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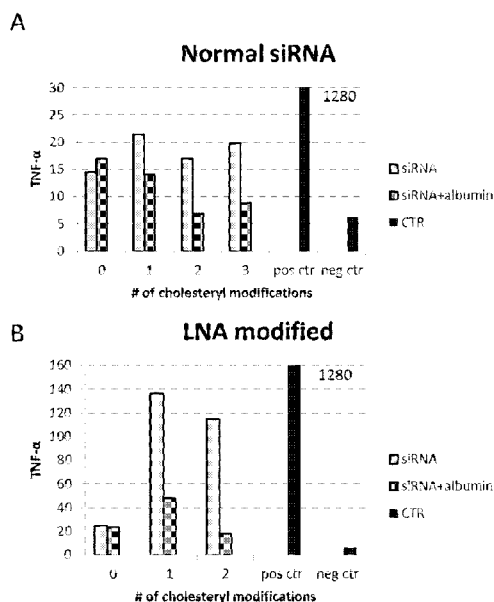
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[Continued on next page]

(54) Title: MODIFIED PAYLOAD MOLECULES AND THEIR INTERACTIONS AND USES

Fig. 68



(57) Abstract: An object of the present invention relates to a composition comprising a payload molecule such as a small interfering nucleic acid (siNA) and albumin or fragments thereof, or fusion polypeptides comprising variant albumin or fragments thereof, of a parent albumin. Another object of the present invention relates to a payload molecule such as a siNA conjugated with one or more cholesterol and/or one or more fatty acid. A further object of the present invention relates to a payload molecule such as a siNA the composition of the present invention for use as a medicament. Yet another object of the present invention relates to a payload molecule such as a siNA or the composition of the present invention for use as in regulating a genetic expression of a transcript or protein associated with a disease.



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Modified payload molecules and their interactions and uses

Technical field of the invention

The present invention relates to modified payload molecules such as short interfering
5 nucleic acids (siNAs) and compositions comprising payload molecules such as a siNAs in
combination with natural or synthetic variants of Human Serum Albumin (HSA) as well
as the uses of such payload molecules and compositions as medicaments.

Background of the invention

10 Concentrations of drug in blood are dependent on the route and interval of
administration, organ clearance, tissue degradation and non-specific accumulation.

Maintenance of drug concentration within the therapeutic range is restricted by rapid
clearance by conventional administration.

15

Effective drug delivery should address one or both of: 1) circulatory half-life control and
2) specific tissue targeting, with an aim to have a zero order drug release profile
(Figure 1) in the tissue of interest. This has led to the development of drug delivery
technologies to improve the efficacy of existing drugs and enable the use of new
20 therapeutics such as peptides and oligonucleotides.

Drug delivery carrier systems incorporating the therapeutic can be composed of
polymers that include poly (lactide-co-glycolide) (PLGA), chitosan, polyethyleneimine
(PEI), lipids and fatty acids. These systems can control the rate of drug release, reduce
25 renal clearance and have the possibility for targeted delivery.

These systems can be referred to as nanoparticles due to size and spherical nature.

Nanoparticles can be targeted to tissues by passive or active targeting.

30

Passive accumulation by the Enhanced Permeability and Retention Effect (EPR-Effect)
has been utilized for delivery to tumours.

Proteins greater than 40-50kDa accumulate in the tumour, due to the leaky vasculature and lack of lymphatic drainage, and function as a source of nutrients.

Synthetic lipid and polymer-based particles have been shown to accumulate in tumours
5 by transport across disrupted endothelium, which has resulted in marketed systems such as Doxil[®] and DaunoXome[®] for the delivery of anticancer agents.

Active targeting can be achieved by incorporation of a targeting moiety to the drug or carrier system. Common targeting molecules are: antibodies and antibody fragments,
10 proteins, peptides, carbohydrates and aptamers.

The addition of targeting moieties can lead to non-specific accumulation or clearance if the targeting molecule is recognized as foreign, and can, therefore, compromise the effect of the carrier system.

15

Targeting can also be achieved by triggered release, meaning that the therapeutic molecule is encapsulated in a liposome, polymer, gel or particle, and released from the carrier in the presence of certain stimuli. The release trigger can be internal *e.g.* driven by pH or enzymes, or external *e.g.* application of heat or magnetism to the site of
20 release.

This results in an increased concentration of the drug in the desired tissue.

Nanoparticles reduce renal clearance because of their size, and can, therefore, reduce
25 the clearance of the incorporated therapeutic.

The particles can, however, be recognized by the Mononuclear Phagocyte System (MPS) composed of circulatory monocytes and fixed tissue macrophages that capture foreign material.

30

This can lead to accumulation in liver and spleen, and subsequent degradation or clearance, and results in short circulatory half-life of the carrier system.

Nanoparticles can be functionalized with hydrophilic polymers such as poly ethylene glycol (PEG) to install "stealth" characteristics.

35

Surface PEG results in a highly hydrated surface, that reduces phagocyte recognition and capture.

Studies have shown, however, an increased immune response after repeated

5 administration of PEG coated particles that may result in an accumulation in liver and spleen over time. There is, therefore, a necessity to investigate alternative methods for improving circulatory half-life of delivery systems.

Despite the properties of synthetic carrier systems, issues of MPS capture and poor

10 targeting restrict the therapeutic application, particularly for the delivery of therapeutics that require site-specific and intracellular delivery, including short interfering nucleic acids (siNAs).

It is difficult to combine properties such as targeting and stealth in synthetic carrier

15 systems, because the addition of targeting moieties can compromise the stealth properties and biocompatibility of the carrier.

An attractive alternative is the application of albumin as a drug carrier. Albumin is a

20 natural carrier with prolonged half-life, which avoids renal clearance and accumulates in tumours.

The protein is biocompatible and biodegradable and, therefore, an attractive candidate for a drug carrier.

25 The successful delivery of modified short interfering nucleic acids (siNAs) with albumin as carrier molecule has until now not been reported.

Summary of the invention

An object of the present invention relates to a composition comprising a payload of one

30 or more (several) molecules such as a short interfering nucleic acid (siNA) alone or in combination with albumin or variant of albumin or fragment thereof, or fusion polypeptide comprising albumin or variant albumin or fragment thereof.

Another object of the present invention relates to a payload of one or more (several)

35 molecules such as a siNA conjugated with one or more cholesteryl and/or one or more fatty acids.

A further object of the present invention relates to a composition of the one or more (several) payload molecules of the present invention for use as a medicament.

- 5 Yet another object of the present invention relates to one or more (several) payload molecules such as a siNA or the composition of the present invention for use in regulating a genetic expression of a transcript or protein associated with a disease.

- A further object of the present invention relates to a method of treating a disease
10 comprising administration of the of one or more (several) payload molecules such as a siNA or the composition of the present invention to a person in need thereof.

- In one embodiment of the present invention the siNA comprises a sense strand and an antisense strand, and wherein said sense strand is conjugated with said one or more
15 cholesteryl and/or one or more fatty acid.

- In another embodiment of the present invention the siNA comprises a sense strand and an antisense strand, and wherein said antisense strand is conjugated with said one or more cholesteryl and/or one or more fatty acid.

20

In a further embodiment of the present invention the of one or more (several) payload molecules such as a siNA comprises at least one terminal nucleic acid conjugation to said one or more cholesteryl and/or one or more fatty acid.

- 25 In yet another embodiment of the present invention the of one or more (several) payload molecules such as a siNA comprises at least two terminal nucleic acid conjugations to said one or more cholesteryl and/or one or more fatty acid.

- In another embodiment of the present invention the siNA comprises at least one mid-
30 sequence conjugation to said one or more cholesteryl and/or one or more fatty acid.

In another embodiment of the present invention the siNA comprises at least two mid-sequence conjugations to said one or more cholesteryl and/or one or more fatty acid.

In another embodiment of the present invention the siNA comprises a combination of at least one mid-sequence conjugation and at least one terminal nucleic acid conjugation to said one or more cholesteryl and/or one or more fatty acid.

- 5 In a further embodiment of the present invention is this fatty acid derived from a carboxylic acid selected from the group consisting of saturated monocarboxylic acids of length c10-18, being decanoic acid, dodecanoic acid, tetradecanoic acid, hexadecanoic acid and octadecoanoic acid.
- 10 In another embodiment of the present invention has the siNA a nucleic acid length selected from the group consisting of, 8-250, 8-120 , 16-50, 18-35, 18-25, 18-21, 18, 21, and 27.

In a further embodiment of the present invention the siNA is a Dicer substrate.

15

In yet another embodiment of the present invention the of one or more (several) payload molecules such as a siNA further has a linker between the cholesteryl and/or one or more fatty acid and the payload molecule.

- 20 In yet another embodiment of the present invention the linker is selected from the group consisting of disulfide moiety, amide, phosphate, phosphate ester, phosphoramidate, polyethylene glycol (PEG), disulphide linker or thiophosphate ester linkers.
- 25 In yet another embodiment of the present invention the payload molecule comprises further modifications selected from the group consisting of a dye label, a radioactive label, PET label, OMe, and drugs such as short peptides or proteins.

In yet another embodiment of the present invention the albumin is wild-type Human

- 30 Serum Albumin (wtHSA), SEQ ID No. 2 or a Human Serum Albumin (HSA) with at least 70% sequence identity to SEQ ID No. 2.

In another embodiment of the present invention, albumin or variant albumin or fragment thereof, or fusion polypeptide comprising albumin or variant albumin or

- 35 fragment thereof has a binding affinity to FcRn, and/or serum half-life, which is altered relative to a reference albumin, e.g. parent albumin, such as HSA (SEQ ID No: 2) or

fragment thereof or to a parent fusion polypeptide, e.g. comprising wild-type albumin or a fragment thereof. The binding affinity of the albumin to FcRn (and/or serum half-life) may be stronger (longer) or weaker (shorter) than that of the reference albumin. For example, the binding affinity to FcRn may be at least 2, 5, 10, 20, 30, 40, 50, 100, 500 or 1000 fold stronger than that of the reference albumin to FcRn. Alternatively, the binding affinity to FcRn may be at most 50%, 20%, 10%, 5%, 4%, 3%, 2%, 1%, 0.2, 0.1 % of the binding affinity of the reference albumin to FcRn. Binding affinity may be measured according to the Surface Plasmon Resonance (SPR) procedure described in WO 2011/051489, incorporated herein by reference in its entirety.

10

In yet another embodiment of the present invention, the albumin is albumin or variant albumin or fragment thereof, or fusion polypeptide comprising albumin or variant albumin or fragment thereof comprising an alteration at one or more (several) positions corresponding to positions 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 of the mature polypeptide of SEQ ID NO: 2. Preferably, the variant is not the variant consisting of SEQ ID NO: 2 with the substitution H464A, D494N, E501K, E503K, E505K, H510A, H535A, H536A, K536E, I537N, K541E, D550G, A, K573E, K574N or K584E.

20

Corresponding positions in other albumins may be identified by aligning the other albumin(s) with SEQ ID NO:2 and identifying the equivalent (or corresponding) amino acid position. The skilled person knows suitable alignment algorithms. One suitable alignment algorithm is the Needleman-Wunsch algorithm as described herein.

25

In yet another embodiment of the present invention the albumin variant has an alteration at position 573 or 500 such as K573A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, Y or K500A, C, D, E, F, G, H, I, L, M, N, P, Q, R, S, T, V, W, Y, particularly K573P, K573Y, K573W, K500A, K500D or K500G.

30

In a further embodiment of the present invention the albumin variant has an alteration selected from the group consisting of K573P, K573Y, and K573W.

In a further embodiment of the present invention the albumin variant has a K573P alteration. In a further embodiment of the present invention the albumin variant has a K573W alteration.

35

In a further embodiment of the present invention the albumin variant has a K573Y alteration.

- 5 In a further preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of EP12191856.9, particularly the alterations which relate to the 'high binders' of SEQ ID NO: 123, SEQ ID NO: 113, SEQ ID NO: 111; more preferred SEQ ID NO: 3 (*i.e.* HSA with substitution K573P), 117, 124; even more preferred SEQ ID NO: 131, 110, 128, 134, 108; most preferred SEQ ID NO: 115 or SEQ
- 10 ID NO: 114. It is preferred that the alterations are in HSA (SEQ ID NO: 2), however since they may be at equivalent (corresponding) positions in other species of albumin. In a preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 123 of EP12191856.9.
- In a preferred embodiment, the albumin variant comprises alterations selected from or
- 15 corresponding to those of SEQ ID NO: 113 of EP12191856.9.
- In a preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 111 of EP12191856.9.
- In a more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 3 of EP12191856.9.
- 20 In a more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 117 of EP12191856.9.
- In a more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 124 of EP12191856.9.
- In an even more preferred embodiment, the albumin variant comprises alterations
- 25 selected from or corresponding to those of SEQ ID NO: 131 of EP12191856.9.
- In an even more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 110 of EP12191856.9.
- In an even more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 128 of EP12191856.9.
- 30 In an even more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 134 of EP12191856.9.
- In an even more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 108 of EP12191856.9.
- In a most preferred embodiment, the albumin variant comprises alterations selected
- 35 from or corresponding to those of SEQ ID NO: 115 of EP12191856.9.

In a most preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 114 of EP12191856.9.

5 An advantage of using a variant of albumin is that it allows the half-life of the albumin to be tuned up or down relative to the half-life of wild-type albumin. This is useful if, for example, an albumin with a longer or shorter half-life is desired. As described in WO 2011/124718, incorporated herein by reference in its entirety, albumin variants comprising K573P, Y or W have a stronger binding affinity to FcRn and therefore are understood to have a longer half-life than wild-type albumin. Conversely, albumin
10 variants comprising K500A, D or G have a weaker binding affinity to FcRn and therefore are understood to have a shorter half-life than wild-type albumin.

In a further embodiment of the present invention the payload molecule such as a siNA optionally in combination with albumin is a payload albumin conjugate such as a siNA
15 albumin conjugate.

In a further embodiment of the present invention the payload optionally in combination with albumin are associated through electrostatic forces, hydrophobic forces or hydrogen bonds.
20

In a further embodiment of the present invention the composition comprises one or more further therapeutic molecules.

In a further embodiment of the present invention the composition comprises one or
25 more excipients, diluents and/or carriers.

In a further embodiment of the present invention the composition is a pharmaceutical composition.

30 A further embodiment of the present invention relates to a payload molecule modified by two or more cholesteryls.

Brief description of the figures

Figure 1. Drug concentrations in plasma depend on the mode of administration. The
35 therapeutic effect of the drug is present when the concentration is within the therapeutic range. When the concentration enters the toxic range, the incidence of

adverse effects is increased. Zero order release allows prolonged concentrations within the therapeutic range without reaching the toxic range. The drug concentrations represent arbitrary numbers.

5 Figure 2. Passive accumulation of radioactively labeled rat serum albumin in ovarian tumours of varying size (A, B) and W-256 carcinoma of the hind leg (C) 72 hours after administration.

Figure 3. The 3-dimensional structure of human serum albumin. The subdomains of the
10 protein are colored in gray scale. Figure was made in PyMol from the Protein Data Bank entry 1AO6.

Figure 4. The secondary structure of human serum albumin. Helical structures are represented as boxes, and numbered according to the subunit location. The figure
15 illustrates the homologous domains and the presence of characteristic double disulfide bridges in each subdomain.

Figure 5. The structure of albumin with warfarin (dark gray) and diazepam (light gray). The location of these ligands represents the location of Sudlows Site I (warfarin) and
20 Site II (diazepam). The ligands are enlarged to emphasize their location. Only the backbone of the protein is shown. The image was made in PyMol from the Protein Data Bank entries 2BXF (diazepam) and 2BXF (warfarin).

Figure 6. The figure shows the 10 binding sites for decanoic acid and 7 for stearic acid
25 revealed by x-ray diffraction studies. The numeration of binding sites is also done according to this publication. Only the backbone of the protein is represented for transparency. The sites 6 and 6' facilitate one decanoic acid molecule each, but one stearic acid together. The image was made in PyMol from the Protein Data Bank entry 1E7E (decanoic acid) and 1E7I (stearic acid).

30

Figure 7. The distribution of albumin between plasma (IV) and extravascular spaces (EV), and the rates of synthesis, exchange and loss. TER: Transcapillary Escape Rate.

Figure 8. Schematic representation of the FcRn recycling pathway, which is highly
35 important for maintaining the high physiological levels and long half-life of albumin. As albumin enters the early endosomes the acidic environment facilitates binding between

albumin and FcRn. Unbound albumin is destined for lysosomal degradation, whereas FcRn bound albumin is returned to the cell membrane, and released to the bloodstream. In this way most albumin avoids lysosomal degradation. FcRn can also facilitate transcytosis of albumin to the extravascular space. Pathway selection is not
5 known.

Figure 9. Plasma insulin levels after administration. Due to interaction with albumin, insulin Detemir exhibits prolonged circulation compared to regular insulin. Half-life of other insulin types is controlled by other means.

10

Figure 10. Illustration of the tumour and cell uptake of Abraxane[®] paclitaxel protein-bound particles for injectable suspension) (albumin-bound). The albumin bound drug complex is transcytosed into the tumour interstitium by the gp60 receptor, where it is bound and internalized by the SPARC protein, overexpressed by the tumour.

15

Figure 11. The INNO-206 prodrug, consisting of a Cys-34 binding maleimide linker, and pH sensitive N-N bond and the Doxorubicin drug. The low pH of solid tumours facilitates the release of free Doxorubicin.

20 Figure 12. Schematic representation of the albumin fusion protein Albinterferon-alpha-2b. With an appropriate linker both protein fold properly, and the interferon benefits from the long circulatory half-life of albumin. Image created from Protein Data Base entries 1E7E and 1ITF.

25 Figure 13. Schematic representation of a siRNA molecule. The double stranded part of 21-23 residues is flanked by 2 residue overhangs at each 3' terminal.

RNAi is thought to have evolved as a defense mechanism against foreign RNA such as viruses. An alternate microRNA (miRNA) pathway, however, exists that regulates cellular processes by expression of endogenous miRNA. These miRNA enter the RNAi
30 pathway using Dicer and RISC, and control mRNA levels by enzymatic cleavage or translational repression. It is possible to study cellular functional genomics by the use of synthetic RNA.

Figure 14. Examples of chemical modifications in an siRNA backbone, A, and in single
35 nucleotides, B-E. The modifications can decrease serum degradation (A, B, C) or

immune response (D), and improve the gene silencing efficiency (E). Modifications can be inserted in discrete positions in the siRNA, and therefore combined.

Figure 15. Representation of the functionalized siRNA designs in this project. Circle
5 palmitoyl, square: cholesteryl. These representations will be used throughout the text. All the siRNA described in this figure are of the same length and sequence.

Figure 16. Structures of the hydrophobically modified nucleotides; A: Cholesteryl
modification, with a triethylene glycol linker between cholesteryl and phosphate. The
10 bend in the molecule does not represent the actual structure, instead it allows the structure to be visualized on one page. B: Palmitoyl modification. The palmitoyl is conjugated to a LNA-thymidine, and the cholesteryl to an unmodified cytosine.

Figure 17. Mobility shift assay binding experiment carried out with single stranded RNA
15 7 to 14. First well of each design contains RNA alone, the second well contains RNA and rAlb. All experiments were done using rAlb. siRNA:rAlb ratio was 1:8. All wells contained 2pmol siRNA.

Figure 18. Mobility shift binding experiments carried out with siRNA 15-26. Well 1
20 contained siRNA alone, well 2 contained siRNA and rAlb. All experiments were done using rAlb. siRNA:rAlb ratios were 1:8. All wells contained 2pmol siRNA.

Figure 19. A) Assessment of the double bands appearing in the binding experiments.
siRNA 24, compared to its components: ssRNA 8 and 10, next to a 25BP Ladder. B)
25 siRNA 18 before (left well) and after adjustment of annealing concentrations. C) siRNA 20 after annealing (left) and after four freeze-thaw cycles. The amount of single stranded material has increased.

Figure 20. Comparison of the binding capacity of rAlb and sAlb. All wells contained
30 2pmol siRNA 26, and the amount of albumin indicated below the gel.

Figure 21. Results of the competition mobility shift experiment. The following siRNA
were used: A: 23; B: 24; C:25 and D:26. The first well contained no albumin, the
remaining contained albumin and siRNA. Identities of the competing ligands are given
35 above the figure.

Figure 22. Schematic representation of an ITC instrument. The reference heater supplies a constant power to the reference cell. In equilibrium the sample heater supplies similar power to ensure $\Delta T=0$ between the cells. The sample heater power is measured throughout the experiment. When sample is injected into the chamber, heat is released, thereby changing the power required to keep $\Delta T=0$. This change in power is recorded and corresponds to the energy consumed or released by the reaction.

