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(54) Title: METHOD FOR SELECTIVE OLIGONUCLEOTIDE MODIFICATION

(57) Abrégé/Abstract:
Method for producing a modified oligonucleotide, wherein at least one polymer, preferably polyalkylene oxide, and/or a compound is covalently bound to the 5´-end or the 3´-end of the oligonucleotide via native ligation forming a native ligation site, with the proviso that the polymer and/or the compound is not a protein or peptide, if only the 5´-end of the oligonucleotide is modified by binding of the polymer or compound via native ligation. The invention is further directed to a modified oligonucleotide obtainable by the inventive method as well as the use of such modified oligonucleotide for the preparation of a medicament for preventing and/or treating a tumor, formation of metastasis, an immune disease or disorder, a cardiovascular disease or disorder, and/or a viral disease or disorder.

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(54) **Title**: METHOD FOR SELECTIVE OLIGONUCLEOTIDE MODIFICATION

(57) **Abstract**: Method for producing a modified oligonucleotide, wherein at least one polymer, preferably polyalkylene oxide, and/or a compound is covalently bound to the 5′-end or the 3′-end of the oligonucleotide via native ligation forming a native ligation site, with the proviso that the polymer and/or the compound is not a protein or peptide, if only the 5′-end of the oligonucleotide is modified by binding of the polymer or compound via native ligation. The invention is further directed to a modified oligonucleotide obtainable by the inventive method as well as the use of such modified oligonucleotide for the preparation of a medicament for preventing and/or treating a tumor, formation of metastasis, an immune disease or disorder, a cardiovascular disease or disorder, and/or a viral disease or disorder.

Method for selective oligonucleotide modification

The present invention is directed to a method for selectively modifying an oligonucleotide, preferably an antisense oligonucleotide, wherein a polymer, in particular a polyalkylene oxide and/or a compound is covalently bound to the 3´- and/or 5´-end of the oligonucleotide, wherein the 5´- or the 3´-end is modified via native ligation forming a native ligation site, and optionally a further polymer and/or compound is bound, preferably to a cysteine residue at the native ligation site of the oligonucleotide conjugate.

Nonimmunogenic and water-soluble polymers have been widely used in biomaterial, biotechnology and medicine (Zhao 1997). In the area of drug delivery, polymer derivatives have been widely used in covalent attachment to proteins for example to reduce immunogenicity (Chirila 2001), proteolysis, kidney clearance and to enhance solubility. Polyethyleneglycol (PEG) for example is a widely used polymer that has been attached to more or less hydrophobic drugs to enhance solubility, reduce toxicity and alter biodistribution. A further aim of linking polymers like polyethyleneglycol to drugs is to enlarge the molecular weight and increase the body halflife time of the drug. In addition, a polymer is suitable as carrier in a pharmaceutical composition or is linked covalently to a drug to enhance cellular uptake and/or to direct the oligonucleotide to a target. Hence, polymers bound to an active agent often result in enhanced penetration and retention of the active agent in a cell or body system, and allow for example directing the agent specifically to a tumor, tumor tissue, or metastasis (targeting) to increase the success of a therapy.

WO 2008/077965 A2 describes oligonucleotide conjugates, wherein an oligonucleotide is connected to a polymer, preferably a polyethylenglycol. These conjugates are produced based on oligonucleotide synthesis, wherein 3´-> 5´synthesis and 5´ -> 3´ synthesis of the oligonucleotide on solid support are varied depending on the type and/or molecular size of the polymer, which is conjugated with the oligonucleotide.

Dawson et al. (Science 1994) originally described native ligation as a method for coupling unprotected polypeptides and/or proteins, respectively, to construct a large polypeptide or protein. Later native ligation was adapted to the coupling of peptides and oligonucleotides resulting for example in peptide-oligonucleotide hybrids, wherein the peptide is bound to the 5´-end of the oligonucleotide (Stetsenko DA et al., 2000; Pierce TL et al., 2005).

It is an object of the present invention to provide a method allowing multiple specific modification(s) of an oligonucleotide in a broad range with a polymer, which is preferably not a peptide or protein if it is bound to the 5´-end of the oligonucleotide using native ligation, and optionally further modification of the oligonucleotide with a compound, wherein the polymer and/or the compound is bound to the 3´-, and/or 5´-end of the oligonucleotide, and/or to the cysteine residue of the native ligation site forming an oligonucleotide conjugate of the invention. Due to the mild reaction conditions, in particular of the native ligation, of the present invention, the method is very efficient, characterized by a very low number of unwanted side products, and the products prepared by this method can easily be used for clinical administration. Moreover, the present method provides the platform for a high variety of conjugates with different polymers and/or compounds at different positions of the oligonucleotide.

Accordingly, it is a further object of the present invention to provide an oligonucleotide conjugate according to the inventive method.

Furthermore, it is an object of the present invention to use the oligonucleotide conjugate of the present invention for the preparation of a medicament for the prophylaxis and/or treatment of a disease, wherein the oligonucleotide of the conjugate hybridizes or interacts with a factor involved in the disease.

Summary

The present invention refers to a method for highly selective modification of an oligonucleotide, preferably an antisense olignucleotide, wherein the 5´- or the 3´-end of the oligonucleotide is modified via native ligation. The oligonucleotide is further modified at the 3´-end, and/or at the 5´-end, and/or at a cysteine residue of the native ligation site. The modification of the oligonucleotide is preferably based on the binding of a polymer and/or a compound to the oligonucleotide at the 5´- , 3´-end of the oligonucleotide and/or at the thiol function of the cysteine residue, i.e., the native ligation site. In one embodiment, the 5´- and the 3´-end are modified via native ligation, wherein the 5´- or 3´-end is modified first, or both ends are modified in parallel. In case of the 5´- and 3´-end modification of the oligonucleotide via native ligation even two additional native ligation sites are created, which allow the combination with one or two additional polymers and/or compounds.

In a preferred embodiment, a polymer such as polyalkylene oxide for example a polyethylene glycol (PEG) is connected to the 5´- or to the 3´-end via native ligation with the cysteine

residue at the native ligation site. This first step results in a conjugate comprising an oligonucleotide and a polymer. In a next step, a compound such as a small molecule, an antibody, an antigen, an enzyme, a part of an antibody, antigen or an enzyme, any peptide, e.g., an internalizing peptide, an aptamer, a chromophor, a marker, preferably a fluorescent marker like FITC, biotin, a hormone, a signal peptide, a lipid, a fatty acid, a sugar, an amino acid, a receptor, a part of a receptor, or any ligand of a receptor or binding molecule, or a polymer for example a polyalkylene oxide, is connected to the native ligation site, preferably to the cysteine residue of the conjugate. Alternatively, a polymer or a compound is connected to the 3´- or the 5´-end of the conjugate. In a further alternative embodiment, a polymer and/or a compound is connected to the native ligation site and to the 3´- or 5´-end of the oligonucleotide. The compound and/or the polymer is permanently bound to the oligonucleotide or is detached from the oligonucleotide, preferably if the modified oligonucleotide, i.e., the conjugate, reaches a target and the conjugate binds to the target. In a preferred embodiment, the protein and peptide, respectively, guides the oligonucleotide to the target, binds to the target and the oligonucleotide, preferably comprising further modifications (polymer and/or compound), is split off from the peptide or protein.

For example the combination of a modification of the 5´- or the 3´-end of an oligonucleotide via native ligation and the modification of the free 3´- or 5´-end using a linker such as an NHS-ester offers a surprisingly simple and reliable method for the production of symmetrically or assymetrically modified oligonucleotides for example a polyalkylated oligonucleotide, preferably a pegylated oligonucleotide. A further advantage of the method is the formation of an additional functional group at the cysteine residue, which allows a further modification of the modified oligonucleotide for example with a further polymer, a small molecule, an antibody, an antigen, an enzyme, a part of an antibody, or an enzyme, any peptide, e.g., an internalizing peptide, an aptamer (based on nucleic acid, protein, or peptide), a spiegelmer, RNAi, shRNA, microRNA (miRNA), a chromophor, a marker, preferably a fluorescent marker like FITC, biotin, a hormone, a signal peptide, a lipid, a fatty acid, a sugar, an amino acid, a receptor, a part of a receptor, or any ligand of a receptor or binding molecule, or glutathion.

In an alternative embodiment, the oligonucleotide modified at the 5´- or 3´-end via native ligation is temporarily or permanently connected to a solid support via the free 3´- or 5´-end of the oligonucleotide, or the native ligation site, preferably the cysteine residue of the native ligation site for example to thiol-sepharose. This embodiment allows further modification of the oligonucleotide at the 5´- and/or 3´-end, and/or the native ligation site, and/or at any site, which is not connected to the support. Alternatively, the connection of the modified

oligonucleotide to a solid support forms the basis for a test system such as a kit, and/or allows purification of the modified oligonucleotide. An example of a solid support is a magnetic bead, which allows for example an easy isolation of the native ligation product via magnetism.

The polymers and compounds, respectively, bound to the oligonucleotide are either identical or differ for example in chemical structure, molecular weight (size), and reactivity. Hence, the method of the present invention results in a high variety of different oligonucleotide conjugates having a broad range of different characteristics allowing adapting the oligonucleotide specifically to any requirement. Hence, the present invention represents a platform technology for the modification of oligonucleotides, in particular antisense oligonucleotides.

Figures

Fig. 1 presents examples of oligonucleotide sequences of exemplaric genes TGF-beta1, TGF-beta2, TGF-beta3, PGE-rec., VEGF, IL-10, c-erbb2 (Her-2), c-jun, c-fos, and MIA.

Fig. 2 shows a general reaction scheme of native ligation, wherein X is a polymer and/or compound, and Y is a modified oligonucleotide comprising a linker. RS^- is the leaving group.

Fig. 3 presents the 5´Cys-Oligo-synthesis. Step 1: synthesis of an oligonucleotide having an amino linker at the 3´-end and a cysteine modifier (OMR) at the 5´-end of the oligonucleotide; step 2: cleaving off of the Fmoc protection group from the cysteine modifier via piperidin, preferably 20 %, in DMF; step 3: removing the oligonucleotide from the support and cleaving off of the permanent protection group via ammonia.

Fig. 4 shows the thioester modification of a polymer and/or compound, which will be coupled to an oligonucleotide via native ligation.

Fig. 5 presents the conjugation of the 5´-end of cysteine-modified oligonucleotide via native ligation.

Fig. 6 shows the protection and activation, respectively of the thiol-function of the cysteine residue (Native Ligation Site) for example via dipyridyldisulfide wherein R is a polymer and/or compound.

Fig. 7 demonstrates the conjugation of the 3´-end of a 5´-end conjugated oligonucleotide for example via NHS (N-hydroxysuccimid) linker, wherein R´ and R´´ is a polymer and/or a compound.

Fig. 8 presents an example of 3´-cysteine oligonucleotide synthesis using for example a cysteine modified solid support.

Fig. 9 demonstrates increasing stability of a modified oligonucleotide against S1 endonuclease in comparison to an unmodified oligonucleotide, wherein Fig. 9a refers to the unmodified oligonucleotide, Fig. 9b to the mPEG400-Oligo-mPEG1000 (mPEG400 at the 5´-end of the oligonucleotide and mPEG1000 at the 3´-end of the oligonucleotide), and Fig. 9c to the mPEG1000-Oligo-mPEG400 (mPEG1000 at the 5´-end of the oligonucleotide and mPEG400 at the 3´-end of the oligonucleotide). Samples were taken at 0h, 1h, 24h, 48h, 72h, and 144h of incubation with S1 endonuclease.

Fig. 10 presents the increased stability of a modified oligonucleotide against 5´-exonuclease in comparison to an unmodified oligonucleotide, wherein Fig. 10a refers to the unmodified oligonucleotide, Fig. 10b to the mPEG400-Oligo-mPEG1000 (mPEG400 at the 5´-end of the oligonucleotide and mPEG1000 at the 3´-end of the oligonucleotide), and Fig. 10c to the mPEG1000-Oligo-mPEG400 (mPEG1000 at the 5´-end of the oligonucleotide and mPEG400 at the 3´-end of the oligonucleotide). Samples were taken at 0h, 1h, 24h, 48h, 72h, and 144h of incubation with 5´-exonuclease.

Fig. 11 shows the increased stability of a modified oligonucleotide against 3´-exonuclease in comparison to an unmodified oligonucleotide, wherein Fig. 11a refers to the unmodified oligonucleotide, Fig. 11b to the mPEG400-Oligo-mPEG1000 (mPEG400 at the 5´-end of the oligonucleotide and mPEG1000 at the 3´-end of the oligonucleotide), and Fig. 11c to the mPEG1000-Oligo-mPEG400 (mPEG1000 at the 5´-end of the oligonucleotide and mPEG400 at the 3´-end of the oligonucleotide). Samples were taken at 0h, 1h, 24h, 48h, 72h, and 144h of incubation with 3´-exonuclease.

Fig. 12 presents the schematic coupling of a 5´-/3´-end PEG modified oligonucleotide, which was modified at the 5´-end via native ligation according to the present invention, and a peptide at the oligonucleotide´s native ligation site. The activated thiol residue of the oligonucleotide reacts with the free thiol residue at the C-terminus of the peptide by forming a disulfide bond and elimination of a pyridinthion.

Fig. 13 shows the results of the reaction of the 3´-end PEG modified oligonucleotide of SEQ ID No. 2461 and penetratin based on the principle presented in Fig. 12 (see Example 14). In **Fig. 13A** samples of the products were loaded on a 19 % PAGE gel, which is silver stained: M is a marker comprising the antisense oligonucleotide (ASO)-2 nucleotides, ASO-1 nucleotide, i.e., the antisense oligonucleotide minus 1 or 2 nucleic acids, ASO, mPEG400-ASO-mPEG400, mPEG1000-ASO-mPEG1000, lane 1 shows the 3´-end PEG modified oligonucleotide without penetratin, lane 2 shows the 3´-end PEG modified oligonucleotide with 0.5 x excess of penetratin, lane 3 shows the 3´-end PEG modified oligonucleotide with 1 x excess of penetratin, lane 4 shows the 3´-end PEG modified oligonucleotide with 2 x excess of penetratin, lane 5 shows the 3´-end PEG modified oligonucleotide with 4 x excess of penetratin, lane 6 shows the 3´-end PEG modified oligonucleotide with 8 x excess of penetratin, and lane 7 shows the 3´-end PEG modified oligonucleotide with 16 x excess of penetratin; depending on the excess of penetratin the amount of PEG-modified oligonucleotid connected to penetratin increases. **Fig. 13B** shows a chromatogram of the 3´-end PEG modified oligonucleotide without penetratin (A) according to lane 1), and with 2x (B) according to lane 4) and 8x (C) according to lane 6) excess of penetratin, which is connected to the natve ligation site, according to Fig. 13A. **Fig. 13C** shows the curves of the chromatogram in more detail, wherein line A presents the 3´-end PEG modified oligonucleotide, line B presents the 3´-end PEG modified oligonucleotide, wherein penetratin is connected to the modified oligonucleotide when a 2x excess of penetratin was added to the modified oligonucleotide, and line C presents the 3´-end PEG modified oligonucleotide, wherein penetratin is connected to the modified oligonucleotide when an 8x excess of penetratin was added to the modified oligonucleotide.

Fig. 14 shows the results of the reaction of the 5´-/3´-end PEG modified oligonucleotide of SEQ ID No. 2030 and penetratin based on the principle presented in Fig. 12 (see Example 14). In **Fig. 14A** samples of the products were loaded on a 19 % PAGE gel, which is silver stained: M is a marker comprising the antisense oligonucleotide (ASO)-2 nucleotides, ASO-1 nucleotide, ASO, mPEG400-ASO-mPEG400, mPEG1000-ASO-mPEG1000, lane 1 shows the 5´-/3´-end PEG modified oligonucleotide without penetratin, lane 2 shows the 5´-/3´-end PEG modified oligonucleotide with 0.5 x excess of penetratin, lane 3 shows the 5´-/3´-end PEG modified oligonucleotide with 1 x excess of penetratin, lane 4 shows the 5´-/3´-end PEG modified oligonucleotide with 2 x excess of penetratin, lane 5 shows the 5´-/3´-end PEG modified oligonucleotide with 4 x excess of penetratin, and lane 6 shows the 5´-/3´-end PEG modified oligonucleotide with 8 x excess of penetratin; depending on the excess of penetratin the amount of PEG-modified oligonucleotid connected to penetratin increases. **Fig. 14B** shows a chromatogram of the 5´-/3´-end PEG modified oligonucleotide without penetratin (A)

according to lane 1), and with 0.5x (B) according to lane 2), 2x (C) according to lane 4), and 8x (D) according to lane 6) excess of penetratin, which is connected to the natve ligation site, according to Fig. 14A. **Fig. 14C** shows the curves of the chromatogram in more detail, wherein line A presents the 5´-/3´-end PEG modified oligonucleotide, line B presents the 5´-/3´-end PEG modified oligonucleotide, wherein penetratin is connected to the modified oligonucleotide when a 0.5x excess of penetratin was added to the modified oligonucleotide, line C presents the 5´-/3´-end PEG modified oligonucleotide, wherein penetratin is connected to the modified oligonucleotide when an 2x excess of penetratin was added to the modified oligonucleotide, and line D presents the 5´-/3´-end PEG modified oligonucleotide, wherein penetratin is connected to the modified oligonucleotide when an 8x excess of penetratin was added to the modified oligonucleotide.

Fig. 15 presents the results of experiments, where 5´-/3´-end PEG modified oligonucleotide of SEQ ID No. 2030 without an accessible native ligation site. Thus, even if penetratin is added to the oligonucleotide, no connection is detectable based on the principle presented in Fig. 12 (see Example 14), where the native ligation site is blocked. The samples of the products were loaded on a 19 % PAGE gel, which is silver stained: M is a marker comprising the antisense oligonucleotide (ASO)-2 nucleotides, ASO-1 nucleotide, ASO mPEG400-ASO-mPEG400, mPEG1000-ASO-mPEG100, lane 1 shows the 5´-/3´-end PEG modified oligonucleotide without penetratin, lane 2 shows the 5´-/3´-end PEG modified oligonucleotide with 0.5 x excess of penetratin, lane 3 shows the 5´-/3´-end PEG modified oligonucleotide with 1 x excess of penetratin, lane 4 shows the 5´-/3´-end PEG modified oligonucleotide with 2 x excess of penetratin, and lane 5 shows the 5´-/3´-end PEG modified oligonucleotide with 4 x excess of penetratin. When the native ligation site is blocked no connection with penetratin is observable independent of the penetratin excess.

Fig. 16 presents the results of the reaction of the 5´-/3´-end PEG modified oligonucleotide and the RGDC peptide based on the principle presented in Fig. 12 (see Example 15). In **Fig. 16A** samples of the products were loaded on a 19 % PAGE gel, which is silver stained: M is a marker comprising the antisense oligonucleotide (ASO)-2 nucleotides, ASO-1 nucleotide, ASO, mPEG400-ASO-mPEG400, mPEG1000-ASO-mPEG100, lane 1 shows the 5´-/3´-end PEG modified oligonucleotide, lane 2 shows the reaction product of RGDC peptide in a 0.5x excess connected to the native ligation site of the 5´-/3´-end PEG modified oligonucleotide, lane 3 shows the reaction product of RGDC peptide in a 2x excess connected to the native ligation site of the 5´-/3´-end PEG modified oligonucleotide, lane 4 in a 8x excess and lane 5 in a 16x excess. **Fig. 16B** shows a chromatogram of the 5´-/3´-end PEG modified oligonucleotide without RGDC (A) according to lane 1) and with 2x (B) according to lane 3)

and 8x (C) according to lane 4) excess of RGDC which is connected to the native ligation site according to Fig. 16A. **Fig. 16C** shows the curves of the chromatogram in more detail, wherein line A presents the 5´-/3´-end PEG modified oligonucleotide, line B presents the 5´-/3´-end PEG modified oligonucleotide, wherein RGDC is connected to the modified oligonucleotide when a 2x excess of RGDC was added to the modified oligonucleotide, and line C presents the 5´-/3´-end PEG modified oligonucleotide, wherein RGDC is connected to the modified oligonucleotide when an 8x excess of RGDC was added to the modified oligonucleotide.

Fig. 17 presents the effect of 5´-/3´-end PEG-modified AP12009 on glioma cells (see Example 16), wherein AP12009 is modified via native ligation at the 5´-end end (derivative #26) in comparison to the effect of 5´-/3´-end PEG- and RGDC-modified AP12009, wherein AP12009 is modified via native ligation at the 5´-end end and RGDC is connected to the native ligation site (derivative #28) according to Fig 14. **Fig. 17A** shows the effect on the proliferation of glioma cells and **Fig. 17B** on the expression of TGF-ß2, i.e., the inhibitory effect of AP12009 and its via native ligation modified forms. Black columns indicate AP12009, light grey columns indicate mPEG400-AP12009-mPEG1000 (derivative #26) and dark grey columns indicate 5´-/3´-end PEG modified AP 12009 connected to RGDC at the native ligation site, i.e., mPEG400-(RGDC-S)-Cys-AP12009-mPEG1000 (derivative #28). Derivative #28 is the most efficient form in suppressing TGF-beta2 expression in glioma cells compared to unmodified AP12009 and derivative #26. The concentrations of AP12009, derivative #26, and #28 used in this experiment were 0 µM, 0.25 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM, or 10 µM.

Fig. 18 shows the results of the reaction of the cystein comprising 5´-/3´-end PEG modified oligonucleotide and Iodoacetamide-Fluorescin as an example for an irreversible binding. In Fig. 15 the educt and the product were loaded on a 19 % PAGE gel, which is silver stained: M is a marker comprising the antisense oligonucleotide (ASO)-2 nucleotides, ASO-1 nucleotide, ASO, mPEG400-ASO-mPEG400, mPEG1000-ASO-mPEG100, lane 1 shows the 5´-/3´-end PEG modified oligonucleotide without Fluorescein (educt), and lane 2 shows the 5´-/3´-end PEG modified oligonucleotide with Fluorescin connected to the native ligation site of the modified oligonucleotide.

Detailed description

The present invention is directed to a method providing a platform for a broad range of oligonucleotide conjugates based on highly specific modifications of the 5´-end of the

oligonucleotide via native ligation. Preferably the 5´-end of the oligonucleotide is modified by binding of a polymer and/or a compound via native ligation, wherein the thiol function of the cysteine residue is called native ligation site. The cysteine residue remaining after native ligation is optionally modified by binding of a polymer and/or a compound. The 3´-end of the oligonucleotide is likewise optionally modified by binding of a polymer and/or a compound, wherein the 3´-end of the oligonucleotide is modified in a first step, in a second step after modification of the 5´-end of the oligonucleotide, or in a third step after modification of the 5´-end and the native ligation site of the oligonucleotide and conjugate, respectively. The native ligation site, which is preferably a cysteine residue of the polymer- and/or compound-oligonucleotide conjugate, is modified by binding of a further polymer and/or compound before or after modification of the 3´-end of the oligonucleotide.

Alternatively, the 3´-end of the oligonucleotide is modified with a polymer and/or compound via native ligation, and optionally, in addition, the 5´-end of the oligonucleotide, and/or the cysteine residue of the native ligation site is modified by binding to a polymer and/or a compound.

In some embodiments, the order of modifying the different positions of the oligonucleotide does not influence the characteristics of the oligonucleotide conjugate. In other embodiments, the order of the steps for modifying the oligonucleotide is essential to reach a specific oligonucleotide conjugate. Preferably, the 5´- or 3´-end of the oligonucleotide is modified via native ligation in a first step before modification of the free 3´- or 5´-end, or before modification of the native ligation site.

In another embodiment, both ends, i.e., the 5´- and the 3´-end of the oligonucleotide are modified via native ligation either one end after the other end, or both ends in parallel. If one end of the oligonucleotide is modified after the other via native ligation, the second end has to be protected when the first end is modified via native ligation. Preferred protecting groups are orthogonal protection groups.

In a further embodiment, the 5´-end and/or the 3´-end of the oligonucleotide and/or the native ligation site, in particular the cysteine residue, or any other residue of the oligonucleotide is modified by binding of a linker before binding of a polymer and/or a compound. The linker provides a high variety of different chemical bindings and allows reversible or irreversible binding of the polymer and/or the compound at each position of the oligonucleotide, preferably at the 5´-, 3´-end and/or native ligation site of the oligonucleotide.

