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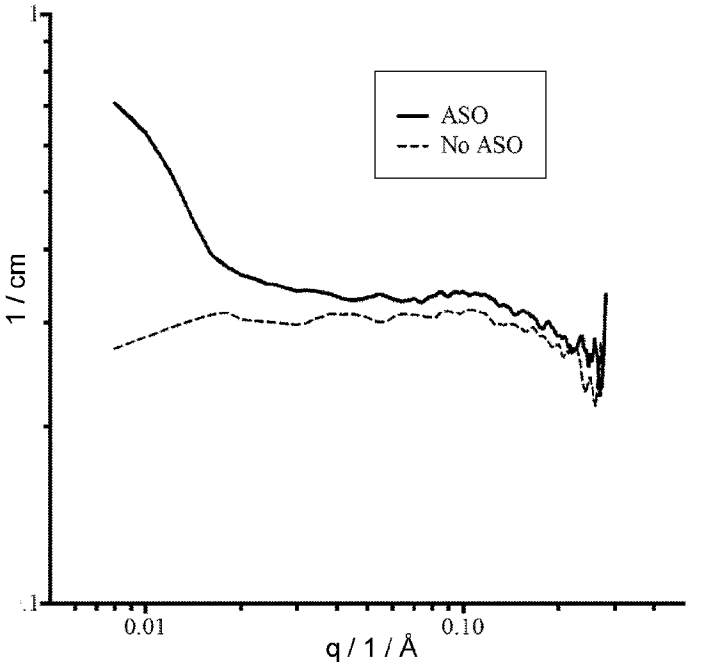

(57) Abrégé/Abstract:
The present invention relates to antisense oligonucleotide (ASO) compositions and particularly to compositions and methods for the cytosolic delivery of antisense oligonucleotides (ASOs). Hybrid ASOs, part single-stranded and part double-stranded, are provided, hybridising to form a double-stranded region that can non-covalently bond to nucleic-acid-binding protein regions. In this way, ASO::protein complexes may be produced that facilitate delivery of antisense DNA into target cells. Such complexes may be used to down-regulate gene expression in cells.

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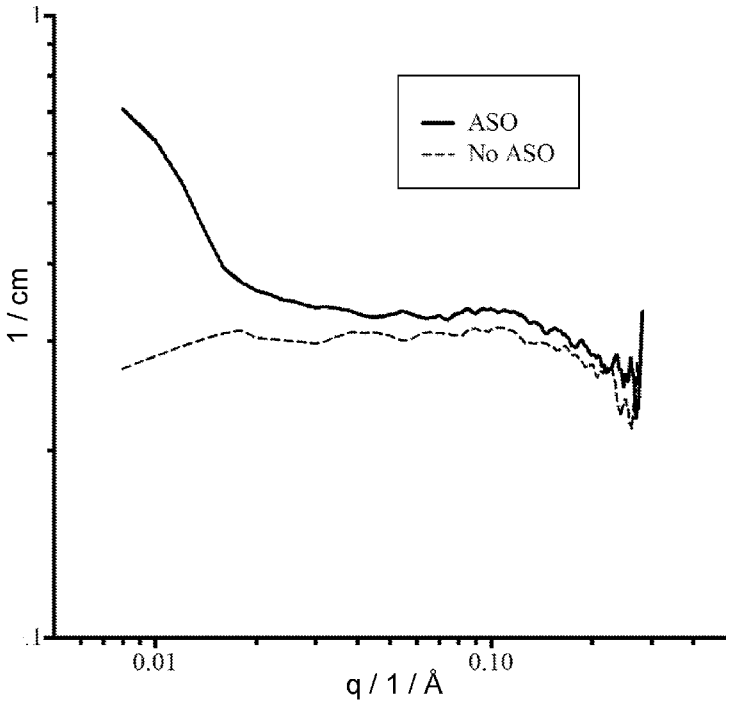

Figure 3

(57) Abstract: The present invention relates to antisense oligonucleotide (ASO) compositions and particularly to compositions and methods for the cytosolic delivery of antisense oligonucleotides (ASOs). Hybrid ASOs, part single-stranded and part double-stranded, are provided, hybridising to form a double-stranded region that can non-covalently bond to nucleic-acid-binding protein regions. In this way, ASO::protein complexes may be produced that facilitate delivery of antisense DNA into target cells. Such complexes may be used to down-regulate gene expression in cells.

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Antisense Oligonucleotide Compositions

The present invention relates to antisense oligonucleotide (ASO) compositions and particularly to compositions and methods for the cytosolic delivery of antisense oligonucleotides (ASOs). Hybrid ASOs, part single-stranded and part double-stranded, are provided, hybridising to form a double-stranded region that can non-covalently bond to nucleic-acid-binding protein regions. In this way, ASO::protein complexes may be produced that facilitate delivery of antisense DNA into target cells. Such complexes may be used to down-regulate gene expression in cells.

Antisense oligonucleotides have been approved by the FDA for use as antivirals, for the treatment of cytomegalovirus-mediated retinitis and chronic ulcerative colitis (Roehr, 1998; Yacychyn *et al.*, 1998). ASOs are comprised of segments of single-stranded DNA, or analogues thereof, that are designed to hybridise to a messenger RNA (mRNA) transcript, derived from a specific gene. The mRNA::ASO hybrids so formed may be degraded by RNAse H. In an organism infected with a virus, the genetic material of the virus enters specific cells in order to replicate, a process requiring the translation of virus-specific mRNA. By treating an infected cell with an antisense oligonucleotide specific for virus mRNA, it is possible to prevent the expression of a target viral gene and to block the viral life cycle.

An important factor in the effectiveness of antisense oligonucleotide treatment is the bioavailability of the oligonucleotides (Biroccio *et al.*, 2003). Bioavailability can be limited by the inability of antisense oligonucleotides to penetrate the plasma membrane or endomembrane system of cells. Since the targets of antisense oligonucleotides (*e.g.*, mRNA) are located within the cytosol of a cell, the oligonucleotides need to be able to traverse cell membranes before they can access the cytosol.

One way to address this problem of cytosolic access of antisense oligonucleotides is to administer the treatment locally rather than systemically, and in particular, to administer the antisense oligonucleotide into a discrete pharmacokinetic compartment, raising the local concentration of the antisense oligonucleotide. For example, an antisense oligonucleotide treatment of cytomegalovirus-mediated retinitis involves administering

fomivirsen (Vitravene®) into a discrete pharmacokinetic compartment by intravitreal injection. However, cytosolic access *i.e.,* access of the antisense oligonucleotides to their target mRNA, remains a substantial limitation, even after intravitreal administration (Lysik and Wu-Pong, 2003).

Other approaches to antisense oligonucleotide intracellular delivery may involve: mechanical and electrical cell damage to the cell membrane, polymeric and lipid carriers (known to non-specifically destabilise membranes), or viral vectors. However, these and similar approaches have not proved to be safe or reliable in the clinic and have not resulted (to date) in any delivery technologies licensed for antisense products in the market place.

The present invention aims to provide compositions and methods for use in a system for the down-regulation of one or more genes within a cell by means of a novel arrangement of an active antisense sequence (e.g., of single-stranded DNA) flanking a partially overlapping second oligonucleotide strand that forms a binding site that can bind to a proteinaceous, nucleic-acid-binding domain. We term these partly single- and partly double-stranded nucleotides, "ASO hybrids". Unexpectedly, we have discovered and have shown empirically (unpublished data):

1) That ASO hybrids can show an equivalent antisense activity profile to the control single-stranded antisense oligonucleotide in a cell-free assay, for example at an ASO concentration of 30 pMol.

2) That ASO hybrids bind to a nucleic-acid-binding domain (such as *S. cerevisiae* GAL4) to form a complex without requiring a polycationic affinity handle (such as poly(L-lysine), used previously (Gaur *et al.,* 2002; WO97/23236)) or other covalent chemical conjugation methods.

3) That if, in an ASO hybrid complex (for example, GAL4::ASO) the nucleic-acid-binding domain is fused to attenuated lethal factor domain 1 (from *Bacillus anthracis, i.e.,* LFn), this supramolecular assembly can pass through a pore derived from a bacterial virulence factor (*Bacillus anthracis* PA63). This is unexpected as it is reported that passage through the PA pore requires the structural disassembly of cargo (molten globular transition) (Zornetta *et al.,* 2010). For a supramolecular complex to transit into the cytosol via the PA pore, molten globular transition of the

cargo, *i.e.*, a transition from an ordered structure to a random coil, must not be taking place. Again this transition has been shown without the use of a polycationic affinity handle (such as poly(L-lysine) (Gaur *et al.*, 2002: WO97/23236)).

It is a particular aim of the present invention to provide a conjugation strategy to enable compositions of antisense oligonucleotides to cross a cell membrane. In this way, antisense oligonucleotides can be delivered to the cytosol of a cell providing direct access to RNA or DNA targets within the cytosol (such as viral mRNA transcripts) via the described antisense-to-protein conjugation strategy.

In one aspect, the present invention provides an antisense oligonucleotide composition comprising a pair of antisense oligonucleotides, wherein the two antisense oligonucleotides hybridise to give at least one single-stranded antisense sequence and at least one double-stranded protein-binding sequence. Preferably the composition further comprises a shuttle protein (*e.g.*, recombinant LFn-GAL4), wherein the shuttle protein comprises a nucleic-acid-binding domain (for example GAL4) that recognises the double-stranded protein-binding sequence and wherein the shuttle protein is non-covalently bonded to the pair of antisense oligonucleotides.