Figure 23. Theoretical isotherms and the dependence on the value c . The optimal range is 10-100, acceptable is 5-500.

10

Figure 24. Binding curve for a representative Albumin-Octanoate experiment. Sample cell albumin concentration = 10 μM , syringe octanoate concentration = 100 μM .

Figure 25. ITC single injection experiments determining the enthalpy of the siRNA-Albumin interaction.

Figure 26. The raw data from a representative ITC binding experiment with siRNA 26 and albumin. The curve represents the baseline reference power. Each downward peak is an injection of siRNA 26 into albumin. Sample cell albumin concentration = 5 μM , syringe siRNA concentration = 50 μM .

Figure 27. ITC curve for the binding of siRNA 26 to albumin, calculated from the raw data shown in Figure 25.

Figure 28. Western blot assessment of albumin antibody efficiency. Lane 1 contained 10 ng rAlb, lane 2 100 ng rAlb. Blot was stained with A) Alexa 488 conjugated secondary antibody, or B) HRP conjugated secondary antibody. The HRP blot was overstained in this case, but clearly shows the presence of albumin.

Figure 29. Confocal microscopy of HepG2 cells (A and B) stained with Hoechst, (dark) and albumin antibodies (bright). Reference experiment using OK cells (C) and the same albumin staining procedure revealed no unspecific staining.

No albumin was added to the cells, therefore, staining represents endogenous albumin produced in by HepG2 cells.

35

Figure 30. Cellular albumin uptake in OK cells. A) No albumin, followed by antibody staining B) FITC albumin and C) rAlb followed by antibody staining. Dark: Hoechst, medium: Rhodamine-Phalloidin, light: Albumin. Uptake carried out for four hours using 1 μ M albumin.

5

Figure 31. Cellular albumin uptake in Caco-2 monolayer. A) No Albumin B) FITC rAlb C + D) rAlb stained with antibodies. Dark: Hoechst, Medium: Rhodamine, Bright: Albumin. Uptake carried out for 24 hours using 1 μ M albumin.

10 Figure 32. Three-dimensional image of cellular albumin uptake in Caco-2 monolayer. The protrusion is approximately 30 μ m high. Dark: Hoechst, Medium: Rhodamine, Bright: Albumin. Uptake carried out for 24 hours using 1 μ M albumin.

Figure 33. Evaluation of siRNA knockdown efficiency. Experiments were four hour
15 transfections with siRNA concentrations of 50nM. All values have been normalized to non-treated samples. Untr: Untreated, Mism: Mismatch. The experiments were carried out in triplicate, using standard protocol.

Figure 34. Optimization of albumin transfection using siRNA 19. Samples were
20 normalized to an untreated sample, and siRNA transfected with TransIT-TKO was included as a control.

Figure 35. 50nM of siRNA was added without (bright) or with (dark) 200nM albumin to cells using the transfection conditions described above.

25

Figure 36: Quantified fluorescence from serum, measured in terminal blood samples at 24, 72 and 120 hours after injection.

Figure 37: Fluorescence in serum samples from blood samples taken at 4, 24 and 48
30 hours after injection.

Figure 38: Total signal from the collected organs at 24 hours after injection.

Figure 39: Total signal from the remaining carcass of the animals at 24, 72 and 120
35 hours after injection. Note the logarithmic axis.

Figure 40: Blood fluorescence measured at a) 0-24 hours and b) 32-72 hours. The data have been normalized. n=2.

Figure 41: Internal organs scanned at 24 hours after injection. n=2.

5

Figure 42: Peripheral tissue fluorescence at 24 hours, normalized to fluorescence per gram of tissue.

Figure 43: Internal organs scanned at T72. n=2.

10

Figure 44: Peripheral tissue fluorescence at 72 hours, normalized to fluorescence per gram of tissue. n=2.

Figure 45: Albumin is detected in rat large intestine after 24 hours. A) control – rat injected with PBS, 20x; B) rat injected with 650 µg of low binding albumin, 20x; C) rat injected with 650 µg of high binding albumin; D) enlarged area from C; arrows indicate accumulated albumin (gray); dark gray-DAPI (nucleus), light gray-ATTO 488-phalloidin (actin); gray – Alexa 680 conjugated albumin. The color coding is done in gray scale.

15

20 Figure 46: Albumin is detected in rat small intestine after 24 hours. A) control – rat injected with PBS, 40x, water; B) rat injected with 650 µg of low binding albumin, 20x; C) rat injected with 650 µg of high binding albumin; D) enlarged area from C; arrows indicate accumulated albumin (gray); dark gray-DAPI (nucleus), light gray-ATTO 488-phalloidin (actin); gray – Alexa 680 conjugated albumin. The color coding is done in
25 gray scale.

Figure 47: HB albumin is detected in rat small intestine after 24 hours. A) Small intestine lumen with visible villas and lamina muscularis, 40x water immersion; B) small intestine lumen with visible villas, 20x, zoomed square shows HB albumin (gray)
30 ; C) orthogonal presentation of zoomed area from B). dark gray-DAPI (nucleus), light gray-ATTO 488-phalloidin (actin); gray – Alexa 680 albumin. The color coding is done in gray scale.

Figure 48: Animals were divided into 3 groups injected with siRNA 56, 57 and 58 shown
35 in the table. The square is cholesteryl, the star is Cy7.

Figure 49: Blood fluorescence following intravenous injection.

Figure 50: Scan of the organs from the 3 groups, A) animals dosed with siRNA 56, B) siRNA 57 and C) siRNA 58. Left animal in each caption is the PBS control. The three
5 captions are depicted on the same intensity scale.

Figure 51: IVIS scans of live animals A) 0.5 hours after injection, from left PBS, LB ("Low Binder"), WT (Wild-Type), HB ("High Binder") B) 2 hours after injection, from left LB, WT, HB C) 4 hours after injection, from left PBS, LB, WT, HB D) 24 hours after
10 injection, from left PBS, LB, WT, HB. The data are represented after spectral unmixing, and represent Alexa 680 signal only. The images are provided at different scales.

Figure 52: Organs at 1 hour and 2 hours after injection, from the left animals dosed with PBS, LB, WT, HB.
15

Figure 53: Fluorescence in the organs. After the successful unmixing, no signal is detected in the PBS animal.

Figure 54: Unmixed Alexa 680 signal. From top, the organs are: Stomach, upper small
20 intestine, lower small intestine, cecum and colon.

Figure 55: The table shows the siRNA used in Phase II, containing various combinations of siRNA modification. Squares indicate cholesteryl modification.

25 Figure 56: Results of binding studies for the single stranded siRNA 30 and 31, and double stranded siRNA 33, 34, 35 and 36, with estimated Kd values. Squares indicate cholesteryl modification.

Figure 57: Schematic representation of the LNA cholesteryl oligoes. Site of modification
30 by cholesteryl is indicated. Squares indicate cholesteryl modification.

Figure 58: Binding of LNA oligoes to albumin.

Figure 59: schematic representations of Cy7 labeled oligoes.

Figure 60: Albumin-siRNA complex formation. Increasing the number of modifications increased the amount of dimers and trimers compared to monomers.

Figure 61: Dimer formation with single stranded siRNA. Left lane: one cholesteryl, right 5 lane: two cholesteryl.

Figure 62: siRNA knockdown efficiency. siRNA 18 is non-modified.

Figure 63: Binding of siRNA 34 to albumin from different species. All the binding gels 10 have been run where the siRNA amount is constant and the albumin amount decreases from right to left. The more clear bands present in the top left, the higher the affinity. Due to the solubility issues with the cholesteryl siRNA, the free siRNA is less visible in the gel than the albumin-bound. Different albumin types are shown.

15 Figure 64: Assessment of binding of cholesteryl siRNA to albumin variants.

Figure 65: Competition study with three albumin variants LB, WT and HB and siRNA 34.

Figure 66: Serum degradation experiment. Top pane: Non-cholesteryl siRNA, bottom 20 pane: 3X cholesteryl siRNA. The arrows show the following: 1) Albumin-siRNA complexes 2) Free siRNA 3) Degraded siRNA. The circle shows the albumin-siRNA complex.

Figure 67: The serum degradation was using the LNA modified siRNA 52, 53 and 54, to 25 determine whether the siRNA: albumin complex stays together over time when incubated in serum. The arrows show the following: 1) Albumin-siRNA complexes 2) Free siRNA 3) Degraded siRNA. The circle shows the albumin-siRNA complex

Figure 68: TNF- α response towards siRNA either with or without albumin. A) Non-LNA 30 modified siRNA, from left siRNA 18 (0 cholesteryl), 34 (1 cholesteryl), 36 (2 cholesteryls) and 35 (3 cholesteryl) B) LNA modified siRNA, from left siRNA 52 (0 cholesteryl), 54 (1 cholesteryl), 53 (2 cholesteryls)

Figure 69: Examples of gel assessment of annealing. The arrows indicate the annealing 35 method.

Figure 70: Gel of albumin annealing. A) Cy7 Fluorescence and B) SYBR Gold stain

Figure 71: Competition experiment to compare behaviour of siRNA 34 annealed

with/without albumin. siRNA 57 was used in this experiment. A) Cy7 scan and B) SYBR

5 Gold stain of siRNA 57 annealed with albumin, C) Cy7 and D) SYBR-Gold stain of siRNA 57 annealed without albumin. The following ligands were added to the wells: 1) no ligand 2) stearic acid and warfarin 3) sodium octanoate 4) fusidic acid 5) sodium myristate 6) lithocholic acid 7) cholesterol 8) myristic acid 9) stearic acid

10 Figure 72: A stearic acid titration was carried out on siRNA 57 annealed with/without albumin . A) Cy7 scan and B) SYBR Gold stain of siRNA 57 annealed with albumin, C) Cy7 and D) SYBR-Gold stain of siRNA 57 annealed without albumin. The amount of stearic acid is in each well: 1) 0 2) 2048 pmol 3) 1024 pmol 4) 512 pmol 5) 256 pmol 6) 128 pmol 7) 64 pmol 8) 32 pmol 9) 16 pmol 10) 8 pmol 11) 4pmol

15

Figure 73: A competition experiment with siRNA 58 annealed with albumin was done.

The control experiment without albumin was not included because the siRNA 58 cannot be annealed efficiently without. A) Competition screen with the following ligands: 1) no ligand 2) stearic acid and warfarin 3) sodium octanoate 4) fusidic acid 5) sodium

20 myristate 6) lithocholic acid 7) cholesterol 8) myristic acid 9) stearic acid and B) Stearic acid titration with the following amount of stearic acid: 1) 0 2) 2048 pmol 3) 1024 pmol 4) 512 pmol 5) 256 pmol 6) 128 pmol 7) 64 pmol 8) 32 pmol 9) 16 pmol 10) 8 pmol 11) 4pmol. Both panes show Cy7 fluorescence

25 Figure 74: Serum degradation of siRNA 58. The numbers below indicate hours of incubation in serum. The 0* is before addition of serum

Figure 75: Binding of siRNA 34 to A) Alexa 680 labeled LB albumin and B)Alexa 680 labeled HB albumin.

30

Figure 76: Schematic representation of SMCC coupling of siRNA and albumin. A) The oligo is activated by SMCC B) The SMCC activated oligo couples covalently to the -SH group of Cys34.

Figure 77: Covalent siRNA:albumin conjugates. The figure shows the same gel, where DNA is visualized by SYBR-Gold on the left, and protein is visualized on the right by Coomassie Blue staining.

5 The present invention will now be described in more detail in the following.

Detailed description of the invention

The present invention relates to one or more (several) payload molecules in particular small interfering nucleic acids (siNAs) or plasmids that have been modified and
10 compositions comprising such siNAs in combination with natural or synthetic variants of Human Serum Albumin (HSA).

A payload molecule can be any molecule that would be suitable for a composition comprising HSA.

15

A payload molecule can also be any molecule that is capable of being modified with cholesteryl and/or fatty acids.

An object of the present invention relates to composition comprising a payload molecule
20 that is less than 25 kDa in size and one or more (several) albumins or fragments thereof, or fusion polypeptides comprising albumin or fragments thereof, wherein the albumin is a wild-type albumin or a variant albumin such as a variant of a parent albumin.

25 The size of the payload molecule may also be less than 10 MDa, less than 5 MDa, less than 20 kDa or even less than 10 kDa.

The size of the payload molecule may also be more than 10 kDa, more than 100 kDa or more than 1 MDa.

30 The payload molecule can also be a plasmid DNA or a vector.

In one embodiment of the present invention, the payload molecule is selected from the group consisting of a small molecule, a synthetic small molecule, a peptide or a siNA.

35 Thus, one aspect of the present invention relates to a composition comprising a short interfering nucleic acid (siNA) and one or several albumins or fragments thereof, or

fusion polypeptides comprising albumin or fragments thereof, wherein the albumin is a wild-type albumin or a variant albumin such as a variant of a parent albumin.

Another aspect of the present invention relates to a composition comprising a payload
5 molecule and one or several albumins or fragments thereof, or fusion polypeptides comprising albumin or fragments thereof, wherein the albumin is a wild-type albumin or a variant albumin such as a variant of a parent albumin.

The small molecules of the present invention are selected from the group consisting of
10 peptides, small molecules, small synthetic molecules, plasmids, vectors and small nucleic acids.

The small molecule can, for example, also be a dye such as Cy7 as exemplified in the present examples.

15

In the fields of pharmacology and biochemistry, a small molecule is a low molecular weight (<1000 Daltons) organic compound that may serve as an enzyme substrate or regulator of biological processes, with a size in the order of 10^{-9} m.

20 In pharmacology, the term 'small molecule' is usually restricted to a molecule that binds to a biopolymer such as protein, nucleic acid, or polysaccharide and acts as an effector, altering the activity or function of the biopolymer. Small molecules may function across a variety of cell types and species (*e.g.* mice and humans) and their study can lead to the development of new therapeutic agents. Some can inhibit a
25 specific function of a multifunctional protein or disrupt protein-protein interactions.

Another aspect of the present invention relates to a composition comprising a peptide and one or several albumins or fragments thereof, or fusion polypeptides comprising albumin or fragments thereof, wherein the albumin is a wild-type albumin or a variant
30 albumin such as a variant of a parent albumin.

In one embodiment of the present invention the small molecule is a GLP-1 analogue or a GLP-2 analogue.

35 The peptide may be any peptide of interest that can be used as a payload for albumin facilitated delivery.

In one embodiment of the present invention the peptide is insulin, *e.g.* an insulin comprising an A and/or B chain, preferably comprising both an A chain and a B chain.

- 5 In another embodiment of the present invention the peptide is less than 300 kDa in size, such as less than 200 kDa in size.

In yet another embodiment of the present invention the peptide is less than 70 kDa in size.

10

In another embodiment of the present invention the peptide is more than 3 kDa in size, such as more than 5 kDa in size, such as more than 10 kDa in size, such as more than 20 kDa in size.

- 15 In another embodiment of the present invention is the peptide more than 10 kDa in size.

Another object of the present invention relates to the payload molecule such as modified siNAs described herein which is not fused or conjugated to albumin.

20 siNAs

The siNAs of the invention are single or double stranded RNA or DNA oligonucleotides capable of binding target RNA or DNA sequences, including endogenous regulatory sequences, thereby inhibiting gene expression.

- 25 The term "short interfering nucleic acid", "siNA", "short interfering RNA", "siRNA", "short interfering nucleic acid molecule", "short interfering oligonucleotide molecule", "chemically-modified short interfering nucleic acid molecule", "microRNA", "miRNA", "sisiRNA", "shRNA", "pre-miRNA", "antagomir", "aptamer", "pri-miRNA", "Fork siRNA", "ss-siRNA", "aiRNA", "Dumbell siRNA", "aiRNA", or "antisense oligonucleotide",
30 "blockmir", miRNA sponges as used herein refers to any nucleic acid molecule capable of inhibiting or down regulating gene expression or viral replication, for example by mediating RNA interference "RNAi" or gene silencing in a sequence-specific manner.

- Chemical modifications described can be applied to any siNA sequence and either
35 strand of the invention.

For example the siNA can be a double-stranded polynucleotide molecule comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide
5 sequence corresponding to the target nucleic acid sequence or a portion thereof.

The siNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (*i.e.* each strand comprises nucleotide sequence that is
10 complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure, for example wherein the double stranded region is about 19 base pairs); the antisense strand comprises a nucleotide sequence that is complementary to a nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense strand
15 comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof.

The siNA can be a polynucleotide with a hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises
20 nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof.

In one embodiment of the present invention the siNA is a short hairpin RNA (shRNA).

In another embodiment of the present invention the siNA is a siRNA composed of an intact antisense strand complemented with two shorter 10–12 nucleotide sense strands. This three-stranded construct is termed small internally segmented interfering RNA (sisiRNA).

The siNA can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to a nucleotide sequence in a target nucleic acid molecule or a portion thereof and the
35 sense region having nucleotide sequence corresponding to the target nucleic acid

sequence or a portion thereof, and wherein the circular polynucleotide can be processed either *in vivo* or *in vitro* to generate an active siNA molecule capable of mediating RNAi.

Examples of such siNAs are pre-miRNAs and miRNAs.

5

The siNA can also comprise a single stranded polynucleotide having nucleotide sequence complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof (for example, where such siNA molecule does not require the presence within the siNA molecule of nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof), wherein the single stranded polynucleotide can further comprise a terminal phosphate group, such as a 5'-phosphate or 5',3'-diphosphate.

In certain embodiments, the siNA molecules of the invention comprise a nucleotide sequence that is complementary to a nucleotide sequence of a target gene.

In another embodiment, the siNA molecules of the invention interact with a nucleotide sequence of a target gene in a manner that causes inhibition of expression of the target gene.

20

Examples of such siNAs are antagomirs or blockmirs.

An antagomir or blockmir is a small synthetic RNA or DNA that is perfectly complementary to the specific miRNA target with either mispairing at the cleavage site of Ago2 or some sort of base modification to inhibit Ago2 cleavage.

Usually, antagomirs have some sort of modification, such as 2' methoxy groups and phosphothioates, to make it more resistant to degradation.

It is unclear how antagomirization (the process by which an antagomir inhibits miRNA activity) operates, but it is believed to inhibit by irreversibly binding the miRNA.

Antagomirs are now used as a method to constitutively inhibit the activity of specific miRNAs.

35

Thus, the term siNA is meant to be equivalent to other terms used to describe nucleic acid molecules that are capable of mediating sequence specific RNAi, for example short interfering RNA (siRNA), double-stranded RNA (dsRNA), micro-RNA (miRNA), short hairpin RNA (shRNA), short interfering oligonucleotide, short interfering nucleic acid, short interfering modified oligonucleotide, chemically-modified siRNA, post-transcriptional gene silencing RNA (ptgsRNA), translational silencing, antagomirs, or others.

In a further embodiment of the present invention the siNA is a Dicer substrate.

A Dicer substrate refers to any siNA that is recognisable by Dicer and that can be processed by Dicer. Usual lengths are 27mers but longer substrate such as 40mers or 70mers could also work.

Thus is one embodiment of the present invention siNAs that are recognisable by Dicer with a length of 27 to 40, such as 40 to 70 nucleotides.

In addition, the term RNAi is meant to be equivalent to other terms used to describe sequence specific RNA interference, such as post transcriptional gene silencing, epigenetics, or antagomirization.

For example, siNA molecules of the invention can be used to epigenetically silence genes at both the post-transcriptional level or the pre-transcriptional level.

In a non-limiting example, epigenetic regulation of gene expression by siNA molecules of the invention can result from siNA mediated modification of chromatin structure to alter gene expression.

The term "nucleic acid molecule" as used herein, refers to a molecule having nucleotides.

The nucleic acid can be single, double, or multiple stranded and can comprise modified or unmodified nucleotides or non-nucleotides or various mixtures and combinations thereof.

By "inhibit" or "down-regulate" it is meant that the expression of the gene, or level of RNAs or equivalent RNAs encoding one or more protein subunits, or activity of one or more protein subunits, such as pathogenic protein, viral protein or cancer related protein subunit(s), is reduced below that observed in the absence of the compounds or
5 combination of compounds of the invention.

In one embodiment, inhibition or down-regulation with an enzymatic nucleic acid molecule preferably is below that level observed in the presence of an enzymatically inactive or attenuated molecule that is able to bind to the same site on the target RNA,
10 but is unable to cleave that RNA.

In another embodiment, inhibition or down-regulation with antisense oligonucleotides is preferably below such as 10, 20, 30, 40, 50, 60, 70, 80, 90 % or, 10, 1, 0.1, 0.01% below that level observed in the presence of, for example, an oligonucleotide with
15 scrambled sequence or with mismatches.