Alternatively, a solid support is bound to the modified oligonucleotide, preferably at the 5´-end, the 3´-end and/or the native ligation site of the oligonucleotide. The binding of a solid support and the modified oligonucleotide forms for example the basis for a kit or purification system resulting for example in a highly purified modified oligonucleotide, which is not achievable with any other method.

A solid support is an insoluble, inert, functionalized, preferably polymeric material to which an oligonucleotide or a oligonucleotide conjugate is attached, optionally via a linker, for example allowing the oligonucleotide or the oligonucleotide conjugate to be readily separated (by filtration, centrifugation, etc.) from excess reagents, soluble reaction byproducts, or solvents. An example of a solid support is a magnetic bead, or a support used for oligonucleotide synthesis such as controlled pore glass, or thiol-activated sepharose for isolating and separating, respectively, of thiol containing conjugates (e.g. thiol-activated sepharose).

The present method allows the construction of an unlimited number of modifications for example all these different modifications explicitly disclosed in the specification, and thus, provides an advantageous platform for tailoring an oligonucleotide for specific requirements such as the combination of drug targeting, e.g., via an antibody, and the increase of the half-life of the oligonucleotide for example via a polymer such as a polyalkylene oxide, optionally in high purity. The method leads to the production of highly specifically modified oligonucleotides, preferably without the use of protection groups, tailored for any desired application and function, respectively.

The method of the present invention is advantageously not limited by the type or size and molecular weight, respectively, of the polymer and/or compound described.

The at least one polymer, for example starch, is either linear or branched, wherein the polymer comprises one or more, e.g., 2, 3, 4, 5, 6, 7, 8, 9, or 10 branching points. The term “branched” further comprises polymers such polyalkylene oxide, in particular PEG, which are combined via a linker, e.g., a Lys core to form a “pseudo-branched” polymer. In this case the polymer represents a monomer of a multi-polymer complex.

A polymer according to the present invention is any class of natural and/or synthetic substances composed of monomers and/or macromolecules that are multiples of monomers wherein the polymer optionally comprises natural and/or synthetic monomers. The monomers need not all be the same or have the same structure forming a homo- or copolymer. Polymers consist of long chains of unbranched or branched monomers, and/or

are cross-linked networks of monomers in two or three dimensions. Their backbones are flexible or rigid. Many important natural materials are for example organic polymers, including polysaccharides, polypeptides, proteins, lignin, rubber, or nucleic acid. Polymers preferably increase the size and molecular weight of the oligonucleotide in the present invention and/or guide the oligonucleotide to the target. Polymers in the context of this application comprise biocompatible materials, such as polyalkylen oxides, more preferred polyethyleneglycol, for example alpha-, omega-dihydroxylpolyethyleneglycol, biodegradable lactone-based polymers, e.g. polyacrylic acid, polylactide acid (PLA), poly(glycolic acid) (PGA), polypropylene, polystyrene, polyolefin, polyamide, polycyanoacrylate, polyimide, polyethylenterephthalat (PET, PETG), polyethylene terephthalate (PETE), polytetramethylene glycol (PTG), or polyurethane as well as mixtures thereof. Mixture refers to the use of different polymers within the same compound as well as it refers to block copolymers. Block copolymers are polymers wherein at least one section of a polymer is build up from monomers of another polymer. Preferred polymers of the present invention are polyalkylene oxides such as PEG as well as polyethylene imide (PEI), or hydroxy ethyl starch (HES), wherein HES is preferably bound to the cysteine residue of the native ligation site via the thiol function. Selection of such materials depends on a number of factors including the stability and toxicity of the polymer.

A compound of the present invention is any substance having functional characteristics such as a small molecule, an antibody for example MUJ591, an antibody binding to glycoprotein IIb/IIa, to CD52, to CD25, to VEGF, to the epidermal growth factor, to CD33, to CD20, or to carbonic anhydrase, an antigen for example an external domain of prostate-specific membrane antigen (PSMA), an enzyme, a part of an antibody, or an enzyme, any peptide, e.g., an internalizing peptide, a cell penetrating peptide such as penetratin (see Example 14), TAT (transactivator or transcription), Transportan (e.g., TP10), R9 peptide, MPG peptide, KALA peptide, a pH (low) insertion peptide, hormonal peptides such as LHRH, bombesin, somatostatin, or a targeting peptide such as RGD (Arg-Gly-Asp), in particular linear or cyclic RGD (see Example 15), an aptamer (based on nucleic acid, protein, or peptide), a spiegelmer, siRNA, e.g., cholesterol-modified siRNA, RNAi, shRNA, microRNA (miRNA), a human serum albumin carrier, a tumor vaccine such as a tumor antigen e.g., PSA, PAP, HIFalpha or an immune stimulatory agent, a cytotoxine such as TMZ, BCNU, DTIC, 5-Fluorouracil (FU), gemcitabine, taxol (pacitaxel), irinoteca, oxaliplatin doxorubicin, cyclophosphamid, folinsäure, a chromophor, a marker for example a fluorescent marker such as FITC, fluorescin (see Example 17), rhodamine, phycoerytherin, phycocynin, allophycocyanin, o-phthaldehyde or fluorescamine, biotin, a contrast agent, a chemiluminescent agent such as luminol, isoluminol, aromatic acridinium ester, imidazole,

acridinium salt, or oxalate ester, a bioluminscent agent such as luciferin, luciferase or aequorin, a hormone, a signal peptide, a lipid, a fatty acid, e.g., conjugated linoleic acid, a sugar, an amino acid, a receptor, a part of a receptor, or any ligand of a receptor for example an antagonist of the CXCR4 chemokine receptor or binding molecule, or glutathione which enhances the delivery of the conjugate via the glutathione transferes for example across the blood-brain-barrier.

An RGD (Arg-Gly-Asp) peptide for example is linear or cyclic and consists of RGD or comprises RGD for example Arg-Gly-Asp-D-Phe-Cys, Arg-Gly-Asp-D-Phe-Glu, Arg-Gly-Asp-D-Phe-Lys, H-Gly-Arg-Gly-Asp-Asn-Pro-OH, or H-Gly-Gly-Gly-Gly-Arg-Gly-Asp-Ser-Pro-OH.

A compound of the present invention is preferably directed to the targeting and/or detecting of the oligonucleotide. Advantageously, the compounds, in particular markers and chromophors, are solidly bound to the oligonucleotide, preferably via native ligation or to the native ligation site, which reduces amongst others the risk of false positive results due to unbound markers, chromophors etc.

In a preferred embodiment, a polymer and/or compound, which is connected to the oligonucleotide via the native ligation site comprises an additional sterically available cysteine residue for example at the end or within the polymer and/or compound like the N-terminus, the C-terminus or within the polymer or compound such as a peptide, preferably for potential further bindings of polymers and/or compounds.

A conjugate of the present invention is an oligonucleotide, preferably an antisense oligonucleotide comprising a polymer and/or a compound at the 5´-end and/or the 3´-end of the oligonucleotide and/or at the native ligation site(s), wherein the 5´-end (Fig. 5) or the 3´-end (Fig. 8) of the oligonucleotide is or both ends are modified via native ligation.
Modification of an oligonucleotide means the binding of a polymer and/or a compound to the oligonucleotide, preferably to the 5´-end and/or the 3´-end of the oligonucleotide and/or the native ligation site, wherein the polymer and/or compound is bound to the 5´- and/or 3´- end of the oligonucleotide via native ligation or to one of the 5´- or 3´- ends with or without a linker.

The free end of an oligonucleotide or of an oligonucleotide conjugate is the 5´-end and 3´-end, respectively, of the oligonucleotide, which is optionally bound to a linker, and is free of a polymer and/or a compound. The free end of the oligonucleotide or the oligonucleotide conjugate is suitable to be bound to a polymer and/or a compound with or without a linker.

The direct binding of at least one polymer and/or at least one compound to an oligoucleotide results in a conjugate, i.e., a polymer-oligonucleotide, a compound-oligonucleotide, or a mixed compound-polymer-oligonucleotide conjugate. Further polymers and/or compounds are optionally bound to the oligonucleotide and conjugate, respectively, either directly at any position of the oligonucleotide for example at a phosphate group, a sugar moiety, and/or a base of the oligonucleotide, preferably at the 5´-, 3´-end and/or native ligation site of the oligonucleotide, or indirectly via a polymer or compound already bound to the oligonucleotide and conjugate, respectively. If only the 5´-end of the oligonucleotide is modified with a polymer or a compound via native ligation, the polymer or the compound is preferably not a peptide or a protein.

In a further embodiment one or more polymers and/or compounds are bound to the oligonucleotide and/or to a polymer and/or compound already bound to the oligonuleotide with or without a linker. The term “linker” comprises any type of linker, preferably cross-linker, which refer to any chemical substance able to bind at least two molecules such as an oligonucleotide and at least one polymer and/or compound. Linkers include zero length linkers, homobifunctional crosslinkers, heterobifunctional cross linkers and the like. Different linkers are usable and combinable, respectively, in a conjugate of the invention. In addition, a linker connects two or more oligonucleotides, which are modified or will be modified. Depending on the linker, the binding of the polymer and/or compound to the oligonucleotide is reversible for example via disulfid binding, or irreversible for example via thioether binding.

An example for a linker, which connectes a polymer and/or a compound, preferably a peptide, to a native ligation site is a Peptide Modifying Reagent (PMR) such as pentafluorophenyl S-benzyl-thiosuccinate (e.g., OPeC® Conjugation Reagents of link technologies).

Further linkers of the invention are for example homobifunctional linkers such as Lomant´s reagent dithiobis (succinimidylpropionate) DSP, 3´3´-dithiobis(sulfosuccinimidyl proprionate (DTSSP), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl)suberate (BS), disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo DST), ethylene glycobis(succinimidylsuccinate) (EGS), disuccinimidyl glutarate (DSG), N, N´-disuccinimidyl carbonate (DSC), dimethyl adipimidate (DMA), dimethyl pimelimidate (DMP), dimethyl suberimidate (DMS), dimethyl-3,3´-dithiobispropionimidate (DTBP), 1,4-di-3´-(2´-pyridyldithio)propionamido)butane (DPDPB), bismaleimidohexane (BMH), aryl halide-containing compound (DFDNB), such as e.g. 1,5-difluoro-2,4-dinitrobenzene or 1,3-difluoro-4,6-dinitrobenzene, 4,4´-difluoro-3,3´-dinitrophenylsulfone (DFDNPS), bis-[ß-(4-

azidosalicylamido)ethyl]disulfide (BASED), formaldehyde, glutaraldehyde, 1,4-butanediol diglycidyl ether, adipic acid dihydrazide, carbohydrazide, o-toluidine, 3,3´-dimethylbenzidine, benzidine, α, α´-p-diaminodiphenyl, diiodo-p-xylene sulfonic acid, N,N´-ethylene-bis(iodoacetamide), N,N´-hexamethylene-bis(iodoacetamide).

The linkage with heterobifunctional linkers is preferred, which increases specificity of the reaction. Examples for heterobifunctional linkers are amine-reactive and sulfhydryl cross-linkers such as N-succinimidyl 3-(2-pyridyldithio)propionate (sPDP), long-chain N-succinimidyl 3-(2-pyridyldithio)propionate (LC-sPDP), water-soluble- long-chain N-succinimidyl 3-(2-pyridyldithio) propionate (sulfo-LC-sPDP), succinimidyloxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (sMPT), sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-sMPT), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sMCC), sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-sMCC), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBs), m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBs), N-succinimidyl(4-iodoacteyl)aminobenzoate (sIAB), sulfosuccinimidyl(4-iodoacteyl)aminobenzoate (sulfo-sIAB), succinimidyl-4-(p-maleimidophenyl)butyrate (sMPB), sulfosuccinimidyl-4-(p-maleimidophenyl)butyrate (sulfo-sMPB), N-(γ-maleimidobutyryloxy)succinimide ester (GMBs), N-(γ-maleimidobutyryloxy)sulfosuccinimide ester (sulfo-GMBs), succinimidyl 6-((iodoacetyl)amino)hexanoate (sIAX), succinimidyl 6-[6-(((iodoacetyl)amino)hexanoyl)amino]hexanoate (sIAXX), succinimidyl 4-(((iodoacetyl)amino)methyl)cyclohexane-1-carboxylate (sIAC), succinimidyl 6-((((4-iodoacetyl)amino)methyl)cyclohexane-1-carbonyl)amino)hexanoate (sIACX), ρ-nitrophenyl iodoacetate (NPIA), carbonyl-reactive and sulfhydryl-reactive cross-linkers such as 4-(4-N-maleimidophenyl)butyric acid hydrazide (MPBH), 4-(N-maleimidomethyl)cyclohexane-1-carboxyl-hydrazide -8 (M_2C_2H), 3-(2-pyridyldithio)propionyl hydrazide (PDPH), amine-reactive and photoreactive cross-linkers such as N-hydroxysuccinimidyl-4-azidosalicylic acid (NHs-AsA), N-hydroxysulfosuccinimidyl-4-azidosalicylic acid (sulfo-NHs-AsA), sulfosuccinimidyl-(4-azidosalicylamido)hexanoate (sulfo-NHs-LC-AsA), sulfosuccinimidyl-2-(ρ-azidosalicylamido)ethyl-1,3´-dithiopropionate (sAsD), N-hydroxysuccinimidyl-4-azidobenzoate (HsAB), N-hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HsAB), N-succinimidyl-6-(4´-azido-2´-nitrophenylamino)hexanoate (sANPAH), sulfosuccinimidyl-6-(4´-azido-2´-nitrophenylamino)hexanoate (sulfo-sANPAH), N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOs), sulfosuccinimidyl-2-(m-azido-o-nitrobenzamido)-ethyl-1,3´-dithiopropionate (sAND), N-succinimidyl-4(4-azidophenyl)1,3´-dithiopropionate (sADP), N-sulfosuccinimidyl(4-azidophenyl)-1,3´-dithiopropionate (sulfo-sADP), sulfosuccinimidyl 4-(ρ-azidophenyl)butyrate (sulfo-sAPB), sulfosuccinimidyl 2-(7-azido-4-

methylcoumarin-3-acetamide)ethyl-1,3´-dithiopropionate (sAED), sulfosuccinimidyl 7-azido-4-methylcoumain-3-acetate (sulfo-sAMCA), ρ-nitrophenyl diazopyruvate (ρNPDP), ρ-nitrophenyl-2-diazo-3,3,3-trifluoropropionate (PNP-DTP), sulfhydryl-reactive and photoreactive cross-linkers such as1-(ρ-Azidosalicylamido)-4-(iodoacetamido)butane (AsIB), N-[4-(ρ-azidosalicylamido)butyl]-3´-(2´-pyridyldithio)propionamide (APDP), benzophenone-4-iodoacetamide, benzophenone-4-maleimide carbonyl-reactive and photoreactive cross-linkers such as ρ-azidobenzoyl hydrazide (ABH), carboxylate-reactive and photoreactive cross-linkers such as 4-(ρ-azidosalicylamido)butylamine (AsBA), and arginine-reactive and photoreactive cross-linkers such as ρ-azidophenyl glyoxal (APG). For more details see Hermanson (1996).

The oligonucleotide, a polymer and/or a compound, optionally including a linker, are covalently bound to each other, wherein the polymer and/or the compound is preferably bound to the 5´-end and/or 3´-end of the oligonucleotide and/or to the native ligation site. Optionally, the oligonucleotide, the polymer and/or the compound form non-covalent bindings such as intermolecular forces, ionic interactcions, e.g., Watson-Crick base pairing, wherein the polymer and/or the compound is likewise preferably bound to the 5´-end and/or 3´-end of the oligonucleotide and/or to the native ligation site.

The size of an oligonucleotide, which is modified according to the present invention, comprises or consists of at least 5 nucleotides, preferably of between 5 and 70 nucleotides, more preferably of between 10 and 60 nucleotides, even more preferably of between 10 and 40 nucleotides, with higher preference of between 12 and 25 nucleotides, and most preferred of between 12 and 20 nucleotides. In particular the oligonucleotide comprises or consists of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 nucleotides. The oligonucleotide is generated in any manner, preferably by chemical synthesis, DNA replication, reverse transcription, or a combination thereof, or isolated from a natural source.

Preferably, the oligonucleotide in the context of the invention comprises any type of oligonucleotide including an oligonucleotide derivative having e.g., an additional functional group for example an amino group for example at one or both ends of the oligonucleotide. These groups form the basis for example for the reaction with a polymer and/or compound leading to a conjugate such as a PEGylated oligonucleotide.

Further, oligonucleotides comprise nucleotide building blocks composed of base, sugar, and phosphate moiety. Oligonucleotides include oligonucleotides having non-naturally occurring oligonucleotide building blocks with similar function. Naturally occurring nucleotides as well

as non-naturally occurring nucleotides, modifications of these nucleotides at the base, the sugar or the backbone as well as spacers instead of a at least one nucleotide are also referred to as nucleotide building block. Modifications of an oligonucleotide are for example phosphorothioate, methylphosphonate, phosphoramidate, or 2´-modifications of the sugar (e.g., 2´-O-methyl oligonucleotide, 2´-O-methoxy-ethyl oligonucleotide, or 2´-deoxy-2´-fluoro oligonucleotide).

The oligonucleotide preferably comprises at least 5 nucleotide building blocks, preferably 5 to 120 nucleotide building blocks, more preferably 8 to 30 nucleotide building blocks, even more preferably 10 to 28 nucleotide building blocks, even more preferred 12 to 26 nucleotide building blocks, most preferred 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26 nucleotide building blocks.

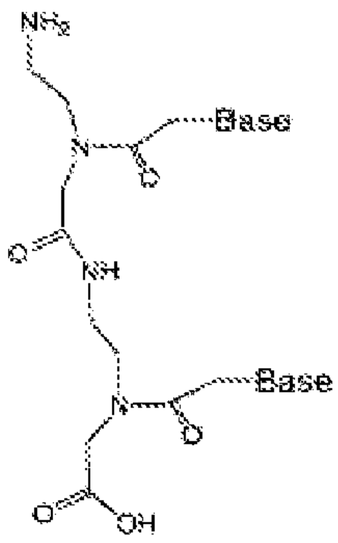
The term nucleotide building block comprises nucleotides composed of naturally-occuring nucleobases, sugars and covalent internucleoside (backbone) linkages, each of these also referred to as portions, as well as oligonucleotides having non-naturally-occuring portions which function similarly, e.g. hybridizing with the same mRNA of a selected target. In one embodiment the base is modified or substituted by a similar molecule. Similar bases are those molecules that are also able to support the hybridization to the mRNA or at least do not affect the hybridization in a negative way. In some embodiments at least one base portion is substituted with a spacer.

In other embodiments the sugar moiety of the nucleotide building block is modified or substituted by another group, structure, or moiety. Examples for sugars are arabinose, xylose, lyxose, allose, altrose, glucose, mannose, gulose, idose, galactose, talose or stabilized modifications of those sugars. In some embodiments a spacer substitutes the sugar.

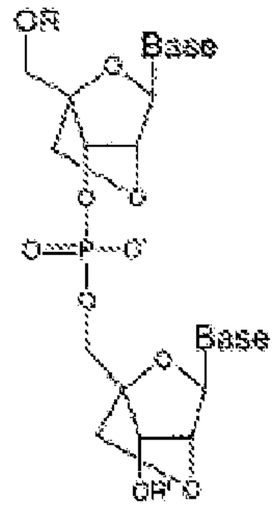
In other embodiments the internucleoside linkage, also referred to a linkage between two nucleotide building blocks, is not a phosphorodiester but another group, structure, or moiety.

Such oligonucleotides with at least one modified nucleotide building block are often preferred over native forms because of desirable properties such as enhanced cellular uptake, enhanced affinity for nucleic acid target, increased stability in the presence of nucleases and/or enhanced therapeutic effectiveness.

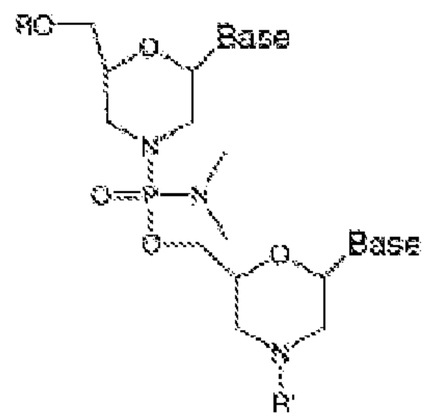
Oligonucleotides, having a modified nucleotide building block, further comprise for example peptide nucleic acid (PNA), locked nucleic acid (LNA), and morpholinos as shown in the following:



Peptide nucleic acid (PNA)



Locked nucleic acid (LNA)



Morpholino

PNA is chemically similar to DNA and RNA, and is generally artificially synthesized. The PNA´s backbone is composed for example of repeating N-(2-aminoethyl)-glycine units linked by peptide bonds. The various purine and pyrimidine bases are preferably linked to the backbone by methylene carbonyl bonds.

LNA is modified RNA, wherein the ribose moiety of an LNA nucleotide is modified with an extra bridge connecting the 2´ and 4´ carbons. The bridge “locks” the ribose in the 3´-endo structural conformations, which is often found in the A-form of DNA or RNA. LNA is combinable with DNA or RNA bases in an oligonucleotide.

Morpholino oligonucleotides are an antisense technology used to block access of other molecules to specific sequences with nucleic acid. Morpholinos block small (about 25 base) regions of the base-pairing surface of RNA or DNA.

In the context of this invention, the term oligonucleotide refers to an oligomer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof also referred to as nucleotide building block-polymers. The term oligonucleotide comprises single and double stranded RNA or DNA. In case of double stranded RNA or DNA the strands are blunt end or with overhanging ends, wherein one strand has an overhang on one end of the other strand or on both ends of the other strand, or one strand has an overhang on one side and the other strand has an overhang on the opposite side.

The term oligonucleotide further comprises aptamers and/or spiegelmers. Aptamers are nucleic acid molecules, comprising at least 5 or 8 nucleotides, preferably 10 to 100 nucleotides, more preferably 25 to 75 nucleotides, even more preferably 30 to 70 nucleotides, most preferred between 40 to 60 nucleotides, having specific binding affinity to molecules through interactions other than classic Watson-Crick base pairing. Aptamers are engineered through repeated rounds of *in vitro* selection or equivalently, SELEX (systemic evolution of ligands by exponential enrichment) to bind to various molecular targets such as small molecules, proteins, nucleic acids, even cells, tissues and organisms, preferably with affinities in the nanomolar to the picomolar range. Aptamers are for example engineered completely in a test tube, are readily produced by chemical synthesis, possess desirable storage properties, and elicit little or no immunogenicity in therapeutic applications. Moreover, aptamers are combinable with ribozymes to self-cleave in the presence of their target molecule.

Spiegelmers are developed on basis of aptamers. They consist of nucleotides having the L-form leading to a high resistance against nucleases. Hence, spiegelmers have all the diversity characteristics of aptamers as well as their binding characteristics, preferably in the low nanomolar to picomolar range, but possess a structure that prevents enzymatic degradation. Aptamers and spiegelmers bind both to extracellular and intracellular molecules such as a receptor or its ligand, to a transcription factor, or a lipid-containing molecule.

Furthermore, the term oligonucleotide comprises siRNA, RNAi, shRNA, and microRNA (miRNA). RNAi is a mechanism for RNA-guided regulation of gene expression in which double-stranded ribonucleic acid inhibits preferably the expression of genes with complementary nucleotide sequences. The RNAi pathway is initiated by the enzyme dicer, which cleaves double-stranded RNA to short double-stranded fragments preferably of 15 to 35 base pairs, more preferably of 20 to 30 base pairs, and most preferably 20 to 25 base pairs. One of the two strands of each fragment, which is the „guide strand“, is incorporated into the RNA-induced silencing complex (RISC) and base-pairs with a complementary sequence. The most-well studied effect of RNAi is post-transcriptional gene silencing, which occurs when the guide strand base pairs with a mRNA and induces degradation of the mRNA for example by argonaute, a catalytic component of the RISC complex. The short fragments are known as small interfering or silencing RNA (siRNA), which are preferably perfectly complementary to the gene, which is to be suppressed. In addition to their role in RNAi pathway, siRNA also act in RNAi-related pathways, e.g., as an antiviral mechanism or in shaping the chromatin structure of a genome. Similar to siRNA, microRNA (miRNA), which are single-stranded RNA molecules of 15 to 30 nucleotides, preferably 20 to 30 nucleotides,

and most preferably 21 to 23 nucleotides, regulate preferably gene expression. miRNA is encoded by genes that are transcribed from DNA, but not translated into protein (non-coding RNA). Instead the miRNA is processed from primary transcripts known as pre-miRNA preferably to short stem-loop structures such as pre-miRNA and finally to functional miRNA. A further type of RNA preferably involved in gene silencing is short hairpin RNA (shRNA). shRNA is RNA which makes a tight hairpin turn that is preferably suitable to silence gene expression via RNAi. shRNA uses a vector introduced into cells and utilizes a promoter, preferably the U6 promoter, to ensure that the shRNA is expressed. The vector is preferably passed on to daughter cells, allowing the gene silencing to be inherited. The cellular machinery preferably cleaves the shRNA hairpin structure, preferably into siRNA, which is then binding to RISC and starts the mechanism as described above.