In another aspect, the present invention provides a system for delivering an antisense oligonucleotide across a membrane of a cell, the system comprising (i) a pair of antisense oligonucleotides, the pair comprising a double-stranded protein-binding sequence (*e.g.*, GAL4) and at least one single-stranded antisense sequence (as shown for example in Figure 1), and (ii) a shuttle protein having a nucleic-acid-binding domain that is capable of recognising a specific sequence (*e.g.*, CGG-N_{11}-CCG) incorporated within the double-stranded protein-binding nucleic acid sequence to non-covalently bind the shuttle protein to the pair of antisense oligonucleotides.

In a further aspect, the present invention provides a method of non-covalently conjugating an antisense oligonucleotide to a shuttle protein (*e.g.*, LFn-GAL4), the method comprising: (i) providing two antisense oligonucleotides, (ii) hybridising the two antisense oligonucleotides to form an antisense hybrid, wherein the antisense hybrid comprises at least one single-stranded antisense sequence and at least one double-stranded protein-

binding sequence, (iii) providing a shuttle protein containing a nucleic-acid-binding domain that recognises a double-stranded protein-binding target sequence, and (iv) non-covalently conjugating the shuttle protein to the antisense hybrid by means of the nucleic-acid-binding domain of the protein and the protein-binding sequence of the antisense hybrid.

The method of conjugation and membrane translocation does not require the use of (polycationic or ionic) DNA condensing agents (such as poly(ethylene imine) or poly(L-Lysine)) as these materials are often toxic and this toxicity is thought to be due, at least in part, to their propensity to destabilise cell membranes (Richardson *et al.*, 1999) (and see Table 1 below). In the context of the present invention, a shuttle protein is defined as a protein that is capable of conveying genetic material through a pore in a cell membrane. The pore may comprise a pore-forming protein, such as *Bacillus anthracis* PA83 or PA63.

The oligonucleotides of the invention comprise nucleic acid sequences, which may be of DNA or DNA analogues, for example sulphonated analogues in which certain oxygen atoms are replaced by sulphur. Other DNA analogues that may be found suitable include those disclosed by Leumann (2002). The sequences may directly adjoin each other or be connected by an intervening DNA sequence, preferably at least partly double-stranded.

In a preferred embodiment, the shuttle protein is an attenuated toxin protein. In particular, the shuttle protein may be *B. anthracis* lethal factor domain I (LFn).

Preferably, in the attenuated toxin, at least one toxin domain, *e.g.*, one or more of toxic domains II-IV of the *B. anthracis* lethal factor protein toxin, is replaced by a nucleic-acid-binding domain. For example, the nucleic-acid-binding domain may be *Saccharomyces cerevisiae* GAL4 (fused with LFn).

Advantageously, the antisense oligonucleotide composition further comprises a pore-forming protein. Preferably, the pore-forming protein is a non-toxic protein. One preferred pore-forming protein is *B. anthracis* virulence factor Protective Antigen (PA). In particular, the pore-forming protein may be *B. anthracis* PA83 or PA63.

In one embodiment, (in the instance of targeting syntaxin5 (Syn5) for down-regulation) one of the oligonucleotides includes the antisense sequence 5' AATTTGTTTGTTGAGGCTA 3' (SEQ ID No: 18). Each member of the antisense oligonucleotide pair comprises one of the two complementary strands that hybridise to form the protein-binding sequence (see Figure 1). The antisense sequence may be downstream (3') or upstream (5') of the protein-binding sequence (or both, if there is more than one antisense sequence in the hybrid that results) (see Figure 1a).

In one embodiment, the protein-binding sequence is CGG-N_{11}-CCG where "N" is any nucleotide comprising a purine or pyrimidine base. Suitably, the protein-binding sequence is 5' **CGG**CTGCTCTGATG**CCG** 3' (SEQ ID No: 19).

Advantageously, the antisense oligonucleotide composition comprises oligonucleotide pairs that hybridise to form a protein-binding sequence that includes the sequence: 5' CGGCATCAGAGCAGCCG 3' (SEQ ID No: 20). Examples of such sequences are SEQ ID Nos 9, 11 and 13 shown below (Example 4).

In one embodiment, the oligonucleotide further comprises a flanking sequence between the protein-binding sequence and the antisense sequence (see Figure 1a).

Advantageously, the oligonucleotides used in the present invention may be sulphonated, thereby increasing their stability. In sulphonated oligonucleotides, one or more non-bridging oxygen atoms in the oligonucleotide chain may be replaced by sulphur atoms. Sulphonation may be effected by various known methods, for example by use of Beaucage Reagent (3H-1,2-benzodithiole-3-one-1,1,-dioxide) (Cieślak *et al.*, 2005). Other ways of increasing stability of oligonucleotides may be employed in the invention: for example, those disclosed in Leumann (2002).

The present invention also provides an *in vitro* method of down-regulating the expression of a target gene in the cytosol of a cell by delivering an antisense oligonucleotide across a membrane of the cell using an antisense oligonucleotide composition as described above.

An alternative aspect of the present invention is an antisense oligonucleotide composition as described above for use in a method of down-regulating the expression of a gene from Human Papilloma Virus (HPV) in a human cervical epithelial cell (see Figure 5 and Example 7).

In a further aspect, the present invention provides an antisense oligonucleotide composition as described above as a potential treatment or prophylaxis for cancer, in particular cancers caused by human papilloma virus (HPV), including cervical cancer and oral cancers.

Many plant and bacterial toxins (virulence factors) have evolved to deliver a toxin, in the form of a macromolecular catalytically-active protein domain, to the cytosol of a cell. Examples include cholera toxin (Sandvig *et al.,* 2005), ricin toxin (Sandvig *et al.,* 2010) and anthrax toxin (Gaur *et al.,* 2002). These agents are known to operate *in vivo,* causing human morbidity and mortality.

The present inventors have prepared a known attenuated version of *Bacillus anthracis* lethal factor (LFn), where the wild-type domains II-IV (responsible for catalytic toxic activity) have been removed using recombinant PCR. These domains (II-IV) have subsequently been replaced with a nucleic-acid-binding domain such as *Saccharomyces cerevisiae* GAL4. All of this is known in the art (Gaur *et al.,* 2002). The inventors have shown that GAL4 can bind, indirectly, to active antisense oligonucleotides under suitable conditions *i.e.,* when two suitable oligonucleotides are hybridised together to form a hybrid containing a single-stranded antisense sequence and a double-stranded (DS) protein-binding sequence. The inventors have further shown that the complete complex (LFn-GAL4::ASO) can be used to translocate antisense oligonucleotides into the cytosol of a cell via interaction with another protein, PA83. Significantly, the processes and materials employed by the inventors to generate the complete antisense oligonucleotide - protein complex mean that condensing agents or polycation affinity handles hitherto regarded as necessary for DNA complex formation in the art (such as poly(L-lysine) (Gaur *et al.,* 2002; WO97/23236), which is toxic and known to destabilise membranes (Richardson *et al.,* 1999)) are not required.

A preferred embodiment of a system in accordance with the present invention provides a single-stranded antisense oligonucleotide sequence 3' and/or 5' to a double-stranded (DS)

protein-binding sequence. Proteins such as LFn may be used in conjunction with pore-forming proteins, for example PA83. PA83 has the capacity to enhance the membrane translocation of LFn. The interaction between LFn and PA83 is described elsewhere (Krantz *et al.*, 2005) though it has been documented to require the molten-globular transition of cargo during pore translocation. We have now shown that it is possible to use a supramolecular assembly as cargo and that an antisense sequence associated with that supramolecular assembly can translocate into the cytosol. This could not have been predicted.

The inventors have further developed the system to use hybridised oligonucleotide containing a double-stranded LFn-GAL4 binding sequence and a 3' and/or 5' overhanging single-stranded antisense sequence, which they have shown to be no less effective than the antisense (single-stranded nucleotide) sequence without any flanking sequence at antisense oligonucleotide (ASO) concentrations above 30pMol. The complex developed by the inventors can be used as an antisense oligonucleotide conjugation system to facilitate antisense oligonucleotide cytosolic delivery without a covalent attachment between the oligonucleotides and the LFn-GAL4 protein. The present invention provides a useful tool for *in vitro* research as well as a basis for an antisense oligonucleotide pharmaceutical composition and a critical part of the delivery methodology of any suitable antisense therapy. In particular, the present invention may form the basis for an antisense antiviral composition for the treatment of HPV-infected cervical epithelial cells.

The dimerisation of the LFn-GAL4 protein in response to the presence of a double-stranded protein-recognition nucleic acid sequence adjacent to the antisense sequence has been shown using small-angle neutron scattering (SANS) (see Example 5). Example 5 shows that the protein LFn-GAL4 forms high molecular weight complexes (protein radius of gyration (R_g) expanding from approximately 5nm to 25nm) upon the addition of the hybrid oligonucleotide compositions of the invention.

In order to test a three-component system in accordance with the present invention, the inventors initially produced a prototype. The system's ability to down-regulate genes was tested in human epithelial cells (Hela). The LFn-GAL4::oligonucleotide composite, when mixed with PA83 facilitates the down-regulation of the specific, selected gene (see

Example 6), corresponding to the antisense sequence. With reference to Example 6, this illustrates the down-regulation of the protein Syntaxin5 by Western blotting and immunodetection (Suga *et al.*, 2005). Gene specificity (as well as controlling for cell number) is further emphasised by normalising the levels of target gene expression to the expression levels of a "housekeeper" gene such as Derlin1 and through the inclusion of "non-sense" controls (see Figure 4). 'Non-sense' controls are controls that include single-stranded DNA that is not complementary to target RNA in the gene being expressed, and accordingly are not expected to inhibit its expression.