In another embodiment, inhibition or down-regulation of viral or oncogenic RNA, protein, or protein subunits with a compound of the instant invention is greater such as 10, 20, 30, 40, 50, 60, 70, 80, 90 % greater or 2, 5, 10, 25, 50, 100, 1000 times in the
20 presence of the compound than in its absence.

In one embodiment of the invention, siNAs of the invention are antisense oligonucleotides.

25 Antisense oligonucleotides encompass single-stranded DNA or RNA that is complementary to a portion of a specific RNA sequence, or alternatively the complementary gene sequence, and reduce or inhibit gene expression.

Non-limiting examples of antisense oligonucleotides include RNA sequences
30 complementary to an mRNA transcript, thereby forming an RNA duplex resulting in reduced levels of translation.

Alternatively, antisense oligonucleotides may encompass a DNA sequence complementary to an mRNA transcript, which hybridizes with the mRNA transcript and
35 serves as a substrate for RNaseH.

Ribozymes are another form of siNAs having potential as a therapeutic. Ribozymes are ribonucleic acids having catalytic activity that can specifically cleave other RNA molecules.

- 5 The siNAs of the invention may be derived from any number of sources, including genomic DNA, cDNA, mRNA, and synthetic oligonucleotides.

The siNAs of the present invention can have different lengths.

- 10 Thus, in another embodiment of the present invention has siNA has a nucleic acid length selected from the group consisting of 8-250, 8-120 , 16-50, 18-35, 18-25, 18-21, 18, 21, and 27.

Linkers

- 15 The payload molecules of the present invention such as siNAs of the present invention can be linked to further therapeutic molecules or protein such as albumin through linking groups.

The payload molecule may also be linked to further therapeutic molecules without any

- 20 link to albumin.

Thus, in another embodiment of the present invention the siNA further has a linker between the cholesteryl and/or one or more fatty acid and the siNA.

- 25 Linking groups of the invention are chemical moieties that link or conjugate reactive groups to siNAs.

The linking groups typically contain between four and twelve carbon atoms, saturated or unsaturated and optionally branched.

30

Linking groups include, but are not limited to, one or more alkyl groups such as methyl, ethyl, propyl, butyl, pentyl, hexyl, heptyl, octyl, nonyl, decyl, undecyl, dodecyl, alkoxy groups, alkenyl groups, alkynyl groups or amino group substituted by alkyl groups, cycloalkyl groups, polycyclic groups, aryl groups, polyaryl groups, substituted aryl

- 35 groups, heterocyclic groups, and substituted heterocyclic groups.

Linking groups may also comprise polyethoxy amino acids such as AEA ((2-amino)ethoxy acetic acid) or a preferred linking group AEEA ([2-(2-amino)ethoxy]] ethoxy acetic acid).

- 5 In one embodiment of the present invention the linking group is a "biodegradable nucleic acid linker molecule".

The term "biodegradable nucleic acid linker molecule" as used herein, refers to a nucleic acid molecule that is designed as a biodegradable linker to connect one molecule to
10 another molecule, for example, a biologically active molecule.

The stability of the biodegradable nucleic acid linker molecule can be modulated by using various combinations of ribonucleotides, deoxyribonucleotides, and chemically modified nucleotides, for example 2'-O-methyl, 2'-fluoro, 2'-amino, 2'-O-amino, 2'-O-
15 allyl, 2'-O-allyl, and other 2'-modified or base modified nucleotides. Such linkers can also be disulphide linkers and acid sensitive linkers.

The linker may also be selected from the group consisting of Poly (β -amino ester), diorthoester, vinyl ether, phosphoramidate, hydrazone, beta-thiopropionate, which are
20 pH sensitive linkages and peptides that are protease sensitive that are to the cleavable spacer parts.

The biodegradable nucleic acid linker molecule can be a dimer, trimer, tetramer or longer nucleic acid molecule, for example an oligonucleotide of about 2, 3, 4, 5, 6, 7, 8,
25 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 nucleotides in length, or can comprise a single nucleotide with a phosphorus based linkage, for example a phosphoramidate or phosphodiester linkage.

The biodegradable nucleic acid linker molecule can also comprise nucleic acid backbone,
30 nucleic acid sugar, or nucleic acid base modifications.

The term "biodegradable" as used herein, refers to degradation in a biological system, for example enzymatic degradation, reducing environment or chemical degradation.

Other linkers of interest include linkers that utilize external activation such as physical cleavage exemplified by magnetism or by dicer processing to cleave siNA or siRNA processing.

- 5 The linkers can be of any length, it can for example include 200 atoms, such as 100 atoms, such as 50 atoms, such as 20 atoms e.g. from 10, 20, 50, 100, 200 to 20, 50, 100, 200, 250.

- 10 In yet another embodiment of the present invention the linker may be selected from the group consisting of disulfide moiety, amide, phosphate, phosphate ester, phosphoramidate, polyethylene glycol (PEG), 2N-hydroxy-propyl-methacrylamide (HPMA), or thiophosphate ester linkers.

Fatty acids and cholesterol

- 15 An object of the present invention relates to a siNA conjugated with one or more cholesteryl and/or one or more fatty acids.

A fatty acid is a carboxylic acid with a long aliphatic tail (chain), which is either saturated or unsaturated.

20

For increased binding and solubility, the carboxylic acid is selected from the group of mono-, di- and poly-unsaturated carboxylic acids of length c16-20, being palmitoleic acid, oleic acid, linoleic acid, linolenic acid and arachidonic acid.

- 25 The two groups of carboxylic acids above contain a single, terminal carboxyl group, which is used as a reactive group for siRNA conjugation.

The carboxyl is therefore consumed in the reaction, resulting in an aliphatic chain modification of the siRNA.

30

For increased binding, the carboxylic acid may be selected from the group of dicarboxylic acids of length c10-18, being decanedioic acid, dodecanedioic acid, tetradecanedioic acid, hexadecanedioic acid and octadecanedioic acid.

- 35 This group of carboxylic acids contains 2 carboxyl groups, one in each terminal of the molecule.

Terminal may be the terminal nucleotide or the second nucleotide from the terminal nucleotide.

- 5 During the conjugation, one carboxyl group is consumed, but the other remains intact. This results in an aliphatic chain with a terminal carboxyl group, which can improve binding.

Cholesteryl refers the radical of cholesterol, formed by removal of the hydroxyl group
10 and is thus the cholesterol substituted to a target molecule.

The cholesteryl may be any variant of cholesteryl for example PEGylated cholesteryl or 2-hydroxypropyl)methacrylamide (HPMA) cholesteryl.

- 15 When the siNAs are siRNAs, the siNAs can be modified with two different types of single strand cholesteryl modifications; 3' modification or 3' within or mid sequence modification which are modification that are not 3. modifications.

These modifications will allow a non-modified antisense strand for improved knockdown
20 efficiency, and a cholesteryl modified single strand for improved albumin binding.

Also, with such siNA it is possible to anneal siNA with up to 3 cholesteryls or even more cholesteryls.

- 25 In an embodiment of the present invention the siNA is modified by at least two cholesteryls, such as from two to five cholesteryls.

In an embodiment of the present invention the siNA is modified by at least two cholesteryls.

30

In an embodiment of the present invention the siNA is modified by at least three cholesteryls.

In an embodiment of the present invention the siNA is modified by at least four cholesteryls.

35

In an embodiment of the present invention the siNA is modified by four cholesteryls.

In an embodiment of the present invention the siNA is modified by five cholesteryls.

In an embodiment of the present invention the siNA is modified by six cholesteryls.

5

Further combinations of cholesteryl and palmitoyl with the cholesteryl modification in the single strand are preferred.

Thus, in one embodiment of the present invention the siNA comprises a sense strand
10 and an antisense strand, and wherein said sense strand is conjugated with said one or more cholesteryl and/or one or more fatty acid.

In another embodiment of the present invention the siNA comprises a sense strand and an antisense strand, and wherein said antisense strand is conjugated with said one or
15 more cholesteryl and/or one or more fatty acid.

In a further embodiment of the present invention the siNA comprises at least one terminal nucleic acid conjugation to said one or more cholesteryl and/or one or more fatty acid.

20

In one embodiment of the present invention the terminal nucleic acid conjugation is a 3' conjugation.

In yet another embodiment of the present invention the siNA comprises at least two
25 terminal nucleic acid conjugations to said one or more cholesteryl and/or one or more fatty acid.

In another embodiment of the present invention the siNA comprises at least one mid-sequence conjugation to said one or more cholesteryl and/or one or more fatty acid.

30

In another embodiment of the present invention the siNA comprises at least two mid-sequence conjugations to said one or more cholesteryl and/or one or more fatty acid.

In another embodiment of the present invention the siNA comprises a combination of at
35 least one mid-sequence conjugation and at least one terminal nucleic acid conjugation to said one or more cholesteryl and/or one or more fatty acid.

A mid-sequence or within-sequence conjugation is defined as a conjugation that is non-terminal *i.e.* modification of any nucleic acid other than the terminal nucleic acid.

- 5 Preferably a mid-sequence conjugation is located at least 20 nucleic acids, such as 10 nucleic acids, such as 5 nucleic acids, such as 3 nucleic acids from the terminal nucleic acid.

10 In one embodiment of the present invention the mid-sequence conjugation is located in the first 50 % of the siNA, such as the first 40 %, such as the first 30 %, such as the first 20 %, such as the first 10 %, wherein "the first" is defined as the 5' end of the nucleic acid.

15 In one embodiment of the present invention the mid-sequence conjugation is located in the last 50 % of the siNA, such as the last 40 %, such as the last 30 %, such as the last 20 %, such as the last 10 %, wherein "the first" is defined as the 5' end of the nucleic acid.

20 Preferably, if two or more mid-sequence conjugations are present, a mid-sequence conjugation is located at from 1 to 50 nucleic acids such as 1 to 20 nucleic acids, such as 1 to 10 nucleic acids such as 10 to 20 nucleic acids such as 10 to 20 nucleic acids such as 5 to 15 nucleic acids, from another mid-sequence conjugation.

25 In the present context the terms "conjugation" and "modification" are used interchangeably.

In a further embodiment of the present invention, the term "one or more" includes an integer selected from the group consisting of 1, 2, 3, 4, 5, 6 or at least one, such as at least one two, such as at least three.

30

In a further embodiment of the present invention are the number of cholesteryl modifications less than four.

35 The payload molecules of the present invention can also be modified by at least one cholesteryl, such as from one to six cholesteryls.

In one embodiment of the present invention the payload molecule of the present invention is modified by at least a single cholesteryl.

In one embodiment of the present invention the payload molecule of the present invention is modified by at least two cholesteryls.

In one embodiment of the present invention the payload molecule of the present invention is modified by at least three cholesteryls.

10 In one embodiment of the present invention the payload molecule of the present invention is modified by at least four cholesteryls.

In one embodiment of the present invention the payload molecule of the present invention is modified by at least five cholesteryls.

15

The peptides of the present invention can similarly be modified by cholesteryls.

In one embodiment of the present invention the peptide of the present invention is modified by at least two cholesteryls.

20

In one embodiment of the present invention the peptide of the present invention is modified by at least three cholesteryls.

In one embodiment of the present invention the peptide of the present invention is modified by at least four cholesteryls.

25

In one embodiment of the present invention the peptide of the present invention is modified by two cholesteryls.

30 In one embodiment of the present invention the peptide of the present invention is modified by three cholesteryls.

In one embodiment of the present invention the peptide of the present invention is modified by four cholesteryls.

35

Modifications

The present invention describes in certain embodiments short interfering nucleic acids that do not require the presence of nucleotides having a 2'-hydroxy group for mediating RNAi and as such, short interfering nucleic acid molecules of the invention optionally do not include any ribonucleotides (*e.g.*, nucleotides having a 2'-OH group).

Such siNA molecules that do not require the presence of ribonucleotides within the siNA molecule to support RNAi can however have an attached linker or linkers or other attached or associated groups, moieties, or chains containing one or more nucleotides with 2'-OH groups. Optionally, siNA molecules can comprise ribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions. The siNA molecules can also comprise deoxyribonucleotides at about 5, 10, 20, 30, 40, or 50% of the nucleotide positions.

Chemically synthesizing nucleic acid molecules with modifications (base, sugar and/or phosphate) that prevent their degradation by serum ribonucleases can increase their potency.

Modifications which enhance their efficacy in cells, and removal of bases from nucleic acid molecules to shorten oligonucleotide synthesis times and reduce chemical requirements are desired.

In another aspect the nucleic acid molecules comprise a 5' and/or a 3'-cap structure.

In another embodiment the 3'-cap includes, for example 4',5'-methylene nucleotide; 1-(beta-D-erythrofuransyl) nucleotide; 4'-thio nucleotide, carbocyclic nucleotide; 5'-amino-alkyl phosphate; 1,3-diamino-2-propyl phosphate, 3-aminopropyl phosphate; 6-aminoethyl phosphate; 1,2-aminododecyl phosphate; hydroxypropyl phosphate; 1,5-anhydrohexitol nucleotide; L-nucleotide; alpha-nucleotide; modified base nucleotide; phosphorodithioate; threo-pentofuransyl nucleotide; acyclic 3',4'-seco nucleotide; 3,4-dihydroxybutyl nucleotide; 3,5-dihydroxypentyl nucleotide, 5'-5'-inverted nucleotide moiety; 5'-5'-inverted abasic moiety; 5'-phosphoramidate; 5'-phosphorothioate; 1,4-butanediol phosphate; 5'-amino; bridging and/or non-bridging 5'-phosphoramidate, phosphorothioate and/or phosphorodithioate, bridging or non bridging methylphosphonate and 5'-mercapto moieties.

In another embodiment of the invention the siNAs are designed to contain locked nucleic acids (LNAs), phosphorothiorates (PS), TINAs and/or unlocked nucleic acids (UNAs).

- 5 SiNAs containing LNAs may have, among other attributes, improved affinity for complementary sequences and increased melting temperatures (hereinafter "T_m").

As used herein, siNA molecules need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

10

In certain embodiments, the short interfering nucleic acid molecules of the invention lack 2'-hydroxy (2'-OH) containing nucleotides.

- 15 In yet another embodiment of the present invention the siNA comprises further modifications selected from the group consisting of a dye label, a radioactive label for example for PET, SPECT, optimal imaging or MRI, OMe, and drugs such as short peptides or proteins.

- 20 PET radionucleotide agents can be such as ¹¹C, ¹³N, ¹⁵O, ¹⁸F, rubidium-⁸² and Fludeoxyglucose (FDG)

SPECT tracers can be such as ¹³¹I, ⁶⁷Ga, ¹¹¹In, ¹²³I, ^{99m}Tc.

- 25 Optical imaging tracers can be such as Quantum dots and fluorophores.

MRI paramagnetic and superparamagnetic particles and Iron and Gadolinium as also possible modifications.

- 30 Modifications may be anywhere in the molecule, for example in a 5' overhang, a 3' overhang or within the molecule at the positions described above in connection with mid-sequence modifications.

Modifications in a 3' overhang are preferred.

- 35 Particularly preferred modifications are addition of one or more (several) such as at least 1, 2, 3, 4, or 6 palmitoyl modifications.

Human serum albumin (HSA)

The payload molecules such as the siNAs, the peptides and the small molecules according to the present invention are particularly useful when combined with natural
5 or synthetic variants of an albumin such as a serum albumin, in particular Human Serum Albumin (HSA).

Albumins are proteins and constitute the most abundant protein in plasma in mammals and albumins from a long number of mammals have been characterized by biochemical
10 methods and/or by sequence information. Several albumins, e.g., human serum albumin (HSA), have also been characterized crystallographically and the structure determined.

HSA is a preferred albumin according to the invention and is a protein consisting of 585
15 amino acid residues and has a molecular weight of 67 kDa. In its natural form it is not glycosylated.

The amino acid sequence of HSA is shown in SEQ ID NO: 2. The skilled person will appreciate that natural alleles may exist having essentially the same properties as HSA
20 but having one or more amino acid changes compared to SEQ ID NO: 2, and the inventors also contemplate the use of such natural alleles as parent albumin according to the invention.

Albumins have generally a long plasma half-life of approximately 20 days or longer,
25 e.g., HSA has a plasma half-life of 19 days. It is known that the long plasma half-life of HSA is mediated *via* interaction with its receptor FcRn, however, an understanding or knowledge of the exact mechanism behind the long half-life of HSA is not essential for the present invention.

30 According to the invention the term "albumin" means a protein having the same, or very similar three dimensional structure as HSA and having a long plasma half-life. As examples of albumin proteins according to the invention can be mentioned human serum albumin, primate serum albumin, (such as chimpanzee serum albumin, gorilla serum albumin), rodent serum albumin (such as hamster serum albumin, guinea pig
35 serum albumin, mouse albumin and rat serum albumin), bovine serum albumin, equine serum albumin, donkey serum albumin, rabbit serum albumin, goat serum albumin,

sheep serum albumin, dog serum albumin, chicken serum albumin and pig serum albumin. HSA as disclosed in SEQ ID NO: 2 or any naturally occurring allele thereof, is the preferred albumin according to the invention.

- 5 The parent albumin, a fragment thereof, or albumin part of a fusion polypeptide comprising albumin or a fragment thereof according to the invention has generally a sequence identity to the sequence of HSA shown in SEQ ID NO: 2 of at least 60%, preferably at least 70%, preferably at least 80%, preferably at least 85%, preferably at least 86%, preferably at least 87%, preferably at least 88%, preferably at least 89%,
10 preferably at least 90%, preferably at least 91%, preferably at least 92%, preferably at least 93%, preferably at least 94%, preferably at least 95%, more preferred at least 96%, more preferred at least 97%, more preferred at least 98% and most preferred at least 99%.
- 15 The relatedness between two amino acid sequences or between two nucleotide sequences is described by the parameter "sequence identity".

For purposes of the present invention, the degree of sequence identity between two amino acid sequences is determined using the Needleman-Wunsch algorithm

- 20 (Needleman and Wunsch, 1970, J. Mol. Biol. 48: 443-453) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice et al., 2000, Trends Genet. 16: 276-277), preferably version 3.0.0 or later.
- 25 The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EBLOSUM62 (EMBOSS version of BLOSUM62) substitution matrix. The output of Needle labelled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:
(Identical Residues x 100)/(Length of Alignment – Total Number of Gaps in Alignment).

30

- For purposes of the present invention, the degree of sequence identity between two deoxyribonucleotide sequences is determined using the Needleman-Wunsch algorithm (Needleman and Wunsch, 1970, supra) as implemented in the Needle program of the EMBOSS package (EMBOSS: The European Molecular Biology Open Software Suite, Rice
35 et al., 2000, supra), preferably version 3.0.0 or later. The optional parameters used are gap open penalty of 10, gap extension penalty of 0.5, and the EDNAFULL (EMBOSS

version of NCBI NUC4.4) substitution matrix. The output of Needle labeled "longest identity" (obtained using the -nobrief option) is used as the percent identity and is calculated as follows:

$$\frac{(\text{Identical Deoxyribonucleotides} \times 100)}{(\text{Length of Alignment} - \text{Total Number of Gaps in Alignment})}$$

Thus, variants with 1 point mutation have 99.8% ID to wtHSA, 2 point mutations = 99.6%, 3 point mutations = 99.4%.

- 10 In another embodiment of the present the albumin variant is a HSA with a sequence identity to SEQ ID No. 2 of at least 70%, such as at least 75, such as at least 80, such as at least 85, such as at least 90, such as at least 95, such as at least 96, such as at least 97, such as at least 98, such as at least 99, such as at least 99.2, such as at least 99.4, such as at least 99.6, such as at least 99.8, such as 100% to wtHSA (SEQ ID No. 15 2).

The parent preferably comprises or consists of the amino acid sequence of SEQ ID NO: 2. In another aspect, the parent comprises or consists of the mature polypeptide of SEQ ID NO: 2.

20

In another embodiment, the parent is an allelic variant of the mature polypeptide of SEQ ID NO: 2.

- 25 In one embodiment of the present invention, the albumin is wild-type albumin or variant albumin or fragment thereof, or fusion polypeptides comprising wild-type albumin or variant albumin or fragment thereof.. A variant albumin may comprise an alteration at one or more (several) positions corresponding to positions 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 of an 30 albumin such as the mature polypeptide of SEQ ID NO: 2. It is preferred that the variant does not consist of SEQ ID NO: 2 with the substitution H464A, D494N, E501K, E503K, E505K, H510A, H535A, H536A, K536E, I537N, K541E, D550G,A, K573E, K574N or K584E.

In yet another embodiment of the present invention the albumin variant has an alteration at position 573 or 500, particularly K573P, K573Y, K573W, K500A, K500D or K500G.

- 5 In a further embodiment of the present invention the albumin variant has an alteration selected from the group consisting of K573P, K573Y, and K573W.

In a further embodiment of the present invention the albumin variant has a K573P alteration.