Additionally, the term oligonucleotide comprises CpG oligonucleotides. CpG motifs induce preferably Toll-like receptor mediated immune response by simulating bacterial DNA. The CpG oligonucleotide preferably activates immune cells such as dendritic cells and B-lymphocytes, and stimulates NF-κB. CpG oligonucleotides are in general not a target sequence specific approach.

Moreover, the term oligonucleotide comprises a decoy oligonucleotide, also known as „decoy“, which is preferably a double-strand oligonucleotide bearing a consensus binding sequence for example of a specific transcription factor for manipulating gene expression.

The oligonucleotide of the present invention preferably hybridizes with mRNA of a molecule negatively influencing a physiological and/or biochemical effect in a cell or hybridizes with mRNA of the receptor of the molecule. In a preferred embodiment, the modified oligonucleotide of the present invention hybridizes with mRNA of TGF-beta1, TGF-beta2, TGF-beta3, VEGF, IL-10, c-jun, c-fos, Her-2, MIA, receptors thereof, and/or prostaglandin E2 receptor, or binds to and interacts with, respectively, a target in form of an aptamer or a spiegelmer. Further preferred embodiments of oligonucleotides are given in the sequence listing, or are oligonucleotides published in WO 94/25588, WO 95/17507, WO 95/02051, WO 98/33904, WO 99/63975, WO 01/68146, WO 01/68122, WO 03/06445, WO 2005/014812, WO 2005/059133, WO 2005/084712 herein incorporated by reference.

Preferred oligonucleotides that are linked with at least one polymer and/or at least one compound according to the present invention comprise or consist of at least one of SEQ ID NO 1 to 435 (cf. Fig. 1), or of example 10.

Especially preferred oligonucleotides consist of or comprise SEQ ID NO. 2030, 2031, 2032, 2057 and/or 2066. Mostly preferred are the oligonucleotides comprising or consisting of SEQ ID No.: 2030 (CGGCATGTCTATTTTGTA) and/or SEQ ID No.: 2057 (CTGATGTGTTGAAGAACA).

In a preferred embodiment, the modified oligonucleotide of the present invention is a novel, improved therapeutic agent for controlling, treating and/or preventing one or more of cellular proliferative and/or differentiating diseases or disorders, diseases or disorders associated with bone metabolism, immune, hematopoietic, cardiovascular, liver, kidney, muscular, hematological, viral, pain, neurological and/or metabolic diseases or disorders, in particular disorders or diseases associated with undesired TGF-beta signaling. The term cancer, carcinoma, or neoplasm includes malignancies of the various organ systems for example such affecting brain, eye, lung, breast, thyroid, lymphoid, gastrointestinal, and genito-urinary tract.

The diseases or disorders, in particular maligne or benign tumors or metastases and their formation, which are controllable, preventable and/or treatable with a modified oligonucleotide of the present invention, or a pharmaceutical composition comprising or consisting of such modified oligonucleotide include, but are not limited to solid tumors, blood born tumors such as leukemias, acute or chronic myelotic or lymphoblastic leukemia, tumor metastasis, benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, pyogenic granulomas, pre-malignant tumors, rheumatoid arthritis, psoriasis, astrocytoma, acoustic neuroma, blastoma, craniopharyngloma, ependymoma, Ewing´s tumor, medulloblastoma, glioma, hemanglioblastoma, Hodgkins-lymphoma, medullablastoma, leukaemia, mesothelioma, neuroblastoma, neurofibroma, non-Hodgkins lymphoma, pinealoma, retinoblastoma, sarcoma (including angiosarcoma, chondrosarcoma, endothelialsarcoma, fibrosarcoma, leiomyosarcoma, liposarcoma, lymphangioandotheliosarcoma, lyphangiosarcoma, melanoma, meningioma, myosarcoma, oligodendroglioma, osteogenic sarcoma, osteosarcoma), seminoma, trachomas, Wilm´s tumor, or is selected from the group of bile duct carcinoma, bladder carcinoma, brain tumor, breast cancer, bronchogenic carcinoma, carcinoma of the kidney, cervical cancer, choriocarcinoma, cystadenocarcinome, embryonal carcinoma, epithelial carcinoma, esophageal cancer, cervical carcinoma, colon carcinoma, colorectal carcinoma, endometrial cancer, gallbladder cancer, gastric cancer, head cancer, liver carcinoma, lung carcinoma, medullary carcinoma, neck cancer, non-small-cell bronchogenic/lung carcinoma, ovarian cancer, pancreas carcinoma, papillary carcinoma, papillary adenocarcinoma, prostate cancer, small intestine carcinoma, prostate carcinoma, rectal cancer, renal cell carcinoma,

skin cancer, small-cell bronchogenic/lung carcinoma, squamous cell carcinoma, sebaceous gland carcinoma, testicular carcinoma, uterine cancer, liver metastasis, lung metastasis, brain metastasis, metastasis of lymphoma.

The modified oligonucleotides of the invention as well as the pharmaceutical compositions comprising or consisting of one or more such modified oligonucleotides are also suitable to control, prevent and/or treat a variety of immune disorders such as autoimmune diseases or disorders, e.g., diabetes mellitus, arthritis, including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriac athritis; multiple sclerosis, encephalomyelitis, myasthenia gravis, systemic lupus erythrematosis, autoimmunethyroiditis, dermatitis, including atopis dermatitis, eczematous dermatitis; psoriasis, Sjgren´s Syndrome, Crohn´s disease, aphthous ulcer, irits, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener´s granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Graves´ disease, sarcoidosis, primary billiary cirrhosis, uveitis posterior, and interstitial lung fibrosis, graft-versus-host disease, cases of transplantation, and allergy such as an atopic allergy. Moreover, the modified oligonucleotide or the pharmaceutical composition is suitable to control, prevent and/or treat cardiovascular diseases or disorders such as hypertension, atherosclerosis, coronary artery spasm, congestive heart failure, coronary artery disease, valvular disease, arrhythmias, and cardiomyopathies; or viral diseases or disorders for example hepatitis A (HVA), hepatitis B (HVB), hepatitis C (HVC), or caused by herpes simplex virus (HSV), HIV, FIV, poliovirus, influenza virus, adenoviruses, papillomaviruses, Epstein-Barr-viruses and small pox virus, or virus-associated cancer such as hepatocellular cancer.

The modified oligonucleotides of the invention as well as the pharmaceutical compositions comprising or consisting of one or more such oligonucleotides are further suitable to control, prevent and/or treat fibrotic diseases or disorders which are for example associated with undesired TGF-beta signaling, which include, without limitation, kidney disorders and (excessive) fibrosis and/or sclerosis, such as glomerulonephritis (GN) of all etiologies, e.g., mesangial proliferative GN, immune GN, crescentic GN; diabetic nephropathy, renal interstitial fibrosis and all causes of renal interstitial fibrosis including hypertension, renal fibrosis resulting from complications of drug exposure, including cyclosporin treatment of transplant recipients, HIV-associated nephropathy, or transplant nephropathy; hepatic

diseases associated with (excessive) scarring and (progressive) sclerosis for example cirrhosis due to all etiologies, disorders of the biliary tree, and hepatic dysfunction; pulmonary fibrosis with consequential loss of gas exchange or ability to efficiently move air into and out of the lungs such as adult respiratory distress syndrome (ARDS), chronic obstructive pulmonary disease (COPD), idiopathic pulmonary fibrosis (IPF), acute lung injury (ALI) or pulmonary fibrosis due to infectious or toxic agents such as smoke, chemicals, allergens, or autoimmune diseases; eye diseases or disorders associated with fibroproliferative states such as fibroproliferative vitreoretinopathy of any etiology or fibrosis associated with ocular surgery, e.g., treatment of glaucoma, retinal reattachement, cataract extraction, or drainage procedures of any kind; excessive or hypertrophic scar formation in the dermis occuring for example during wound healing resulting from trauma or surgical wounds. The modified oligonucleotides, and compositions of the invention, respectively, are particularly designed to target genes associated with particular diseases or disorders, preferably TGF-ß such as TGF-ß1, -ß2, or -ß3.

The oligonucleotide conjugate of this invention is administerable by different routes, which include, but are not limited to electroporation, epidermal, impression into skin, intra-arterial, intra-articular, intra-cranial, intra-thecal, intra-cerebral, intra-dermal, intra-lesional, intra-muscular, intra-nasal, intra-ocular, intra-peritoneal, intra-prostatic, intra-pulmonary, intra-spinal, intra-tracheal, intra-tumoral, intra-venous, intra-vesical placement within cavities of the body, nasal inhalation, oral, pulmonary inhalation (e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer), subcutaneous, subdermal, transdermal, or topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery). Topical administration further comprises administration of the skin, the eyes and the ears.

In one embodiment, a cytokine and/or a chemotherapeutic is co-administered with one or more oligonucleotide conjugates of the invention. The cytokine and/or the chemotherapeutic increases the effect of the oligonucleotide and/or of one or more compounds bound to the oligonucleotide for example a small molecule, an antibody for example MUJ591, an antibody binding to glycoprotein IIb/IIa, to CD52, to CD25, to VEGF, to the epidermal growth factor, to CD33, to CD20, or to carbonic anhydrase, an antigen for example an external domain of prostate-specific membrane antigen (PSMA), an enzyme, a part of an antibody, or an enzyme, any peptide, e.g., an internalizing peptide, a cell penetrating peptide such as penetratin, TAT (transactivator or transcription), Transportan (e.g., TP10), R9 peptide, MPG peptide, KALA peptide, a pH (low) insertion peptide, hormonal peptides such as LHRH, bombesin, somatostatin, or a targeting peptide such as RGD (Arg-Gly-Asp), an aptamer

(based on nucleic acid, protein, or peptide), a spiegelmer, siRNA, e.g., cholesterol-modified siRNA, RNAi, shRNA, microRNA (miRNA), a human serum albumin carrier, a tumor vaccine such as a tumor antigen e.g., PSA, PAP, HIFalpha or an immune stimulatory agent, a cytotoxine such as TMZ, BCNU, DTIC, 5-Fluorouracil (FU), gemcitabine, taxol (pacitaxel), irinoteca, oxaliplatin doxorubicin, cyclophosphamid, folinsäure, a chromophor, a marker for example a fluorescent marker such as FITC, rhodamine, phycoerytherin, phycocynin, allophycocyanin, o-phthaldehyde or fluorescamine, biotin, a contrast agent, a chemiluminescent agent such as luminol, isoluminol, aromatic acridinium ester, imidazole, acridinium salt, or oxalate ester, a bioluminscent agent such as luciferin, luciferase or aequorin, a hormone, a signal peptide, a lipid, a fatty acid, e.g., conjugated linoleic acid, a sugar, an amino acid, a receptor, a part of a receptor, or any ligand of a receptor for example an antagonist of the CXCR4 chemokine receptor or binding molecule, or glutathione which enhances the delivery of the conjugate via the glutathione transferase for example across the blood-brain-barrier. A compound of the present invention is preferably directed to the targeting and/or detecting of the oligonucleotide. Preferred cytokines are for example interferones such as α-, β- or γ-interferone (IFN), interleukins (IL) such as IL-1α, -1β, -2, -3 -4, -5, -6, -7, -8, -9, -10, -11, -12, -13, -16, -17, -18, -22, -23, or 31, or colony stimulating factors (CSF) such as CSF1, -2 (GM-CSF or sargramostin), or -3 (G-CSF or filgrastim) , or a combination thereof. Preferred chemotherapeutics are for example alkylating agents, antimetabolites, anthracyclines, alkaloids, topoisomerase inhibitors, and antitumor agents, in particular temozolomide, gemcitabine, avastin, or a combination thereof.

In a preferred embodiment, a polymer such as polylakylene oxide, e.g., PEG is bound to the 5´-end and/or 3´-end of an oligonucleotide via native ligation resulting in a native ligation site, preferably comprising a cysteine residue, which is either protected to avoid interaction of a further polymer and/or compound with the native ligation site, or is bound to a polymer such as PEG, or a compound such as a small molecule, an antibody, an antigen, an enzyme, a part of an antibody, or an enzyme, any peptide, e.g., an internalizing peptide, an aptamer (based on nucleic acid, protein, or peptide), a spiegelmer, RNAi, shRNA, microRNA (miRNA) a chromophor, biotin, a hormone, a signal peptide, a lipid, a fatty acid, a sugar, an amino acid, a receptor, a part of a receptor, or any ligand of a receptor or binding molecule, optionally via a reversible or irreversible linker.

Preferably, hydroxy protecting groups are for example t-butyl, t-butoxymethyl, methoxymethyl, tetrahydropyranyl, 1-ethoxyethyl, l-(2-chloroethoxy)ethyl, 2-trimethylsilyl ethyl, p-chlorophenyl, 2,4-dinitrophenyl, benzyl, 2,6-dichlorobenzyl, diphenylmethyl, p, p´-dinitrobenzylhydryl, triphenylmethyl, trimethylsilyl, triethylsilyl, t-butyldimethylsilyl, t-butyldiphenylsilyl, triphenylsilyl, benzoylformate, acetate, chloroacetate, trichloroacetate,

trifluoroacetate, pivaloate, benzoate, p-phenylbenzoate, 9-fluorenylmethyl carbonate, mesylate and tosylate.

Amino protecting groups are for example dimethoxytrityl (DMT), monomethoxyethyl (MMT), 2-trimethylsilylethoxycarbonyl (Teoc), 1-methyl-1-(4-biphenylyl)ethoxycarbonyl (Bpoc), t-butoxycarbonyl (BOC), allyloxycarbonyl (Alloc), 9-fluorenylmethyloxycarbonyl (Fmoc), benzyloxycarbonyl (Cbz); amide protecting groups such as formyl, acetyl, trihaloacetyl, benzoyl, and nitrophenylacetyl; sulfonamide protecting groups such as 2-nitrobenzenesulfonyl, or imine and cyclic imide protecting groups such as phthalimido and dithiasuccinoyl.

Carbonyl protecting groups are for example acetals, ketals, acylals, or dithianes. Carboxylic acid protecting groups are for example methyl esters, benzyl esters, tert-butyl esters, or silyl esters.

Thiol protecting reagents are for example ethyliodoacetate, an irreversible capping reagent, resulting in thioether, or dipyridyldisulfide, a reversible protection group, resulting in disulfide, Benzyl-S or tert. Butyl-S etc.

Equivalents of the protecting groups such as hydroxy protecting groups, carbonyl protecting groups, carboxylic acid protecting groups, thiol protecting groups or amino protecting groups are also encompassed by the conjugates or compounds and the methods of their production.

In a further preferred embodiment, the oligonucleotide conjugate of the present invention based on native ligation comprises a polyalkylene oxide, e.g., PEG at the 5´-and/or 3´-end, and optionally a polymer such as PEG and/or a compound like penetratin and/or an RGD peptide at the native ligation site, wherein the polyalkylene oxide such as PEG at both ends is identical in size or differs in size.

Polymers for example polyalkylene oxide, in particular PEG is available as polydispers or monodispers material. The polydispers material comprises dispers distribution of different molecular weight of the material, characterized by mean weight (weight average) size and dispersity. The monodispers PEG comprises one size of molecules. Polyalkylene oxide, preferably PEG, of the present invention is poly- or monodispers and the indicated molecular weight represents an average of the molecular weight of the polyalkylene oxide, e.g., PEG, molecules. Moelcular weights indicated for polymers, in particular PEGs are often rounded; alternatively, polymers, in particular PEGs are indicated according to the number of

monomers the polymer comprises, for example PEG4 comprises 4 monomers and has a molceular weight of 200.

For example the molecular weight of a polymer for example polyalkylene oxide such as PEG, which is linked to the 5´-end of the oligonucleotide is 200 (e.g., PEG 4), 300, 400 (e.g., PEG 8), 500, 600, 700, 800, 900, 1000 (e.g. PEG 24), 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500 or 4750, 5000, 10000, 20000, 50000 or 100000 Da and the molecular weight of the polyalkylene oxide linked to the 3´-end of the oligonucleotide is 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500 or 4750, 5000, 10000, 20000, 50000 or 100000 Da, or the molecular weight of the polymer for example polyalkylene oxide such as PEG linked to the 3´-end of the oligonucleotide is 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500 or 4750, 5000, 10000, 20000, 50000 or 100000 Da and the molecular weight of the polymer for example polyalkylene oxide such as PEG linked to the 5´-end of the oligonucleotide is 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500 or 4750, 5000, 10000, 20000, 50000 or 100000 Da, or the molecular weight of the polymer for example polyalkylene oxide such as PEG linked to the 5´-end and/or the 3´-end of the oligonucleotide is 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500 or 4750, 5000, 10000, 20000, 50000 or 100000 Da and the molecular weight of the polymer for example polyalkylene oxide such as PEG linked to the native ligation site is 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500 or 4750, 5000, 10000, 20000, 50000 or 100000 Da, or the molecular weight of the polymer for example polyalkylene oxide such as PEG linked to the native ligation site is 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500 or 4750, 5000, 10000, 20000, 50000 or 100000 Da and the molecular weight of the polymer for example polyalkylene oxide such as PEG linked to the 5´-end and/or the 3´-end of the oligonucleotide is 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1250, 1500, 1750, 2000, 2250, 2500, 2750, 3000, 3250, 3500, 3750, 4000, 4250, 4500 or 4750, 5000, 10000, 20000, 50000 or 100000 Da. Polymers, in particular PEGs of the present invention are preferably monodispers polymers and PEGs, respectively.

In a further preferred embodiment, the modified oligonucleotide comprises a polymer for example polyalkylene oxide such as PEG, and a compound for example an antibody and an internalizing peptide such as penetratin or an RGD peptide. The antibody guides the

modified oligonucleotide to the target, and the PEG avoids amongst others and reduces, respectively, degradation of the modified oligonucleotide for example via 5´- and/or 3´-exonuclease, and/or endonuclease. Once the target is reached, the antibody and/or the PEG is/are separated from the modified oligonucleotide, preferably via a reversible linker such as a disulfide binding, and the internalizing peptide promotes entrance of the oligonucleotide into the target cell. Alternatively, the modified oligonucleotide comprises a polymer such as PEG at the 5´-end or at the 5´- and 3´-end of the oligonucleotide, and a compound such as an antibody at the 3´-end or at the native ligation site of the oligonucleotide for example. Once the target is reached, the modified oligonucleotide interacts with the surface of the target cell.

In a further alternative, an oligonucleotide comprises a polymer for example PEG at its 5´- and 3´-end, which are connected to the oligonucleotide via native ligation, resulting in two native ligation sites at the 5´- and the 3-end of the oligonucleotide. A compound like an antibody, e.g., for targeting, and an internalizing peptide such as penetratin are connected independently to one of the native ligation sites. Once the target is reached, the antibody is removed from the oligonucleotide and/or the compounds, e.g., the PEGs are removed and the penetratin improves the incorporation of the oligonucleotide into the cell.

In another embodiment, a solid phase is connected to the 3´-end and/or the native ligation site of the modified oligonucleotide comprising a polymer and/or a compound at the 5´-end. Such combination forms for example the basis for a test kit using the modified oligonucleotide for the detection of a cell or cell factor.

The following examples will further illustrate the present invention without limiting the subject matter of the present invention to these examples.

Examples

The present invention is further described with references to the following examples, wherein the invention is not limited to the examples. The oligonucleotides used in the following examples were preferably SEQ ID No. 2030, 2031, 2032, 2057, 2066, and 2461, in particular SEQ ID No. 2030.

Example 1 – Synthesis of a 5´-cysteine-modified oligonucleotide-thiophosphate (Fig. 3)

Synthesis of an oligonucleotide having a phosphorothioate backbone on a DNA/RNA sythesizer according to a modified synthesis protocol comprising double coupling cycles on an amino-on-support in a 2µmol scale (2µmol net weight of functional groups of the support per cartridge). In the last step comprising a single coupling step, a cysteine modifier such as OMR (oligonucleotide-modifying reagens for example Link-technologies Ltd., Lanarkshire, Scotland) dissolved in 0.1 M acetonitril was connected to the 5´-end of the oligonucleotide, wherein the OMR is protected with a Fmoc and a tertiary Butyl-S-(tBu-S) protecting group. Once, the synthesis of the oligonucleotide is finished, the Fmoc protecting group is removed from the OMR using 20 % piperidine in dimethyl formamide (DMF). Piperidine is then removed by washing the oligonucleotide in pure DMF, which is finally removed by acetonitril. The support is removed from the cartridge, dried at 37 °C then the oligonucleotide is cleaved from the solid support by ammonia overnight, preferably by concentrated ammonia at 55 °C.

The final product is purified via HPLC, wherein the eluate is collected and the volume of the eluate is reduced via ultracentrifugation or ultrafiltration, quantified, dried preferably in a speedvac and stored at -20 °C. The identity of the product is controlled via MALDI-TOF-MS.

Example 2 – Production of a PMR-modified PEG component (Fig. 4)

Starting material for the PEG modified with a thioester is for example common amino functionalized PEG and PMR (peptide-modifying reagents (pentafluorophenyl-S-benzyl-thiosuccinat) for example Link-technologies Ltd., Lanarkshire, Scotland). 100µmol methoxy-PEG-NH2 of MW 400 (mPEG400-NH2), i.e., 400µl of a 250mM solution in DMF, is mixed with a 1.5 x excess of PMR, i.e., 150µmol (58mg) in 100µl DMF, and added to 500µl solution of 30µmol (4,6mg) HOBt (N-hydroxybenzotriazole). The composition is incubated for 30 min. at 37 °C. The reaction is controlled using an analytic RP-HPLC, wherein the mPEG400-PMR forms a sharp peak, and the isolation of mPEG400-PMR was likewise performed on RP-HPLC. The isolated PEG400-PMR is dried, ultrafiltrated, and/or ultracentrifuged and stored at -20 °C.

Example 3 – Modification of the 5´-end of a 5´-OMR-modified oligonucleotide via native ligation (Fig. 5)

The modification of the 5´-end of the oligonucleotide is based on the reaction of the thioester of the PEG-PMR component, for example the mPEG400-PMR component (see example 2), with the cytseine residue of the OMR-modified oligonucleotide (see example 1). In a first step an intermediate thioester bond is formed followed by an intramolecular acyl rearrangement resulting in an amide bond, i.e., native ligation.

The reaction buffer for the native ligation comprises 100mM Tris(carboxyethyl)phosphine (TCEP), 50mM Triethylammonium acetate (TEAA) pH7.5, and 25mM Mercaptophenyl acetic acid (MPAA) for an oligonucleotide concentration of 1nmol/µl and an mPEG400-PMR concentration of 10nmol/µl.

In a first step the 5´-OMR-modified oligonucleotide comprising cysteine is chemically reduced via TCEP to remove the tertiary Butyl-S (tBu-S) protecting group from the cysteine residue of the 5´-OMR-modified oligonucleotide. The mixture is incubated for 1 h at 37 °C, and the reaction is controlled using RP-HPLC.

Parallel, in a second independent step, the PEG-PMR-compound / -polymer such as the mPEG400-PMR is activated via MPAA by interacting with the mPEG-PMR and exchanging the Benzyl-S (Bn-S) group of the thioester; this mixture is likewise incubated for 1 h at 37 °C, and the reaction is controlled using RP-HPLC.

The combination of the reduced oligonucleotide of step one and the activated PEG-PMR-compound / -polymer of step two, and the incubation at 37 °C leads to native ligation resulting in the final product of the reaction comprising an amide bond. Reaction time is between 24 and 200 h.

