination of sequence length isn’t easily possible. The purification of the some sequences was also successfully done with gel electrophoresis Analytical HPLC confirmed successful purification, but isolation yields from the gels were rather low. The HPLC method for the respective samples was adjusted with respect to methanol gradient. As expected the PEGylation of oligonucleotides resulted in later elution from the reversed phase column. Methanol was used as a polar compound of mobile phase for elution.

CD spectroscopy was used to obtain information about the secondary structures of the PEGylated oligonucleotide by direct comparison to their unmodified counterparts. By measurement of the melting temperatures the denaturation of desoxyoligoribonucleotide duplexes was determined.

In figure 4.03 the additional band on the PAGE indicates that the PEGylation at the 5’-end was successful. By visual comparison of the intensity of both bands it can be determined that after 120 minutes reaction time yielded higher product formation.

The HPLC analysis showed an additional peak corresponding to the modified oligonucleotide.

PEGylation at the 3’-end generally gave higher yields. After preparative purification with gel electrophoresis, HPLC of the fractions showed good separation of conjugated from unconjugated oligonucleotide.

It can be concluded that optimization of the attachment can be achieved either by longer reaction time or higher concentration of components.

The incomplete reaction of phosphordiesters is likely due to buffer or sample impurities with amines, such as residual ammonia from the deprotection step. Amines compete with the aminohexyl linker for reaction of NHS-PEG. Possibly, ammonium salts sticking tightly to

the backbone phosphates hindered the reaction. Instead of carrying out comprehensive attempts to further optimize the procedure, it was decided to purify the PEGylated compounds from the reaction and proceed with biophysical and *in vitro* characterization. Interestingly, the reaction with phosphorothioates proceeded in higher yields. Since the work up procedure is identical to phosphorodiesters, it can be speculated that ammonium salts of phosphorothioates are less stable, leading to a lower concentration of competing amine containing impurities.

Comparisons of the CD spectra of PEGylated and unPEGylated single strands show very similar curve shapes but some differences in intensity. The slightly higher intensity of PEGylated phosphordiester oligonucleotide can supposedly be attributed to a more rigid secondary structure. Phosphorothioate single strands and double strands show identical curve shapes and nearly identical intensities compared to the phosphordiester. Overall, the CD spectra show no distortion of the secondary structure is caused by attachement of the PEG chains at either the 3'- or the 5'-end. The measurement of melting temperature showed only minor differences between PEGylated and unPEGylated compounds of phosphordiesters. The PEGylation of phosphorothioates resulted in higher melting temperature comparison to their unPEGylated counterpart. The increase in hybridization affinity can possibly be explained by the amphiphilic properties conferred by the PEG ligands, which decrease overall lipophilicity of the phosphorothioates.

5.2 Cell culture assays

PEGylated and unPEGylated oligonucleotides were transferred into mammalian cells and the down regulation of target bcl-2 and galectin mRNAs were examined with a Dual-Luciferase reporter assay. In this test Renilla and Firefly luciferase signals are measured of each sample. In the psiCHECK2 plasmid, Renilla luciferase is fused with the gene of interest. The alteration of expression of the target gene leads accordingly to change of the Renilla gene. Firefly luciferase serves as internal standard thus the rate of Renilla/Firefly activity enables quick analysis of gene silencing in a high throughput manner.

It permits normalisation of effects such as variations in transfection effectiveness and cytotoxicity. The Stop&Glo substrate, as a reagent for the firefly luciferase reaction offers quenching of the firefly luciferase signal thus prevent an intervention with the Renilla luminescence and enables generation of the Renilla luciferase reaction.

A dual injector luminometer for this process was used because of a short half life of the reagents of approximately 2 hours. The assay was repeated several times, and the results of a representative assay are reported. Optimization of plasmid transfection was necessary in order to achieve sufficiently high values of luminescence and in turn reduce the standard variation resulting from poor signal to noise ratios. Transfection of both phosphordiester and phosphorothioate antisense agents showed equal target down regulation at most tested concentrations of PEGylated and unPEGylated agents. This indicates that the PEG chain does not interfere with cellular uptake events, counter strand hybridization, or RNase H activation. At the highest tested concentrations (10 pMol per well, figure 4.08) and on the basis of the observed t-test a significant difference between both groups was found. In this group the effectiveness (Effect size: 0.77) was also measured, which enables quantitative assessment of the strength and extent of the relationship in the population. Cohen provided a simple guide for the clinical implication of phenomena (relationship between modified and unmodified oligonucleotides). Correlation values were divided in the three groups; in the range of 0.10 to 0.29, values were considered small effects, values from 0.30 to 0.49 possess medium effects and correlation values across 0.50 were considered large effects [29]. According to the Cohen guideline, the effectiveness of 0.77 was considered large effects. These results are indicative of a higher effect of PEGylated phosphorothioates which could be due to better intracellular

dissociation from the transfection reagent, higher intracellular stability, or enhanced hybridization to the mRNA.