10

In a further embodiment of the present invention the albumin variant has a K573Y alteration.

- 15 In a further embodiment of the present invention the albumin variant has a K573W alteration.

In a further embodiment of the present invention the albumin variant has an alteration selected from the group consisting of K500A, K500D or K500G.

- 20 In a further embodiment of the present invention the albumin variant has a K500A alteration.

In a further embodiment of the present invention the albumin variant has a K500D alteration.

25

In a further embodiment of the present invention the albumin variant has a K500G alteration.

- 30 In another embodiment of the present invention, albumin or variant albumin or fragment thereof, or fusion polypeptide comprising albumin or variant albumin or fragment thereof has a binding affinity to FcRn, and/or serum half-life, which is altered relative to a reference albumin, *e.g.* parent albumin, such as HSA (SEQ ID No: 2) or fragment thereof or to a parent fusion polypeptide, *e.g.* comprising wild-type albumin or a fragment thereof. The binding affinity of the albumin to FcRn (and/or serum half-
35 life) may be stronger (longer) or weaker (shorter) than that of the reference albumin. For example, the binding affinity to FcRn may be at least 2, 5, 10, 20, 30, 40, 50, 100,

500 or 1000 fold stronger than that of the reference albumin to FcRn. Alternatively, the binding affinity to FcRn may be at most 50%, 20%, 10%, 5%, 4%, 3%, 2%, 1%, 0.2, 0.1 % of the binding affinity of the reference albumin to FcRn. Binding affinity may be measured according to the Surface Plasmon Resonance (SPR) procedure described in

5 WO 2011/051489, incorporated herein by reference in its entirety.

In a further preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of EP12191856.9, particularly the alterations of SEQ ID NO: 123, SEQ ID NO: 113, SEQ ID NO: 111; more preferred SEQ ID NO: 3 (*i.e.* HSA
10 with substitution K573P) , 117, 124 ; even more preferred SEQ ID NO: 131, 110, 128, 134, 108 ; most preferred SEQ ID NO: 115 or SEQ ID NO: 114. It is preferred that the alterations are in HSA (SEQ ID NO: 2), however they may be at equivalent (corresponding) positions in other species of albumin.

15 In a preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 123 of EP12191856.9.

In a preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 113 of EP12191856.9.

20

In a preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 111 of EP12191856.9.

25 In a more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 3 of EP12191856.9.

In a more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 117 of EP12191856.9.

30 In a more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 124 of EP12191856.9.

In an even more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 131 of EP12191856.9.

35

In an even more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 110 of EP12191856.9.

In an even more preferred embodiment, the albumin variant comprises alterations
5 selected from or corresponding to those of SEQ ID NO: 128 of EP12191856.9.

In an even more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 134 of EP12191856.9.

10 In an even more preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 108 of EP12191856.9.

In a most preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 115 of EP12191856.9.

15

In a most preferred embodiment, the albumin variant comprises alterations selected from or corresponding to those of SEQ ID NO: 114 of EP12191856.9.

The term "fragment" means a polypeptide having one or more (several) amino acids
20 deleted from the amino and/or carboxyl terminus of an albumin and/or an internal region of albumin that has retained the ability to bind to FcRn. Fragments may consist of one uninterrupted sequence derived from HSA or may comprise two or more sequences derived from HSA.

25 The fragments according to the invention have a size of more than approximately 20 amino acid residues, preferably more than 30 amino acid residues, more preferred more than 40 amino acid residues, more preferred more than 50 amino acid residues, more preferred more than 75 amino acid residues, more preferred more than 100 amino acid residues, more preferred more than 200 amino acid residues, more
30 preferred more than 300 amino acid residues, even more preferred more than 400 amino acid residues and most preferred more than 500 amino acid residues.

Useful fragments of HSA are domains *e.g.* D1, D2, D3, D1+D2, D2+D3, D1+D3, D3+D3, D1+D3+D3, D1+D2+D3+D3. Fragments may or may not comprise one or
35 more of the amino acid sequence alterations described herein.

Such fragments and other variants as well as fusion variants are described in WO 2011/124718 which hereby is incorporated by reference in its entirety.

Relative binding affinity of a variant to FcRn (preferably human FcRn as described herein) may be calculated by comparing an "RU" value (RU: Response Units) generated for a reference albumin (preferably wild-type albumin, SEQ ID NO: 2) with an "RU" value generated for the variant. It is more preferred that relative binding affinity is calculated by comparing a "KD" value ($KD = K_d/K_a$, where KD = binding affinity K_d = dissociation and K_a = association) generated for reference albumin (preferably wild-type albumin, SEQ ID NO: 2) with a "KD" value generated for a variant.

A particularly preferred method is: SPR analyses were performed on a Biacore 3000 instrument (GE Healthcare). Immobilisation was carried out on CM5 chips coupled with shFcRn (GeneArt lot#1177525) using GE Healthcare amine coupling chemistry as per the manufacturer's instructions. Immobilised levels of shFcRn-HIS (shFcRn with a 6-His tail on the C-terminus of beta-2-microglobulin) were 1200 - 2500RU and achieved by injecting 20µg/mL shFcRn diluted using sodium acetate pH4.5 (G E Healthcare). Chip surface was left to stabilize with a constant flow (5µL/min) of running buffer - Di-basic/Mono-basic phosphate buffer pH5.5 at 25 °C overnight. After ligand stabilization, the chip surface was conditioned by injecting 5-12 x 45µL Di-basic/Mono-basic phosphate buffer at 30µL/min followed by HBS_EP (0.01 M HEPES, 0.15 M NaCl, 3mM EDTA, 0.005% surfactant P20) at pH 7.4 (GE Healthcare)) regeneration steps (12s) in between each injection. Surfaces were then checked for activity by injecting 3x45µL positive control at 30µL/min, followed by 12s regeneration pulse. Kinetic measurements were performed by injecting dilutions (100µM – 0.016µM) of HSA and HSA variants at 30µL/min over immobilised shFcRn, at 25°C. The reference cell value was then subtracted and Biaevaluation software 4.1 used to obtain kinetic data and confirm KD values.

The variants were wild-type albumin (SEQ ID NO: 2) and variant albumins. The variants were analysed by SPR to determine their binding response (RU) to shFcRn. Some variants were further characterized to determine KD values. The relative binding affinity of albumin variants compared to wild-type albumin was calculated by calculating the mean of duplicate measurements for each variant and for wild-type albumin (SEQ ID NO: 2).

An embodiment of the present invention relates to the compositions or payload molecules of the present invention that have an enhanced or weaker binding to the FcRn receptor.

- 5 The variants of albumin or fragments thereof according to the invention may also be fused with a non-albumin polypeptide fusion partner.

The term 'fusion' means a genetic fusion to a fusion partner.

- 10 The fusion partner may in principle be any polypeptide but generally it is preferred that the fusion partner is a polypeptide having therapeutic or diagnostic properties. Fusion polypeptides comprising albumin or fragments thereof are known in the art. It has been found that such fusion polypeptide comprising albumin or a fragment thereof and a fusion partner polypeptide have a longer plasma half-life compared to the unfused fusion partner polypeptide. According to the invention it is possible to alter the plasma half-life of the fusion polypeptides according to the invention compared to the corresponding fusion polypeptides of the prior art.

- One or more therapeutic polypeptides may be fused to the N-terminus, the C-terminus of albumin, inserted into a loop in the albumin structure or any combination thereof. It may or it may not comprise linker sequences separating the various components of the fusion polypeptide.

The term 'conjugation' means a chemical conjugation to a conjugation partner.

25

The term 'linked' covers both genetic fusions and chemical conjugations.

- "Allelic variant" means any of two or more alternative forms of a gene occupying the same chromosomal locus. Allelic variation arises naturally through mutation, and may result in polymorphism within populations. Gene mutations can be silent (no change in the encoded polypeptide) or may encode polypeptides having altered amino acid sequences.

- An allelic variant of a polypeptide is a polypeptide encoded by an allelic variant of a gene.

35

The alteration at one or more position may independently be selected among substitutions, insertions and deletions, where substitution are preferred.

In one embodiment of the present invention the albumin is a recombinant albumin.

5

The variants of albumin or fragments thereof according to the invention may be conjugated to a second molecule such as the siNAs of the present invention using techniques known within the art.

- 10 Said second molecule may comprise a diagnostic moiety, and in this embodiment the conjugate may be useful as a diagnostic tool such as in imaging; or the second molecule may be a therapeutic compound and in this embodiment the conjugate may be used for therapeutic purposes where the conjugate will have the therapeutic properties of the therapeutic compound as well as the long plasma half-life of the
- 15 albumin.

Conjugates of albumin and a therapeutic molecule are known in the art and it has been verified that such conjugates have long plasma half-life compared with the non-conjugated, free therapeutic molecule as such.

20

The conjugates may conveniently be linked *via* a free thiol group present on the surface of HSA (amino acid residue 34 of mature HSA, or corresponding amino acid residue in other species of albumin) using well known chemistry.

- 25 Thus, in a further embodiment of the present invention the payload molecule such as a siNA optionally in combination with albumin is a payload albumin conjugate such as a siNA albumin conjugate.

The variants of albumin or fragments thereof may further be used in form of

- 30 "associates". In this connection the term "associate" is intended to mean a compound comprising a variant of albumin or a fragment thereof and another compound bound or associated to the variant albumin or fragment thereof by non-covalent binding.

The payload molecule such as an siNA optionally in combination with albumin can be

- 35 non-covalently linked by ionic interactions, hydrogen bonding, van der waals interactions, hydrophobic interactions, and/or stacking interactions.

Thus means "conjugate" that the payload molecule can be in combination with another molecule in either covalent or non-covalent links.

- 5 Thus, in a further embodiment of the present invention the payload molecule such as a siNA optionally in combination with albumin is associated through electrostatic forces.

A further embodiment of the present invention relates to a composition comprising several albumins, such as two or more albumins that are linked using a linker as
10 described above and payload molecule such as a siNA of the present invention.

Preferably the albumin is a recombinant albumin such as from a fungus such as a yeast, preferably from *Pichia* or *Saccharomyces*, most preferably from *Saccharomyces cerevisiae*.

15

Preferably the albumin has a lower level of post-translational covalent modification, such as glycation, than serum derived albumin.

Preferably the albumin has, prior to conjugation or association with payload molecule
20 such as a siNA, low levels of covalently bound molecules to Cys34. For example, it is preferred that less than 30%, more preferably less than 25, 20, 15, 10, 5, 4, 3, 2, 1 % of the albumin carries a molecule covalently bound to Cys34.

The result of the conjugation of a payload molecule such as a small molecule, a peptide
25 or a siNA to one of the above mentioned albumins can result in dimer or trimer formation.

Examples of such dimers and trimers can be seen in the present examples.

- 30 There are several benefits from using the albumin i.e. in a conjugate or as a target for the payload molecule of the present invention. These benefits include increased stability and a reduced immune response as exemplified in the examples.

Thus, in one embodiment of the present invention the result of the conjugation is a
35 dimer.

In another embodiment of the present invention the result of the conjugation is a trimer.

"The payload molecules of the present invention have the unique property that they are able to form soluble conjugates with albumin. Specifically have the present inventors in the example "Albumin annealing of siRNA" shown that siRNAs with two cholesterols and a Cy7 dye are capable of annealing in the presence of albumin, see figures 71 and 72, whereas the molecule precipitates out due to hydrophobicity without albumin presence, resulting in poor annealing efficiency, see figures 69 and 70.

10

Thus one aspect of the present invention relates to a method for forming a conjugate comprising a highly hydrophobic payload molecule, for example the two cholesteryl and Cy7 modified siRNA described in the example, and an albumin, where the formulation with albumin increases the solubility and stability of the payload molecule."

15 A further aspect of the present invention relates to the use of an albumin as disclosed herein for the formation of a conjugate comprising the albumin and a payload molecule modified by at least two cholesterols.

Further therapeutic molecules

In a further embodiment of the present invention the composition comprises one or

20 more further therapeutic molecules.

Such molecules can be other payload molecules, siNAs, natural or synthetic proteins, small molecules, hormones or similar.

25 Such further therapeutics can be conjugated directly to the siNAs of the present invention and can also be included in the compositions of the present invention either by conjugation to a siNA or the albumin(s).

Excipients, diluents and carriers

30 Pharmaceutical compositions comprising the payload molecules such as siNAs such as alone or optionally in combination with albumin may be administered in a physiologically acceptable medium (*e.g.*, deionized water, phosphate buffered saline (PBS), saline, aqueous ethanol or other alcohol, plasma, proteinaceous solutions, mannitol, aqueous glucose, vegetable oil, or the like).

35

Thus a further embodiment of the present invention relates to a composition comprising siNAs alone or in combination with albumin which constitutes a pharmaceutical composition.

- 5 Buffers may also be included, particularly where the media are generally buffered at a pH in the range of about 5 to 10, where the buffer will generally range in concentration from about 50 to 250 mM salt, where the concentration of salt will generally range from about 5 to 500 mM, physiologically acceptable stabilizers, and the like.
- 10 The compounds may be lyophilized for convenient storage and transport.

Thus, in a further embodiment of the present invention the composition comprises one or more excipients, diluents and/or carriers.

- 15 Aqueous suspensions may contain the active materials in admixture with excipients suitable for the manufacture of aqueous suspensions.

- Such excipients include suspending agents, for example sodium carboxymethylcellulose, methylcellulose, hydropropyl-methylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia; dispersing or wetting agents can be a naturally-occurring phosphatide, for example, lecithin, or condensation products of an alkylene oxide with fatty acids, for example polyoxyethylene stearate, or condensation products of ethylene oxide with long chain aliphatic alcohols, for example heptadecaethyleneoxycetanol, or condensation products of ethylene oxide with partial esters derived from fatty acids and a hexitol such as polyoxyethylene sorbitol monooleate, or condensation products of ethylene oxide with partial esters derived from fatty acids and hexitol anhydrides, for example polyethylene sorbitan monooleate.
- 20
25

- The aqueous suspensions can also contain one or more preservatives, for example ethyl, or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents, and one or more sweetening agents, such as sucrose or saccharin.
- 30

- Oily suspensions can be formulated by suspending the active ingredients in a vegetable oil, for example arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin.
- 35

The oily suspensions can contain a thickening agent, for example beeswax, hard paraffin or cetyl alcohol. Sweetening agents and flavoring agents can be added to provide palatable oral preparations. These compositions can be preserved by the addition of an anti-oxidant such as ascorbic acid.

5

Dispersible powders and granules suitable for preparation of an aqueous suspension by the addition of water provide the active ingredient in admixture with a dispersing or wetting agent, suspending agent and one or more preservatives.

- 10 Suitable dispersing or wetting agents or suspending agents are exemplified by those already mentioned above. Additional excipients, for example sweetening, flavoring and coloring agents, can also be present.

Compositions of the invention can also be in the form of oil-in-water emulsions.

15

The oily phase can be a vegetable oil or a mineral oil or mixtures of these.

- Suitable emulsifying agents can be naturally-occurring gums, for example gum acacia or gum tragacanth, naturally-occurring phosphatides, for example soy bean, lecithin,
20 and esters or partial esters derived from fatty acids and hexitol, anhydrides, for example, sorbitan monooleate, and condensation products of the said partial esters with ethylene oxide, for example polyoxyethylene sorbitan monooleate. The emulsions can also contain sweetening and flavoring agents.

- 25 Compounds of the invention can be administered parenterally in a sterile medium.

The siNA alone or in combination with albumin, depending on the vehicle and concentration used, can either be suspended or dissolved in the vehicle.

- 30 Advantageously, adjuvants such as local anesthetics, preservatives and buffering agents can be dissolved in the vehicle.

Administration mode

- The payload molecules such as a siNAs, peptides, small molecules or compositions
35 comprising siNAs, peptides or small molecules optionally in combination with albumin

are generally administered parenterally, such as intravascularly (IV), intraarterially (IA), intramuscularly (IM), subcutaneously (SC), mucosally, orally or the like.

Administration may also be made by transfusion, or it may be mucosal, oral, nasal, rectal, transdermal or aerosol, where the nature of the conjugate allows for transfer to the vascular system.

Usually a single injection will be employed, although more than one injection may be used, if desired.

10

The payload molecule such as siNAs, peptides or small molecules or compositions comprising siNAs peptides or small molecules optionally in combination with albumin may be administered by any convenient means, including syringe, trocar, catheter, or the like.

15

The particular manner of administration will vary depending upon the concentration to be administered, whether a single bolus or continuous administration, or the like.

The administration can be intravascularly, where the site of introduction is not critical to this invention, preferably at a site where there is rapid blood flow, (e.g., intravenously, peripheral or central vein).

Preferably the route of administration is mucosal or oral.

25 Other administration routes may be useful, e.g. where the administration is coupled with slow release techniques or a protective matrix.

The intent is that the siNAs or compositions are effectively distributed in the blood, so as to be able to react with the mobile proteins.

30

Dosage levels of the order of from about 0.1 mg to about 140 mg per kilogram of body weight per day are useful in the treatment of the described conditions (about 0.5 mg to about 7 g per patient per day).

The amount of active ingredient that can be combined with the carrier materials to produce a single dosage form may vary depending upon the host treated and the particular mode of administration.

- 5 The concentration of the modified siNA for administration may vary, generally ranging from about 1 pg/ml to 100 mg/ml, pre-administration.

The total amount administered intravascularly will generally be in the range of about 0.1 mg to about 500 mg, more usually about 1 mg to about 250 mg.

10

The composition of the present invention may be formulated for the intended use.

Thus relates one embodiment of the present invention to a composition of the present invention that is formulated for oral or mucosal administration.

15

Medical use and treatment

The payload molecule such as siNAs, peptides or small molecules and compositions of the invention may be used to treat diseases in a mammal in which inhibition of gene expression of a particular gene is beneficial.

20

"Mammal" for purposes of treatment refers to any animal classified as a mammal, including humans, domestic and farm animals, and zoo, or sports, animals, such as dogs, horses, cats, cows, etc. Preferably, the mammal is human.

- 25 The diseases include, but are not limited to, cancer, autoimmune diseases, viral and bacterial infections, endocrine system disorders, neural disorders including central and peripheral nervous system disorders, cardiovascular disorders, pulmonary disorders, and reproductive system disorders.

- 30 Thus a further object of the present invention relates to a payload molecule such as a siNA, peptides or small molecules or the composition of the present invention for use as a medicament.

In one particular embodiment of the invention, the payload molecule such as siNAs,

- 35 peptides or small molecules and compositions of the invention are useful for the amelioration and/or treatment of cancers and other hyperproliferative disorders.

Cancer cells are usually characterized by aberrant expression of a gene.

Cancers and other hyperproliferative disorders for which this invention provides therapy
5 include, but are not limited to, neoplasms associated with connective and
musculoskeletal system tissues, such as fibrosarcoma, rhabdomyosarcoma,
myxosarcoma, chondro sarcoma, osteogenic sarcoma, chordoma, and liposarcoma,
neoplasms located in the abdomen, bone, brain, breast, colon, digestive system,
endocrine glands (adrenal, parathyroid, pituitary, testicles, ovary, thymus, thyroid),
10 eye, head and neck, liver, lymphatic system, nervous system (central and peripheral),
pancreas, pelvis, peritoneum, skin, soft tissue, spleen, thorax, and urogenital tract,
leukemias (including acute promyelocytic, acute lymphocytic leukemia, acute
myelocytic leukemia, myeloblastic, promyelocytic, myelomonocytic, monocytic,
erythroleukemia), lymphomas (including Hodgkins and non-Hodgkins lymphomas),
15 multiple myeloma, colon carcinoma, prostate cancer, lung cancer, small cell lung
carcinoma, bronchogenic carcinoma, testicular cancer, cervical cancer, ovarian cancer,
breast cancer, angiosarcoma, lymphangiosarcoma, endotheliosarcoma,
lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's sarcoma,
leiomyosarcoma, squamous cell carcinoma, basal cell carcinoma, pancreatic cancer,
20 renal cell carcinoma, Wilm's tumor, hepatoma, bile duct carcinoma, adenocarcinoma,
epithelial carcinoma, melanoma, sweat gland carcinoma, sebaceous gland carcinoma,
papillary carcinoma, papillary adenocarcinoma, glioma, astrocytoma, medulloblastoma,
craniopharyngioma, ependymoma, pinealoma, emangioblastoma, acoustic neuroma,
oligodendroglioma, meningioma, neuroblastoma, retinoblastoma, bladder carcinoma,
25 embryonal carcinoma, cystadenocarcinoma, medullary carcinoma, choriocarcinoma, and
seminoma.

Thus an aspect of the present invention relates to the use of a payload molecule such
as a siNA or siNA-albumin conjugate as described herein for treatment of diseases that
30 benefit from intestinal delivery *e.g.* cancer, inflammatory disease as described above.