Example 4 - Protection of the thiol residue via dipyridyldisulfide (Fig. 6)

The thiol function of the cysteine-residue of the PEG-PMR-oligonucleotide intermediate product, which is the direct product of native ligation (see example 3), is protected by adding an 1000 x molar excess of dipyridyldisulfide (1M Dithiopyridin, Py2S2 dissolved in methanol) to the reaction buffer of example 3; the mixture is incubated at 37 °C for about 20 h; alternatively DTNB or Ellman´s reagens were used. The resulting product, which is for example a mPEG400-PMR, 5´-end PEG modified oligonucleotide comprising cysteine having

a protecting group at the cysteine residue, is isolated via ultrafiltration and RP-HPLC; the final product is dried and stored at -20 °C.

Example 5 - Modification of the 3´-end of a native ligation modified oligonucleotide (Fig. 7)

The modification of the 3´-end of the 5´-end native ligation modified oligonucleotide comprising a pyridyl-disulfid protected cysteine (see example 4) is based on the reaction of the 3´-terminal amino function with for example PEG-NHS such as mPEG2000-NHS. Reaction buffer is 0.3M $NaHCO_3$ pH 8.5 for an oligonucleotide concentration of 1 mM. About 10 times 3 x excess of mPEG-NHS is added to the 5´-end PEG modified oligonucleotide, and is incubated at 37°C for 10 min. The reaction is controlled via RP-HPLC, and the final product is isolated via RP-HPLC, IE-FPLC, or ultrafiltration which is subsequently dried for example in a speedvac, and stored at -20 °C.

Example 6 – Stability of an unmodified oligonucleotide against endonuclease, 5´-exonuclease, and 3´-exdonuclease, respectively

An unmodified oligonucleotide comprising 18 nucleotides and a phosphate or a thiophosphate backbone was incubated with S1 endonuclease (0.005 U/µl, i.e., 0.05 U per nmol oligonucleotide) in 70 mM Tris-HCl, 50 mM NaCl, 20 mM $MgCl_2$, pH 7 at 37 °C.

A second sample of the unmodified oligonucleotide was incubated with 5´-exonuclease (for example phosphodiesterase II (PDE II)) in 0.1 M TEAA buffer pH 6.5 (0.018 U/µl, i.e., 0.18 U per nmol oligonucleotide), and a third group of the unmodified oligonucleotide was incubated with 3´-exonuclease (for example phosphodiesterase I (PDE I)) in 100 mM Tris-HCl, 100 mM NaCl, 14 mM $MgCl_2$, pH 8.8 (0.0001 U/µl, i.e., 0.001 U per nmol oligonucleotide).

The results are presented in the following, wherein the amount of oligonucleotide at t=0 was set 100 %. The degradation of the oligonucleotides of all three samples was tested by RP-HPLC directly at the start of the experiment (t=0), after 1 h, 24 h, 48 h, and 144 h. Table 1 shows the results of the endonuclease, table 2 of the 5´-exonuclease, and table 3 of the 3´-exonuclease.

Table 1: Effect of the endonuclease (S1)

	Unmodified oligonucleotide comprising phosphate backbone	Unmodified oligonucleotide comprising thiophosphate backbone
0 h	100 %	100 %
1 h	16 %	21 %
24 h	-*	4 %
48 h	-*	-*
144 h	-*	-*

*No oligonucleotide detectable at 24 h, 48 h and 144 h

Table 2: Effect of the 5´-exonuclease (PDE II)

	Unmodified oligonucleotide comprising phosphate backbone	Unmodified oligonucleotide comprising thiophosphate backbone
0 h	100 %	100 %
1 h	24 %	100 %
24 h	16 %	86 %
48 h	-*	79 %
144 h	-*	63 %

*No oligonucleotide detectable at 48 h and 144 h

Table 3: Effect of the 3´-exonuclease (PDE I)

	Unmodified oligonucleotide comprising phosphate backbone	Unmodified oligonucleotide comprising thiophosphate backbone
0 h	100 %	100 %
1 h	30 %	96 %
24 h	-*	66 %
48 h	-*	54 %
144 h	-*	20 %

*No oligonucleotide detectable at 24 h, 48 h and 144 h

Example 7 - Stability of the modified oligonucleotides against endonuclease

A modified oligonucleotide comprising a thiophosphate backbone was bound to mPEG400 at the 5´-end of the oligonucleotide via native ligation and to mPEG2000 (mPEG400-Oligo-mPEG2000) at the 3´-end via NHS ester coupled to the 3´-aminofunction of the oligonucleotide and incubated with S1 endonuclease (0.005 U/µl, i.e., 0.05 U per nmol oligonucleotide) in 70 mM Tris-HCl, 50 mM NaCl, 20 mM $MgCl_2$, pH 7 at 37 °C. The

degradation of the oligonucleotide was tested by RP-HPLC directly at the start of the experiment (t=0), after 1 h, 24 h, 48 h, and 144 h. Table 4 shows the results of these experiments.

Table 4: Effect of the endonuclease (S1)

	mPEG400-Oligo-mPEG2000
0 h	100 %
1 h	100 %
24 h	90 %
48 h	85 %
144 h	66 %

The modified oligonucleotides show a higher stability than the unmodied oligonucleotides of table 1.

Example 8 – Testing stability of the modified oligonucleotides against endonuclease on PAGE (Fig. 9a - c)

An unmodified oligonucleotide and modified oligonucleotides comprising a thiophosphate backbone were incubated with S1 endonuclease (0.005 U/µl, i.e., 0.05 U per nmol oligonucleotide) in 70 mM Tris-HCl, 50 mM NaCl, 20 mM $MgCl_2$, pH 7 at 37 °C. The modified oligonucleotides were either bound to mPEG400 at the 5´-end of the oligonucleotide via native ligation and to mPEG1000 (mPEG400-Oligo-mPEG1000) at the 3´-end via NHS ester or to mPEG1000 at the 5´-end of the oligonucleotide via native ligation and to mPEG1000 (mPEG1000-Oligo-mPEG400) at the 3´-end via NHS ester. The degradation of the oligonucleotides was tested by PAGE directly at the start of the experiment (t=0), after 1 h, 24 h, 48 h, 72 h, and 144 h. 1 µl (0.01 nmol) of each sample was mixed with 5 µl formamide and the samples were separated on a polyacrylamid gel via polyacrylamid gel electrophoresis (PAGE) comprising 6.3 g urea, 7.5 ml acrylamid/bisacrylamid 19:1 (40%), 1.5. ml TBE buffer (10x), 1.5 ml water, 90 µl Amoniumpersulfat (APS) and 7 µl N, N, N´, N´-Tetramethylenethylenediamine (TEMED). Current time of the gel was about 2.5 h at 5 mA. The weight marker on the gel comprises 0.01 nmol mPEG1000-Oligo-mPEG1000, mPEG400-Oligo-mPEG400, unmodified oligonucleotide, unmodified oligonucleotide (n-1), and unmodified oligonucleotide (n-2) (decreasing size).

Fig. 9a shows the results of the unmodified oligonucleotide, Fig. 9b of the mPEG400-Oligo-mPEG1000, and Fig. 9c of the mPEG1000-Oligo-mPEG400, which demonstrate an increased stability of mPEG400-Oligo-mPEG1000 and mPEG1000-Oligo-mPEG400 against S1 endonuclease incubation.

Example 9 – Stability of the modified oligonucleotides against 5´-exonuclease (PDE II)

A modified oligonucleotide comprising a thiophosphate backbone was bound to mPEG400 at the 5´-end of the oligonucleotide via native ligation and to PEG1000 at the 3´-end of the oligonucleotide via reaction of an NHS ester with the 3´-aminofunction (mPEG400-Oligo-mPEG1000) and was incubated with 5´-exonuclease in 0.1 M TEAA buffer pH 6.5 (0.018 U/µl, i.e., 0.18 U per nmol oligonucleotide), at 37°C. The degradation of the oligonucleotide was tested directly at the start of the experiment (t=0), after 1 h, 24 h, 48 h, and 144 h. Table 5 shows the results of these experiments.

Table 5: Effect of the 5´-exonuclease (PDE II)

	mPEG400-Oligo-mPEG1000
0 h	100 %
1 h	97 %
24 h	95 %
48 h	92 %
144 h	91 %

The modified oligonucleotides show a higher stability than the unmodied oligonucleotides of table 2.

Example 10 – Testing stability of the modified oligonucleotides against 5´-exonuclease (PDE II) on PAGE (Fig. 10a – c)

An unmodified oligonucleotide and modified oligonucleotides comprising a thiophosphate backbone were incubated with 5´-exonuclease (0.018 U/µl, i.e., 0.18 U per nmol oligonucleotide) in 0.1M TEAA buffer pH 6.5 at 37 °C. The modified oligonucleotides were either bound to mPEG400 at the 5´-end of the oligonucleotide via native ligation and to mPEG1000 (mPEG400-Oligo-mPEG1000) at the 3´-end via NHS ester or to mPEG1000 at the 5´-end of the oligonucleotide via native ligation and to mPEG1000 (mPEG1000-Oligo-mPEG400) at the 3´-end via NHS ester. The degradation of the oligonucleotides was tested

by RP-HPLC directly at the start of the experiment (t=0), after 1 h, 24 h, 48 h, 72 h, and 144 h. The samples were tested on PAGE according to Example 8.

Fig. 10a shows the results of the unmodified oligonucleotide, Fig. 10b of the mPEG400-Oligo-mPEG1000, and Fig. 10c of the mPEG1000-Oligo-mPEG400, which demonstrate an increased stability of mPEG400-Oligo-mPEG1000 and mPEG1000-Oligo-mPEG400 against 5´-exonuclease incubation.

Example 11 – Stability of the modified oligonucleotides against 3´-exonuclease (PDE I)

A modified oligonucleotide comprising a thiophosphate backbone was bound to mPEG400 at the 5´-end of the oligonucleotide via native ligation and to mPEG1000 at the 3´-end of the oligonucleotide via NHS ester (mPEG400-Oligo-mPEG1000) and was incubated with 3´-exonuclease in 100 mM Tris-HCl, 100 mM NaCl, 14 mM $MgCl_2$, pH 8.8 (0.0001 U/µl, i.e., 0.001 U per nmol oligonucleotide) at 37 °C. The degradation of the oligonucleotide was tested by RP-HPLC directly at the start of the experiment (t=0), after 1 h, 24 h, 48 h, and 144 h. Table 6 shows the results of these experiments.

Table 6: Effect of the 3´-exonuclease (PDE I)

	PEG400-Oligo-PEG1000
0 h	100 %
1 h	98 %
24 h	79 %
48 h	62 %
144 h	44 %

The modified oligonucleotides show a higher stability than the unmodied oligonucleotides of table 3.

Example 12 - Testing stability of the modified oligonucleotides against 3´-exonuclease (PDE I) on PAGE (Fig. 11a – c)

An unmodified oligonucleotide and modified oligonucleotides comprising a thiophosphate backbone were incubated with 3´-exonuclease (0.0001 U/µl, i.e., 0.001 U per nmol oligonucleotide) in 100mM Tris-HCl buffer 100 mM NaCl, 14 mM $MgCl_2$, pH 8.8 at 37 °C. The modified oligonucleotides were either bound to mPEG400 at the 5´-end of the oligonucleotide

via native ligation and to mPEG1000 (mPEG400-Oligo-mPEG1000) at the 3´-end via NHS ester or to mPEG1000 at the 5´-end of the oligonucleotide via native ligation and to mPEG1000 (mPEG1000-Oligo-mPEG400) at the 3´-end via NHS ester. The degradation of the oligonucleotides was tested by PAGE directly at the start of the experiment (t=0), after 1 h, 24 h, 48 h, 72 h, and 144 h. The samples were tested on PAGE according to Example 8. Fig. 11a shows the results of the unmodified oligonucleotide, Fig. 11b of the mPEG400-Oligo-mPEG1000, and Fig. 11c of the mPEG1000-Oligo-mPEG400, which demonstrate an increased stability of mPEG400-Oligo-mPEG1000 and mPEG1000-Oligo-mPEG400 against 3´-exonuclease incubation.

Example 13 – Oligonucleotides

The TGF-beta1, TGF-beta2 and TGF-beta3 oligonucleotides, in particular antisense oligonucleotides, of this example are bound to a polymer and/or compound according to the method of the present invention, which are suitable for controlling, preventing, and/or treating of unwanted neoplasms, formation of metastases, fibrosis or viral diseases or disorders such as HIV as described in this invention.

TGF-beta1, -2 and -3 antisense oligonucleotides:

gtgccatcaatacctgcaaa(SEQ ID No. 1), catcagttacatcgaaggag(SEQ ID No. 2), tcttgggacacgcagcaagg(SEQ ID No. 3), gaaatcaatgtaaagtggac(SEQ ID No. 4), catgaactggtccatatcga(SEQ ID No. 5), gaggttctaaatcttgggac(SEQ ID No. 6), gcactctggcttttgggttc(SEQ ID No. 7), tagctcaatccgttgttcag(SEQ ID No. 8), ccctagatccctcttgaaat(SEQ ID No. 9), accaaggctctcttatgttt(SEQ ID No. 10), tcgagtgtgctgcaggtaga(SEQ ID No. 11), tgaacagcatcagttacatc(SEQ ID No. 12), gctgggttggagatgttaaa(SEQ ID No. 13), agaggttctaaatcttggga(SEQ ID No. 14), cgccggttggtctgttgtga(SEQ ID No. 15), ctgctttcaccaaattggaa(SEQ ID No. 16), aagtatagatcaaggagagt(SEQ ID No. 17), tgctcaggatctgcccgcgg(SEQ ID No. 18), gtgctgttgtagatggaaat(SEQ ID No. 19), agggcggcatgtctattttg(SEQ ID No. 20), taagcttattttaaatccca(SEQ ID No. 21), tagctgcatttgcaagactt(SEQ ID No. 22), tctgttgtgactcaagtctg(SEQ ID No. 23), aagcaataggccgcatccaa(SEQ ID No. 24), tcaatgtaaagtggacgtag(SEQ ID No. 25), attttagctgcatttgcaag(SEQ ID No. 26), tgtagatggaaatcacctcc(SEQ ID No. 27), ttaacactgatgaaccaagg(SEQ ID No. 28), attgtacccttgggttcgt(SEQ ID No. 29), agatccctcttgaaatcaat(SEQ ID No. 30), tgtaaagtggacgtaggcag(SEQ ID No. 31), ccattcgccttctgctcttg(SEQ ID No. 32), tgttaaatctttggacttga(SEQ ID No. 33), gaagggcggcatgtctattt(SEQ ID No. 34), gaccctgctgtgctgagtgt(SEQ ID No. 35), gaactagtaccgccttttca(SEQ ID No. 36), cgatcctcttgcgcatgaac(SEQ ID No. 37), ccggccaaaagggaagagat(SEQ ID No. 38), aaagagacgagtggctatta(SEQ ID No. 39), aagtggaaatattaatacgg(SEQ ID No. 40), agatcaaggagagttgtttg(SEQ ID No. 41), agttgtttttaaaagtcaga(SEQ ID No. 42), tgtaacaactgggcagacag(SEQ ID No. 43), ggtgttgtaacaactgggca(SEQ ID No. 44), tacccacagagcacctggga(SEQ ID No. 45), gggatggcatcaaggtaccc(SEQ ID No. 46), tcgtcatcatcattatcatc(SEQ ID No. 47), aagggtgcctattgcatagc(SEQ ID No. 48), ctcactgttaactctaagag(SEQ ID No. 49), gcaaagtatttggtctccac(SEQ ID No. 50), caagttccttaagccatcca(SEQ ID No. 51), ttatcttaatgcagactttc(SEQ ID No. 52), cttacaagaagcttccttag(SEQ ID No. 53), actggtgagcttcagcttgc(SEQ ID No. 54), acttgagaatctgatatagc(SEQ ID No. 55), aggttcctgtctttatggtg(SEQ ID No. 56), gtgtatccatttccacccta(SEQ ID No. 57), cagcacagaagttggcattg(SEQ ID No. 58), gcaaggagaagcagatgctt(SEQ ID No. 59), agcaaggagaagcagatgct(SEQ ID No. 60), ttttccaagaattttagctg(SEQ ID No. 61), ttcttgttacaagcatcatc(SEQ ID No. 62), ttaaagaaggagcggttcgg(SEQ ID No. 63), ctgggctgaaatttatatat(SEQ ID No. 64), gggcagacagctaggagttt(SEQ ID No. 65), gtgtactcaccaaggtaccc(SEQ ID No. 66), cccagcactttgggaggccg(SEQ ID No. 67),

ggctcacgcctgtaatccca(SEQ ID No. 68), tgaccgtgaactcactattt(SEQ ID No. 69), atagtggtgatggctataca(SEQ ID No. 70), ttttggttacctgcaaatct(SEQ ID No. 71), gaacactcaccctgctgtgc(SEQ ID No. 72), gaatggctctttaaacccta(SEQ ID No. 73), gaagaaatggagttcagtgt(SEQ ID No. 74), tttctcctggaagggagagg(SEQ ID No. 75), aaatgcaacgcgttcccaac(SEQ ID No. 76), aatacgaaacttttgcaaag(SEQ ID No. 77), actagtaattctcagagcgg(SEQ ID No. 78), aagaaactagtaattctcag(SEQ ID No. 79), agtgcatgtttttaaaagga(SEQ ID No. 80), cagtagtgcatgtttttaaa(SEQ ID No. 81), ctcagcacacagtagtgcat(SEQ ID No. 82), agatgcaggagcaaaaaggt(SEQ ID No. 83), caggtagacagactgagcgc(SEQ ID No. 84), gcctcgatcctcttgcgcat(SEQ ID No. 85), gcggatggcctcgatcctct(SEQ ID No. 86), ctcaggatctgcccgcggat(SEQ ID No. 87), gctccggatagtcttccggg(SEQ ID No. 88), agatggaaatcacctccggg(SEQ ID No. 89), gttgtagatggaaatcacct(SEQ ID No. 90), ctggtactgttgtagatgga(SEQ ID No. 91), aggcggctgccctccggctt(SEQ ID No. 92), aacctccttggcgtagtact(SEQ ID No. 93), attttataaacctccttggc(SEQ ID No. 94), cggcatgtcgattttataaa(SEQ ID No. 95), cgggatggcattttcggagg(SEQ ID No. 96), gtagggtctgtagaaagtgg(SEQ ID No. 97), tgaagtagggtctgtagaaa(SEQ ID No. 98), attctgaagtagggtctgta(SEQ ID No. 99), aagcggacgattctgaagta(SEQ ID No. 100), cccaggttcctgtctttgtg(SEQ ID No. 101), ggcagtgtaaacttatttta(SEQ ID No. 102), ccatcaatacctgcaaatct(SEQ ID No. 103), aggtgccatcaatacctgca(SEQ ID No. 104), agttttctgatcaccactgg(SEQ ID No. 105), ttatagttttctgatcacca(SEQ ID No. 106), cctagtggactttatagttt(SEQ ID No. 107), acattagcaggagatgtggg(SEQ ID No. 108), agggcaacaacattagcagg(SEQ ID No. 109), actccagtctgtaggagggc(SEQ ID No. 110), tcctgcacatttctaaagca(SEQ ID No. 111), cagcaattatcctgcacatt(SEQ ID No. 112), atgtaaagagggcgaaggca(SEQ ID No. 113), ctcttaaaatcaatgtaaag(SEQ ID No. 114), ccaagatccctcttaaaatc(SEQ ID No. 115), cctttgggttcatggatcca(SEQ ID No. 116), gcattgtaccctttgggttc(SEQ ID No. 117), gcacagaagttagcattgta(SEQ ID No. 118), ctgaggactttggtgtgttg(SEQ ID No. 119), tcctgggacacacagcaagg(SEQ ID No. 120), tttagctgcatttacaagac(SEQ ID No. 121), caaggactttagctgcattt(SEQ ID No. 122), gtcattgtcaccgtgatttt(SEQ ID No. 123), ccagttttaacaaacagaac(SEQ ID No. 124), agatgccagttttaacaaac(SEQ ID No. 125), gttcattatatagtaacaca(SEQ ID No. 126), atgaaaggttcattatatag(SEQ ID No. 127), ttccaagggtaatgaaaggt(SEQ ID No. 128), cttaagccatccatgagttt(SEQ ID No. 129), cctggcttatttgagttcaa(SEQ ID No. 130), ttagtcctataacaactcac(SEQ ID No. 131), gcaaagaaccatttacaatt(SEQ ID No. 132), cttgcttaaactggcaaaga(SEQ ID No. 133), acatgtaaagtagttactgt(SEQ ID No. 134), acacattacatgtaaagtag(SEQ ID No. 135), taagatctacacattacatg(SEQ ID No. 136), attcaaaggtactggccagc(SEQ ID No. 137), tttgtagtgcaagtcaaaat(SEQ ID No. 138), catgtcattaaatggacaat(SEQ ID No. 139), cctacatttgtgcgaacttc(SEQ ID No. 140), ttccccctttgaaaaactca(SEQ ID No. 141), tttttaatcagcctgcaaag(SEQ ID No. 142), actgggcagacagtttcgga(SEQ ID No. 143), taacaactgggcagacagtt(SEQ ID No. 144), tgttgtaacaactgggcaga(SEQ ID No. 145), cacagagcacctgggactgt(SEQ ID No. 146), gtacccacagagcacctggg(SEQ ID No. 147), tcaaggtacccacagagcac(SEQ ID No. 148), tggcatcaaggtacccacag(SEQ ID No. 149), ggcgggatggcatcaaggta(SEQ ID No. 150), tttgcaggtattgatggcac(SEQ ID No. 151), ctccttcgatgtaactgatg(SEQ ID No. 152), ccttgctgcgtgtcccaaga(SEQ ID No. 153), tcgatatggaccagttcatg(SEQ ID No. 154), gtcccaagatttagaacctc(SEQ ID No. 155), gaacccaaaagccagagtgc(SEQ ID No. 156), atttcaagagggatctaggg(SEQ ID No. 157), aaacataagagagccttggt(SEQ ID No. 158), tctacctgcagcacactcga(SEQ ID No. 159), tcacaacagaccaaccggcg(SEQ ID No. 160), ttccaatttggtgaaagcag(SEQ ID No. 161), actctccttgatctatactt(SEQ ID No. 162), ccgcgggcagatcctgagca(SEQ ID No. 163), caaaatagacatgccgccct(SEQ ID No. 164), tgggatttaaaataagctta(SEQ ID No. 165), cagacttgagtcacaacaga(SEQ ID No. 166), ttggatgcggcctattgctt(SEQ ID No. 167), ctacgtccactttacattga(SEQ ID No. 168), ggaggtgatttccatctaca(SEQ ID No. 169), ccttggttcatcagtgttaa(SEQ ID No. 170), acgaacccaaagggtacaat(SEQ ID No. 171), attgatttcaagagggatct(SEQ ID No. 172), ctgcctacgtccactttaca(SEQ ID No. 173), caagagcagaaggcgaatgg(SEQ ID No. 174), tcaagtccaaagatttaaca(SEQ ID No. 175), aaatagacatgccgcccttc(SEQ ID No. 176), acactcagcacagcagggtc(SEQ ID No. 177), tgaaaaggcggtactagttc(SEQ ID No. 178), gttcatgcgcaagaggatcg(SEQ ID No. 179), atctcttccctttggccgg(SEQ ID No. 180), taatagccactcgtctcttt(SEQ ID No. 181), ccgtattaatatttccactt(SEQ ID No. 182), caaacaactctccttgatct(SEQ ID No. 183), ctgtctgcccagttgttaca(SEQ ID No. 184), tgcccagttgttacaacacc(SEQ ID No. 185), tcccaggtgctctgtgggta(SEQ ID No. 186), gatgataatgatgatgacga(SEQ ID No. 187), gctatgcaataggcaccctt(SEQ ID No. 188), ctcttagagttaacagtgag(SEQ ID No. 189), gtggagaccaaatactttgc(SEQ ID No. 190), gaaagtctgcattaagataa(SEQ ID No. 191), ctaaggaagcttcttgtaag(SEQ ID No. 192), gcaagctgaagctcaccagt(SEQ ID No. 193), tagggtggaaatggatacac(SEQ ID No. 194),

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Example 14 - Combination of a 5´- and 3´-end PEG modified conjugate with a third component penetratin

Starting product was a pyridyl-activated 5´-/3´-end PEG modified oligonucleotide, wherein the 5´-end was modified via native ligation, which is described as mPEG400-NH-Cys(SPy)-Oligo-NH-mPEG1000. The oligonucleotides connected to penetratin were selected from the group consisting of sequences of Fig. 1 and Example 13, preferably of SEQ ID No. 2030, 2031, 2032, 2057, 2066, and 2461;.