In addition antisense activity is also shown against the expression of the HPV gene E7 (see Example 7). HPV E7 is critical to the propagation of the HPV viral life cycle and is a target for therapeutic intervention (Jonson *et al.*, 2008).

The arrangement of two oligonucleotide strands to provide a double-stranded protein-binding region or sequence, whilst leaving free single-stranded regions of sequence that have antisense activity provides a convenient conjugation method that can be used to bind the antisense domains to agents already described in the art (such as LFn-GAL4 and PA83). This facilitates the cytosolic delivery of the antisense agents that can mediate the down-regulation of genes coding for potential drug targets, for example HPV E7.

Specific embodiments to the invention are now described in more detail with reference to the accompanying drawings, in which:

Figure 1 comprises Figures 1a to 1e showing five different compositions according to the invention, illustrating different topologies for creating a double-stranded protein-binding site from a pair of single-stranded antisense oligonucleotides;

Figure 2 compares inhibitory activity of a composition according to the invention with other antisense compositions in a cell-free assay.

Figure 3, by using small-angle neutron scattering (SANS), shows that in the presence of antisense compositions of the invention, LFn-GAL4 dimerise to form compositions of higher molecular weight.

Figures 4 and 5 illustrate how the described system can down-regulate expression of two different genes: down-regulating the expression of Syntaxin5 is shown in Figure 4 and of HPV (serotype 18) Early (E)7 is shown in Figure 5;

Figure 6 is the LFn-GAL4 nucleotide sequence (SEQ ID No: 1);

Figure 7 is the LFn (LF domain I) protein sequence (SEQ ID No: 2);

Figure 8 is the GAL4 (amino acids 1-147) protein sequence (SEQ ID No: 3);

Figure 9 is the V5-LFn-GAL4-6His DNA sequence (SEQ ID No: 4);

Figure 10 is the V5-LFn-GAL4-6His protein sequence (SEQ ID No: 5);

Figure 11 is the MRGS-6His-PA83 DNA sequence (SEQ ID No: 6); and

Figure 12 is the MRGS-6His-PA83 protein sequence (SEQ ID No: 7).

Example 1: Formation of shuttle protein

LFn-GAL4 was enriched from cultures of *E. coli* with a yield of approximately 5 mg/l. The DNA sequence coding for the protein LFn-GAL4 is SEQ ID No: 4 (Figure 9) . This sequence is within the plasmid pET151/D (Invitrogen), an *E.coli* expression cassette.

The DNA sequence for LFn (LF domain 1) and GAL4 (amino-acids 1-147) (known in the art - Gaur *et al.,* 2002) was sub-cloned into the pET151/D bacterial expression cassette. The addition of a V5 epitope tag at the N-terminus and 6x histidine affinity tag at the C-terminus allowed for the rapid immunodetection and affinity purification, respectively, of the fusion protein from bacterial lysate.

Recombinant protein production: Chemically competent bacteria (*E.coli* BL21*DE3 (Invitrogen)) were transformed with purified plasmid (described above) and cultured overnight (10 mL) in 2x yeast extract tyrptone (2xYT) bacterial culture, containing ampicillin (200 μg/ml) media prior to sub-culturing in large volumes (1L) of 2xYT also containing a similar concentration of ampicillin. Bacterial cultures were then incubated in an orbital shaker set at 180 rpm (at 37°C) for 3 hours. Subsequently isopropyl β-D-1-thiogalactopyrnoside (IPTG) was added to a final concentration of 500 μM and incubated for a further 3 hours. Bacterial pellets were prepared by centrifugation (6 000xg for 10 min at 4°C) and subject to lysis using a French Press set to 1500 PSI. Bacterial lysates were subjected to further centrifugation (20 000xg for 20 min). The resultant supernatant was passed over a 6xHis (Co^{2+} Tallon® resin (Clontech)) affinity chromatography column.

LFn-GAL4 was eluted using 150 mM imidazole in fractions of 1 mL. Fractions were analysed for protein purity and concentration, pooled and dialysed in phosphate buffered saline. The final protein preparation was evaluated by SDS-PAGE and subjected to Coomassie staining (to determine protein purity) and Western blot analysis (using an antibody specific to V5 and 6xHis).

Example 2: Production of pore-forming protein

PA83 was enriched from cultures of *E. coli* BL21*DE3 with a yield of approximately 5 mg/l. A plasmid containing the known DNA sequence encoding PA83 was obtained as a gift from Professor Les Baillie (University of Cardiff). It contained the PA83-encoding sequence sub-cloned into the bacterial expression vector pQE30 providing an N-terminal 6x histidine affinity tag (Baillie *et al.*, 2010). Chemically competent bacteria were transformed with purified plasmid and cultured overnight as before. During the growth phase, the 1*l* bacterial cultures were incubated in an orbital shaker set at 180 rpm (at 37°C) for 3 hours. Subsequently IPTG was added to a final concentration of 500 μM and incubated for a further 2 hours. Bacterial pellets were prepared by centrifugation (6 000xg for 10 min) and subjected to lysis using a French Press as before. Bacterial lysates were subjected to further centrifugation (20 000xg for 20 min). The resultant supernatant was passed over a 6x histidine affinity chromatography column. PA was eluted using 150 mM imidazole in fractions of 1 mL. Protein fractions were analysed for purity and concentration, pooled, and dialysed in phosphate-buffered saline (PBS). The final protein preparation was evaluated by SDS-PAGE and subjected to Coomassie staining (to determine purity) and western blot analysis (using an antibody specific to PA).

The amino-acid sequence for PA83 is shown in SEQ ID No: 7 (Figure 12 -MRGS-6His-PA83 protein).

Example 3: Formation of antisense oligonucleotide with double-stranded protein-binding region

Two complementary oligonucleotides, each encoding one strand of a GAL4 recognition sequence, were annealed, to form a double-stranded (GAL4) protein-binding sequence with flanking single-stranded antisense sequences. The ASO compositions described (SEQ ID Nos: 8 & 9: see Example 4) were used in this instance. The resulting composition is

shown in Figure 1a. Antisense oligonucleotide hybridization was performed by repeated (x10) thermal cycling, melting (1 min. at 94°C) and re-annealing (1 min. at 55°C) the two partially overlapping oligonucleotides, leaving single-strand antisense oligonucleotide sequence free to interact with mRNA in a sequence-specific manner.

Figures 1a - 1e show several possible topologies of the protein-binding (DS) sequence of DNA (or DNA analogues) in relation to the antisense nucleic acid sequence(s) using the protein::oligonucleotide conjugation strategy disclosed herein. Figure 1a shows two sequences SEQ ID Nos: 8 and 9 oriented in opposite senses and bonded together by complementary sections – bases 23 to 48 in SEQ ID No: 8 bonding with bases 48 to 23 of SEQ ID No. 9 (SEQ ID No. 9 is shown 3' to 5' in Figure 1a). The ASO hybrids of our invention may or may not include an additional DNA sequence flanking the antisense sequence(s). As shown in Figures 1a - 1e, the double-stranded (protein-binding) region of the ASO hybrid can be formed in a number of different ways. For example, the double-stranded region can be positioned between two antisense sequences (which may be identical or different), as in Figure 1a. Alternatively, the double-stranded region can be positioned at one side of a single antisense oligonucleotide, as in Figures 1b-1e.

Example 4: Production of DNA sequences useful for inhibiting expression of specific proteins

Antisense oligonucleotide sequence specific for Syntaxin5 with flanking and GAL4 binding sequence:

Forward oligonucleotide (SEQ ID No: 8)

5’ AATTTGTTTGTTGAGGCTAATGCATGCCGGCTGCTCTGATGCCGGCAT 3’

Reverse oligonucleotide (SEQ ID No: 9)

5’ AATTTGTTTGTTGAGGCTAATGCATGCCGGCATCAGAGCAGCCGGCAT 3’

Antisense oligonucleotide sequence specific for HPV (serotype 18) Early (E) 7 mRNA transcripts, with flanking and GAL4 binding sequence:

Forward oligonucleotide (SEQ ID No: 10)

5’ GGTCGTCTGCTGAGCTTTCTATGCATGCCGGCTGCTCTGATGCCGGCAT 3’

Reverse oligonucleotide (SEQ ID No: 11)

5' GGTCGTCTGCTGAGCTTTCTATGCATGCCGGCATCAGAGCAGCCGGCAT 3'

Antisense oligonucleotide sequence specific for TurboGFP mRNA transcripts, with flanking and GAL4 binding sequence:

Forward oligonucleotide (SEQ ID No: 12)

5' GGTGCTCTTCATCTTGTTGGTATGCATGCCGGCTGCTCTGATGCCGGCAT 3'

Reverse oligonucleotide (SEQ ID No: 13)

5' GGTGCTCTTCATCTTGTTGGTATGCCGGCATCAGAGCAGCCGGCATGCAT 3'

The above oligonucleotides (SEQ ID Nos: 8-13) were synthesised with phosphorothioate modification to enhance the lifetime of the antisense oligonucleotide.