Another aspect of the present invention relates to the use of a payload molecule such
as a siNA or siNA-albumin conjugate as described herein for intestinal delivery of a drug.

Yet another object of the present invention relates to payload molecule such as a siNA or the composition of the present invention for use as in regulating a genetic expression of a transcript or protein associated with a disease.

- 5 A further object of the present invention relates to a method of treating a disease comprising administration of the payload molecule such as a siNA or the composition of the present invention to a mammal in need thereof.

Examples

- 10 Poor control of the circulatory half-life and biodistribution limits the use of many therapeutic molecules, which are rapidly cleared or degraded after administration. Drug blood concentrations are dependent on the route and interval of administration, organ clearance, tissue degradation and non-specific accumulation. Maintenance of drug concentration within the therapeutic range (Figure 1) is restricted by rapid clearance by
15 conventional administration. Effective drug delivery should address: 1) circulatory half-life control and 2) specific tissue targeting, with an aim to have a zero order drug release profile (Figure 1) in the tissue of interest.

- This has led to the present invention of a drug delivery technology to improve the
20 efficacy of existing drugs and enable the use of new therapeutics such as peptides and oligonucleotides.

- Drug delivery carrier systems incorporating the therapeutic can be composed of polymers that include poly (lactide-co-glucolide) (PLGA), chitosan, polyethyleneimine
25 (PEI) or lipid-based. These systems can control the rate of drug release, reduce renal clearance and have the possibility for targeted delivery. They can be referred to as nanoparticles due to size and spherical nature.

- Nanoparticles can be targeted to tissues by passive or active targeting. Passive
30 accumulation by the Enhanced Permeability and Retention Effect (EPR-Effect) has been utilized for delivery to tumours. Proteins greater than 40-50kDa accumulate in the tumour, due to the leaky vasculature and lack of lymphatic drainage, and function as a source of nutrients. Synthetic lipid and polymer-based particles have been shown to accumulate in tumours by transport across disrupted endothelium, which has resulted
35 in marketed systems such as Doxil[®] and DaunoXome[®] for the delivery of anticancer agents.

Active targeting can be achieved by incorporation of a targeting moiety to the drug or carrier system. Common targeting molecules are: antibodies and antibody fragments, proteins, peptides, carbohydrates and aptamers. The addition of targeting moieties can lead to non-specific accumulation or clearance if the targeting molecule is recognized as foreign, and can therefore compromise the effect of the carrier system.

Targeting can also be achieved by triggered release, meaning that the therapeutic molecule is encapsulated in a liposome, polymer, gel or particle, and released from the carrier in the presence of certain stimuli. The release trigger can be internal; driven by pH or enzymes, or external; application of heat or magnetism to the site of release. This results in an increased concentration of the drug in the desired tissue.

Nanoparticles reduce renal clearance because of their size, and can therefore reduce the clearance of the incorporated therapeutic. The particles can, however, be recognized by the Mononuclear Phagocyte System (MPS) composed of circulatory monocytes and fixed tissue macrophages that capture foreign material. This can lead to accumulation in liver and spleen, and subsequent degradation or clearance, and results in short circulatory half-life of the carrier system.

Nanoparticles can be functionalized with hydrophilic polymers such as poly ethylene glycol (PEG) to install "stealth" characteristics. Surface PEG results in a highly hydrated surface, that reduces phagocyte recognition and capture. Studies have shown, however, an increased immune response after repeated administration of PEG coated particles, that may result in an accumulation in liver and spleen over time. There is, therefore, a necessity to investigate alternative methods for improving circulatory half-life of delivery systems.

Despite the properties of synthetic carrier systems, issues of MPS capture and poor targeting restrict the therapeutic application, particularly for the delivery of therapeutics that require site-specific and intracellular delivery, including small interfering RNA (siRNA). It is difficult to combine properties such as targeting and stealth in synthetic carrier systems, because the addition of targeting moieties can compromise the stealth properties and biocompatibility of the carrier. An attractive alternative is the application of albumin as a drug carrier. Albumin is a natural carrier with prolonged half-life, which avoids renal clearance and accumulates in tumours (Figure 2). The protein is

biocompatible and biodegradable and therefore an attractive candidate for a drug carrier.

Albumin

- 5 Human Serum Albumin (HSA) is emerging as a protein carrier in drug delivery. It is an important transport and depot protein in human plasma and extravascular spaces. HSA is synthesized in the liver and secreted into plasma, where it is responsible for about 80% of the osmotic. It is the most abundant plasma protein, with a concentration reaching 40 g/L or 0.6 mM.
- 10 It is a globular protein that consists of a single, non-glycosylated 585 amino-acid chain, and has a molecular weight of 67 kDa. The protein has 3 homologous domains, held together by a total of 17 disulfide bonds, with Cys-34 as the single free sulfhydryl. The binding and release of fatty acids and other cargos is facilitated by allosteric changes in the molecule, making the protein a very dynamic carrier. HSA has an extraordinary long circulatory half-life of 19-23 days due to its high stability, interaction with recycling receptors and rescue from renal clearance. The protein is present in extravascular tissues and organs in addition to plasma.
- 20 The properties of albumin make the protein an attractive candidate for drug delivery. Many conventional drugs interact with the binding domains of albumin with consequential changes in the drug bioavailability and circulatory half-life. The inherent biodistribution and trafficking properties of albumin also offer the possibility for cargo delivery to different tissues. The following sections will discuss the binding and biodistribution properties of albumin relevant for its application in drug delivery.

Ligand binding and allostery

- The transport and depot properties of albumin can be attributed to the unique binding capacity of the protein. The hydrophilic and charged surface of the protein makes it very soluble in water and hydrophobic binding pockets facilitate binding of hydrophobic ligands. By interaction with HSA, the solubility of ligands is increased, enabling circulation in solution. In this way HSA improves the solubility and transports natural ligands such as fatty acids, bile salts, steroids, metal ions and bilirubin.
- 35 In addition to natural ligands, HSA interacts with many known drugs such as Diazepam, Digitoxin, Warfarin, Cisplatin and Taxol. Dependent on the drug type, over 99 % of the

drug can be bound to serum albumin, thereby significantly lowering the amount of free drug in circulation. The next sections will describe the binding sites, which enable these interactions.

5 Subdomains

Albumin consists of 3 homologous domains; I, II and III (sometimes referred to as 1, 2 and 3), further divided into subdomains A and B (see Figure 3). These domains have predominantly helical structure, (67 % of entire protein) with a total of 17 disulfide bonds between helices (see Figure 4). The 3 homological domains are not only similar
10 in helical arrangement, but also in 3-dimensional structure. Due to the arrangement of the domains, however, albumin is a highly asymmetric protein. Domains I and II are perpendicular to each other, and have a large contact region with hydrophobic interactions between IIA and IA-IB interfaces. In contrast, domain III interacts only with IIB, and domains I and III are separated by a large hydrophobic cleft. Due to the
15 flexible intradomain connections the entire molecule is a very adaptable structure, adjusting and changing shape depending on surroundings.

Cysteines and disulfide bridges

The albumin molecule contains a total of 35 cysteines, 34 of which are involved in
20 formation of 17 disulfide bridges. Sixteen of these form characteristic double bridges, where two adjoining cysteines form disulfide bridges to two different helices. Due to this arrangement, each subdomain has a rigid and stable structure (Figure 4). The remaining Cys-34 is located on a loop in subdomain IA, close to the protein surface, but with the sulfur-atom pointing inward and somewhat sterically hindered from interaction
25 with large molecules. In the bloodstream 30-40% of HSA is oxidized by cysteine or glutathione. Cys-34 is a potent antioxidant, because it can form a sulfenic acid by accepting oxygen when subjected to oxidative pressure, and plays an important role in binding and trafficking of NO and SH-containing compounds.

30 Despite these interactions, Cys-34 is located some distance away from the hydrophobic pockets of albumin, and plays no role in binding of the most common ligands and drugs. This has made the cysteine a common target for covalent modifications of albumin for labeling, surface modification and other purposes.

35 Binding sites

Albumin interacts with many ligands *via* a panel of binding sites distributed over the protein surface. Compared to other proteins, albumin has high content of the ionic amino acids glutamic acid and lysine, resulting in ~185 ions per albumin molecule, and a net negative charge at pH 7 of -17. This makes the protein very soluble in water, and solutions up to 50% w/v can be prepared. Most of these hydrophilic residues are on the surface of the protein, and the core is generally hydrophobic. There are a number of clefts, cavities and pockets of different size and shape, through which hydrophobic ligands can interact with the hydrophobic core and bind.

Due to the effect of albumin on drug efficacy and fatty acid trafficking, ligand binding has been studied extensively. The two main drug binding sites have been described after a series of experiments with fluorescent probes.

These experiments showed that some of the used probes could displace each other, and resulted in the description of the two binding sites called Sudlow's site I and II. X-ray diffraction studies have revealed the location and architecture of these binding sites.

Sudlow's site I and II

Sudlow's site I is a pocket comprising all six helices of subdomain IIA, with the entrance to the pocket restricted by subdomain IIIA (Figure 5). The pocket is predominantly hydrophobic, with a few hydrophilic residues that further stabilize binding of ligands, and positive residues close to the protein surface. The best-known ligand for this site is warfarin, but many other ligands have been identified to bind to this site (see Table 1). Ligands of sizes smaller than 310 Da do not induce changes in the binding site, however, binding of larger ligands promotes dislocation of residues around the binding site.

Site II is similar to site I in structure, but smaller. It facilitates binding for many ligands (see Table 1), most notable Ibuprofen, but no drug ligands are shared with site I. Binding of Diazepam ($M = 285\text{Da}$) and larger ligands induces structural changes around this binding site. It is located in subdomain IIIA, and consists of a hydrophobic cavity comprised of all 6 helices of this subdomain. The difference between site I and II is mainly caused by the polar residues protruding into the sites, which vary between the two binding sites.

Together these sites are responsible for most interactions with small molecules.

However, there are several other hydrophobic clefts and cavities on albumin, facilitating binding of fatty acids and other larger molecules.

5 Table 1. Some of the known ligands of Sudlow's site I and II. N.D: Not determined.

Site I	$K_1(M^{-1})$	Site II	$K_1(M^{-1})$
Exogenous ligands			
Warfarin	3.4×10^5	Diazepam	3.8×10^5
Azapropazone	2.8×10^5	Ketoprofen	2.5×10^6
Phenylbutazone	1.5×10^6	Chlofibrate	7.6×10^5
Acenocoumarol	2.2×10^5	Iopanoate	6.7×10^6
Salicylate	1.9×10^5	Ibuprofen	2.7×10^6
Indomethacin	1.4×10^6	Pirprofen	3.9×10^5
Tolbutamide	4.0×10^4	S-Naproxen	3.7×10^6
Chlorpropamide	3.3×10^5	Ethacrynate	1.7×10^6
Iophenoxate	7.7×10^7	Chlorothiazide	5.5×10^4
Iodipamide	9.9×10^6	Diclofenac	3.3×10^6
Sulfadimethoxine	9.0×10^4	S(2)-Thiamylal	8.1×10^4
Sulfathiazole	2.5×10^4	S-Etodolac	2.0×10^5
Furosemide	1.9×10^4	Carprofen	$>10^6$
Tenoxicam	3.7×10^5	Suprofen	N.D.
R-Sulbenicillin	1.4×10^6	Benoxaprofen	N.D.
Valproate	4.0×10^4	Cicloprofen	N.D.
Piretanide	3.3×10^5	Azidodeoxythymidine	N.D.
Spironolactone	7.7×10^7	Norharmaline	N.D.
Canrenoate	9.9×10^6	Propofol	N.D.
Quercetin	9.0×10^4	Flurbiprofen	N.D.
Benzylthiouracil	4.1×10^4		
Oxyphenbutazone	3.5×10^5		
S-Carbenicillin	2.4×10^3		
R-Sulbenicillin	5.2×10^3		
Cosalane	N.D.		
Endogenous ligands			
Bilirubin	9.5×10^7	Octanoate	1.6×10^6
Eicosanoids	N.D.	L-Tryptophan	4.4×10^4
3-Carboxy-4-methyl-5-propyl-2-furanpropanoate	1.3×10^3	Monooleoyl glycerol	4.0×10^5
		L-Thyroxine	3.0×10^5
		Chloride ion	7.2×10^2
		Indol-3-acetate	2.1×10^5
		Indoxylsulfate	1.6×10^6
		Hippurate	1.0×10^4

Other binding sites

Under normal, physiological conditions, there are between 0.1-2 fatty acid (FA) molecules bound per albumin molecule, but there is rapid exchange of FAs between albumins and between different binding sites within the same albumin. FA affinity for albumin increases with chain length, from octanoate ($K \sim 10^5 \text{ M}^{-1}$) to stearate ($K \sim 7 \cdot 10^8 \text{ M}^{-1}$). Fatty acids as long as 28 carbon atoms have been shown to bind to albumin, but these interactions are less studied. Binding studies show there is one high affinity site for shorter FAs (octanoate, decanoate) and two for longer (laurate, myristate) and several sites with lower affinity (see Table 2).

- 10 Table 2. The affinities of fatty acids for the different fatty acid binding sites of albumin. This data is from binding experiments, and, therefore, does not represent an order of binding sites.

Fatty acid	K1 ($\text{M}^{-1} \cdot 10^6$)	K2 ($\text{M}^{-1} \cdot 10^6$)	K3 ($\text{M}^{-1} \cdot 10^6$)	K4 ($\text{M}^{-1} \cdot 10^6$)	K5 ($\text{M}^{-1} \cdot 10^6$)	K6 ($\text{M}^{-1} \cdot 10^6$)
Palmitate	145	130	71	12	8	3
Stearate	689	515	130	120	70	1
Oleate	118	118	80	55	23	6
Linoleate	135	82	18	11	10	1.5

X-ray diffraction studies of albumin conjugated to a surplus of fatty acid have revealed as many as ten binding sites for decanoic acid and seven for stearic acid (see Figure 6). Diffraction studies reveal all possible binding sites, but give a static picture of saturated binding, and thus no information about affinities of the different sites. Competition studies and binding experiments with recombinant albumins indicate that the high affinity site for short FAs is localized in subdomain IIIA. For the longer FA, the high affinity binding site varies, laurate binds in subunits IIIA and IIIB, whereas myristate binds in IB and IIIB. This indicates that even though each binding site can accept different FA, each binding site is specialized to facilitate strong binding to a particular FA type.

- 25 In close proximity of the FA binding site in subdomain IB, there is an additional binding pocket called the heme cleft. According to crystallographic data, this is the binding site for heme, bilirubin and fusidic acid, and is the only binding site for these ligands. Previous data from binding studies using albumin fragments, however, indicate that the binding site of bilirubin is in subdomain IIA, showing that data from different approaches not always correlates. Although the binding sites have been well studied binding information for many ligands is incomplete.

Allostery

The flexibility of the intradomain regions enables conformational changes to facilitate binding. The long loops between subdomains allow large movements of the regions relative to each other. Large structural changes occur in the protein during pH changes, where 5 different shapes have been observed. The Normal (N), heart shaped form of the protein is present at pH 4.3-8. The Fast (F) conformation below pH 4.3 and the Basic (B) above pH 8 show decreased helical content and some degree of denaturation. The B conformation increases the binding strength of warfarin, indicating that the structure of the binding site is only partially altered and not destroyed by this pH. At pH 10 above 10 or below 2.7 the protein is further denatured.

The binding of fatty acids induces conformational changes in the overall protein structure, as well as local changes within the binding sites. In this way, FA binding can regulate binding of ligands to other sites. One of the high affinity sites of myristate overlaps to some extent with Sudlows site I, and myristate regulates drug binding to this site both allosterically and competitively. Conversely, binding of heme to the heme site inhibits binding of drugs to site I.

X-ray diffraction data of bound ligands confirm changes in the conformation, and ligand co-binding studies indicate that the conditions surrounding the binding sites are also altered by these conformational changes. Most known ligands, which have been crystalized, show allosteric changes in the protein, indicating that the allostery is a sensitive and highly regulated system, which continuously modulates the binding properties of albumin.

It is a difficult task to compose a comprehensive description of ligand interactions and binding sites. Crystallographic data provide precise information about binding sites and their location, but the information is static and far from physiological conditions. In contrast, binding experiments such as equilibrium dialysis reveal thermodynamic data but no information about binding site location. This is further complicated by the allosteric properties of the protein, which make albumin a dynamic and very complex protein to study. Studies utilizing albumin fragments and recombinant albumins with altered binding sites have given much information, but many questions still remain unanswered.

Synthesis and biodistribution

Albumin is synthesized by the liver as a single peptide chain containing a signal peptide, a pro-peptide and the albumin itself. The signal peptide is cleaved off directly after translation, but ensures that the protein is transferred into the endoplasmatic reticulum (ER). The resulting protein, proalbumin, is transported *via* the Golgi

5 apparatus to secretory vesicles, where the 6 amino acid large propeptide is cleaved off before secretion of the mature albumin from the cell.

In a healthy adult, approximately 13.6 g albumin is synthesized and secreted daily.

Figure 7 shows the amount of albumin present in the bloodstream (IV) and in the
10 extravascular spaces (EV), and the steady state exchange rates between the compartments. The extravascular albumin is mainly localized in skin, muscle, liver, gut and in subcutaneous space. Albumin has a wide tissue distribution, and there is a dynamic exchange of protein between different organs and compartments

15 The mechanisms of transcapillary escape are less studied, however, it is clear that several mechanisms exist and contribute to the albumin biodistribution. In the liver and spleen, containing sinusoids with fenestrated endothelium, the increased vascular permeability accounts for most of the albumin escape to EV.

20 In organs with continuous endothelium the albumin transport is mediated by active, receptor-mediated transcytosis. Several putative albumin binding proteins have been identified, including SPARC, gp60, gp30 and gp18. SPARC (Secreted Protein Acidic and Rich in Cysteine), also known as osteonectin, is usually present in bone, promoting mineralization.

25

The role of SPARC in albumin transport in healthy tissue is unclear. The protein is, however, upregulated in many cancers, where it promotes uptake and degradation of albumin for nutrition. gp30 and gp18 have been associated with scavenger receptors, and have never been shown to transport albumin. Altered and distorted conformations
30 of albumin increase binding to these proteins compared to native albumin and it has, therefore, been proposed that they are responsible for degradation of non-functioning albumin, and not transcytosis.

gp60 is proposed as the main transport mediator for albumin where gp60 binds albumin
35 and subsequently binds to caveolin-1 to initiate invagination of the cell membrane. The consequent clustering of the gp60–albumin complex during vesicle formation reduces

receptor affinity for albumin, which permits the release of albumin and any bound ligands to the abluminal side of the cell (John *et al*, 2003, *Am. J. Physiol. Lung Cell Mol. Physiol.* 284:L187-L196. This protein is expressed in many different endothelial tissues, and blocking of the protein with antibodies lowers the albumin transport in
5 endothelial cell monolayers by 90 %.

The affinity of the albumin-gp60 interaction is regulated by allosteric changes in albumin. When albumin is saturated with fatty acids, the albumin transport across endothelium can be increased as much as 200 %. This indicates a preference for
10 transcytosis of albumin carrying cargo, and further improves the ability of albumin to transport fatty acids to tissues. The gp60 receptor has never been sequenced or crystallized, and little is known about the mechanism of binding and uptake. It is possible that other proteins or pathways are involved in the transport of albumin across endothelium.

15 Circulatory half-life

Albumin exhibits an extended half-life of ~20 days. This can be attributed to the stability of the protein and to two receptor-mediated pathways, which promote the rescue of the protein from renal excretion or degradation: 1) Reabsorption of albumin
20 by megalin-cubilin from renal proximal tubules and 2) recycling from lysosomal degradation by the neonatal Fc receptor (FcRn).

The rescue from renal clearance is primarily attributed to the megalin-cubilin protein complex. Megalin is a transmembrane transport protein expressed in the renal proximal
25 tubules. It is associated with the formation of coated pits and the internalization of materials. Cubilin interacts with megalin, and has binding sites for apolipoproteins, immunoglobulin light chain and albumin. Albumin filtered into the proximal tubuli binds to cubilin, is internalized and released back into the bloodstream *via* the megalin-mediated pathway, thereby avoiding urinal excretion.

The second pathway is the FcRn-mediated recycling pathway. FcRn is an intracellular receptor complex expressed in a wide range of tissues, including most endothelial cells. The complex plays a role in maternofetal protein transfer, and also protects
30 immunoglobulins from degradation. There is little interaction between the FcRn receptor and albumin at physiological pH, but the interaction is strongly pH dependent. The binding strength increased by two orders of magnitude when the pH is lowered to 6.