Penetratin had the following sequence: H_2N-RQIKIWFQNRRMKWKKC-COOH, wherein the cysteine at the C-terminus was additionally added to improve the connection between penetratin and the native ligation site of mPEG400-NH-Cys(SPy)-Oligo-NH-mPEG1000.

20nmol of mPEG400-NH-Cys(SPy)-Oligo-NH-mPEG1000 were dissolved in formamide to a final oligonucleotide concentration of 0.05 nmol/µl (0.05 mM), which was added to 1 nmol penetration. The mixture of mPEG400-NH-Cys(SPy)-Oligo-NH-mPEG1000 and penetratin was incubated at 37 °C for 30 min, and afterwards purified via anion exchange column and a reverse phase HPLC. The results are shown in Fig. 13A to C, wherein the oligonucleotide of SEQ ID No. 2461 was the selected.

Using the same, above mentioned conditions, a PEG-modified oligonucleotide of SEQ ID No. 2030 was connected to penetration via the native ligation site. SEQ ID No. 2030 like SEQ ID No. 2461 show a higly efficient combination with penetratin. The results for SEQ ID No. 2030 are shown in Fig. 13A to C and 14A to C.

The final products mPEG400-Cys(S-Pen)-Oligo-NH-mPEG1000 were confirmed in a MALDI-TOF MS having a matrix of 2,6 dihydroxyacetophenone (DHAP) and diammoniumhydrogencitrate.

Example 15 - Combination of a 5´- and 3´-end PEG modified conjugate and the RGDC peptide

Starting product was a pyridyl-activated 5´-/3´-end PEG modified oligonucleotide, wherein the 5´-end was modified via native ligation, which is described as mPEG400-Cys(SPy)-Oligo-mPEG1000. The oligonucleotides connected to RGDC were selected from the group consisting of sequences of Fig. 1 and Example 13, preferably of SEQ ID No. 2030, 2031, 2032, 2057, 2066, and 2461.

The activated RGDC peptide comprises an additional C-terminal cysteine and had the following sequence: H_2N-RGDC-COOH.

50nmol of mPEG400-Cys(SPy)-Oligo-mPEG1000 were dissolved in 0.1 M TEAA buffer, pH 7.5 to a final oligonucleotide concentration of 0.05 nmol/µl (0.05 mM). An aequous solution of RGDC peptide (10 nmol/µl) was added to the modified oligonucleotide in a 0.5x, 2x, 8x, and 16x excess. The mixture of mPEG400-Cys(SPy)-Oligo-mPEG1000 and RGDC peptide was incubated at 37 °C for 1 h, and afterwards purified via reverse phase HPLC. The results are shown in Fig. 16A to C for an oligonucleotide of SEQ ID No. 2030.

The final product mPEG400-Cys(S-CDGR)-Oligo-mPEG1000 was confirmed in a MALDI-TOF MS having a matrix of 2,6 dihydroxyacetophenone (DHAP) and diammoniumhydrogencitrate.

Example 16 – Effects of PEG- and RGD-modified oligonucleotides on glioblastoma cells

The commercially available human glioblastoma cell line A-172 (Accession no. CRL-1620) was established from a 53-year old man with a glioblastoma by Giard in the year 1973.

A-172 cells were obtained from Cell Lines Service (CLS). These adherent cells were grown in monolayers.

A-172 glioblastoma cells (7000 cells/well; three wells for each condition) were seeded in growth medium (DMEM-medium supplemented with 10% fetal bovine serum) in 48-well tissue culture plates and cultivated at 37°C in 5% CO_2 atmosphere with a relative humidity > 90%.

6 hours after cell seeding, the supernatants were removed and replaced by treatment solution consisting of growth medium (untreated cells) or growth medium supplemented with 0 µM, 0.25 µM, 0.5 µM, 1 µM, 2.5 µM, 5 µM, or 10 µM of AP12009 or derivative #26, or #28. Derivative #26 is 5´-/3´-end PEG modified AP12009 via native ligation, i.e., mPEG400-AP12009-mPEG1000 and derivative #28 is 5´-/3´-end PEG modified AP 12009 connected to RGD at the native ligation site, i.e., mPEG400-Cys(S-CDGR)-AP12009-mPEG1000.

Replacement of treatment solutions was repeated 2 days and 4 days after the first treatment. On day 7 supernatants were collected, cleared of cellular components by centrifugation (300 x g, 5 min, ambient temperature), and frozen immediately at -20°C. Cell number was determined using for example the CyQuant Direct Cell Proliferation Assay kit (Invitrogen (Karlsruhe, Germany) according to the instructions of the manufacturer.

Secreted TGF-beta2 was measured using for example the Quantikineâ Human TGF-β2-ELISA Kit, (R&D Systems) according to the instructions of the manufacturer.

Derivative #26 (light grey column) is more effective than AP12009 (black column) at 1 and 2.5 µM in suppressing TGF-beta2 expression as shown in Fig. 17A. Derivative #28 (dark grey column) is even more effective than derivative #26 in suppressing TGF-beta2 expression, especially at lower concentration ranges, e.g., 0.5 µM. In the concentration range of 0 to 10 µM AP12009, derivative #26, or derivative #28 have almost no effect on the proliferation of these cells as shown in Fig. 17B. The comparison of proliferation and TGF-beta suppression indicates a specific TGF-beta suppression independent from proliferation.

Example 17 - Combination of a 5´- and 3´-end PEG modified conjugate and Fluorescein

Starting product was a 5´-/3´-end PEG modified oligonucleotide, wherein the 5´-end was modified via native ligation, which is described as mPEG400-Cys(SPy)-Oligo-mPEG1000. The oligonucleotides connected to RGDC were selected from the group consisting of sequences of Fig. 1 and Example 13, preferably of SEQ ID No. 2030, 2031, 2032, 2057, 2066 and 2461.

43 nmol of mPEG400-Cys(SPy)-Oligo-mPEG1000 were dissolved in 50 mM Tris/ 150 mM NaCl / 5 mM EDTA pH 8.5 resultign in an oligonucleotide concentration of 0.25 nmol/µl. 129 nmol Fluorescein-Iodoacetat in 50 mM Tris / 150 mM NaCl / 5 mM EDTA pH 8.5 were added to the modified oligonucleotide and incubated for 30 min. at room temperature. Afterwards the samples were purified via reverse phase HPLC. The results are shown in Fig. 19 for an oligonucleotide of SEQ ID No. 2030.

The final product mPEG400-Cys(S-Fluorescein)-Oligo-mPEG1000 was confirmed in a MALDI-TOF MS having a matrix of 2,6 dihydroxyacetophenone (DHAP) and diammoniumhydrogencitrate.

Claims

1. Method for producing a modified oligonucleotide, wherein at least one polymer, preferably polyalkylene oxide, and/or a compound is covalently bound to the 5´-end or the 3´-end of the oligonucleotide via native ligation forming a native ligation site, with the proviso that the polymer and/or the compound is not a protein or peptide, if only the 5´-end of the oligonucleotide is modified by binding of the polymer or compound via native ligation.

2. Method according to claim 1, wherein a further polymer and/or compound is bound to the native ligation site and/or the 3´-end of the 5´-end native ligation modified oligonucleotide, or to the ligation site and/or the 5´-end of the 3´-end native ligation modified oligonucleotide.

3. Method according to claim 1 or 2, wherein the 5´-end or the 3´-end of the oligonucleotide is bound to a polymer and/or a compound via native ligation in a first step, the free 3´-end or the free 5´-end of the oligonucleotide is bound to a polymer and/or a compound in a second step, and the native ligation site is optionally bound to a polymer and/or a compound in a third step, or wherein the 5´-end or the 3´-end of the oligonucleotide is bound to a polymer and/or a compound via native ligation in a first step, the native ligation site is bound to a polymer and/or a compound in a second step and the free 3´-end or the free 5´-end of the oligonucleotide is optionally bound to a polymer and/or a compound in a third step, or
wherein the 3´-end or the 5´-end of the oligonucleotide is bound to a polymer and/or a compound in a first step, the free 5´-end or the free 3´-end of the oligonucleotide is bound to a polymer and/or a compound via native ligation in a second step, and the ligation site is optionally bound to a polymer and/or a compound in a third step.

4. Method according to any of claims 1 to 3, wherein the antisense oligonucleotide is a TGF-ß2, TGF-ß1, TGF-ß3, VEGF, IL-10, c-jun, c-fos, c-erbb2 (Her-2), or MIA antisense oligonucleotide and/or an antisense oligonucleotide hybridizing with one or more of the receptors of TGF-ß2, TGF-ß1, TGF-ß3, VEGF, IL-10, c-jun, c-fos, c-erbb2 (Her-2), or MIA.

5. Method according to any of claims 1 to 4, wherein the polymer is polyethylene glycol, preferably, PEG-400, PEG-1000, PEG-2000, PEG-5000, or PEG10000.

6. Method according to any of claims 1 to 5, wherein the compound is a small molecule, an antibody, an antigen, an enzyme, a part of an antibody, or an enzyme, any peptide, e.g., an internalizing peptide, an aptamer, a spiegelmer, RNAi, shRNA, miRNA a chromophor, a marker, biotin, a hormone, a signal peptide, a lipid, a fatty acid, a sugar, an amino acid, a receptor, a part of a receptor, or any ligand of a receptor or binding molecule.

7. Method according to any of claims 1 to 6, wherein the polymer and/or compound is bound to the oligonucleotide, with or without a linker.

8. Method according to any of claims 1 to 7, wherein the linker is a reversible or irreversible linker.

9. Method according to any of claims 1 to 8, wherein the modified oligonucleotide is bound to a solid support via the 5´- and/or 3´-end of the oligonucleotide and/or the ligation site.

10. An oligonucleotide modified via native ligation, comprising a polymer and/or a compound at the 5´- and/or 3´-end of the oligonucleotide and/or at the ligation site with the proviso that the polymer and/or the compound is not a protein or peptide, if only the 5´-end of the oligonucleotide is modified by binding of the polymer or compound via native ligation.

11. Oligonucleotide according to claim 10 for use in preventing and/or treating a tumor, formation of metastasis, an immune disease or disorder, a cardiovascular disease or disorder, and/or a viral disease or disorder.

12. Oligonucleotide according to claim 11, wherein the tumor is selected from the group consisting of solid tumors, blood born tumors, leukemias, tumor metastasis, hemangiomas, acoustic neuromas, neurofibromas, trachomas, pyogenic, granulomas, psoriasis, astracytoma, acoustic neuroma, blastoma, Ewing´s tumor, craniopharyngloma, ependymoma, medulloblastoma, glioma, hemangloblastoma, Hodgkins-lymphoma, medullablastoma, leukaemia, mesothelioma, neuroblastoma, neurofibroma, non-Hodgkins lymphoma, pinealoma, retinoblastoma, sarcoma, seminoma, trachomas, Wilm´s tumor, bile duct carcinoma, bladder carcinoma, brain tumor, breast cancer, bronchogenic carcinoma, carcinoma of the kidney, cervical cancer, choriocarcinoma, cystadenocarcinome, embryonal carcinoma, epithelial carcinoma,

esophageal cancer, cervical carcinoma, colon carcinoma, colorectal carcinoma, endometrial cancer, gallbladder cancer, gastric cancer, head cancer, liver carcinoma, lung carcinoma, medullary carcinoma, neck cancer, non-small-cell bronchogenic/lung carcinoma, ovarian cancer, pancreas carcinoma, papillary carcinoma, papillary adenocarcinoma, prostata cancer, small intestine carcinoma, prostate carcinoma, rectal cancer, renal cell carcinoma, skin cancer, small-cell bronchogenic/lung carcinoma, squamous cell carcinoma, sebaceous gland carcinoma, testicular carcinoma, and uterine cancer, and/or

the immune disease or disorder is selected from the group consisting of diabetes mellitus, arthritis, including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriac athritis; multiple sclerosis, encephalomyelitis, myasthenia gravis, systemic lupus erythrematosis, autoimmunethyroiditis, dermatitis, including atopis dermatitis, eczematous dermatitis; psoriasis, Sjgren´s Syndrome, Crohn´s disease, aphthous ulcer, irits, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener´s granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Graves´ disease, sarcoidosis, primary billiary cirrhosis, uveitis posterior, and interstitial lung fibrosis, graft-versus-host disease, cases of transplantation, and allergy such as an atopic allergy, and/or

the cardiovascular disease or disorder is selected from the group consisting of hypertension, atherosclerosis, coronary artery spasm, congestive heart failure, coronary artery disease, valvular disease, arrhythmias, and cardiomyopathies, and/or

the viral disease or disorder is selected from the group consisting of hepatitis A (HVA), hepatitis B (HVB), hepatitis C (HVC), or caused by herpes simplex virus (HSV), HIV, FIV, poliovirus, influenza virus, adenoviruses, papillomaviruses, Epstein-Barr-viruses and small pox virus, or virus-associated cancer.

13. Method for preventing and/or treating a tumor, formation of metastasis, an immune disease or disorder, a cardiovascular disease or disorder, and/or a viral disease or disorder by administration of an oligonucleotide according to claim 10.

14. Pharmaceutical composition comprising a modified oligonucleotide according to claim 10, and optionally a pharmaceutically acceptable carrier with the proviso that the

polymer and/or the compound is not a protein or peptide, if only the 5´-end of the oligonucleotide is modified by binding of the polymer or compound via native ligation.

15. Pharmaceutical composition according to claim 14 further comprising a cytokine and/or a chemotherapeutic.

Figure 1

Target	SEQ ID NO	Sequences
TGF-beta 2	2030	cggcatgtctatttgta
	2031	gctttcaccaaattggaagc
	2032	ctggctttgggtt
	2033	cacacagtagtgca
	2034	gcacacagtagtgc
	2035	gcttgctcaggatctgc
	2036	tactcttcgtcgct
	2037	cttggcgtagtact
	2038	gtaaacctccttgg
	2039	gtctatttgtaaacctcc
	2040	gcatgtctatttgtaaacc
	2041	ggcatcaaggtacc
	2042	ctgtagaaaagtggg
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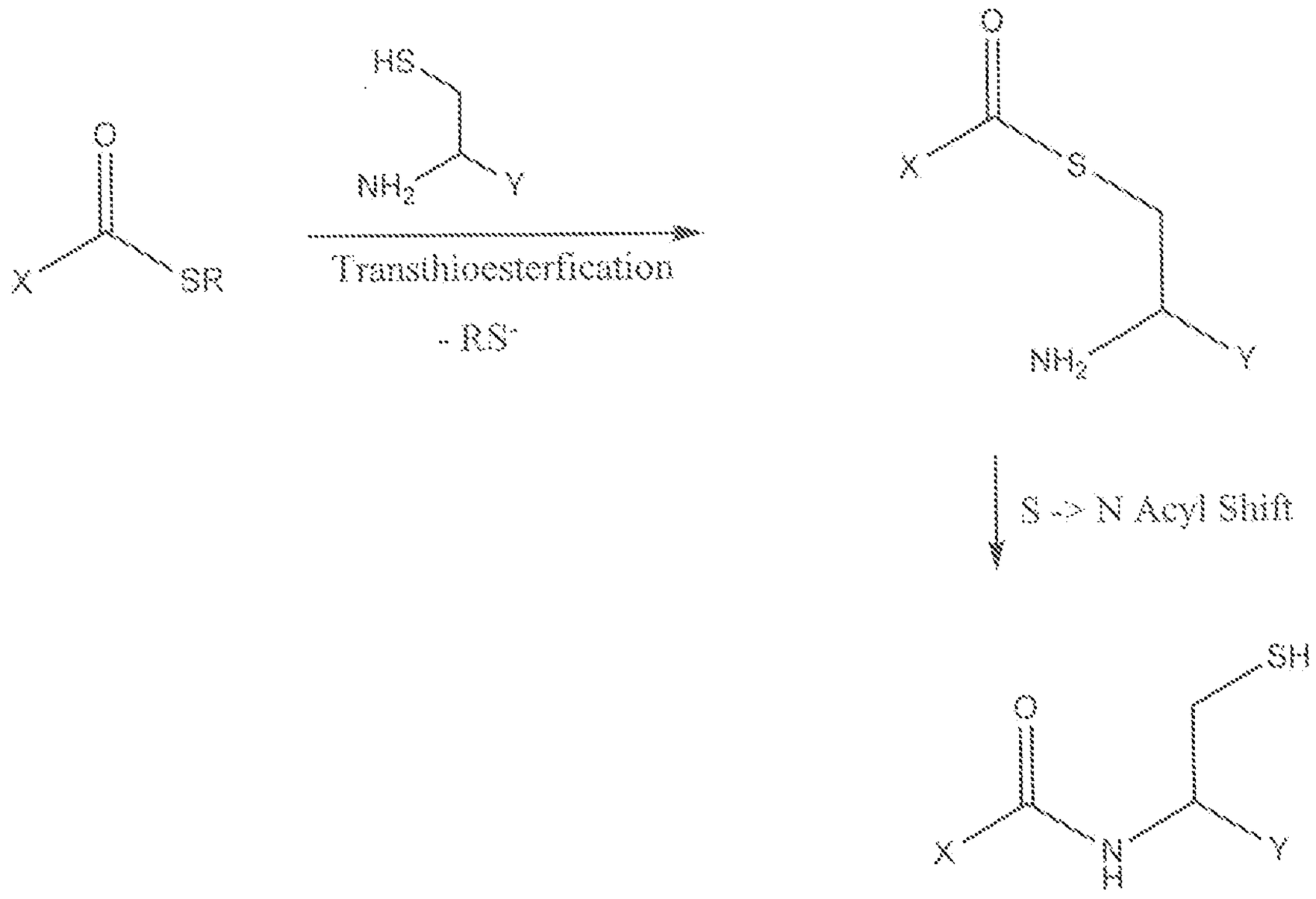

Figure 2: General Reaction Mechanism of Native Ligation

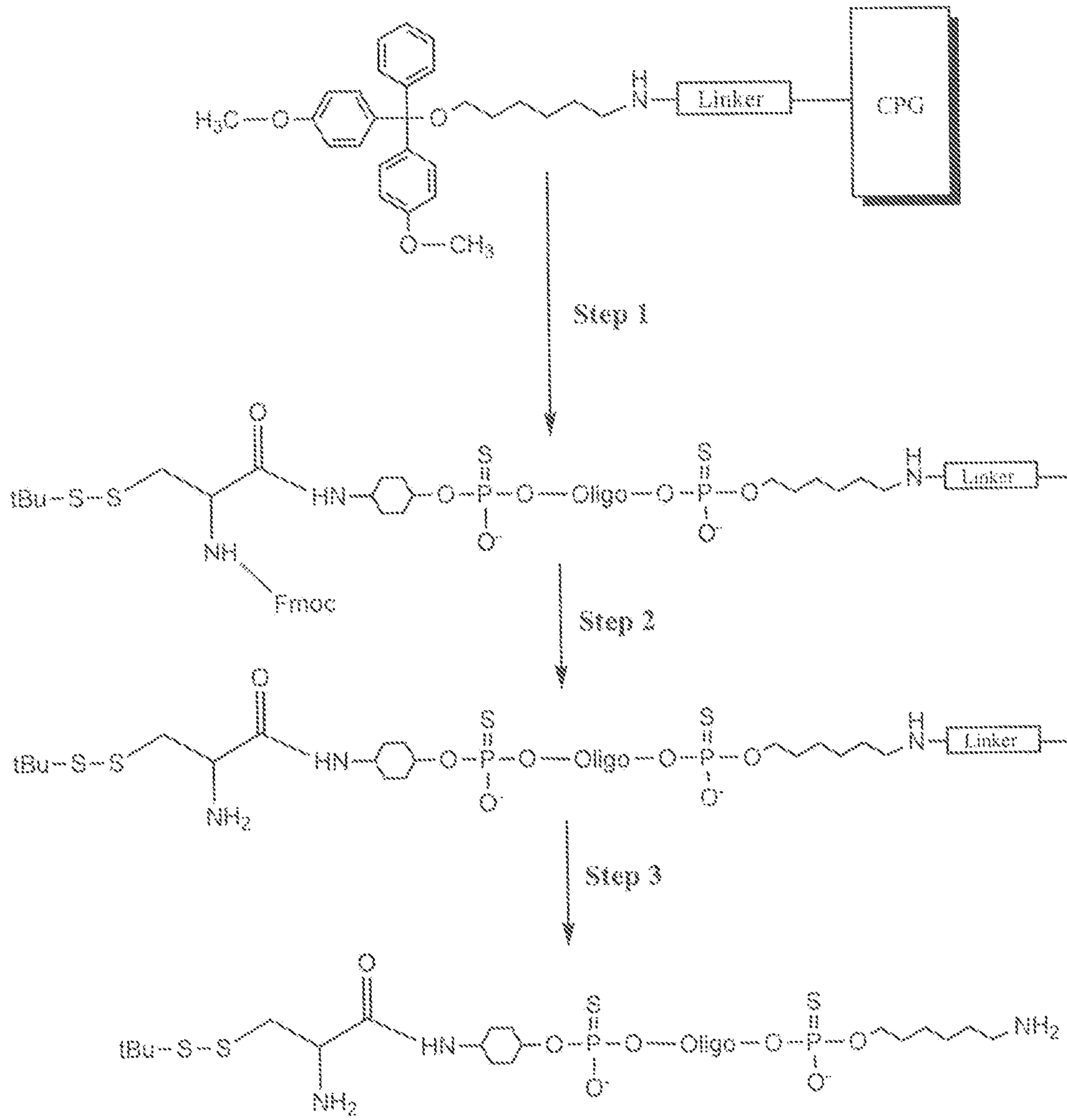

Fig. 3: 5'Cys-Oligo-Synthesis

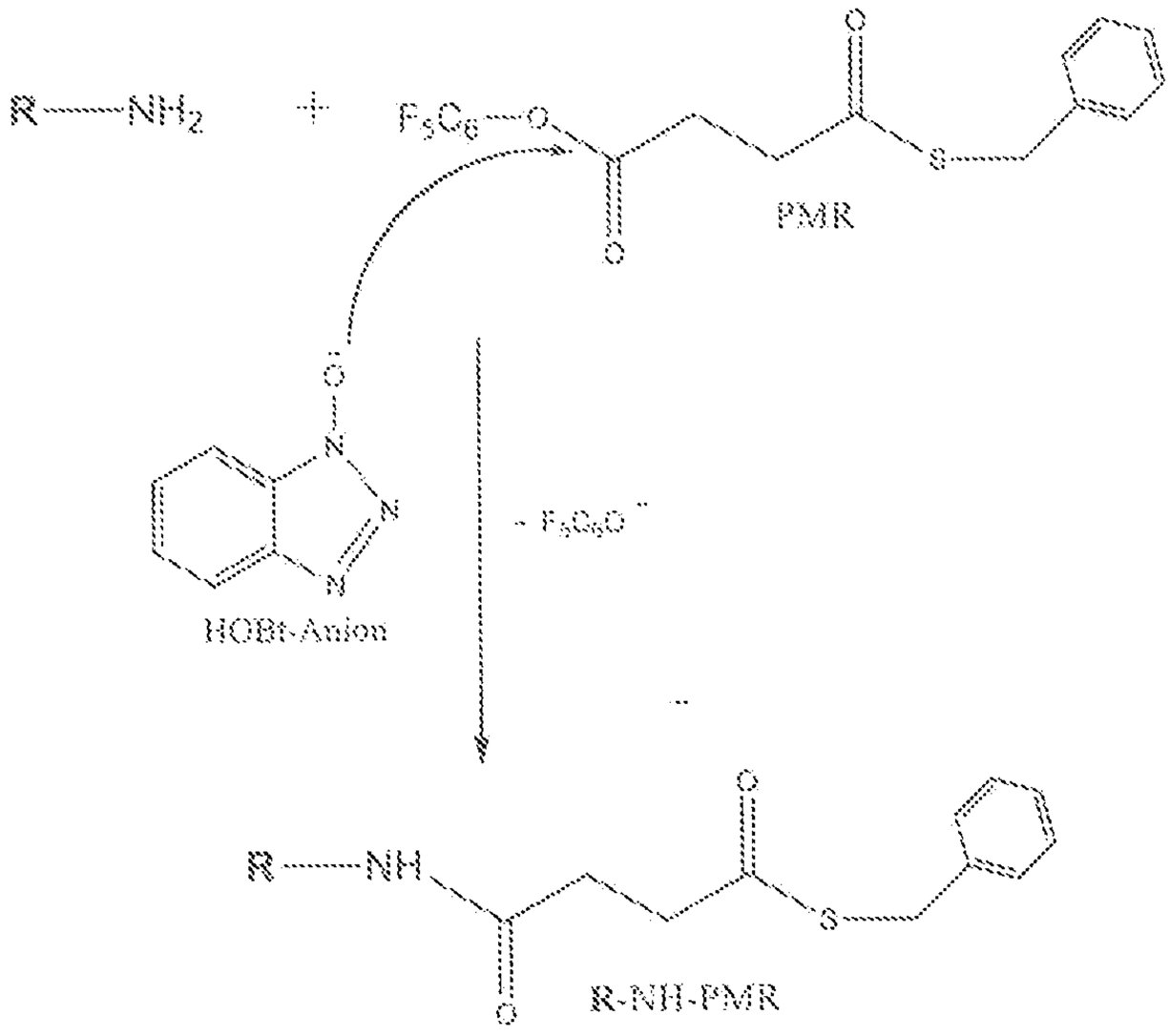

Fig. 4: General Thioestermodification of Amin-containing Compounds for native ligation