Forward and reverse primer pairs (SEQ ID Nos 8 with 9; 10 with 11; 12 with 13) were hybridized using a PCR cycler using the following conditions: Heated to 94°C for 1 min., and cooled to 55°C for 1 min., x10. This annealing process produced hybrid single- and double-stranded DNA sequences according to the invention.

Example 5: Formation of an antisense oligonucleotide-shuttle (LFn-GAL4) complex

Annealed ASO hybrids were produced (as in Example 4). These ASO hybrids were co-incubated with LFn-GAL4 in phosphate-buffered saline (PBS) for 30 min at room temperature at the appropriate stoichiometry (*i.e.,* at approximately a 3:1 protein to oligonucleotide molar ratio).

One such product so obtained was investigated by small-angle neutron scattering (SANS). The profile resulting is compared in Figure 3 with that obtained in the absence of any antisense oligonucleotide. Figure 3 shows LFn-GAL4 dimerisation as measured by SANS in response to the addition of the antisense oligonucleotide composition shown in Figure 1a - small angle neutron scattering by LFn-GAL4 and LFn-GAL4::DS-antisense oligonucleotide in PBS (at approximately 0.5 mg/ml of LFn-GAL4 in each instance). Units of the x-axis of Figure 3 are q/l/angstrom and of the y-axis are 1/cm. The upper thick black line shows results in the presence of the ASO, in comparison with the lower thin line where the ASO is absent. The differential in scattering profile between 0.01 and 0.04 (q/1/Å) is attributable to the LFn-GAL4 dimerising, caused by association with the double-stranded

protein-binding sequence forming part of the oligonucleotide (Figure 1a). In this instance 2mm cuvettes (Hellman Analytics, Essex, UK) were loaded with LFn-GAL4 and LFn-GAL4::oligonucleotide stoichiometry of 3:1 respectively (molar ratio).

The intensity of such scattered radiation is a reflection of the scatterer size and shape, as well as composition (*i.e.*, LFn-GAL4). Both the individual proteins and their blends were examined. For the individual components, generally no scattering was observed, indicating their relatively small size. In the mixture of LFn-GAL4 and the hybridised oligonucleotide, scattering was observed, confirming that an interaction was occurring to form larger structures (*i.e.*, LFn-GAL4::ASO complexes).

Example 6: Down-regulation of target gene expression (Syntaxin5)

One of the complexes produced in Example 5 was employed to target the expression of the gene Syntaxin5 in Hela cells. The pore-forming protein PA83 was used to help mediate antisense oligonucleotide membrane translocation via an interaction with LFn-GAL4. The ASO portion of the complex consisted of two annealed (as in Example 4) ASO sequences (SEQ ID Nos: 8 & 9). Hela cells produce Syntaxin5 when cultured under normal conditions. Hela cells were treated in various ways at a range of ASO complex concentrations: 0 (control), 1, 10, 50, 100 and 200 pMol per litre.

In Figure 4: the black filled bar represents Hela cells treated with PA83, LFn-GAL4 and a "non-Syntaxin5" (GFP specific) ASO (formed from hybridised SEQ ID No:12 and 13). The bar with no fill represents Hela cells treated with PBS only. These two treatments show sequence specificity for Syntaxin5 down-regulation (since the anti-GFP ASO sequence here is inactive against Syntaxin5) and serve as negative controls. Bars filled with horizontal hatching represent Hela cells electroporated with hybrid ASO specific for Syntaxin5 (formed from SEQ ID Nos: 8 and 9). Bars filled with vertical hatching represent Hela cells treated with PA83, LFn-GAL4 and hybrid ASO specific for Syntaxin5 (formed from SEQ ID Nos: 8 and 9). The bars filled with diagonal hatching represent Hela cells treated with hybrid ASO specific for Syntaxin5 (formed from SEQ ID Nos: 8 and 9) mixed with Oligofectamine (Invitrogen), following the manufacturer's recommendations.

Syntaxin5 expression levels in the cells were measured after 24 hours using immunoblotting. Results are shown as % expression of control (Hela cells treated with

PBS only) (y axis). The data show that compositions according to the invention down-regulate Syntaxin5 expression in a gene-specific manner. This is shown at a population level by Western blotting and immunodetection. The graph of Figure 4 displays Syntaxin5 expression levels and shows the effect of loading antisense oligonucleotide hybrids (3.2μg/mL) (SEQ ID Nos: 8 and 9) onto LFn-GAL4 (50μg/mL) co-incubated with PA (50μg/mL) on Hela cells relative to the expression of a housekeeper gene, Derlin1 (to control for any variation in cell number). The data shown is derived from 3 separate experiments and demonstrates that the addition of the compositions described can mediate the knockdown of a specific gene antisense oligonucleotide encoding an antisense sequence (*i.e.*, Syntaxin5). The data represent three repeats of each experiment and the error bars represent the standard error of the mean.

Example 7: Down-regulation of target gene expression (HPV18 E7)

An experiment to examine the efficacy of anti-HPV E7 antisense oligonucleotides against the expression of Human Papilloma Virus (serotype 18) Early 7 in Hela cells was undertaken as described in Example 6 above, but using different oligonucleotide sequences. In this instance the Syntaxin5-specific ASO hybrids (formed from SEQ ID No: 8 and SEQ ID No: 9) of Example 6 were replaced with HPV-E7-specific ASO hybrid also containing the hybrid protein-binding region and a small flanking region (formed from SEQ ID No:10 and SEQ ID No:11). In this example 200 pM of oligonucleotide, associated with LFn-GAL4 and PA83, was used (this is the black bar on the graph in Figure 5). The result is shown in Figure 5: % gene expression is shown on the y axis. The control treatment, PBS, is taken as 100%: this is the white bar on the graph in Figure 5. The data represent three repeats of each experiment and the error bars represent the standard error of the mean. There was a statistical difference between the two treatments ($p = 0.0104$ as measured by a one-tailed, unpaired t-test).

Example 8: *In vitro* toxicity of compositions of the invention compared with poly(ethyleneimine)

The ASO hybrid-shuttle complexes produced as in Example 5 were tested for toxicity in relation to poly(ethyleneimine) (PEI) (Table 1). These data show a differential in toxicity relative to cells exposed to varying concentrations of either PEI, a cationic polymer well characterised in the literature as being able to mediate transfection, and LFn-GAL4 protein

after hybridisation with ASO hybrids in the presence of 50μg/mL PA over 72h. Cell viability was normalised to untreated cells and measured by the addition of MTT (Richardson *et al.*, 1999).

Table 1

	IC_{50} in HeLa (micrograms/ml)	IC_{50} in Vero (micrograms/ml)
25KDa Branched PEI	2.9 +/- 0.6	7.3 +/- 0.1
0.8KDa Branched PEI	2.4 +/- 0.2	7.4 +/- 0.3
20KDa Linear PEI	3.0 +/- 0.1	6.9 +/- 0.5
PA83	>100	>100
ASO	>100	>100
GAL4-LFn	>100	>100
ASO ::GAL4-LFn+PA83	>100	>100

Example 9: Antisense inhibition of an *in vitro* translation assay using different antisense oligonucleotide compositions

Figure 2 shows the effect of compositions of the invention as compared with component ASOs in a cell-free assay (where membrane translocation is not rate-limiting).

Control reactions were performed using a "One step human high yield mini- *in vitro* translation kit" (Thermo Scientific), in conjunction with a control plasmid encoding the protein turboGFP (green fluorescent protein – Evrogen). The reactions were performed in 4.5 μl volumes (incubated for 3h at 30°C) and the expression of turboGFP was monitored by Western immunoblotting using an anti-6His primary antibody (1:1000 dilution) and an anti-mouse HRP-conjugated secondary antibody (1:1000). Detection was performed using a Picostable ECL kit (GE Healthcare). Three anti-turbo GFP antisense oligonucleotide (ASO) compositions were added to the reaction at differing concentrations at the beginning of the 3h incubation period previously described.

Results are shown in Figure 2, (n=3 ± SEM) relative to a control containing no antisense oligonucleotides. In Figure 2 the y-axis represents the expression of turboGPF (%) relative to an untreated control, while the x-axis represents concentration of antisense

oligonucleotide added to the reaction (in pMol). The data points represented with a square were derived from a single-stranded ASO oligonucleotide, SEQ ID No: 12, consisting of the turboGPF antisense sequence and (one strand of the) protein-binding sequence. The data points represented by triangles represent data generated from only the antisense sequence incorporated into SEQ ID No: 12 (*i.e.*, the 21-mer GGTGCTCTTCATCTTGTTGGA, SEQ ID No: 21). The data points represented by circles were generated from the ASO hybrid of the invention formed by annealing together SEQ ID Nos: 12 and 13. The results shown in Figure 2 illustrate that (*in vitro*) the presence of the protein-binding sequence in the ASO hybrid does not inhibit the activity of the antisense sequence above an ASO concentration of 30 pMol.

Our invention may be used to produce antisense compositions having substantially no significantly reduced antisense activity (as measured in cell-free assay), relative to an antisense control having no protein-binding sequence or flanking sequence, at or above a 30pMol concentration of antisense oligonucleotide (ASO). Example 9 above (Figure 2) illustrates this.