By fluid phase endocytosis and gp60 mediated uptake, albumin is brought into the cell. As the endosomes follow the pathway to lysosomal degradation the pH is lowered, and albumin affinity for the FcRn receptor increases. By binding to FcRn receptor the
5 albumin is rescued from the lysosomes degradation and returned to the cell membrane, where pH is physiological and the albumin is released. The albumin can be recycled to the apical side into the bloodstream, or released into the basolateral side in the extravascular space (Figure 8). The mechanisms determining which route is selected are not known.

10

Albumin in disease

In healthy adults, the albumin plasma levels are generally stable, and levels below 30 g/L (hypoalbuminemia) or above 55 g/L (hyperalbuminemia) are considered
15 pathological. Hyperalbuminemia is normally related to dehydration, but can also be linked to high blood pressure, high body weight and an unhealthy diet.

Hypoalbuminemia can result from lower synthesis rate, loss of albumin or a combination of both. It is often related to disease, and the albumin concentration is used as a diagnostic screening tool. The most severe cases, with levels below 20 g/L,
20 are caused by nephrosis and other diseases of kidney and gastrointestinal tract which cause extensive albumin loss. Less acute hypoalbuminemia can be related to hepatitis, rheumatoid arthritis and other infections, which lower the rate of albumin synthesis.

Hypoalbuminemia is a marker of many cancers, and some tumours utilize albumin as a
25 source of energy and nutrients. The albumin is accumulated in tumour tissue passively by the EPR effect and actively by binding to the SPARC protein receptor. In contrast to healthy tissue, the albumin is not recycled but internalized and degraded, resulting in albumin loss. The accumulation of albumin in tumours makes albumin an attractive drug carrier to target cancer.

30

In rare cases of analbuminemia, very little or no albumin is present. The condition is caused by mutations in the albumin gene. When a stop-codon is introduced, the resulting truncated protein does not exhibit the same circulatory or binding properties as native albumin and is, therefore, rapidly degraded. Despite the role of albumin in
35 trafficking and transport, analbuminemia is generally tolerated as other transport proteins are upregulated and take over the transport role of albumin.

Improved half-life and passive targeting of drugs with albumin

The long circulatory half-life of albumin can be utilized to improve the circulatory time of therapeutic molecules. By attachment of a drug cargo to albumin, the drug benefits from the same recycling and rescuing pathways as albumin, and the circulation time is prolonged. There are several approaches for cargo attachment:

- Hydrophobic interactions
- Micro- and nanosphere formulations
- Covalent coupling
- Albumin/therapeutic peptide co-expression as fusion polypeptides

The choice of approach depends on the mode of action of the therapeutic and target tissue. The hydrophobic interaction approach features reversible attachment facilitated by hydrophobic interactions with the albumin binding sites. The cargo can, therefore, be released into the free form over time or from binding competition by other endogenous ligands. The covalent coupling and co-expression approaches are less versatile due to irreversible attachment that may require albumin degradation for drug release. These covalent approaches can, therefore, only be used if the molecule can fulfill the therapeutic role whilst attached to albumin, or a release mechanism can be incorporated. Each of these approaches has benefits and drawbacks, which will be discussed in the following sections.

Hydrophobic interactions

If a drug is not a natural ligand of albumin, it may be functionalized with a compound that interacts reversibly with the hydrophobic binding sites of albumin. The most notable example is the insulin analog Detemir, produced by Novo Nordisk under the trade name Levemir. This insulin is functionalized with a myristic acid moiety protruding from the B chain of the insulin. Detemir is formulated and injected in the same manner as standard insulin, but it binds reversibly to subcutaneous albumin *via* the myristic acid after injection. As the binding is reversible, clearance of free insulin changes the equilibrium of binding and releases bound insulin. Consequently, bound insulin is slowly released into the free, active form, giving an extended period of activity (Figure 9).

In this approach, the ligand binds to endogenous albumin after injection, so there is no requirement for preformulation with albumin prior to injection. There is no overall depletion of albumin, since the drug dissociates over time and causes no albumin

degradation. This enables multiple administrations and high drug concentrations without compromising albumin concentrations, making the approach ideal for insulin.

Micro- and nanosphere formulations

- 5 Albumin micro- and nanoparticles can be prepared by covalent crosslinking or by ionic or hydrophobic interactions under appropriate conditions. Chemically cross-linked albumin microspheres (1-5 μ m) encapsulating a drug were extensively researched in the 1980's, but none were commercialized.
- 10 Current systems focus on smaller nanoparticles (50-200nm) produced without covalent cross-linking. Albumin particles containing 99Technetium or other radioactive compounds have been used for *in vivo* cancer detection. Albumin particle formulations have also been used for drug delivery. The most successful has been Nanoparticle Albumin Bound (NAB) Paclitaxel, sold under the name Abraxane[®]. Without chemical
- 15 crosslinking the anticancer drug Paclitaxel is emulsified under pressure with albumin to form particles ranging from 100-200nm (mean size is 130nm).

Paclitaxel in free form is water insoluble, but is soluble in this formulation without the necessity to include toxic excipients.

20

- In addition to improved half-life and solubility, Abraxane[®] is proposed to improve targeting to tumours. Endothelial binding and transcytosis of Abraxane[®] is higher than that of free paclitaxel, and this difference can be negated by inhibiting the gp60 albumin uptake pathway, indicating an albumin specificity. It is also suggested that the
- 25 particles accumulate in tumour tissue in a non-receptor mediated way by the EPR effect.

- Within the tumour interstitium, the albumin particles bind to the overexpressed SPARC protein, and are internalized into cells (Figure 10). It is not known whether the entire
- 30 particles are taken up, or the particles dissociate to albumin-paclitaxel conjugates before internalization, but it has been shown that the particles dissociate over time.

- The superior performance of Abraxane over other paclitaxel formulations is attributed to albumin properties. The NAB technology is proposed as a general anticancer drug
- 35 formulation method to lower adverse effects and improve efficiency. Despite the large

commercial success of Abraxane[®], however, no other NAB-based drugs have been commercialized.

Covalent coupling of drug

- 5 In this approach the therapeutic molecule is covalently coupled to albumin. One common method is conjugation through the SH group of Cys-34. Since there is only one unpaired cysteine, the conjugation product is homogeneous and reproducible. Cys-34 is not located in the direct proximity of any ligand binding site, nor does covalent attachment alter the binding capabilities of the important drug binding sites. Due to the
- 10 irreversibility of the covalent attachment, the drug is not readily released from the albumin requiring a cleavable spacer such as pH and enzyme degradable for drug release. A single prodrug of this type, INNO-206, has been through phase I clinical trials. This prodrug consists of a maleimide derived albumin binding domain, an acid sensitive linker and the anticancer-drug doxorubicin (Figure 11). The prodrug reduces
- 15 systemic adverse effects whilst increasing the concentration of the drug at the tumour site, where the pH sensitive bond is cleaved. The product is currently under phase II clinical evaluation.

Genetic fusions of albumin

- 20 This approach has been proposed for therapeutic proteins or peptides. The albumin and the therapeutic protein, or peptide, are expressed together as one single protein chain. If sufficient linker space is provided, the albumin is able to fold correctly, and thus retain its functions and long half-life. A notable example is the fusion protein Albinterferon- α -2b, which is a fusion protein of albumin and interferon- α -2b. Human
- 25 Genome Sciences developed this molecule for the treatment of hepatitis C. The fusion protein has a half-life of around 6 days, which is lower than that of native albumin, but substantially higher than that of interferon (2-5 hours).

- Initial phase III clinical studies proved successful in treating hepatitis, with
- 30 administration intervals as long as 4 weeks. It was found, however, that high doses of albinterferon can be associated with nausea and coughing, which resulted in termination of the clinical trials. There are no reports that the adverse effects were attributed to the albumin component and, therefore, in spite of the failure of Albinterferon, the fusion method of extending half-life is a very promising strategy.

35

Active Targeting

It has been reported that surface changes can influence the biodistribution of albumin, which is a promising method of target-specific delivery. Mannosylated albumin is rapidly cleared from circulation, due to recognition by the mannose receptors of Kupffer cells and hepatic endothelial cells, and up to 70 % of an injected dose accumulates in these cell types in two hours after injection.

In contrast, lactosylated albumin is accumulated in hepatocytes and albumin modified with cyclic peptides localizes to the stellate cells of the liver. It is, therefore, possible to modulate albumin delivery to certain cell types by surface modifications. However, modifications can result in the loss of albumin extended circulatory half-life and recycling capacity.

Even though these methods alter the properties of albumin, this approach offers the possibility for specific targeting of albumin to desired tissues. It is possible that more subtle modifications such as addition of targeting peptides can alter the biodistribution of albumin without compromising the attractive properties of the protein.

Albumin delivery of siRNA

Albumin is an attractive yet still fledgling drug carrier with huge potential. The interest for the application of albumin for drug delivery is steadily increasing, and improving methods of characterization and engineering make it possible to further improve and develop this potential.

Short interfering RNA (siRNA) is an emerging class of nucleic acid-based drug. Delivery, however, is key to the clinical translation of these drugs. The next section will outline the mechanism of action of siRNA and highlight delivery challenges to development of an albumin-based delivery solution.

RNA interference (RNAi)

RNA interference (RNAi) is a post-transcriptional gene silencing (PTGS) process mediated by double stranded RNA. Central to the RNAi pathway is the protein complex RISC (RNA-induced silencing complex), and the endonuclease Dicer. When double stranded RNA is present in the cytoplasm of a cell, it is recognized by Dicer and cleaved into 20-25 nucleotide fragments with 2 nucleotide overhangs on the 3' ends (see Figure 13). The RISC complex recognizes these siRNA fragments, and one strand, the guide strand or antisense strand (AS), is assembled into the RISC complex, leading

to degradation of the other strand, sense strand (SS), by the RISC component Argonaute 2 (Ago 2). After the degradation of the SS, the AS functions as a guide or template for recognition of mRNA targets by the RISC complex, by complementary base-pairing between the guide strand and the mRNA. After engagement into RISC the mRNA is degraded by Ago2, resulting in gene silencing.

Small interfering RNA (siRNA)

siRNA is a class of synthetic, double stranded RNAs of less than 30 base-pairs, which are able to enter the RNAi pathway. They can be designed as 27-mer Dicer substrates, that are recognized by Dicer and cleaved, or shorter 21 to 23-mers, which directly enter RISC.

Despite of the enormous potential of siRNA therapeutics, systemic delivery and cellular uptake pose a great challenge. Renal clearance, serum degradation and nonspecific accumulation are associated with systemic delivery of siRNA.

Naked, non-modified siRNA has a serum half-life of minutes. In addition, macromolecular size and polyanionic charge restrict cellular uptake, which is required for gene silencing.

20

The introduction of chemical modifications in siRNA (Figure 14) can be used to reduce nuclease degradation and control pharmacokinetics. A large variety of modifications, from structural changes in the RNA backbone or single nucleotides (Locked nucleic acid (LNA), phosphorothiorate (PS) or unlocked nucleic acid (UNA), Figure 14), to addition of sugars or cholesterol have been developed. Such modifications can be inserted in discrete positions in the siRNA, and many different combinations are possible.

Extensive modification, however, can lead to increased sterical hindrance that reduces the silencing efficiency. This can be overcome by introduction of UNA that exhibit less steric hindrance than native RNA. Chemical modifications can be introduced in discrete positions during synthesis, and combined to achieve desired properties. Chemical modifications can significantly increase the serum stability, but they cannot alone solve the problem of renal clearance and cellular delivery.

Different synthetic nanoparticle-based carrier systems for siRNA have been proposed, including nanoparticles functionalized with PEG. Some of these approaches have proven

successful; however, many show accumulation in liver, possibly as a consequence of MPS capture, which restricts the clinical application.

The inventors propose that albumin may act as an effective systemic carrier for siRNA due to its long circulatory half-life, renal clearance avoidance, and cellular uptake properties.

The inventors have developed albumin as a natural drug carrier for systemic delivery of drugs using siRNA as the model therapeutic molecule. The stepwise synthesis of siRNA enables a high degree of functionalization on the molecular level, meaning that siRNA can be modified and designed reproducibly and homogenously. The great therapeutic potential of siRNA makes it a relevant molecule to investigate. The inventors propose that the challenges of siRNA delivery; renal clearance, degradation and non-specific accumulation can be overcome with albumin.

The aim of this work is to utilize albumin for siRNA delivery. The work can be divided into two categories 1) development of albumin binding siRNA and 2) the investigation of albumin uptake and trafficking in cells.

1. Albumin binding siRNA

Different siRNA containing fatty acid and/or cholesterol modifications were designed. Binding of these modified siRNAs to albumin was investigated using gel mobility shift assay and isothermal titration calorimetry. The goal was modification of siRNA in such a way, that a desired albumin binding strength was obtained.

2. Albumin uptake and trafficking

Albumin uptake and trafficking in cells was investigated. Fluorescently labeled albumin in combination with confocal microscopy or flow cytometry was used to evaluate the uptake of the protein, and, therefore, the potential as a cellular drug delivery system.

This platform can also include labeled siRNA, to visualize both carrier and cargo.

Further experiments included the use of an EGFP expressing cell line for evaluation of the siRNA uptake and silencing efficiency.

This series of approaches and experiments was designed to determine the potential of albumin as a versatile, customizable drug carrier for siRNA. By investigating the protein-siRNA interaction as well as the behavior in cells of the protein, the inventors

have established albumin as a natural carrier system. The design rules established with siRNA forms the basis of a generic albumin delivery platform for a range of therapeutics.

5 siRNA designs towards albumin binding

In the initial phase of this project was functionalized siRNA for interaction with the ligand binding sites of albumin designed. Hydrophilic siRNA is not a natural ligand for albumin and was, therefore, functionalized with hydrophobic moieties. Two different hydrophobic modifications, palmitoyl and cholesteryl were used. The palmitoyl

10 functionalization targets the fatty acid binding sites of the albumin. Cholesterol itself does not bind to albumin, but cholesteryl modified siRNA has been reported to increase circulatory half-life, which can possibly be attributed to albumin interaction. In addition to determining the effect of a particular type of functionalization, the effect of the following was also studied:

- 15 - Varying position of the modification in the siRNA sequence
- Varying number of modifications per siRNA
-

For this purpose, the siRNA molecules presented in Figure 15 were designed.

20 All the siRNA molecules mentioned above contained the same sequence and length:

3'	U	C	T	5'	A	C	U	U	G	U	G	G	C	C	G	U	U	U	A	C	G	U	C	G	C	U	3'
					U	G	A	A	C	A	C	C	G	G	C	A	A	A	U	G	C	A	G	5'			

This sequence (SEQ ID NO: 3) has been shown to provide efficient knockdown of EGFP (Enhanced Green Fluorescent Protein) in cells expressing this protein.

Results and discussion

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This section is divided into 2 main parts:

Albumin-siRNA binding studies

Cellular albumin and albumin-siRNA studies

The first part includes characterization of albumin-siRNA interactions using mobility

30 shift assay and isothermal titration calorimetry (ITC). The second part includes cellular uptake of albumin and EGFP knockdown studies.

Each section will include an introduction to the methods used, results and a discussion.

Binding

The electrophoretic mobility shift assay and isothermal titration calorimetry were used to investigate and characterize the binding between siRNA and albumin.

Electrophoretic mobility shift assay

- 5 The electrophoretic mobility shift assay (EMSA) is a simple and sensitive method of separating proteins and nucleic acids by size or charge. EMSA was used as the primary technique to study the interactions between modified siRNA and albumin. The method was chosen because it allows experiments on native, non-labeled and non-modified protein and siRNA and requires small amounts of material.
- 10 The experiments were based on the difference in migration between siRNA and albumin in polyacrylamide gels, resulting in two distinct bands when there is no siRNA-albumin interaction. siRNA migration is retarded when siRNA binds to albumin, resulting in a significant shift in siRNA localization. By staining for siRNA in the gels, these experiments allow qualitative determination of siRNA-albumin binding (see materials
- 15 and methods for details).

Single stranded RNA binding

- To determine whether the range of siRNA designs could interact with albumin, a series of mobility shift binding experiments were carried out with ssRNAs 7-14. Single
- 20 stranded material was used to screen the different modifications prior to investigations with double stranded combinations. These experiments were performed using recombinant albumin (rAlb, Novozymes Biopharma). siRNA and albumin were mixed in 1:8 molar ratios and incubated for 30 min prior to gel loading.
- 25 The results clearly identify designs which successfully bind to albumin (see Figure 17). The non-modified SS RNA 7 showed no retardation of the RNA band and, therefore, suggests no interaction. The non-modified AS RNA 14 showed very weak interaction. This RNA is complementary in sequence to RNA 7, and the weak interaction can be caused by sequence specificity of this single stranded RNA. AS RNA 12 and SS RNA 13
- 30 contain a terminal 3' palmitoyl, and both interacted weakly with albumin. SS RNA 8, with the palmitoyl placed within the sequence of the RNA, showed no binding, indicating that this design can sterically hinder the interaction with albumin compared to terminal modifications. When the number of palmitoyl modifications was increased to two in the same strand (SS RNA 9), an almost complete displacement of siRNA was observed,
- 35 which suggests strong albumin binding.

Even stronger binding, with no free RNA present was observed for RNA 10, which contains 3' cholesteryl modification, indicating that this modification binds with high affinity to albumin. The results show that both palmitoyl and cholesteryl modifications can promote interaction with albumin. The cholesteryl exhibits the strongest interaction with full displacement, whereas the palmitoyl modification binds significantly weaker. The introduction of a second palmitoyl seemingly provides a synergistic effect, and binding is increased.

The second band observed in SS RNA 7 and 9 of this RNA is probably caused by RNA self-annealing, which is sequence specific and can be observed in ssRNA.

siRNA binding

A series of experiments was carried out using the range of double stranded RNA 15-26 (Figure 18) to investigate the binding capacity of the modified siRNA.

The same trend of binding was observed here, as for the ssRNA binding (Figure 17). The non-modified siRNA 18 showed no binding. Neither did siRNA 15, 16 and 22, which all contain a single palmitoyl modification. For siRNA 17, 19 and 20, containing two palmitoyl modifications, binding was observed. It is evident that the location of the modifications is an important parameter for binding, because siRNA 17 and 20 showed less displacement than siRNA 19.

The insertion of the palmitoyl within the RNA sequence (siRNA 17 and 20) may cause in steric hindrance from the RNA, which results in the observed lower albumin binding. The two terminal modifications of siRNA 19, therefore, promote stronger binding than the in-sequence and terminal modifications of siRNA 17 and 20. There is almost full displacement, when the number of palmitoyl modifications is increased to three (siRNA 21), indicating a further increase in binding strength.

A complete displacement of siRNA to the albumin band (albumin presence was confirmed in this band by coomassie staining, data not shown) was observed for all four cholesteryl modified siRNA's 23-26. The finding that the cholesteryl modification alone (siRNA 26) facilitates binding correlates with the binding of ssRNA 10 (Figure 18) and indicates that the cholesteryl modification binds with high affinity. Since there is already complete displacement in siRNA 26, it cannot be determined whether the presence of additional palmitoyl modifications (siRNA 23-25) further increases binding, however, the presence of palmitoyl modifications does not inhibit cholesteryl induced interaction.

In the lanes containing siRNA 21 and 23-25, a second RNA band is apparent above the albumin-siRNA band. This may indicate that the siRNA promotes albumin dimerization.

This data indicates that native siRNA is not a natural ligand for albumin and that hydrophobic functionalization is needed to promote binding. By using different modifications, and by changing the number and position of the modifications within the RNA molecule it is possible to alter the binding strength of siRNA towards albumin.

Double bands in gels

Two distinct bands were observed for certain siRNA (see Figure 18, siRNA 18, 20, 21 and 24-26). Mobility shift experiments using ssRNA, siRNA and RNA size ladders were carried out to determine the band size and the origin of the second band. An experiment which included ssRNA 10, 8 and siRNA 24 (Figure 19 A) revealed that the lower band visible in siRNA 24 is non-annealed single stranded RNA 10. This also correlates with the fact that this band disappears when the siRNA is incubated with albumin (Figure 18, siRNA 24-26).

This indicates that the two components of the siRNA are not present at a ratio of 1:1 during annealing. Therefore, the concentrations of the ssRNA 7-14 were measured by absorption at 260 nm. To verify the results, the experiments were carried out using two different machines: GeneQuant and NanoDrop. The results are summarized in Table 4.