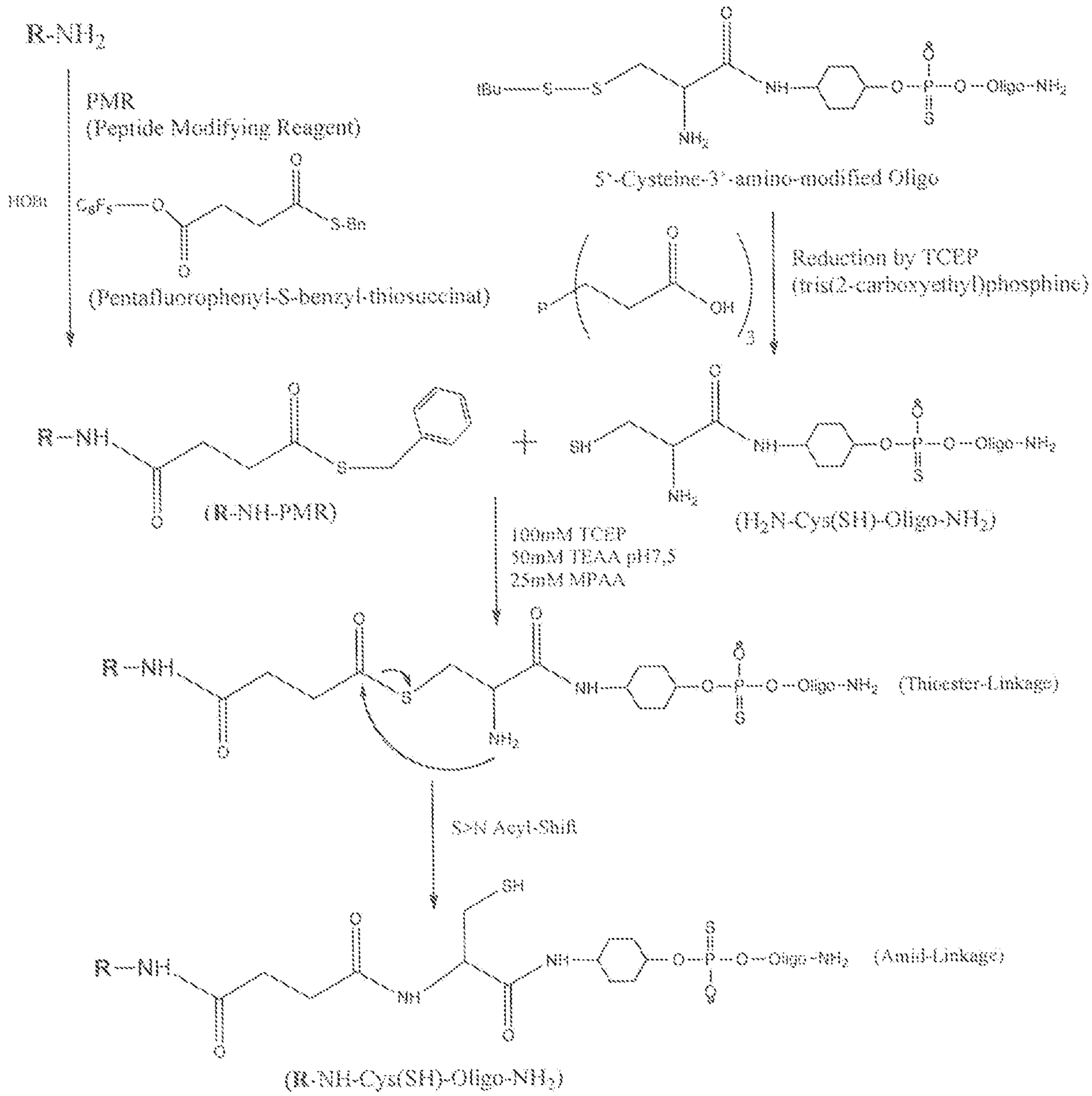

Fig. 5: Conjugation of the 5'-end of Cysteine-modified Oligonucleotide via Native Ligation te of a modified oligonucleotide of SEQ ID No. 2030

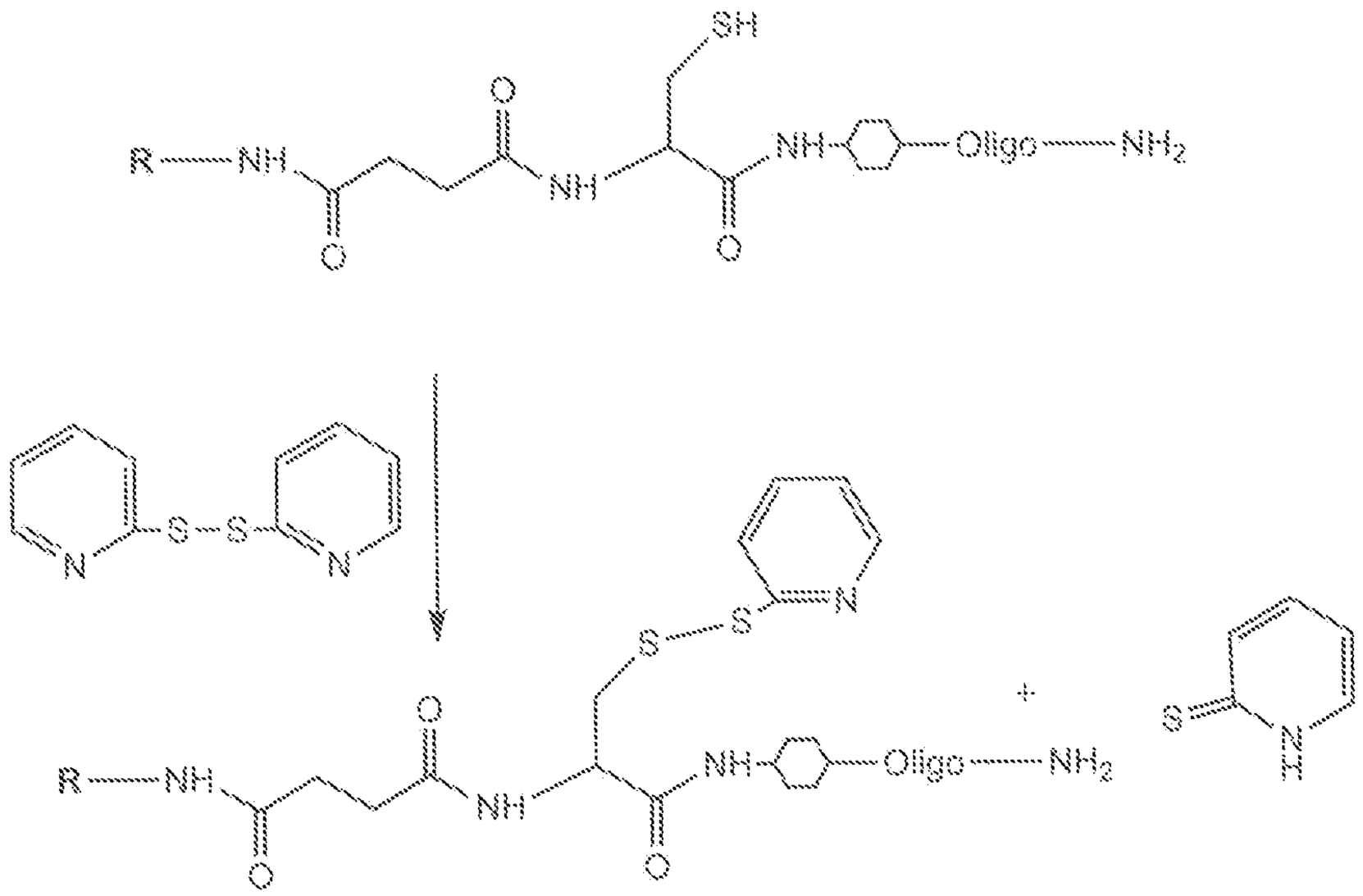

Fig. 6: Protection/Activation of the remaining Thiolfunction of the cysteine-residue after Native Ligation

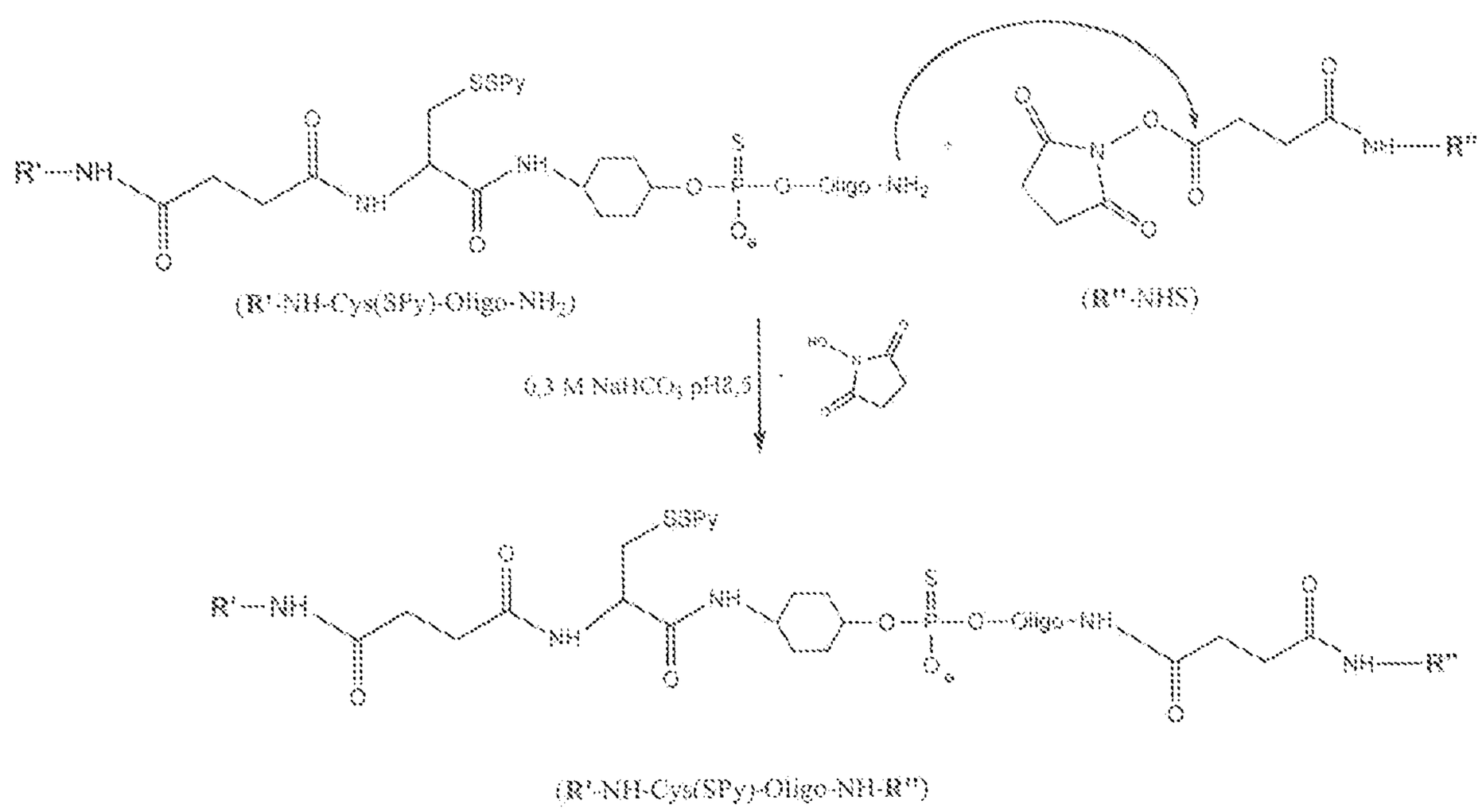

Fig. 7: Conjugation of the 3'-end of a 5'-conjugated Oligonucleotide by NHS ester

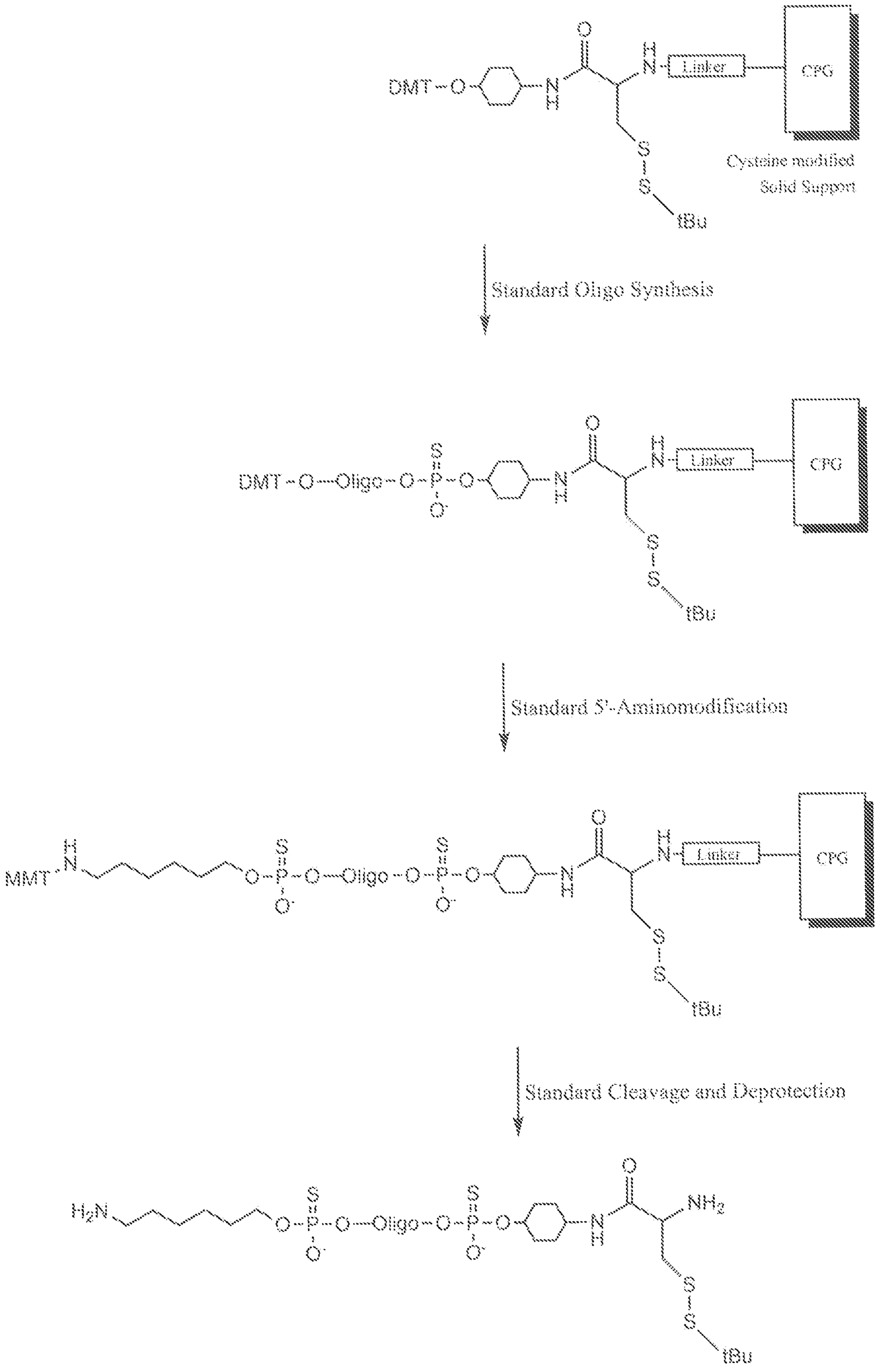

Fig. 8: 3'-Cys-Oligo-Synthesis

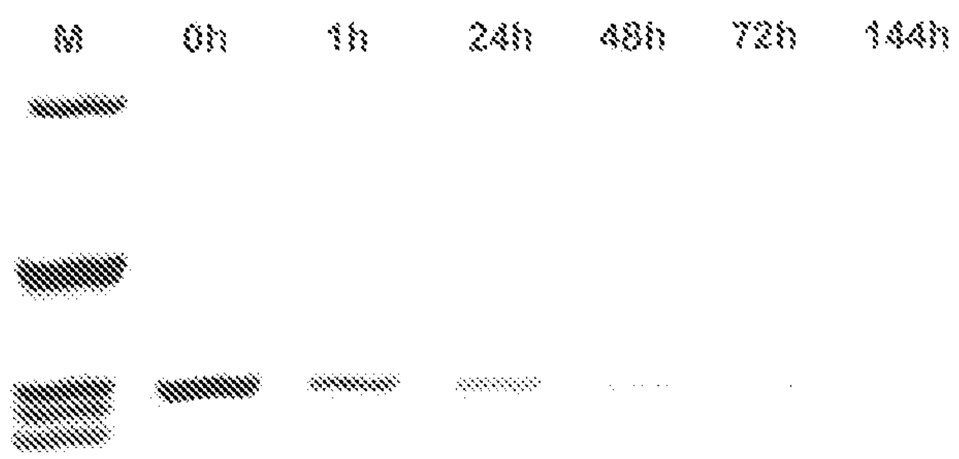

Fig. 9a: Unmodified oligonucleotide incubated with S1 endonuclease according to the indicated times

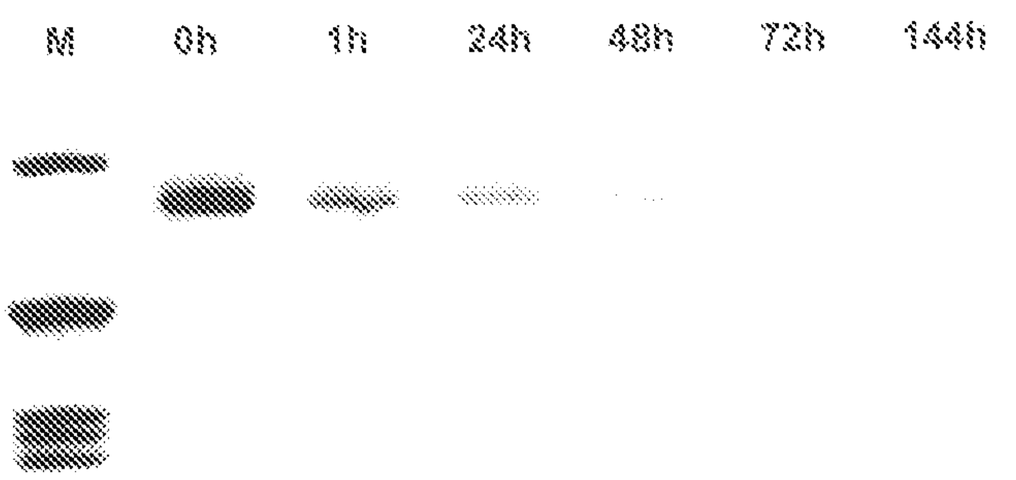

Fig. 9b: PEG-modified oligonucleotide (mPEG400-Oligo-mPEG1000) incubated with S1 endonuclease according to the indicated times

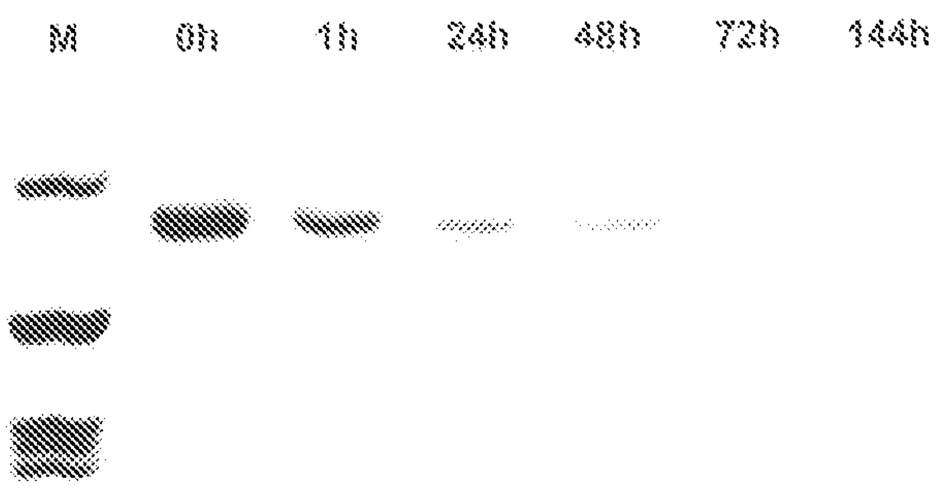

Fig. 9c: PEG-modified oligonucleotide (mPEG1000-Oligo-mPEG400) incubated with S1 endonuclease according to the indicated times

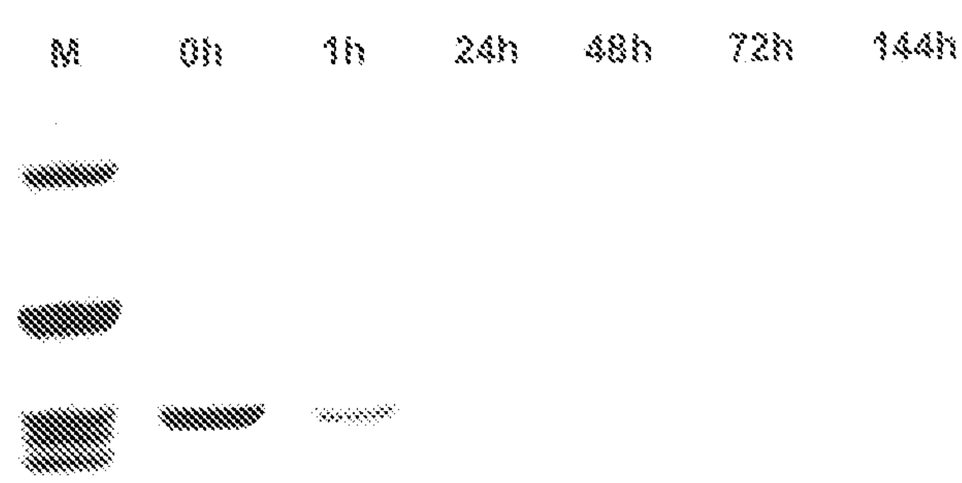

Fig. 10a: Unmodified oligonucleotide incubated with 5´exonuclease according to the indicated times