No biological effect of naked application of phosphorodiesters was detected. This is likely to due to the absence of any unassisted cellular uptake and quick degradation in the cell culture medium by serum nucleases. Phosphorothioates on the other hand are known to be taken up in a slow process when applied in high concentrations [30]. This was proved by the good yield down regulation achieved with a concentration of 10 pMol per well. PEGylated phosphorothioates were slightly more efficient at those concentrations, but the difference was not statistically significant.

5.3 Cell proliferation and cytotoxicity test

PEGylated and unPEGylated oligonucleotides were transferred into mammalian cells and using MTT-test was examined proliferation and toxicity of cells. In this test was measured metabolic activity of living cells which during test MTT in formazan converted. Untreated cells were used as a control. Through the comparison of individual samples values with untreated values can be elevated that the encountered weak toxicity is dependent on the phosphorothioate backbone [28]. For example, according to the table 4-16 and 4.17 and in consideration of observed significance of phosphorothioates (in 1pMol concentration 0.33 and when used 10 pMol concentration 0.99), can be stated that the existing toxicity is evoked by the phosphorothioate backbone. Direct comparision of the proliferation data of isosequential PEGylated and unPEGylated phosphorothioates even suggest a protective effect in some cases.

5.4 Conclusion

Poor pharmacokinetic properties of natural oligonucleotides were improved by chemical modifications such as phosphorothioates, which activate RNAse.H.

However phosphorothioates show only minimal membrane permeability due to their multiple negative charges. Thus PEGylation might represent an efficient approach for enhancing pharmacokinetic parameters and cellular uptake. PEGylation with short PEG-NHS esters of defined structure proceeded smoothly at the 3'-end, but at the 5'-end it was successful only after elution of the modified oligonucleotides from Sephadex gel, likely because of contamination with amine-containing impurities. The PEGylation at the 3'-end of phosphorothioates gave higher yields than phosphordiesters.

Both PEGylated phosphorodiesters and phosphorothioates were successfully prepared after optimization of the reaction conditions.

Methods for the tracking of the reactions were established using gel electrophoresis and RP-HPLC. CD spectrometric analyses showed only minor influences of the attachment of short PEG chains and no distortion of base-pairing properties.

The *in vitro* effect of phosphodiesters was equal to that of the unmodified compounds. In the case of phosphorothioates, at the highest tested concentration, a significantly higher pharmacologic effect was found for the PEGylated compound. Experiments with application of naked oligonucleotides onto cells showed no effect for phosphodiesters, which are quickly degraded and not taken up in cultured cells. The phosphorothioate backbone modification enhances cellular uptake, but the PEG chains did not further improve the biological effect.

The results indicate that the attachment of short PEG chains of defined length is a feasible chemical modification for antisense oligonucleotides. Although cellular uptake is not significantly enhanced, these compounds may prove to have value in the modulation of pharmacokinetic properties, or in combination with supramolecular delivery systems. Finally, we prove that attachment of larger linkers and ligands is possible at the 3'- and 5'-end of phosphorothioate oligonucleotides without loss of their pharmacologic activity.

6 List of abbreviations

ASO	antisense oligonucleotide
APS	ammonium pyrophosphate
Bcl-2	B-Cell Lymphoma-2
Bp	base pair
cDNA	copy DNA
CPG	controlled Pore Glass
ddH_2O	double. distilled water
DMD	duchenne muscular dystrophy
DEPC	diethylpyrocarbonate
DMEM	Dulbecco's Modofied Eagle Medium
DMF	dimethylformamide
DMT	dimethoxytrityl
DNA	desoxyribonucleic acid
ds	double strand
EDTA	ethylenediaminetetraacetic acid
FB	formamid buffer
HPLC	High Performance Liquid Chromatography
LP	lipofectamin
MCF-7	Michigan Cancer Foundation-7
miRNA	micro RNA
mRNA	messenger RNA
NHS	N-hydroxysuccinimide
ODNs	oligodesoxynucleotides
PAGE	Polyacrylamide Gel Electrophoresis
PEG	polyethylene glycol
PBS	phosphate buffered saline
PS	phosphorothioate
RNA	ribonucleic acid
RNase H	ribonuclease

ssDNA	single strand DNA
ssRNA	single strand RNA
siRNA	small interfering RNA
TBE	Tris/Borate/EDTA
TEA	triethanolamine
TEAA	triethylammonium acetate
TEMED	N, N, N‘,N‘-tetramethylethylenediamine

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8 Curriculum vitae

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