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CLAIMS:

1. An antisense oligonucleotide composition comprising:

a pair of partially complementary, single-stranded oligonucleotides, wherein the two oligonucleotides hybridise to give at least one single-stranded antisense sequence and at least one double-stranded protein-binding sequence;

and a shuttle protein capable of conveying genetic material through a pore in a cell membrane, the shuttle protein comprising a nucleic-acid-binding domain that recognises the double-stranded protein-binding sequence and non-covalently binds thereto, wherein the shuttle protein contains *B. anthracis* lethal factor (LF) domain I (LFn); and

a pore-forming protein which is *B. anthracis* virulence factor Protective Antigen PA83.

2. The antisense oligonucleotide composition as claimed in claim 1, comprising an oligonucleotide that is modified by sulphonation to increase stability.

3. The antisense oligonucleotide composition as claimed in claim 1 or 2, wherein the protein-binding sequence is CGG-N11-CCG, where "N" is any purine or pyrimidine base.

4. The antisense oligonucleotide composition as claimed in any one of claims 1-3, wherein the protein-binding sequence is:

5'-CGGCTGCTCTGATGCCG-3' or 5'-CGGCATCAGAGCAGCCG-3'.

5. The antisense oligonucleotide composition as claimed in any one of claims 1-4, wherein at least one of domains (II-IV) of *B. anthracis* lethal factor (LF) is replaced by the nucleic-acid-binding domain.

6. The antisense oligonucleotide composition as claimed in any one of claims 1 to 5, wherein the nucleic-acid-binding domain of the shuttle protein is *Saccharomyces cerevisiae* GAL4.

7. The antisense oligonucleotide composition as claimed in any one of claims 1 to 6 in which the antisense sequence is single-stranded DNA designed to hybridise to messenger RNA derived from a target gene.

8. The antisense oligonucleotide composition as claimed in claim 7 in which the target gene is expressed by a virus.

9. The antisense oligonucleotide composition as claimed in claim 7 or 8 in which the target gene is expressed by Human Papilloma Virus (HPV).

10. An *in vitro* method of downregulating the expression of a target gene in the cytosol of a cell comprising delivering the antisense oligonucleotide composition as defined in any one of claims 1 to 9, and across the membrane of the cell.

11. The antisense oligonucleotide composition as claimed in any one of claims 1 to 9 for use in medical treatment.

12. The antisense oligonucleotide composition as claimed in claim 11, additionally containing at least one additive selected from the group consisting of pharmaceutically acceptable excipients and carriers.

13. The antisense oligonucleotide composition as claimed in claim 11 or 12, for use in treating a subject infected with Human Papilloma Virus (HPV).

14. The antisense oligonucleotide composition as claimed in claim 11 or 12, for use in the treatment of cancer.

15. The antisense oligonucleotide composition as claimed in claim 14, wherein the cancer is cervical or oral cancer.

16. A method of non-covalently conjugating an antisense oligonucleotide to a shuttle protein, the method comprising:

(i) providing two partially complementary single-stranded oligonucleotides,

(ii) hybridising the two oligonucleotides to form an oligonucleotide hybrid, wherein the hybrid comprises at least one single-stranded antisense sequence and at least one double-stranded protein-binding sequence,

(iii) providing a shuttle protein capable of conveying genetic material through a pore in a cell membrane and having a nucleic-acid-binding domain that

recognises the double-stranded protein-binding sequence, and providing a pore-forming protein, wherein the shuttle protein contains *B. anthracis* lethal factor (LF) domain I (LFn) and the pore-forming protein is *B. anthracis* virulence factor Protective Antigen PA83; and

(iv) non-covalently conjugating the shuttle protein to the oligonucleotide hybrid by means of the nucleic-acid-binding domain of the protein and the protein-binding sequence of the oligonucleotide hybrid.

a)

Flanking sequence — Flanking sequence

SEQ ID 8 5′**AATTTGTTTGTTGAGGCTA**atgcatgCCGGctgctctgatgCCGgcat 3′

SEQ ID 9 3′tacgGCCgacgagactacGGCcgtacgta**ATCGGAGTTGTTTGTTTAA**5′

Antisense sequence — Protein binding sequence — Antisense sequence

b)

SEQ ID 8 5′**AATTTGTTTGTTGAGGCTA**atgcatgCCGGctgctctgatgCCGgcat 3′

SEQ ID 14 3′tacgGCCgacgagactacGGCcgta 5′

c)

SEQ ID 16 5′ tacgGCCgacgagactacGGCcgtacgta**AATTTGTTTGTTGAGGCTA** 3′

SEQ ID 14 3′ atgcCGGctgctctgatgCCGgcat 5′

d)

SEQ ID 15 5′ atgcCGGctgctctgatgCCGgcat 3′

SEQ ID 9 3′ tacgGCCgacgagactacGGCcgtacgta**ATCGGAGTTGTTTGTTTAA** 5′

e)

SEQ ID 15 5′atgcCGGctgctctgatgCCGgcat 3′

SEQ ID 17 3′**ATCGGAGTTGTTTGTTTAA**atcgtacgGCCgacgagactacGGCcgta 5′

Figure 1

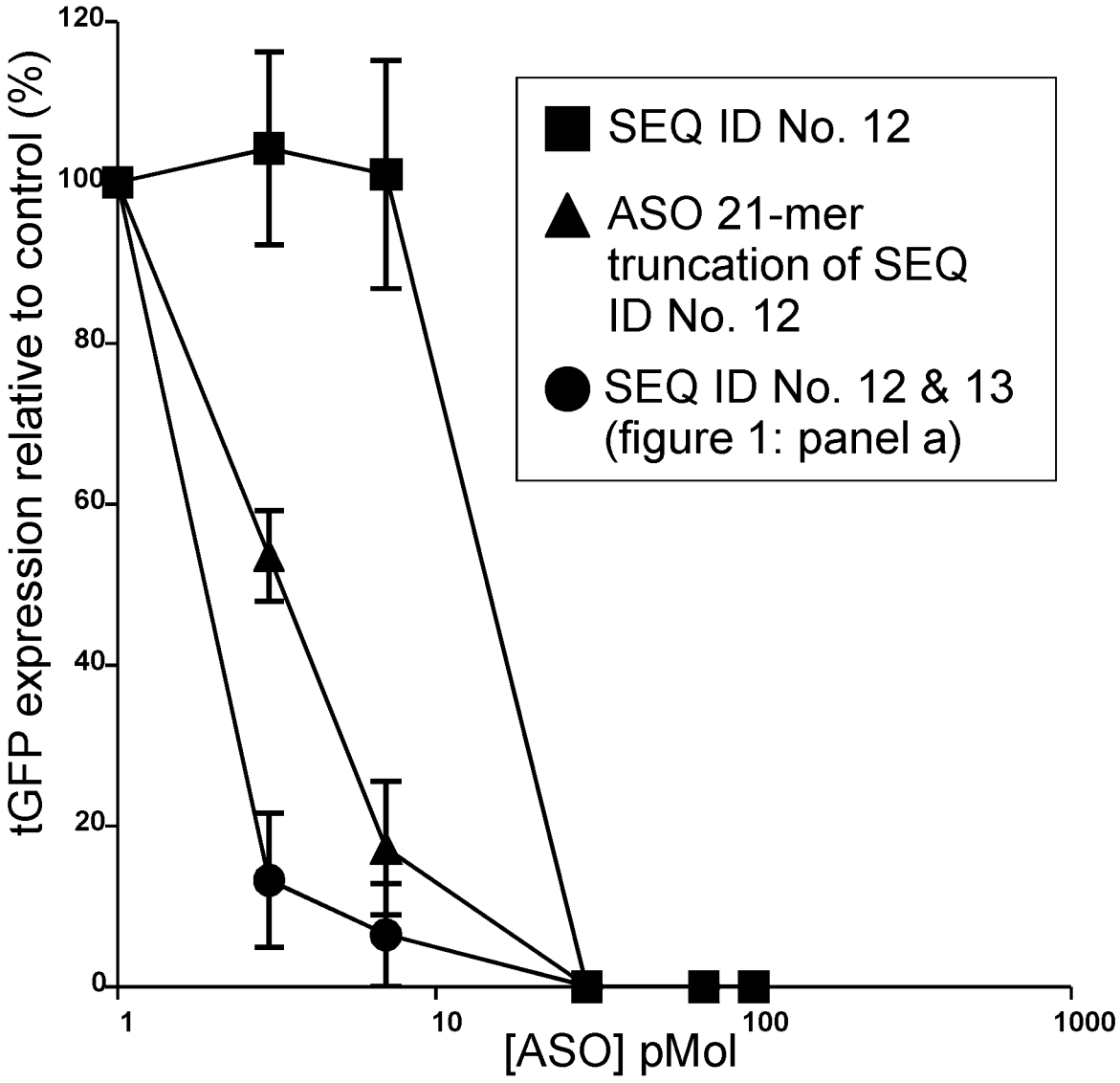
120
100
80
60
40
20
0
tGFP expression relative to control (%)
1
10
100
1000
[ASO] pMol
SEQ ID No. 12
ASO 21-mer truncation of SEQ ID No. 12
SEQ ID No. 12 & 13 (figure 1: panel a)