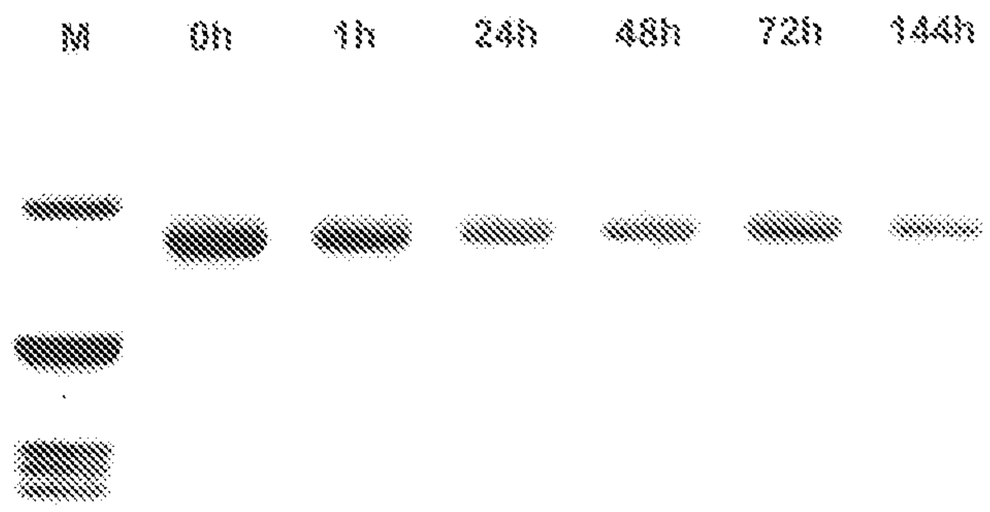

Fig. 10b: PEG-modified oligonucleotide (mPEG400-Oligo-mPEG1000) incubated with 5'exonuclease according to the indicated times

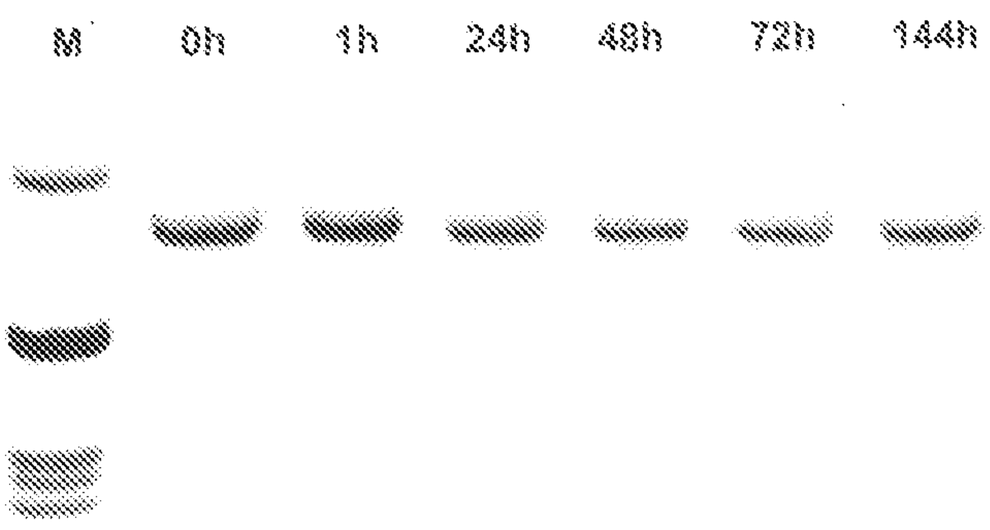

Fig. 10c: PEG-modified oligonucleotide (mPEG1000-Oligo-mPEG400) incubated with 5'exonuclease according to the indicated times

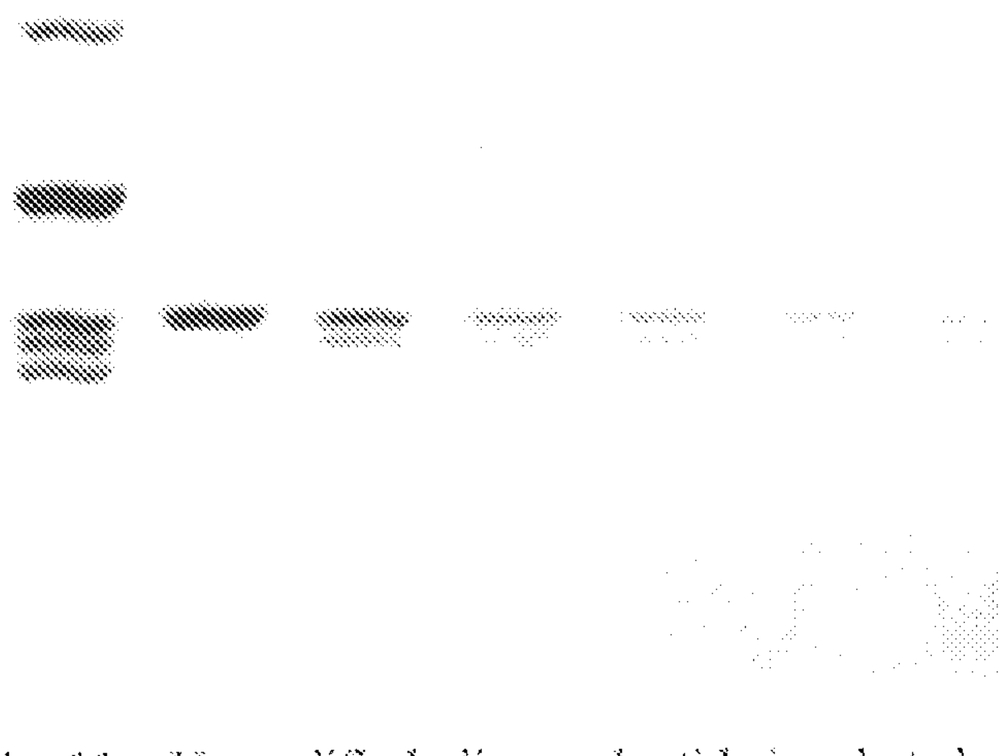

Fig. 11a: Unmodified oligonucleotide incubated with 3´exonuclease according to the indicated times

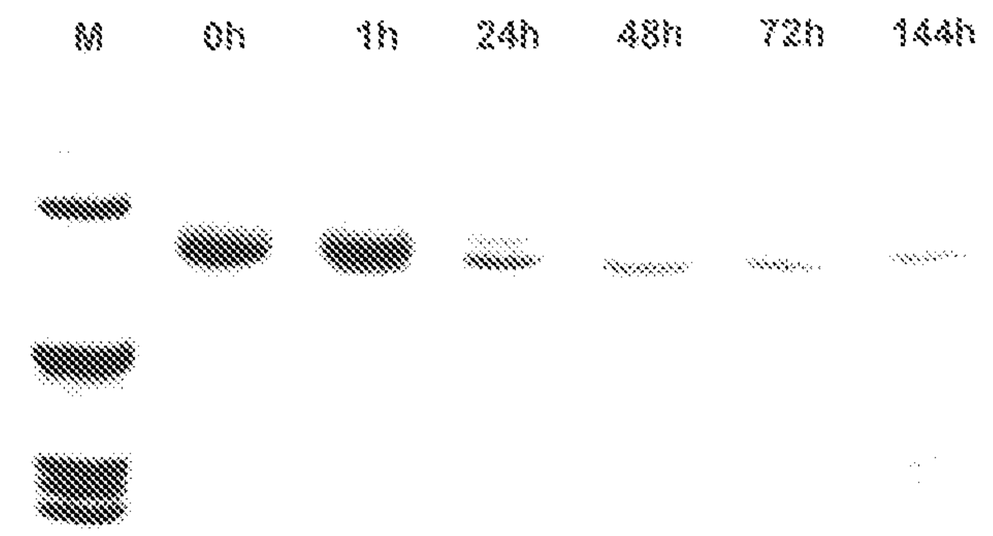

Fig. 11b: PEG-modified oligonucleotide (mPEG400-Oligo-mPEG1000) incubated with 3´exonuclease according to the indicated times

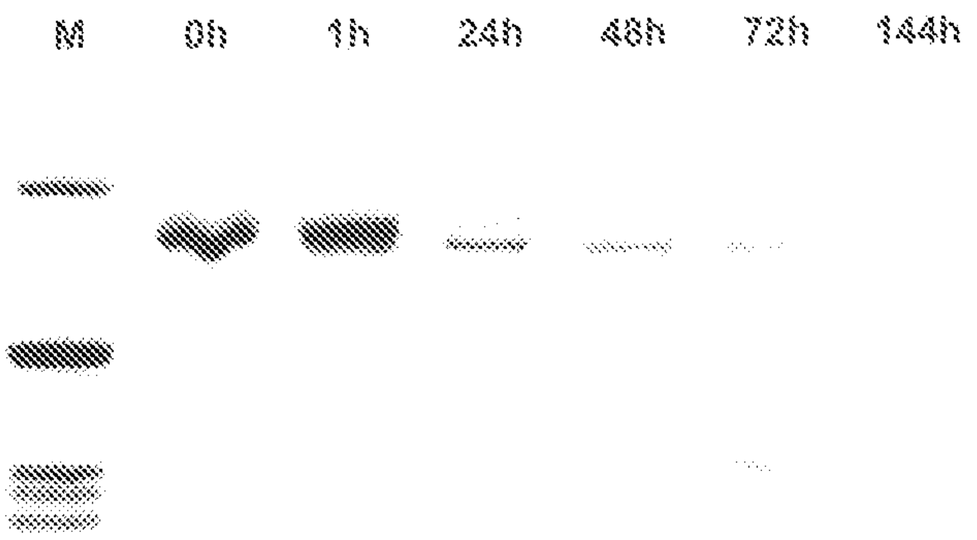

Fig. 11c: PEG-modified oligonucleotide (mPEG1000-Oligo-mPEG400) incubated with 3´exomuclease according to the indicated times

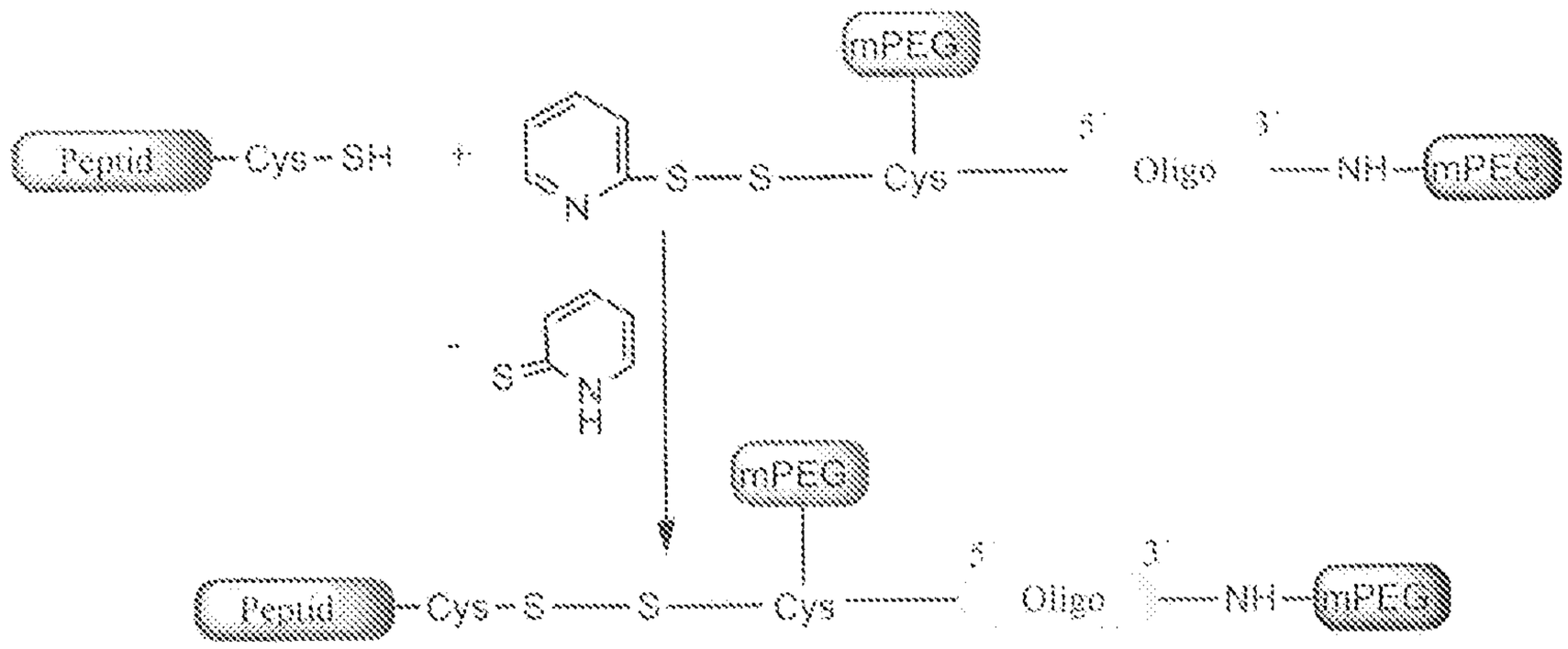

Fig. 12: Schematic presentation of the binding of an activated peptide to the native ligation site of a modified oligonucleotide

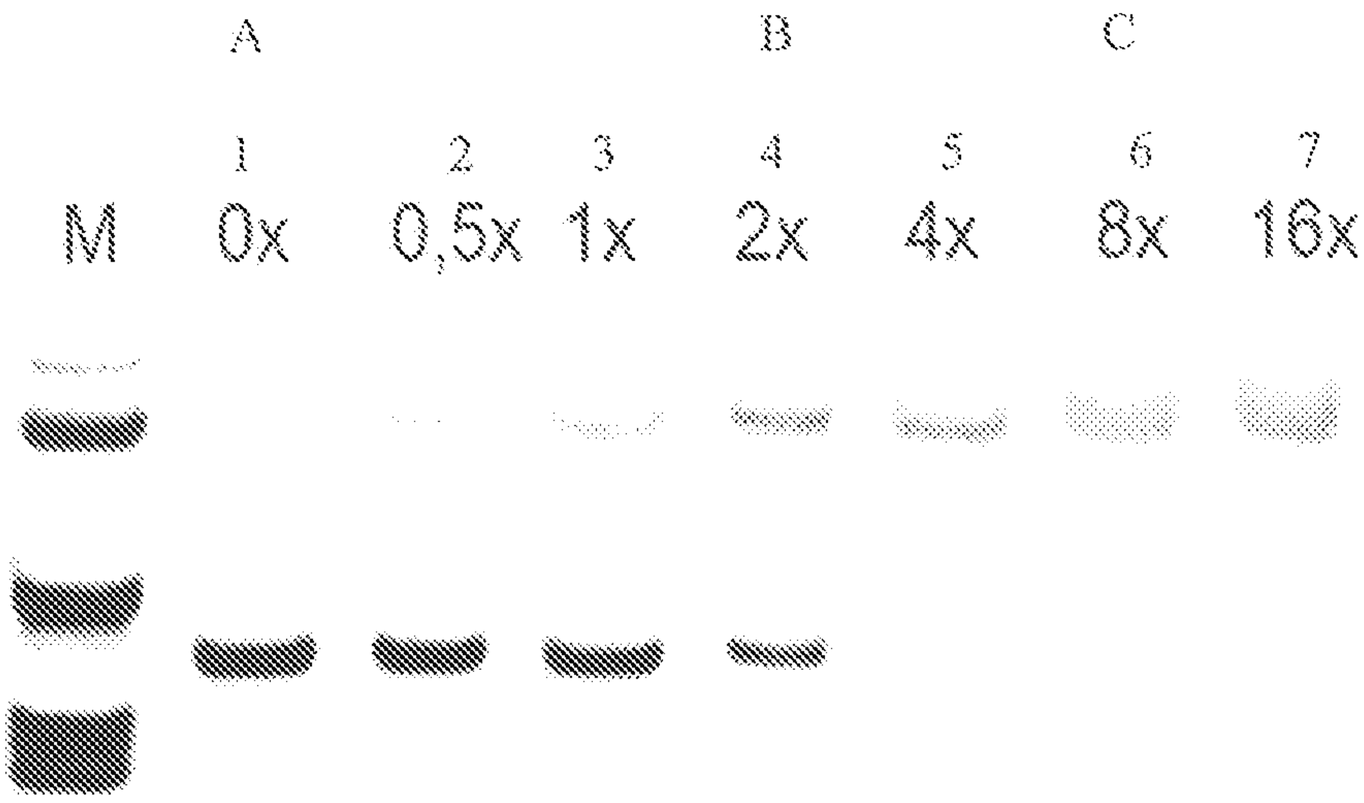

Fig. 13A: Silver stained PAGE gel showing educts and the product of the binding of penetratin to the native ligation site of a modified oligonucleotide of SEQ ID No. 2461

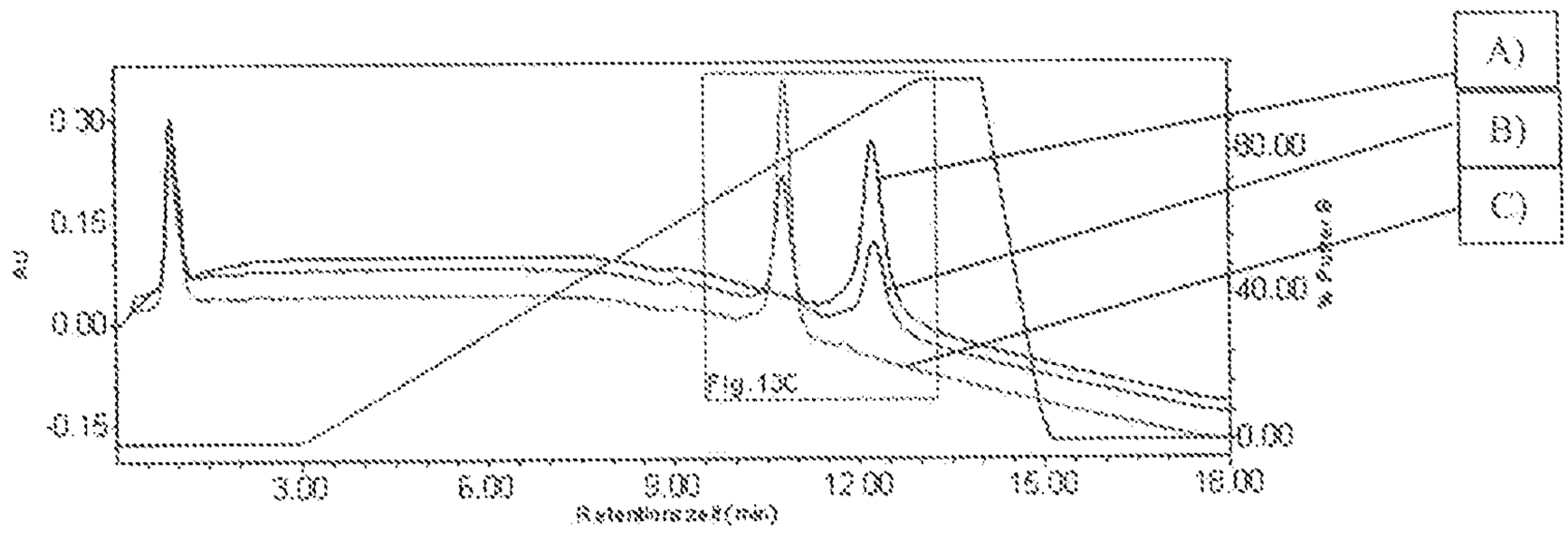

Fig. 13B: Chromatogram showing educts and products of silver stained PAGE indicated as lines A, B, and C in Fig. 13A

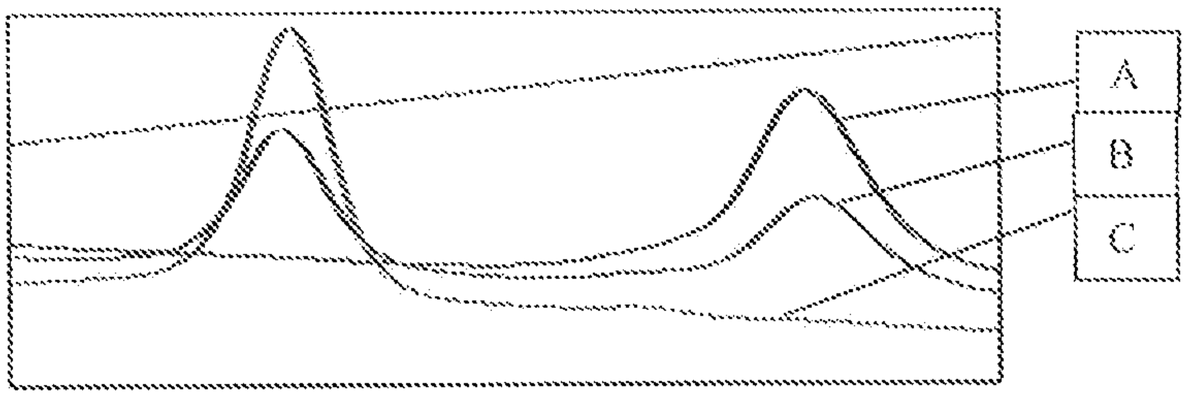

Fig. 13C: Detailed presentation of lines A, B and C of Fig. 13B

	A	B		C		D
M	1	2	3	4	5	6
	0x	0.5x	1x	2x	4x	8x

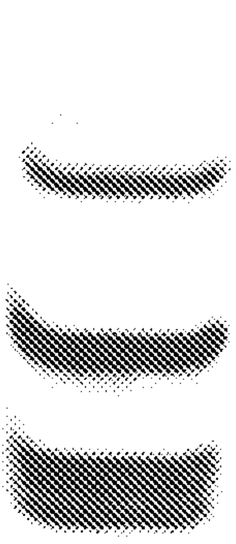

Fig. 14A: Silver stained PAGE gel showing educts and the product of the binding of penetratin to the native ligation site of a modified oligonucleotide of SEQ ID No. 2030

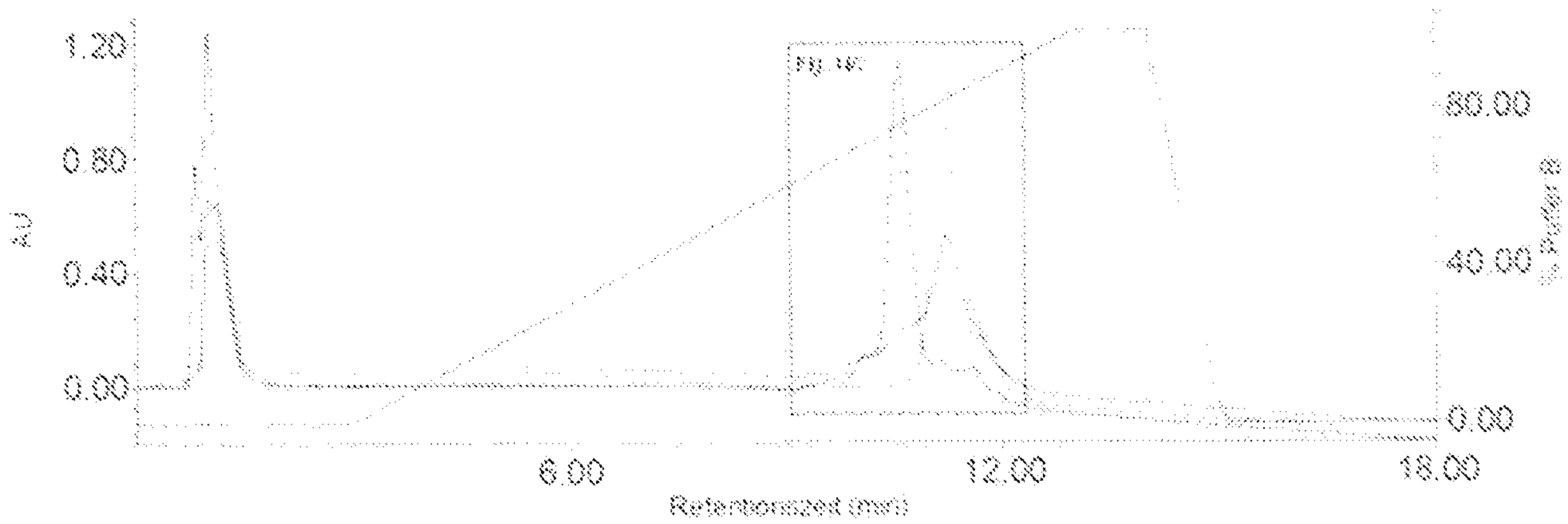

Fig. 14B: Chromatogram showing educts and products of silver stained PAGE indicated as lines A, B, C and D in Fig. 14A