Table 4. ssRNA concentration measurements for ssRNA 7-14. The experiments were performed in triplicate using both machines. The expected concentrations were 200µM.

siRNA	GeneQuant Concentration/ µM	NanoDrop Concentration/ µM	Average Concentration/ µM
7	424.4	418.1	421.3
8	151.1	146.3	148.7
9	131.8	128.4	130.1
10	617.2	610.7	614.0
12	252.8	221.9	237.3
13	218.1	203.2	210.6
14	264.0	259.9	261.9

The concentrations were expected to be 200 µM, therefore, siRNA containing ssRNA 7 and 10 were initially annealed in ratios different than 1:1, accounting for the additional bands present in the binding studies. Reannealing in the appropriate ratios provided a single band on the gel (Figure 19 B).

Another possibility for the double bands is the decreased stability of the siRNA due to the hydrophobic modifications present. The amount of single stranded RNA increased after four freeze-thaw cycles (Figure 19 C). The additional bands can, therefore, be avoided by preparing new aliquots of siRNA when experiments are carried out, and by carefully controlling the concentration prior to annealing. The presence of the double bands does not change the results of the qualitative mobility shift binding experiments, but will change results of quantitative studies and should be avoided.

Albumin dimerization

Albumin was present as a single band when conjugated to siRNA 26 (Figure 18). However, when siRNA 21 and 24 were used, a second band appeared above the albumin band. This was further emphasized with siRNA 23, 25, where the second band was clearly visible. The presence of protein in these bands was confirmed by coomassie staining (data not shown).

15

These results indicated that the addition of more functional moieties to a single siRNA can result in dimerization of albumin. This is likely due to interaction of each modification with distinct albumin molecules, thereby, effectively cross-binding albumins to form dimers or even larger assemblies.

It is possible that this effect can be utilized to improve the half-life of the siRNA *in vivo*. If the siRNA is surrounded by albumin molecules, it is better protected against recognition and degradation.

Comparison of albumin types

Experiments were carried out to compare the efficiency of binding of recombinant albumin from *S. cerevisiae* (rAlb, Novozymes Biopharma) with defatted albumin purified from human serum (sAlb, Sigma), for details see 4.2. siRNA 26 was used in these experiments because it showed binding to albumin with high affinity with only one modification.

30

This data indicated that the cholesteryl modified siRNA binds better to the rAlb than to sAlb. The sAlb is purified from plasma and, therefore, contains posttranslational covalent modifications such as glycation, happening over the lifetime of the protein. In addition, it is reported that 30 % of serum albumin carries a molecule covalently bound to Cys-34, and it is likely that such modifications can alter the binding capacity of the

albumin. rAlb is therefore a more homogenous product, and most likely better suited for therapeutic applications than albumin from serum.

The properties of albumin as a carrier for functionalized siRNA can be utilized in two

5 ways, by:

1) preparation of a preformulation of albumin with siRNA prior to injection or 2) injection of siRNA to assemble with native albumin in situ. These experiments indicate that the choice of albumin for preformulation is important, since the siRNA binding affinity varies with albumin type. For assembly in situ the binding affinity can be lower
10 than indicated by these experiments because of competition from endogenous ligands. Choice of formulation method and albumin type is, therefore, important for the function of the albumin-based siRNA delivery system.

Competition mobility shift assay

15 From the results above it was concluded that the cholesteryl modified siRNA 23, 24, 25 and 26 bind extensively to albumin. With this result in mind, competition experiments were designed to determine the location of the binding site. For this purpose a series of marker ligands with known high affinity binding sites were used:

20 Sodium octanoate
Sodium myristate
Myristic acid
Stearic acid
Fusidic acid
Sodium salicylate
25 Lithocholic acid
Cholesterol
Warfarin
L-Thyroxine

30 Warfarin and octanoate bind with high affinity to Sudlow's site I and II, respectively (see Table 1). Fusidic acid binds to the heme binding cleft. Myristate has two binding sites of similar affinity, FA site 1 and 5, respectively (see Figure 6). Stearic acid is thought to bind with high affinity to the same two sites as myristic acid.

Crystallographic data has revealed four binding sites for thyroxine, but it has not been
35 determined which site has the highest affinity. Two of these sites are in subdomain IIIB near FA site 5, and the remaining are Sudlow's site I and II. Finally it is proposed that

the high affinity site for salicylate is located in domain II, but is distinct from the warfarin binding site. Cholesterol has not been reported to bind to albumin, but was relevant for this study as a control for siRNA modified with cholesteryl. Lithocholic acid is a bile salt of similar structure to cholesterol, but less hydrophobic. Because of the similar structure, lithocholic acid was included in the experiment. It is reported to bind to albumin, but the binding sites are unknown.

The ligands described above cover the important high affinity binding sites of albumin. By competition studies with these ligands the inventors aimed to determine the binding site for the cholesteryl modified siRNA.

Discussion

The competition studies revealed that stearic acid is the ligand most effectively displacing cholesteryl modified siRNA. Myristic acid and myristate were also able to displace the siRNA, but not as efficiently. This is consistent with the lower affinity of myristic acid than stearic acid for albumin.

Interestingly, neither lithocholic acid nor cholesterol was able to displace the siRNA, indicating that these molecules bind to different binding sites despite the similarity in structure to the cholesteryl modification. There was no synergistic effect of the combination of warfarin and stearic acid.

There was no effect of the ligands salicylate, octanoate or fusidic acid, indicating that the binding sites of these ligands are not related to the cholesteryl-siRNA binding site. Furthermore it was noted that thyroxine had a displacing effect, most pronounced with siRNA 24 (Figure 21 B), which was also displaced by warfarin. These effects varied between the different siRNA.

These results indicated that cholesteryl modified siRNA have common binding sites with stearic and myristic acid, being FA sites 1 and 5. Since the heme binding cleft overlaps with FA site 1, and fusidic acid did not displace the siRNA, it is probable that FA site 5 is the binding site. This is also consistent with the open structure of this binding site being able to facilitate binding of a large molecule such as siRNA (see Figure 6).

Thyroxine is able to displace the siRNA 24 and 26, but not 23 and 25. Thyroxine has two binding sites close to FA site 5, and it was found that it can displace siRNA from this site. siRNA 23 and 25 are not displaced by thyroxine, which can be related to the SS 3' palmitoyl modification in both these siRNA. It is possible that this modification

5 either 1: further improves the binding strength to FA site 5, making displacement more difficult, or 2: allows binding with lower affinity to a secondary site, where the siRNA is not displaced by thyroxine.

The pattern of displacement is similar whether the siRNA contains palmitoyl modifications or not, indicating that it is the cholesteryl modification which drives the
10 binding and the palmitoyl plays a secondary role.

The high affinity binding of the cholesteryl modified siRNA to a FA binding site is a surprising result. There are few structural similarities between these molecules, and because neither cholesterol nor lithocholic acid can displace the siRNA, the inventors
15 hypothesize that it is not the cholesteryl modification itself, but the entire siRNA construction including cholesteryl, linker and RNA (see Figure 16) which causes the interaction.

Isothermal Titration Calorimetry (ITC)

20 EMSA binding studies were supplemented by experiments using Isothermal Titration Calorimetry (ITC). ITC determines all parameters (n , K , ΔH and ΔS) of a binding reaction in a single experiment and is widely used to determine protein-protein or protein-ligand binding.

25 Contrary to other binding characterization techniques, including Biacore or Micro Thermophoresis, ITC characterizes molecules without the need for labeling. Albumin changes conformation upon binding or attachment of molecules, and these conformational changes alter the properties of binding sites on the protein.

It is, therefore, important to use the albumin in the non-modified form for binding
30 experiments.

Albumin-octanoate binding was used as an introduction and control system for ITC experiments. This reaction was chosen because it is well characterized in the literature, and was used to evaluate the ITC method. Following initial albumin-octanoate studies,
35 experiments were carried out studying the binding of modified and non-modified siRNA to albumin.

Isothermal Titration Calorimetry Equipment

The ITC instrument consists of two identical cells (sample cell and reference cell) and an injection syringe (Figure 22). The sample cell contains dissolved albumin, the reference cell contains buffer and the siRNA is loaded into the syringe. Each cell has a heater, and the heaters are controlled so that the temperatures in both cells are equal. A constant power (reference power) is added to the reference cell, and the sample heater supplies a similar power (measured power) to keep temperatures equal. The two cells are surrounded by a thermostated jacket, which absorbs the excess heat from the heaters, keeping temperature constant.

During experiments, small amounts of siRNA are injected from the syringe into the sample cell under stirring. The heat released by the reaction causes a temperature change in the sample cell compared to the reference cell. This temperature change is compensated for by the sample cell heater, which ensures constant temperature. This change over time in sample cell heater power, therefore, reflects the energy released during the reaction following each injection. The sample cell heater power is recorded during the experiment, and this is the data output of ITC. Due to this setup, a temperature rise following an injection will result in a drop in sample heater power, because less power is required to keep the temperature constant.

In this project, ITC equipment manufactured by MicroCal and software supplied by MicroCal (LLC ITC for Origin 7, Microcal) was used to carry out calculations and analysis of the data. The work was carried out on three identical VP-ITC instruments (one in Daniel Otzen Lab, Aarhus University; two at Peter Westh Lab, Roskilde University), and preliminary testing was carried out on an ITC200 (Medical Biochemistry, Aarhus University). The VP-ITC is the most commonly used ITC, and is considered the golden standard of ITC measurement. There can be very small variations between specific ITC machines, but it is generally considered that data is reproducible.

Designing experiments and experimental setup

General guidelines for experimental design can be found in the VP-ITC manual (MicroCal, GE Healthcare).

Approximate concentrations of ligand and protein should be calculated before ICT experiments are conducted to ensure the appropriate experimental settings. The critical parameter which, determines the shape of the binding curve in an ITC experiment, is the unitless constant c defined as:

5 Equation 1: $c = K * M_{tot} * n$

K = association constant, n = # of binding sites, M_{tot} = Protein concentration

High c values lead to tight binding and, therefore, steep, rectangular isotherms, whereas low c values result in nearly horizontal isotherms from which no data can be calculated. The acceptable range of c is 5-500, but the optimal is 10-100 (Figure 23).

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Albumin-octanoate

Albumin-Octanoate binding was used as a control binding system to establish the ITC method. Depending on the source and method of characterization, the binding constant of the albumin-octanoate interaction is reported to be between $K \sim 3.4 * 10^4 \text{ M}^{-1}$ and

15 $1.6 * 10^6 \text{ M}^{-1}$, with a single high affinity binding site ($n=1$). From these values, approximate protein concentrations can be calculated:

Equation 2: $M_{tot} = c / (K * n)$

Equation 3: $M_{totMAX} = 100 / (3.4 * 10^4 \text{ M}^{-1} * 1) = 2.94 * 10^{-3} \text{ M}$

20

Equation 4: $M_{totMIN} = 10 / (1.6 * 10^6 \text{ M}^{-1} * 1) = 6.25 * 10^{-6} \text{ M}$

ITC experiments were designed within this concentration range to ensure appropriate binding isotherms. Figure 24 shows a representative binding curve for albumin-octanoate binding. A sample cell albumin concentration of 10 μM and a syringe

25 octanoate concentration of 100 μM were used for this experiment.

Table 5. n and K values calculated on the basis of the results presented in Figure 23, and compared to literature.

	N	$K (\text{M}^{-1})$
Result of this study	1.01 ± 0.02	$2.41 * 10^5 \pm 0.29 * 10^5$
Literature	1	$3.4 * 10^4 - 1.6 * 10^6$

30 These experiments showed that ITC produces data consistent with the literature. This type of experiment was used as a control before each experimental ITC series to ensure that the ITCs were calibrated and produced reproducible data.

Single injection experiments

Binding experiments by ITC were carried out for a representative selection of siRNA. Single injection experiments were used to screen the siRNA for binding. These experiments feature a single injection instead of an injection series and provide information about the enthalpy change of the reaction. The results obtained are summarized in Figure 25.

These results showed the same binding trend as the mobility shift experiments. The cholesteryl modified siRNA (23-26) exhibited the strongest interactions, whereas very weak interactions are observed with the non-modified RNA 7, 10 and siRNA 18. The palmitoyl modifications show intermediate binding, however, the variation between individual siRNA does not entirely reflect the mobility shift results. These experiments confirm that siRNA can be designed to interact with albumin with varying strength depending on the location and type of modification.

Binding experiments

In addition to the single injection experiments, full binding curve experiments were carried out using siRNA 26 (single cholesteryl modification, 3' antisense strand) and rAlbumin:

Table 6. Summary of the results obtained from Figure 27 showing the binding of siRNA 26 to albumin.

	N	K (M^{-1})	ΔH (cal/mol)
siRNA 26	0.65±0.01	$9.18 \cdot 10^6 \pm 1.79 \cdot 10^6$	$-2.14 \cdot 10^4$ $0.54 \cdot 10^4$

The values from these experiments (Table 6) indicated a strong interaction between albumin and siRNA 26. Only few ligands are reported to have such a high K value (Table 1), showing that the cholesteryl modification is particularly efficient in promoting albumin binding. The data confirmed the result from the gel experiments, which also showed efficient binding of the cholesteryl modified siRNA.

Thus, the inventors have confirmed that the cholesteryl modified siRNA is a high affinity ligand for albumin binding. The inventors also show that albumin binding can be adjusted and controlled by different combinations of modifications.

Cellular uptake of albumin

An efficient carrier system for siRNA must facilitate intracellular delivery. Experiments were therefore designed to study the uptake of albumin in relevant cell lines, investigating the potential of albumin for intracellular delivery. Three different cell lines were used, summarized in Table 7.

Table 7. Cell lines used in cellular albumin uptake studies.

Name	#	Origin	Growth media	Notes
HepG2	HB-8065	Hepatocellular carcinoma	DMEM	Secrete albumin
OK		Opossum kidney	MEM	Megalin-Cubilin uptake
Caco-2	ECACC	Colorectal adenocarcinoma	MEM	Intestinal model

HepG2 cells are known to express and secrete albumin and they have also been shown to bind the protein. They were used to assess albumin antibody specificity for immunocytochemistry (ICC) using fluorescently labeled secondary antibodies for labeling. Opossum Kidney (OK) cells are derived from proximal tubuli, express the megalin and cubilin proteins, and are reported to bind albumin. Further experiments were carried out using the colorectal Caco-2 cell line, which is utilized to evaluate intestinal epithelial permeability. The aim was to investigate the potential of albumin to cross intestinal epithelium and, therefore, the potential of the albumin-based delivery system for oral delivery.

The cellular uptake of albumin was investigated by two different approaches 1) uptake of fluorescent albumin and 2) immunocytochemistry (ICC). Studies with fluorescently labeled albumin were carried out using FITC labeled rAlbumin (rAlb, Novozymes Biopharma). Prior to the uptake experiments, the efficiency of the antibodies used for cellular albumin staining was evaluated.

Evaluation of albumin antibody specificity

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To evaluate the albumin antibodies western blotting was carried out using serum albumin (sAlb, Sigma), and rabbit anti-human serum albumin antibody (Abcam, #ab2406) according to standard protocol. Results are shown in Figure 28.

30 After transfer to a polyvinylidene fluoride membrane and staining with primary antibody, the blot was split and stained by two different procedures: 1) Alexa488 conjugated secondary AB (Figure 28 A) by same chemistry as for Alexa 680 and 2) Horseradish Peroxidase (HRP) conjugated secondary AB (Figure 28 B). These blots confirm that the antibodies recognize albumin with the possibility to detect as little as

10 ng of protein. The Alexa488 experiment was carried out as a method control experiment. The HRP method is much more sensitive, but it requires incubation with substrate, which is time-consuming. Therefore, if sufficient protein is present, it is possible to save time by using fluorescent antibodies and reading the blot in a

5 fluorescence scanner.

Using confocal microscopy, the antibodies were evaluated in ICC experiments using the HepG2 cell line, which expresses and secretes albumin (Figure 29 A and B). OK cells were included as a control for unspecific staining (Figure 29 C). No albumin was

10 added to the cells in these experiments.

The ICC experiments show specific albumin staining in the albumin expressing HepG2 cells (Figure 29 A and B), and no staining unspecific staining in the control experiment using OK cells (Figure 29 C). The localization and shape of albumin containing

15 compartments in HepG2 cells suggests Golgi apparatus and secretory pathways, where endogenous albumin is present prior to secretion.

It can be concluded that the antibodies can be used in both western blotting and ICC for the specific recognition of albumin.

20 Albumin uptake in OK cells

Cellular uptake of albumin was investigated in the opossum kidney cell line. These cells originate in the proximal tubuli, and are responsible for the megalin-cubilin mediated reabsorption from urine, and, therefore, play an important role in promoting the long half-life of albumin.

25 Uptake was carried out for four hours in serum free media, with albumin concentrations of 1 μ M. Either FITC labeled rAlb or non-labeled rAlb, followed by antibody staining, was used. All samples were stained with Hoechst (Blue) and Rhodamine (Red) and prepared for confocal microscopy, as described in materials and methods. Rhodamine is a common actin stain, which enables visualization of the cell borders. It was included to
30 help visualize the localization of albumin in the uptake studies.

Antibody staining of rAlb in OK cells (Figure 30 C) indicated some albumin localized on the surface of the cells, however, optical sections revealed no intracellular material.

Incubation with FITC labeled albumin resulted in a very weak signal (Figure 30 B),

35 whilst antibody staining without prior albumin incubation revealed no signal (Figure 30 A). FITC rAlb contains one fluorophore per albumin molecule, whereas antibody labeling

can result in several secondary antibodies, leading to signal amplification from antibody staining.

The inventors concluded that no intracellular albumin could be observed in these experiments, however, some albumin appeared to be localized on the cell surface.

5

Albumin uptake in Caco-2 monolayers

Cultured Caco-2 monolayers are commonly used as a model to investigate the potential of albumin for transport across intestinal epithelium. The cells were cultured on porous membrane inserts (0.4 μm , Polycarbonate, Costar). Albumin (either FITC labeled rAlb, 10 1 μM , or non-labeled rAlb, 1 μM) was incubated with cells in serum containing media for 24 hours. Samples with non-labeled albumin were stained with antibodies. The samples were stained and prepared using the same procedures as described previously for OK cell uptake experiments.

15 Initial images revealed a well-ordered cell culture, growing predominantly in a monolayer (Figure 31 A). FITC labeled albumin uptake (Figure 31 B) revealed presence of albumin, however, the signal was weak and the localization of albumin could not be determined. The antibody labeled albumin uptake revealed a clear presence of albumin in certain cells (Figure 31 C). The albumin was present within the actin-lined borders, 20 indicating an intracellular localization. It was noted, however, that only a subpopulation of cells showed this pattern of albumin uptake.

In some areas of the porous membrane the cells had formed protrusions, where cells grew in several layers (Figure 31 D). A three-dimensional image revealed presence of albumin within such a growth structure (Figure 32).

25

These results indicate that albumin is taken up by a subpopulation of cells in the Caco-2 monolayer model. Additional experiments are required to determine the nature of this subpopulation and the uptake mechanisms, however, the experiments illustrate the potential of albumin for intestinal cell entry.

30

Gene silencing experiments

To investigate the ability of albumin to transport siRNA into the cell and facilitate gene silencing, transfection experiments were carried out using a H1299 cell line, which 35 stably expresses enhanced green fluorescent protein (EGFP). The modified siRNAs 15-26 where designed with a sequence specific to EGFP, and can therefore knockdown

expression of this protein. Successful cellular delivery and consequent gene silencing by siRNA can be monitored by the expression levels of EGFP using flow cytometry.

Evaluation of the effects of modifications on gene silencing

5 siRNA modifications, especially in the antisense strand, can reduce incorporation into RISC and, therefore, the knockdown efficiency of siRNA. An initial screen was, therefore, conducted with the modified siRNAs 15-26 to determine whether the hydrophobic modifications had altered siRNA silencing efficiency. In these experiments, two different commercially available transfection agents were used to transfect the
10 cells: TransIT-TKO Transfection Reagent (Mirus) and Lipofectamine 2000 (Invitrogen). Both are widely and routinely used transfection agents and have been used to transfect cell lines of human origin. The transfections were done according to manufacturers guidelines. Cells were transfected for four hours in serum free media using 50 nM siRNA. A mismatch siRNA with unspecific sequence was included as negative control.

15

The non-modified siRNA 18 resulted in potent knockdown. The presence of the mid-sequence palmitoyl (siRNA 16-17, 20-21 and 24-25) reduced knockdown significantly, possibly due to steric hindrance of RISC interactions of in-sequence modifications compared to 3' terminal modifications.

20 The knockdown efficiency of the remaining siRNAs varied, and there were large differences between the TransIT-TKO[®] (Mirus) and Lipofectamine[®] (Invitrogen) results. The components of these commercial transfection agents are not well described, however, both are based on cationic lipids designed to bind highly hydrophilic siRNA. It is, therefore, plausible, that the introduction of hydrophobic modifications not only
25 possibly alters the efficiency of the siRNA, but also the ability of the transfection agent to form the complexes necessary for cellular delivery of the siRNA. Further transfection experiments using albumin, therefore, included all siRNA modifications.