Figure 2

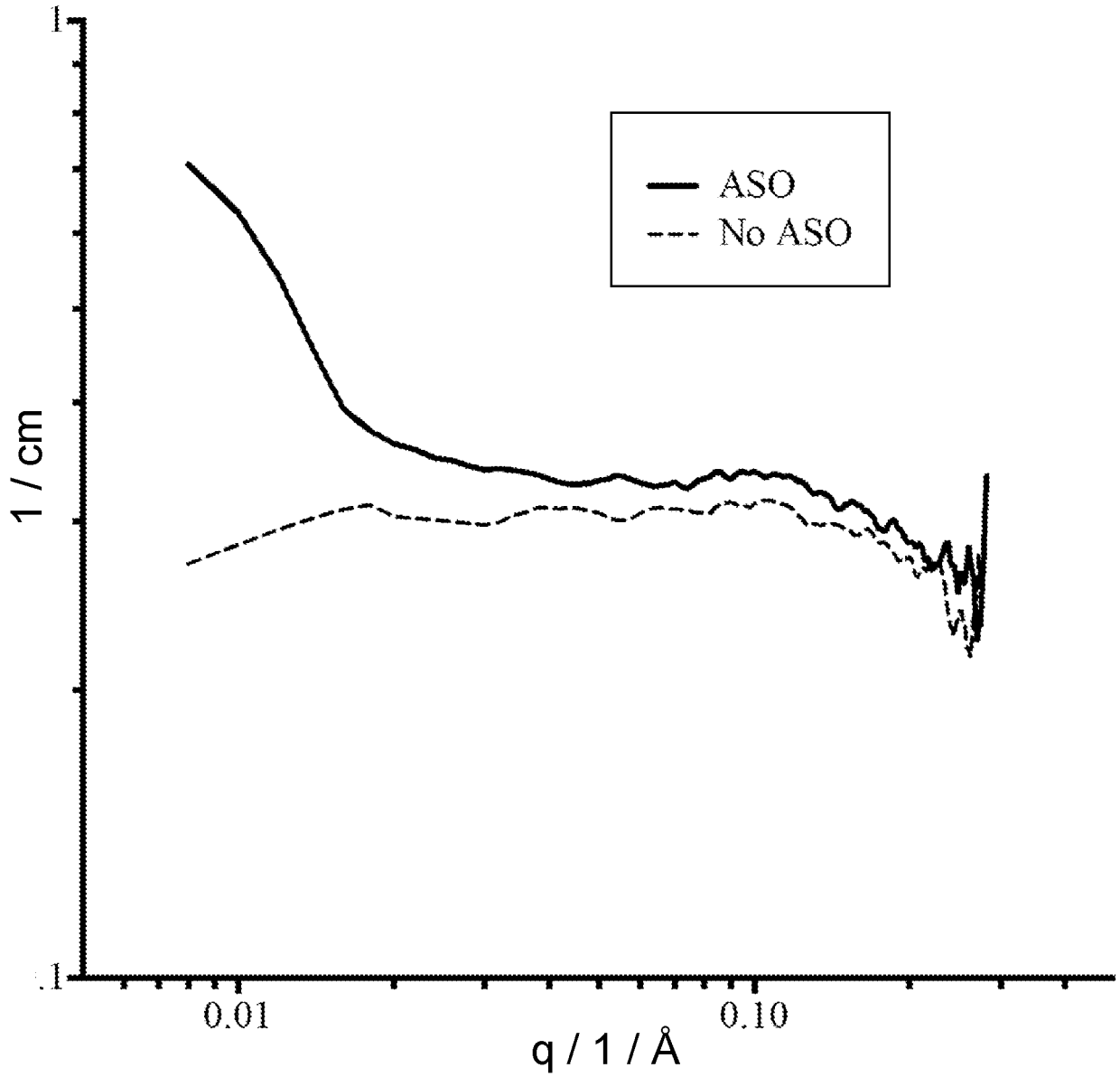

Figure 3

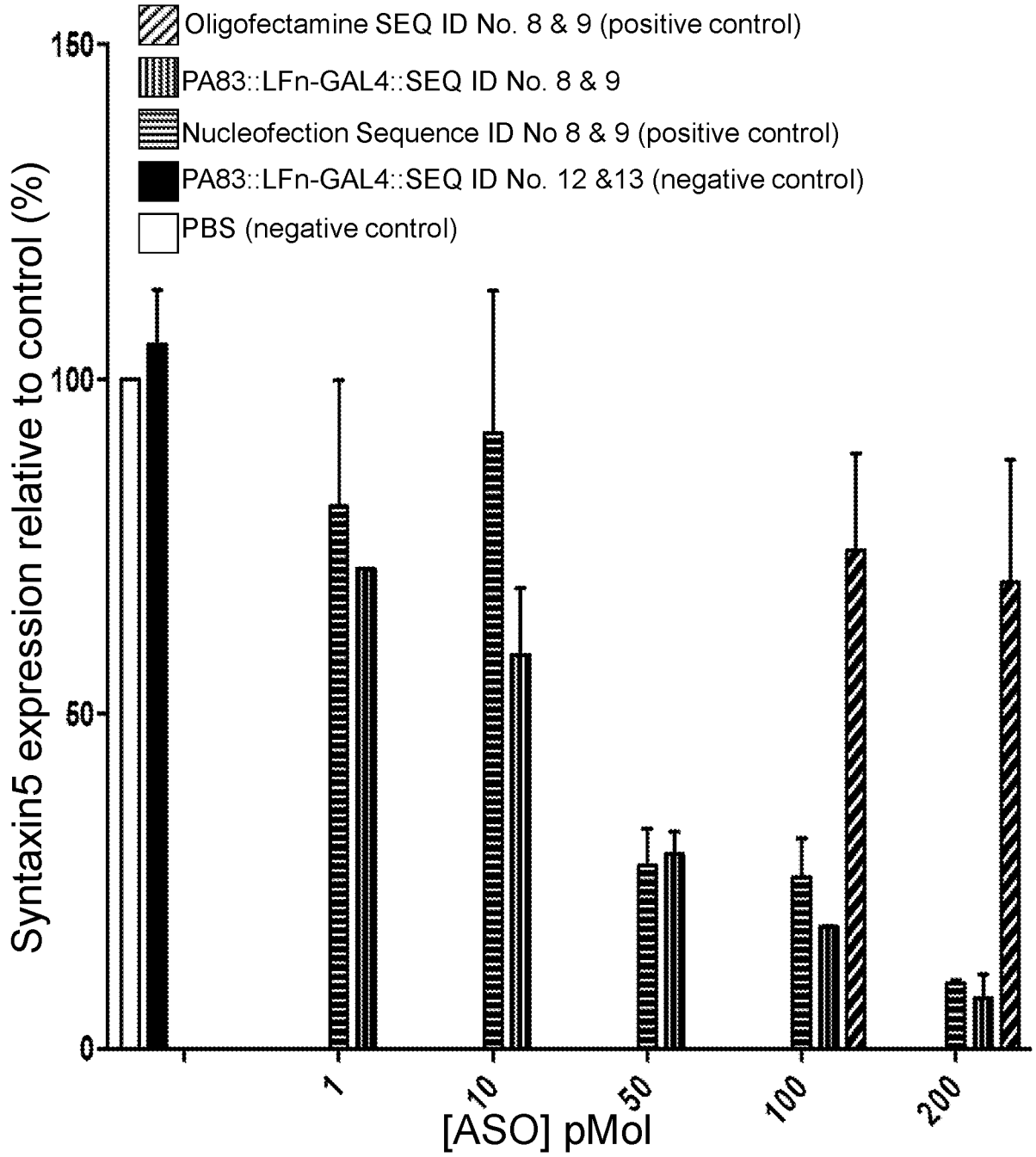
Oligofectamine SEQ ID No. 8 & 9 (positive control)
PA83::LFn-GAL4::SEQ ID No. 8 & 9
Nucleofection Sequence ID No 8 & 9 (positive control)
PA83::LFn-GAL4::SEQ ID No. 12 &13 (negative control)
PBS (negative control)
Syntaxin5 expression relative to control (%)
150
100
50
0
1
10
50
100
200
[ASO] pMol

Figure 4

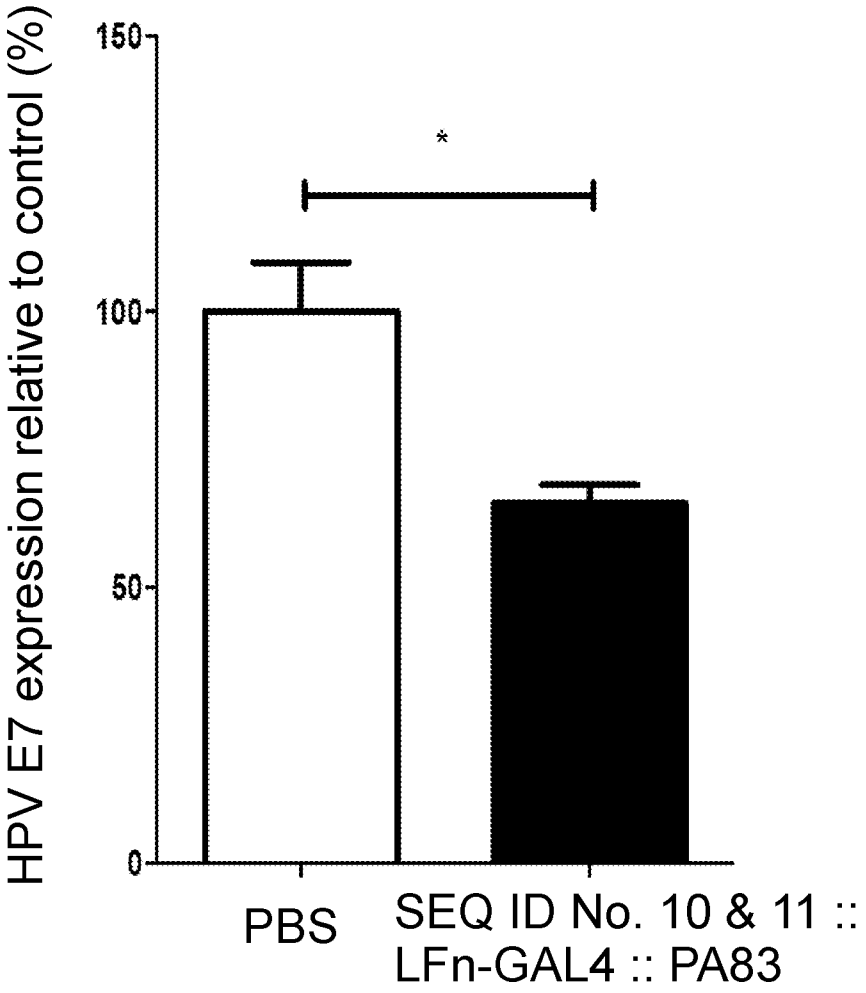
HPV E7 expression relative to control (%)
150
100
50
0
*
PBS
SEQ ID No. 10 & 11 :: LFn-GAL4 :: PA83