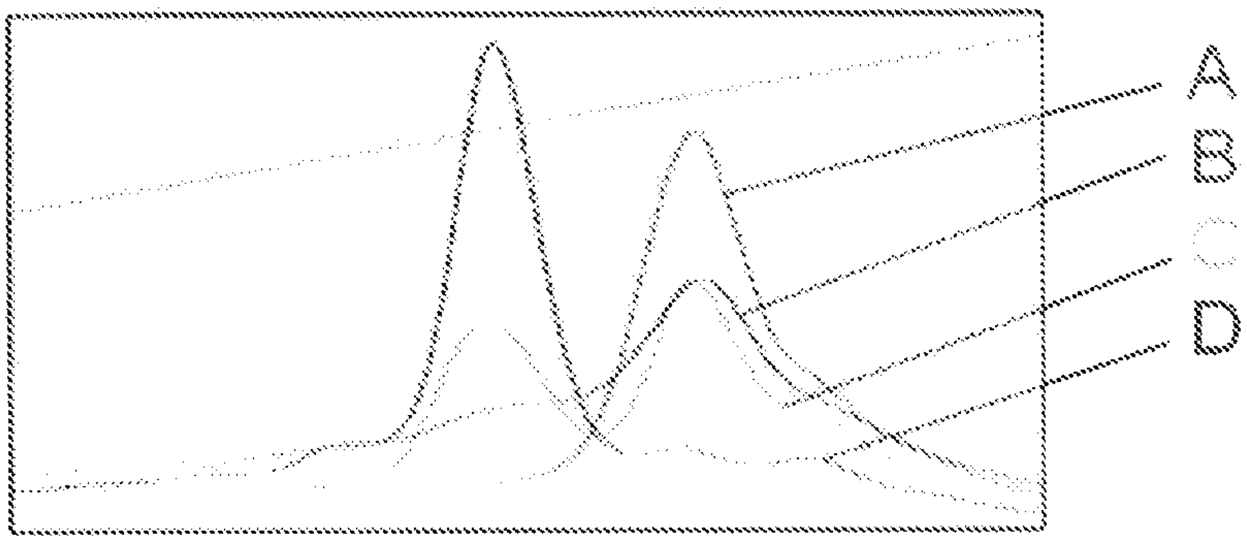

Fig. 14C: Detailed presentation of lines A, B, C and D of Fig. 14B

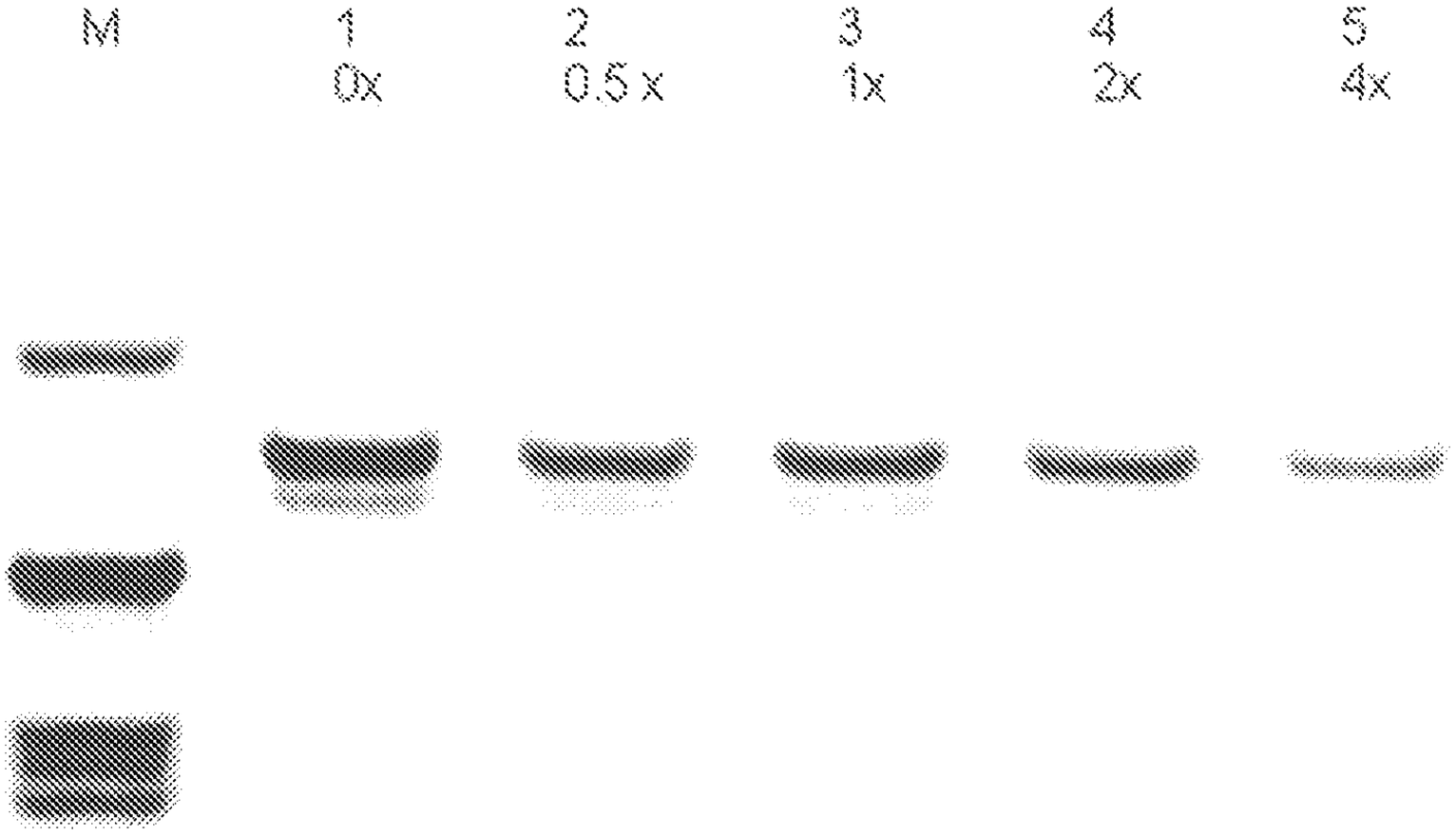

Fig. 15: Silver stained PAGE gel showing an experiment for coupling of penetratin to the native ligation site of a modified oligonucleotide of SEQ ID No. 2030 comprising an inaccessible native ligation site

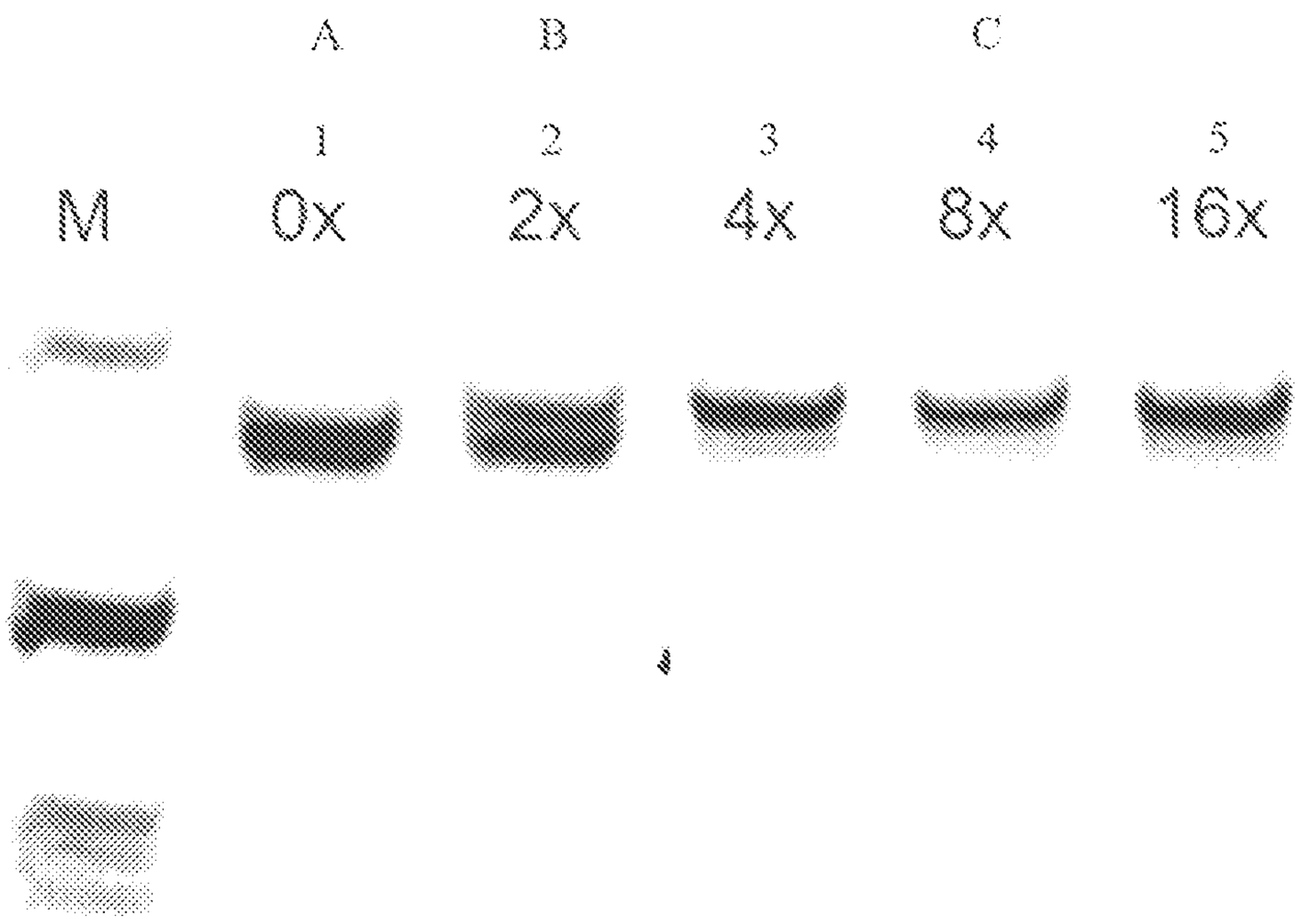

Fig. 16A: Silver stained PAGE gel showing educts and the product of the binding of RGDC peptide to the native ligation site of a modified oligonucleotide of SEQ ID No. 2030

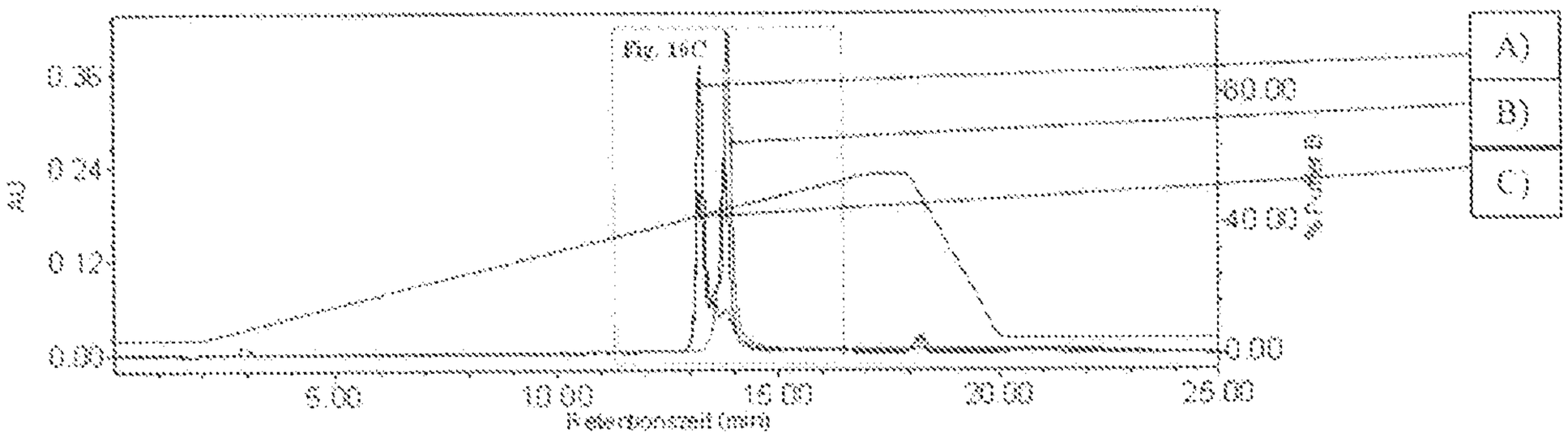

Fig. 16B: Chromatogram showing educts and products of silver stained PAGE indicated as lines A, B, and C in Fig. 16A

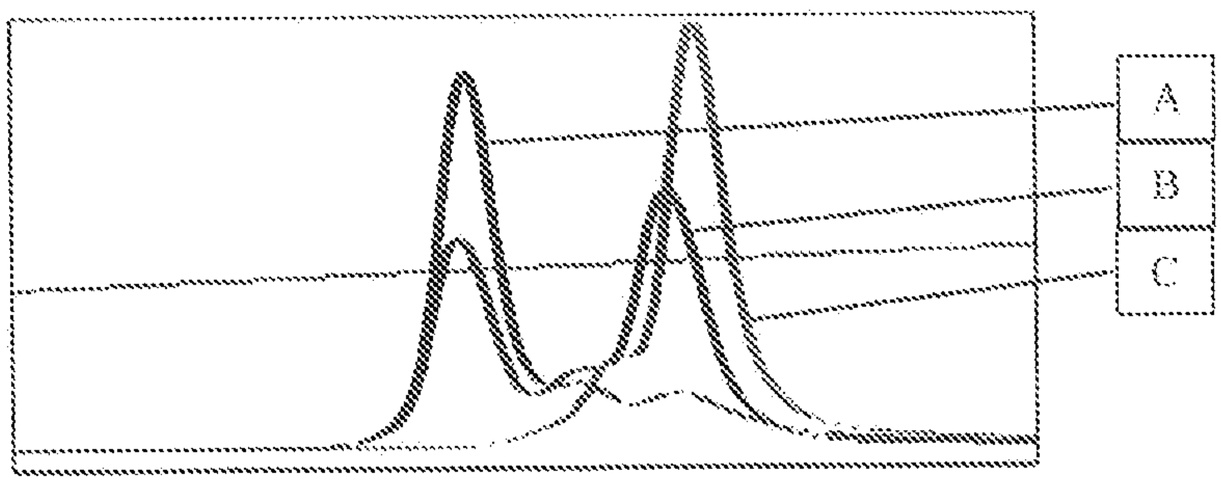

Fig. 16C: Detailed presentation of lines A, B and C of Fig. 16B

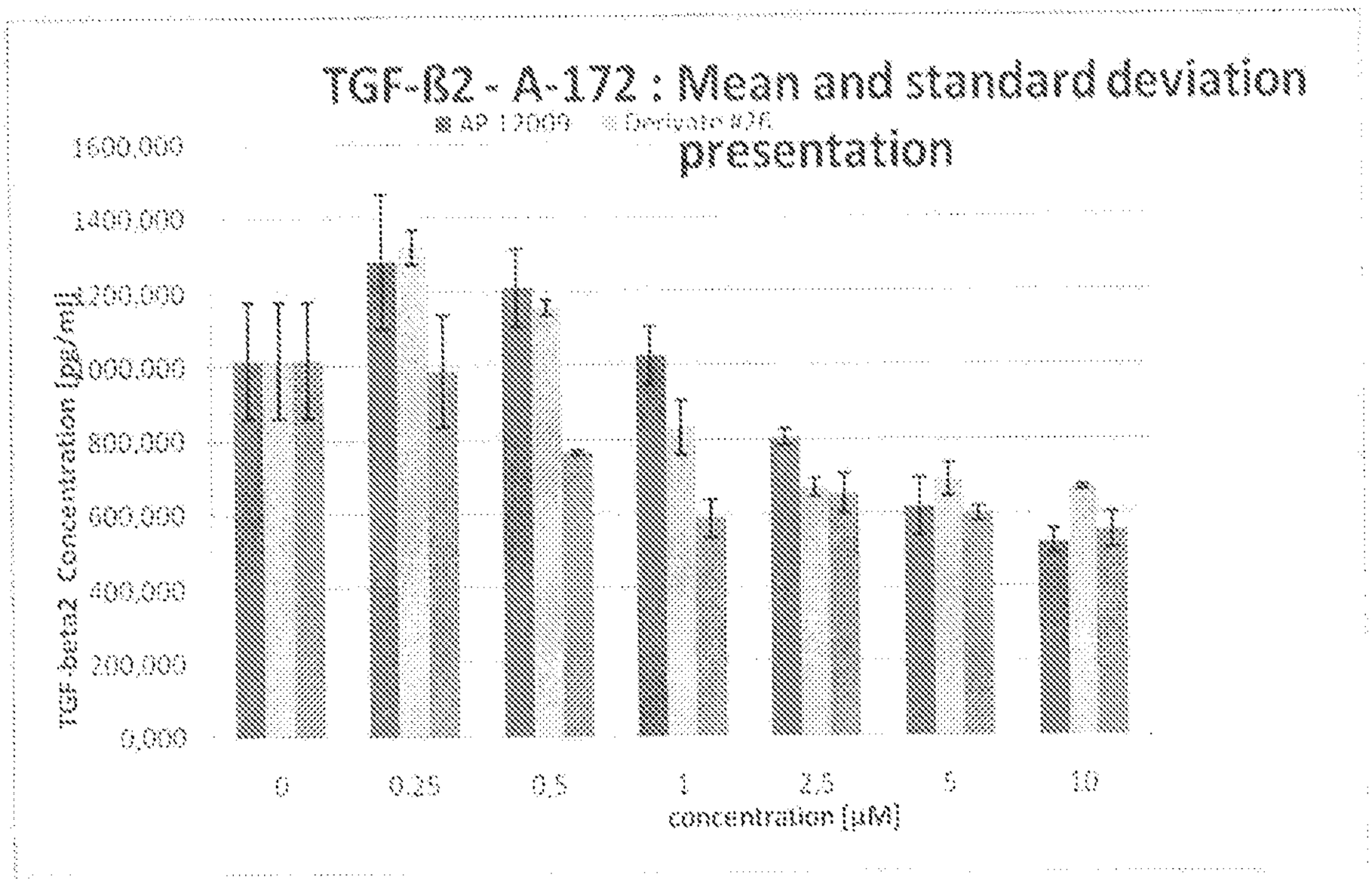

Fig. 17A: TGF-ß2 inhibition of glioma cells in contact with 5´-/3´-end PEG-modified oligonucleotide of SQ ID No. 2030 connected to RGD at the native ligation site

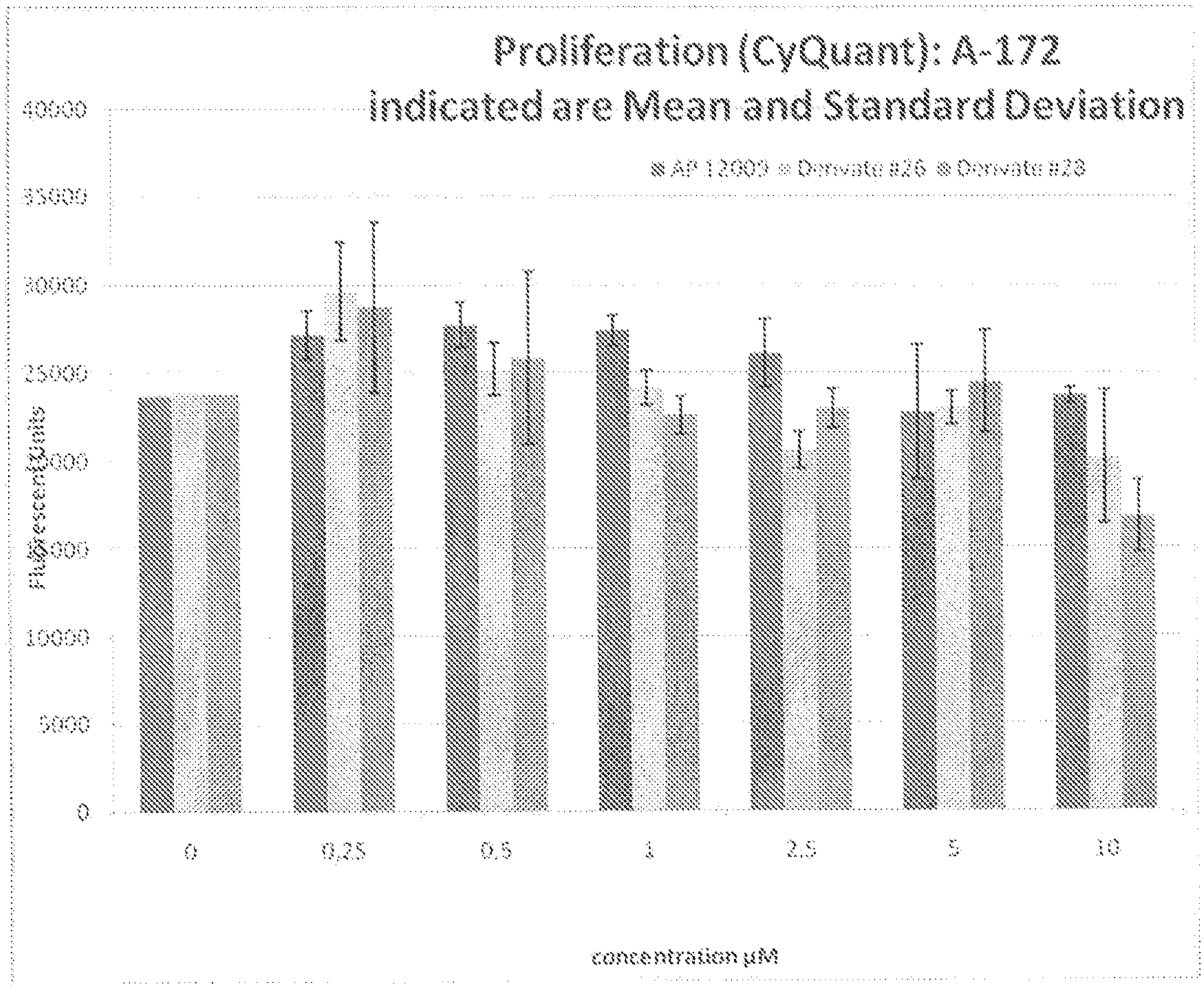

Fig. 17B: Proliferation of glioma cells in contact with 5´-/3´-end PEG-modified oligonucleotide of SEQ ID No. 2030 connected to RGD at the native ligation site

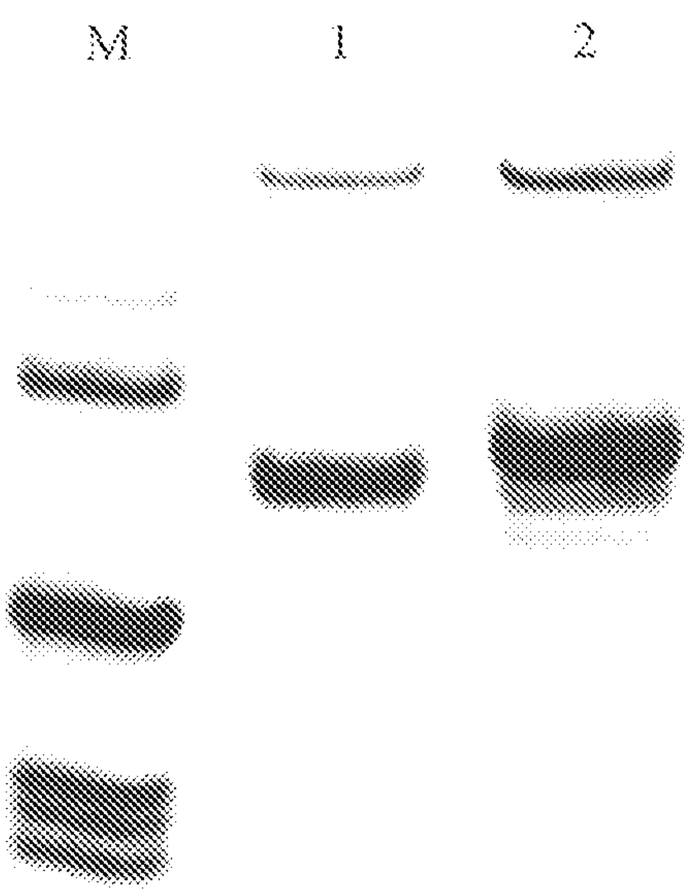

Fig. 18: Silver stained PAGE gel showing educt and the product of the binding of Fluorescein to the native ligation site of a modified oligonucleotide of SEQ ID No. 2030