Figure 5

SEQ ID No: 1

ATGGAACGCAACAAAACTCAGGAGGAACACCTGAAAGAGATCATGAAACACATTGTTAAAAT
CGAGGTGAAAGGCGAAGAAGCGGTTAAAAAGGAGGCTGCCGAAAAGCTGCTGGAGAAGGT
ACCGTCTGATGTGCTGGAAATGTATAAAGCGATTGGTGGCAAAATCTACATCGTGGATGGTGAT
ATTACCAAACACATCTCCCTGGAAGCACTGAGCGAAGACAAGAAGAAGATCAAAGATATCTAC
GGCAAGGACGCGCTGCTGCACGAGCATTACGTTTACGCAAAAGAAGGTTACGAACCGGTGCT
GGTTATCCAGTCCAGCGAGGATTACGTCGAAAATACGGAAAAAGCTCTGAACGTATATTACGA
AATTGGTAAAATCCTGTCTCGTGACATTCTGAGCAAAATTAACCAACCTTATCAGAAGTTCCTG
GACGTTCTGAACACCATCAAAAACGCTTCTGACTCCGACGGCCAGGACCTGCTGTTCACTAAT
CAGCTGAAAGAACATCCGACCGATTTCTCTGTAGAATTCCTGGAACAGAACTCTAACGAGGTC
CAAGAAGTTTTTGCCAAAGCATTCGCGTACTACATCGAGCCGCAGCATCGCGACGTGCTGCAG
CTGTACGCTCCAGAAGCCTTCAACTATATGGACAAATTCAATGAACAAGAAATCAACCTGTCTA
TGAAACTGCTGTCCTCCATCGAACAGGCTTGCGATATCTGTCGTCTGAAGAAACTGAAATGCTC
TAAAGAAAAACCGAAATGCGCGAAATGCCTGAAAAACAACTGGGAATGTCGCTATTCCCCTAA
AACCAAACGTTCTCCACTGACCCGTGCGCACCTGACCGAAGTAGAATCCCGTCTGGAACGTCT
GGAGCAGCTGTTTCTGCTGATTTTCCCGCGTGAAGACCTGGACATGATCCTGAAAATGGATAG
CCTGCAGGATATCAAAGCACTGCTGACCGGTCTGTTTGTGCAGGACAACGTCAACAAAGACG
CTGTTACTGATCGCCTGGCGAGCGTTGAAACTGATATGCCGCTGACCCTGCGTCAGCACCGTAT
CTCCGCAACGAGCTCCAGCGAAGAATCTAGCAACAAAGGTCAGCGCCAGCTGACCGTTAGC

Figure 6

SEQ ID No: 2

MERNKTQEEHLKEIMKHIVKIEVKGEEAVKKEAAEKLLEKVPSDVLEMYKAIGGKIYIVDGDITKHI
SLEALSEDKKKIKDIYGKDALLHEHYVYAKEGYEPVLVIQSSEDYVENTEKALNVYYEIGKILSRDILSK
INQPYQKFLDVLNTIKNASDSDGQDLLFTNQLKEHPTDFSVEFLEQNSNEVQEVFAKAFAYYIEPQ
HRDVLQLYAPEAFNYMDKFNEQEINLS

Figure 7

SEQ ID No: 3

MKLLSSIEQACDICRLKKLKCSKEKPKCAKCLKNNWECRYSPKTKRSPLTRAHLTEVESRLERLEQLF
LLIFPREDLDMILKMDSLQDIKALLTGLFVQDNVNKDAVTDRLASVETDMPLTLRQHRISATSSSEE
SSNKGQRQLTVS

Figure 8

SEQ ID No: 4

ATGTAAACCGATTCCGAACCCGCTGCTGGGCCTGGACTCTACTATGGAACGCAACAAAACTCA
GGAGGAACACCTGAAAGAGATCATGAAACACATTGTTAAAATCGAGGTGAAAGGCGAAGAA
GCGGTTAAAAAGGAGGCTGCCGAAAAGCTGCTGGAGAAGGTACCGTCTGATGTGCTGGAAA
TGTATAAAGCGATTGGTGGCAAAATCTACATCGTGGATGGTGATATTACCAAACACATCTCCCT
GGAAGCACTGAGCGAAGACAAGAAGAAGATCAAAGATATCTACGGCAAGGACGCGCTGCTG
CACGAGCATTACGTTTACGCAAAAGAAGGTTACGAACCGGTGCTGGTTATCCAGTCCAGCGA
GGATTACGTCGAAAATACGGAAAAAGCTCTGAACGTATATTACGAAATTGGTAAAATCCTGTCT
CGTGACATTCTGAGCAAAATTAACCAACCTTATCAGAAGTTCCTGGACGTTCTGAACACCATCA
AAAACGCTTCTGACTCCGACGGCCAGGACCTGCTGTTCACTAATCAGCTGAAAGAACATCCGA
CCGATTTCTCTGTAGAATTCCTGGAACAGAACTCTAACGAGGTCCAAGAAGTTTTTGCCAAAG
CATTCGCGTACTACATCGAGCCGCAGCATCGCGACGTGCTGCAGCTGTACGCTCCAGAAGCCT
TCAACTATATGGACAAATTCAATGAACAAGAAATCAACCTGTCTATGAAACTGCTGTCCTCCATC
GAACAGGCTTGCGATATCTGTCGTCTGAAGAAACTGAAATGCTCTAAAGAAAAACCGAAATGC
GCGAAATGCCTGAAAAACAACTGGGAATGTCGCTATTCCCCTAAAACCAAACGTTCTCCACTG
ACCCGTGCGCACCTGACCGAAGTAGAATCCCGTCTGGAACGTCTGGAGCAGCTGTTTCTGCTG
ATTTTCCCGCGTGAAGACCTGGACATGATCCTGAAAATGGATAGCCTGCAGGATATCAAAGCA
CTGCTGACCGGTCTGTTTGTGCAGGACAACGTCAACAAAGACGCTGTTACTGATCGCCTGGCG
AGCGTTGAAACTGATATGCCGCTGACCCTGCGTCAGCACCGTATCTCCGCAACGAGCTCCAGC
GAAGAATCTAGCAACAAAGGTCAGCGCCAGCTGACCGTTAGCCACCACCATCACCACCACTAA
GG

Figure 9

SEQ ID No: 5

MGKPIPNPLLGLDSTMERNKTQEEHLKEIMKHIVKIEVKGEEAVKKEAAEKLLEKVPSDVLEMYKA
IGGKIYIVDGDITKHISLEALSEDKKKIKDIYGKDALLHEHYVYAKEGYEPVLVIQSSEDYVENTEKAL
NVYYEIGKILSRDILSKINQPYQKFLDVLNTIKNASDSDGQDLLFTNQLKEHPTDFSVEFLEQNSNE
VQEVFAKAFAYYIEPQHRDVLQLYAPEAFNYMDKFNEQEINLSMKLLSSIEQACDICRLKKLKCSKE
KPKCAKCLKNNWECRYSPKTKRSPLTRAHLTEVESRLERLEQLFLLIFPREDLDMILKMDSLQDIKAL
LTGLFVQDNVNKDAVTDRLASVETDMPLTLRQHRISATSSSEESSNKGQRQLTVSHHHHHH

Figure 10

SEQ ID No: 6

ATGAGAGGATCGCATCACCATCACCATCACGGATCCGAAGTTAAACAGGAGAACCGGTTATTAAAT
GAATCAGAATCAAGTTCCCAGGGGTTACTAGGATACTATTTTAGTGATTTGAATTTTCAAGCACCCAT
GGTGGTTACCTCTTCTACTACAGGGGATTTATCTATTCCTAGTTCTGAGTTAGAAAATATTCCATCGG
AAAACCAATATTTTCAATCTGCTATTTGGTCAGGATTTATCAAAGTTAAGAAGAGTGATGAATATACA
TTTGCTACTTCCGCTGATAATCATGTAACAATGTGGGTAGATGACCAAGAAGTGATTAATAAAGCTTC
TAATTCTAACAAAATCAGATTAGAAAAAGGAAGATTATATCAAATAAAAATTCAATATCAACGAGAA
AATCCTACTGAAAAAGGATTGGATTTCAAGTTGTACTGGACCGATTCTCAAAATAAAAAAGAAGTG
ATTTCTAGTGATAACTTACAATTGCCAGAATTAAAACAAAAATCTTCGAACTCAAGAAAAAAGCGAA
GTACAAGTGCTGGACCTACGGTTCCAGACCGTGACAATGATGGAATCCCTGATTCATTAGAGGTAG
AAGGATATACGGTTGATGTCAAAAATAAAAGAACTTTTCTTTCACCATGGATTTCTAATATTCATGAA
AAGAAAGGATTAACCAAATATAAATCATCTCCTGAAAAATGGAGCACGGCTTCTGATCCGTACAGTG
ATTTCGAAAAGGTTACAGGACGGATTGATAAGAATGTATCACCAGAGGCAAGACACCCCCTTGTGG
CAGCTTATCCGATTGTACATGTAGATATGGAGAATATTATTCTCTCAAAAAATGAGGATCAATCCACA
CAGAATACTGATAGTCAAACGAGAACAATAAGTAAAAATACTTCTACAAGTAGGACACATACTAGTG
AAGTACATGGAAATGCAGAAGTGCATGCGTCGTTCTTTGATATTGGTGGGAGTGTATCTGCAGGAT
TTAGTAATTCGAATTCAAGTACGGTCGCAATTGATCATTCACTATCTCTAGCAGGGGAAAGAACTTG
GGCTGAAACAATGGGTTTAAATACCGCTGATACAGCAAGATTAAATGCCAATATTAGATATGTAAATA
CTGGGACGGCTCCAATCTACAACGTGTTACCAACGACTTCGTTAGTGTTAGGAAAAAATCAAACAC
TCGCGACAATTAAAGCTAAGGAAAACCAATTAAGTCAAATACTTGCACCTAATAATTATTATCCTTCT
AAAAACTTGGCGCCAATCGCATTAAATGCACAAGACGATTTCAGTTCTACTCCAATTACAATGAATT
ACAATCAATTTCTTGAGTTAGAAAAAACGAAACAATTAAGATTAGATACGGATCAAGTATATGGGAA
TATAGCAACATACAATTTTGAAAATGGAAGAGTGAGGGTGGATACAGGCTCGAACTGGAGTGAAG
TGTTACCGCAAATTCAAGAAACAACTGCACGTATCATTTTTAATGGAAAAGATTTAAATCTGGTAGA
AAGGCGGATAGCGGCGGTTAATCCTAGTGATCCATTAGAAACGACTAAACCGGATATGACATTAAA
AGAAGCCCTTAAAATAGCATTTGGATTTAACGAACCGAATGGAAACTTACAATATCAAGGGAAAGA
CATAACCGAATTTGATTTTAATTTCGATCAACAAACATCTCAAAATATCAAGAATCAGTTAGCGGAAT
TAAACGCAACTAACATATATACTGTATTAGATAAAATCAAATTAAATGCAAAAATGAATATTTTAATAA
GAGATAAACGTTTTCATTATGATAGAAATAACATAGCAGTTGGGGCGGATGAGTCAGTAGTTAAGG
AGGCTCATAGAGAAGTAATTAATTCGTCAACAGAGGGATTATTGTTAAATATTGATAAGGATATAAG
AAAAATATTATCAGGTTATATTGTAGAAATTGAAGATACTGAAGGGCTTAAAGAAGTTATAAATGAC
AGATATGATATGTTGAATATTTCTAGTTTACGGCAAGATGGAAAAACATTTATAGATTTTAAAAAATAT
AATGATAAATTACCGTTATATATAAGTAATCCCAATTATAAGGTAAATGTATATGCTGTTACTAAAGAAA
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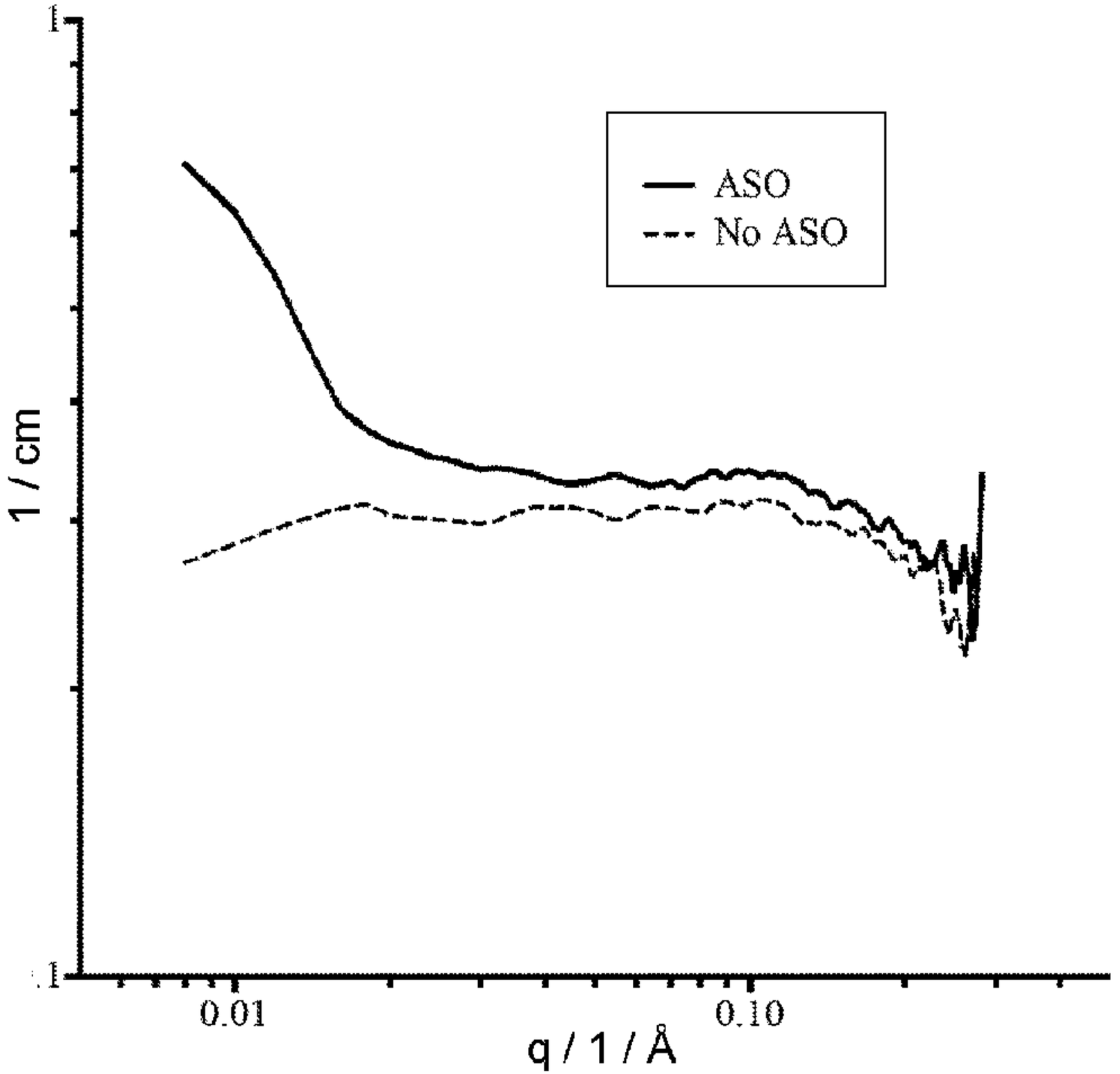
Figure 11

SEQ ID No: 7

MRGSHHHHHHGSEVKQENRLLNESESSSQGLLGYYFSDLNFQAPMVVTSSTTGDLSIPSSELENI
PSENQYFQSAIWSGFIKVKKSDEYTFATSADNHVTMWVDDQEVINKASNSNKIRLEKGRLYQIKI
QYQRENPTEKGLDFKLYWTDSQNKKEVISSDNLQLPELKQKSSNSRKKRSTSAGPTVPDRDNDGI
PDSLEVEGYTVDVKNKRTFLSPWISNIHEKKGLTKYKSSPEKWSTASDPYSDFEKVTGRIDKNVSP
EARHPLVAAYPIVHVDMENIILSKNEDQSTQNTDSQTRTISKNTSTSRTHTSEVHGNAEVHASFF
DIGGSVSAGFSNSNSSTVAIDHSLSLAGERTWAETMGLNTADTARLNANIRYVNTGTAPIYNVLP
TTSLVLGKNQTLATIKAKENQLSQILAPNNYYPSKNLAPIALNAQDDFSSTPITMNYNQFLELEKTK
QLRLDTDQVYGNIATYNFENGRVRVDTGSNWSEVLPQIQETTARIIFNGKDLNLVERRIAAVNPS
DPLETTKPDMTLKEALKIAFGFNEPNGNLQYQGKDITEFDFNFDQQTSQNIKNQLAELNATNIYT
VLDKIKLNAKMNILIRDKRFHYDRNNIAVGADESVVKEAHREVINSSTEGLLLNIDKDIRKILSGYIV
EIEDTEGLKEVINDRYDMLNISSLRQDGKTFIDFKKYNDKLPLYISNPNYKVNVYAVTKENTIINPSE
NGDTSTNGIKKILIFSKKGYEIG

Figure 12


ASO
No ASO
1 / cm
0.01
0.10
q / 1 / Å