Albumin mediated gene silencing

30 The aim of these experiments was to determine whether using albumin as a carrier can facilitate gene silencing of hydrophobically modified siRNA. Albumin mediated gene silencing is not a standard protocol, therefore, an initial optimization experiment was carried out. For this experiment siRNA 19 (3' palmitoyl on both SS and AS) was chosen because of the good result of this RNA in both the control knockdown (Figure 33) and
35 good binding in the gel experiments (Figure 17). siRNA concentrations between 50-200 nM and albumin concentrations between 200-1600nM were used in these transfections.

The different siRNA and albumin concentrations and ratios used to determine the optimal transfection ratio are presented in Table 8.

Table 8. siRNA and albumin ratios and concentrations used to optimize albumin mediated transfection, using siRNA 19 and rAlb. Experiments done in triplicate.

siRNA/nM	Albumin/nM	siRNA:albumin
200	200	1:1
100	200	1:2
50	200	1:4
50	400	1:8
50	800	1:16
50	1600	1:32

siRNA and albumin complexes were mixed in 20 μ L nuclease free water (Ambion) and incubated for 4 hours before addition to the cells. Cells were transfected for 16 hours in serum free media, cultured for additional 32 hours with growth media, collected and prepared for flow cytometry.

Albumin transfected samples showed reduced EGFP expression compared to the non-treated control, but experiments result in less efficient knockdown than using TransIT-TKO transfectant. From this experiment a siRNA:albumin ratio of 1:4, with 50 nM siRNA and 200 nM albumin was selected to take forward in screening the panel of siRNA modifications. Transfection experiments using siRNA designs 15-26, were therefore carried out using these concentrations. The siRNA:albumin complexes were incubated for four hours and added to cells for 16 hours with serum containing media. For each siRNA, a sample transfected with 50 nM siRNA without albumin was included as negative control, to evaluate uptake of free siRNA.

As seen from Figure 35, albumin did not facilitate silencing of the non-modified siRNA 18, which was expected, as no binding was shown in the binding experiments for this siRNA. There was a slight increase in knockdown in the presence of albumin in the remaining siRNA, except 17. siRNA 21, with 3 palmitoyl modifications, showed most knockdown activity. This siRNA showed knockdown without albumin, but presence of albumin clearly improved transfection efficiency.

For the cholesteryl modified siRNA 23-26, the presence of albumin lowered the EGFP expression between 10-15%. This is a moderate knockdown, which may reflect

insufficient release of siRNA due to high albumin affinity or ineffective incorporation into RISC.

Conclusion

- 5 The aim of this work was to study the potential of albumin as a carrier for siRNA. siRNA is not a natural ligand for albumin, but binding was achieved by functionalization with palmitoyl and/or cholesteryl modifications. Binding was analyzed using electrophoretic mobility shift assay (EMSA) and isothermal titration calorimetry (ITC). Initial EMSA studies revealed that siRNA functionalized with cholesteryl and/or palmitoyl show
10 increased albumin binding compared to non-modified siRNA.

The results indicated that the number and location of palmitoyl modifications play a role. Increasing the number of modifications increases binding strength, and modifications in the 3' overhangs are most effective.

15

- Cholesterol modified siRNA was shown to bind with highest affinity, leading to full displacement of the siRNA into the albumin band in the gels, and the presence of both palmitoyl and cholesteryl in the same siRNA did not reduce binding. siRNA with several modifications promoted albumin dimerization, which can potentially be utilized for
20 further protection of siRNA.

Competition studies pointed to FA binding site 5 as the main binding site for cholesteryl modified siRNA, and ITC experiments revealed binding to one binding site with $K=9.18 \times 10^6 \text{ M}^{-1}$, indicating a high affinity for this binding site.

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The inventors have, therefore, successfully designed functionalized siRNA, which bind to albumin with high affinity. The inventors propose that by interacting with albumin, the siRNA will utilize the trafficking properties of albumin to avoid renal clearance, thereby improving the half-life of the siRNA.

30

If preformulation is used, the data indicated that recombinant albumin expressed in *S. cerevisiae* (Novozymes Biopharma) promotes more efficient binding of siRNA than albumin purified from serum.

- 35 siRNA functionalized with cholesterol has previously been suggested to interact with albumin, however, fatty acid modifications were identified as more efficient albumin

ligands. We, therefore, present the first direct evidence of high affinity interactions between albumin and cholesterol modified siRNA and suggest binding to the non-obvious FA binding site 5.

- 5 Fatty acid modifications, including palmitoyl, have previously been shown to associate with albumin, and this has been connected with improved gene silencing.

The work was extended by introducing several palmitoyl modifications, or combinations of palmitoyl/cholesteryl modifications in the same siRNA, and propose that this can
10 optimize the interaction further.

Cellular uptake studies were carried out to determine the uptake and accumulation of albumin in OK and Caco-2. Albumin uptake was confirmed in a subpopulation of Caco-2 cells, indicating the wide potential of albumin as a transport molecule in different
15 tissues.

Transfection studies were carried out to show the effect of albumin on the cellular uptake of modified siRNA. Initial studies using commercial transfection agents showed that the transfection efficiency of siRNA was lowered by introduction of hydrophobic
20 modifications, compared to non-modified siRNA. It could not be concluded whether this was caused by lowered knockdown or lowered cellular uptake.

Albumin was used as a transfection agent for the functionalized siRNA. The knockdown efficiency was in the range of 10-15 %, indicating the need of further optimization, but
25 nonetheless, these experiments show that albumin has an improving effect on the knockdown efficiency.

Because unmodified siRNA showed no knockdown in the presence of albumin, these experiments indicated that albumin increases the cellular delivery of hydrophobically
30 modified siRNA. The high affinity of the cholesteryl modified siRNA can be a limiting factor in the efficient knockdown, if the siRNA is not released from albumin.

This work provides a novel albumin-based siRNA delivery system. The inventors have successfully designed siRNA with high affinity for albumin, and shown that albumin can
35 improve the cellular uptake of such siRNA. The inventors therefore conclude that albumin can be utilized as a carrier for siRNA in the *in vitro* settings used in this project.

Due to the success of existing albumin-based drug delivery systems for other drugs, it is clear this system can be applied to *in vivo* applications for siRNA delivery.

Materials and methods

5 *siRNA*

The siRNA used in this project are described in Figure 15. ssRNA were dissolved in Nuclease-Free water (Ambion) to 200µM solutions, evaluated using GeneQuant II (Pharmacia Biotech) or Implen NanoDrop (AH Diagnostics). For annealing, ssRNAs were mixed 1:1, incubated for 1 min at 95°C for 1min, 1 hour at 37°C, and stored at -20°C.

10 *Albumin*

Four different albumin types were used, as described in Table 9.

Table 9. Different albumins used in the project. Stripped indicates an albumin which is defatted, without long chain FAs or stabilizing octanoate.

Type	Description	Provider	Stripped	Organism	Form	Notes
Recombinant albumin, Recombumin® Alpha (formerly Albucult®)	rAlb	Novozymes Biopharma	No	<i>S. cerevisiae</i>	In solution	
Recombinant albumin, Recombumin® Alpha (formerly Albucult®)	Stripped rAlb	Novozymes Biopharma	Yes	<i>S. cerevisiae</i>	In solution	
Recombinant albumin, Recombumin® Prime (formerly Recombumin®)	F5M rAlb	Novozymes Biopharma	No	<i>S. cerevisiae</i>	In solution	Cys34 F5M labeled
Human serum albumin	sAlb	Sigma, #A1887	Yes	Human	Lyophilized powder	

15

Recombinant albumin was provided by Novozymes Biopharma. The non-stripped rAlb (100mg/mL) contains 145 mM sodium chloride and 8 mM sodium octanoate. Stripped rAlb contains no excipients.

20 Trade names for Novozymes Biopharma recombinant Albumin types are:

Recombumin® Prime (formerly Recombumin®), Recombumin® Alpha (formerly Albucult®), Recombumin® Flex.

Initial experiments were carried out using albumin purified from human serum, bought from Sigma (#A1887). These experiments are not shown, with the exception of Figure 19.

- 5 All later experiments were carried out using different versions of the recombinant albumin (rAlb) supplied from Novozymes Biopharma. Some experiments (Figure 19) where done using both Sigma sAlb and NZ rAlb, resulting in similar binding patterns, however, with the rAlb performing better.

10 *Binding experiments*

The binding experiments were carried out using the defatted rAlb from Novozymes Biopharma.

- The defatted rAlb is also referred to as stripped rAlb, because excipients are removed
15 from the rAlb formulation. All binding and competition experiments are carried out using the defatted Novozymes Biopharma rAlb material.

Cell work

- All cell work was done using the normal, non-defatted rAlb, in some cases labeled with
20 Fluorescein-5-Maleimide (F5M)

rAlbumin product characteristics and specification

- The recombinant albumin (rAlb) may have one or more of the following characteristics:
Sodium: preferably 100 to 200 mM, more preferably 120 to 160 mM or 140 to 150 mM,
25 most preferably about 145 mM.

Fatty acid, such as Octanoate: preferably 2 to 40 mM, more preferably 4 to 36 mM such as 28 to 36 mM or 4 to 12 mM.

- 30 Detergent: such as Polysorbate 80: preferably 5 to 50 mg/L, more preferably 10 to 50 mg/L such as 10 to 20 mg/L or about 50 mg/L.

Endotoxin: preferably less than 0.5 EU/ml (for example, as measured by LAL)

pH: preferably pH 5.5 to 8.0, more preferably 6.0 to 7.8, most preferably 6.4 to 7.4.

Purity: preferably at least 90% pure, more preferably at least 95% pure, most preferably at least 99.0% pure (e.g. as measured by native PAGE).

Polymer content: preferably less than or equal to 5% (w/w), more preferably less than or equal to 2% (w/w), most preferably less than or equal to 1% (w/w) (e.g. as measured by GP.HPLC).

Host cell protein: preferably less than or equal to 150 ng/g, 15 ng/g, 5 ug/g, 0.3 ug/g protein, most preferably less than or equal to 0.15 ug/g protein (e.g. as measured by ELISA).

Electrophoretic mobility shift assay

All gel experiments were carried out using the XCell SureLock™ Mine-Cell Electrophoresis System, using Novex™ 8% polyacrylamide gels 1X Tris/Borate/EDTA (TBE) buffer, 12 wells (Invitrogen). Samples were loaded using Novex™ TBE Running Buffer (5X) (Invitrogen, #LC6675). Gels were run in 1X TBE buffer (From 10X stock, Gibco, #15581-044). Staining for siRNA was done using SYBR-Gold nucleic acid stain (Invitrogen, #S-11494) following standard protocol. Staining for protein was done using SimplyBlue Coomassie Stain (Invitrogen, #LC6060) following standard protocol. For competition assays, the following ligands were used: Sodium octanoate (Sigma, #C5083), fusidic acid (Sigma, #F0881), sodium myristate (Sigma, #M8005), sodium salicylate (Merck, #106600), lithocholic acid (Sigma, #L6250), cholesterol (Sigma, #C8667), myristic acid (Sigma, #M3128), stearic acid (Fluka, #85680), warfarin (Sigma, #A2250) and L-thyroxine (Sigma, #T2376). For experimental details below regarding "Competition mobility shift assay".

Isothermal titration calorimetry (ITC)

ITC experiments were carried out on VP-ITC (MicroCal, GE Healthcare) using the siRNA and albumin described previously, diluted in PBS (pH 7.4) or water. Samples were degassed at 37°C without magnetic stirring for 10 min. The VP-ITC was thermostated and run at 37°C. Contrad-70 5% (Decon Labs Inc., #1002), methanol and double distilled water were used between experiments for cleaning, according to manufacturer specification. For settings see Table 12, below, regarding "ITC".

Cell culture

Cells were cultured in the following media: Caco-2: DMEM (Gibco, #41965), 10 % fetal bovine serum (FBS), 1x non-essential amino acids (Gibco #11140), penicillin, 100µg/ml; streptomycin 100 µg/mL. H1299: RPMI 1640 (Gibco, #61870), 10 % FBS, 5 penicillin 100 µg/mL; streptomycin 100µg/mL, 500 µg/mL geneticin. OK: MEM (Gibco, #41090), 10 % FBS, penicillin, 100 µg/ml; streptomycin 100 µg/mL. HepG2: DMEM (Gibco, #41965), 10 % FBS, penicillin, 100 µg/ml; streptomycin 100 µg/mL.

Cells were cultured according to American Type Culture Collection (ATCC) associated 10 with each cell type, and always for minimum three passages following thawing prior to experiment. By serum free media (SFM) is understood the appropriate media for the cell type without FBS.

Prior to experiments the cells were cultured on porous membrane inserts (0.4µm, 15 polycarbonate, Costar #3407) for three weeks, until monolayers with tight junctions were formed. Trans Epithelial Electrical Resistance (TEER) was used to evaluate cell monolayer formation during culturing.

Gene silencing experiments

20 H1299 cells were seeded at a density of 5×10^4 cells per well in 24 well plates (Nunc) 24 hours before transfection, and supplied with 1mL growth media. Mirus: transfectant was prepared by mixing 50 µL SFM + 3 µL TransIT-TKO® reagent (Mirus, #MIR2154), incubating 15 min, adding 2.5 µL 5 µM siRNA, incubating 15 min. Transfectant added to cells with 200 µL SFM for total transfection volume of 250 µL per well and siRNA 25 concentration of 50 nM. Lipofectamine: Mix 50 µL SFM + 1 µL Lipofectamine™ 2000 reagent (Invitrogen, 11668-019), incubate for 5 min, and mix 50 µL SFM + 2.5 µL 5µM siRNA, incubate 5 min. Combine the two dilutions, incubate for 20 min, add to cells with 150 µL SFM for total transfection volume of 250 µL pr. well and siRNA concentration of 50 nM.

30

Albumin transfectants were prepared by mixing the albumin and siRNA to a total volume of 20 µL in Nuclease-Free water, incubating for 4 hours and adding to cells with 230 µL SFM. Transfections were carried out for 4 (TransIT-TKO, Lipofectamine) or 16 hours (Albumin).

35

Transfectant was removed and 1 mL growth media was added. Cells were harvested 24 hours later using trypsin 0.05 % EDTA (Gibco, #25300), resuspended in 1 mL media, centrifuged at 1500 rpm for 5 min, and resuspended in 500 µL 2% FBS PBS for flow cytometry (Gallios flow cytometer, Beckman Coulter Inc.). 10000 cells were quantified from each well, all transfections were done in triplicate.

Western blotting

Gels were run as described in 4.3. Page Ruler™ Prestained Protein Ladder (Fermentas, #SM0671) was used as size marker. Electrotransfer to Immobilon-FL PVDF (Millipore, 0.45 µm, #IPFL00010) membrane was performed using XCell II™ Blot Module (Invitrogen) in 1x Transfer Buffer (0.3 % Trizma base, 1.44 % Glycine, 20 % EtOH (96 %)), 25 V for 2 hours in 4 °C. Before electrotransfer the membrane was activated in 96 % ethanol for 30 sec, followed by 3 min in water. After electrotransfer, proteins were visualized using 0.5% Ponceau Red in acidic acid, confirming transfer. Membrane was blocked in 5 % milk with PBS-Tween 0.005 % (Sigma, #P1379) for 1 hour at RT (Room Temperature), followed by Rabbit-anti-albumin antibody (1:1000; 1 µg/mL) (Abcam, #ab2406) incubation in 5 % milk with PBS-Tween 0.005 % for 1 hour at RT. This was followed by incubation in anti-rabbit antibodies with either HRP (1:2000; 0.5 µg/mL) or Alexa 488 1:1000; 1 µg/mL) in 5 % milk with PBS-Tween 0.005 % for 1 hour at RT (1:2000). The blots were scanned for chemiluminescence or fluorescence, respectively, using Typhoon Trio+ scanner (GE Healthcare).

Cellular uptake studies

Cells were cultured in Lab-Tek 4-Chamber Slides (Nunc, #177399) or 24-well plates (Nunc) with glass coverslips (Menzel Gläser). Labeled albumin was added for 4 hours in SFM. Recombinant albumin uptake was carried out for 4 hours in SFM or 24 hours in serum containing media. Cells were fixed with 4 % paraformaldehyde, permeabilized with 1 % Triton X-100 in PBS. Cells were stained with rhodamine-phalloidin 1:50 in PBS (Invitrogen, R415) and Hoechst 33342 1:10000 (Invitrogen, H35701), and preserved on SuperFrost Microscope Slides (Menzel Gläser) in fluorescent mounting media (Dako, S302380). For detailed protocol below regarding "ICC".

Immunocytochemistry (ICC)

Cells were cultured as in above "cellular uptake studies". Albumin uptake was carried out for 4 hours in SFM or 24 hours in serum containing media. Cells were fixed with 4 % paraformaldehyde, permeabilized with 1 % Triton X-100 in PBS. Cells were stained

with primary AB 1:1000 (ab2406), secondary AB 1:300 (Alexa 488), rhodamine-phalloidin 1:50 in PBS (Invitrogen, R415) and Hoechst 33342 1:10000 (Invitrogen, H35701). Cells were preserved as in 4.8. For detailed protocol see below regarding "ICC".

5

Protocols

Mobility shift assay

Initial experiments indicated that 2-4 pmol siRNA per well is the optimal amount for gels stained with SYBR-Gold nucleic acid stain. Experiments were generally designed to have siRNA concentrations within this range. Each sample was prepared in a total volume of 12.5 μ L (10 μ L sample in nuclease free water (Ambion) +2.5 μ L loading buffer (5X)). siRNA and albumin were incubated at RT for 30 min and gels were run according to this protocol:

10

- After preparation, vortex samples and incubate for 1 hour at RT to ensure albumin-siRNA binding
- Place gel in chamber, fill up with 1X TBE buffer and load on the precast gel
- Run gel at 150 V for 25-35 min, until the lower band of the loading buffer is 2 cm above the bottom of the gel
- Remove gel from casing and place in 50 mL SYBR-Gold diluted 10000X in 1X TBE buffer
- Incubate for 15 min under gentle shaking
- Wash 3X in dd water to remove excess SYBR-Gold stain.
- Scan the gel in a fluorescence scanner, using the 488/526 nm wavelengths and resolution of 50 μ m

15

20

25

- Coomassie staining can be carried out after the scanning without washing.

Competition mobility shift assay

Optimization studies revealed that the optimal resolution for competition was achieved with siRNA:albumin:ligand molar ratios of 1:8:128. This ensured efficient binding of siRNA:albumin, and sufficient surplus of ligand to induce dissociation of siRNA. Experiments with lower ligand concentrations provided the same patterns, however leaving smears between the bands.

30

Due to issues with solubility the ligands were prepared as 1 mg/mL stock solutions.

35

Table 10 describes the solvents used for each ligand.

Table 10. Ligands and solvents used to make 1 mg/mL stock solutions.

Ligand	Solvent
Sodium octanoate	Water
Fusidic acid	Water 5
Sodium myristate	Water
Sodium salicylate	Water
Lithocholic acid	Ethanol 96 %
Cholesterol	Ethanol 96 %
Myristic acid	Ethanol 96 % 10
Stearic acid	Ethanol 96 %
Warfarin	NaOH 0.5 M
L-Thyroxine	NaOH 0.5 M

- 15 These solvents ensured that all ligand could be dissolved at the given concentration. Stock solutions were dissolved to final solutions of 1280 μM , from which 0.2 μL per well was added.

The samples were prepared in the order and amounts stated in Table 11.

20

Table 11. The samples for the competition assay were prepared in the following way.

Step	1. Water	2. Albumin	3. Loading buffer	4. siRNA	5. Ligand
Concentration	-	8 μM	-	0.5 μM	128 μM
Volume added	3.8 μL	2 μL	2.5 μL	4 μL	0.2 μL

Isothermal titration calorimetry (ITC)

Single injection experiments were carried out using the settings listed in Table 12.

25

Table 12. Experimental settings for ITC single injection experiments.

Setting	Value
Total # injections	2
First injection	5 μL
Second injection	50 μL
Injection spacing	600 s
Cell temperature	37°C
Reference power	14 μcal
Initial delay	900 s
Syringe concentration	1.66 μM
Cell concentration	1.5 μM

Stirring speed	310 rpm
----------------	---------

An initial injection of 2-5 μL is recommended by ITC protocols [114] to stabilize the experiment, and, therefore, this response should be excluded from calculations. 70 μL of siRNA solution and 2 mL albumin solution were degassed for 10 min without
5 magnetic stirring. Samples were loaded and program initiated according to manual.

Full binding curve experiments were carried out using the settings listed in Table 13.
Table 13. Experimental settings for ITC binding curve experiments.

Setting	Value
Total # injections	35
First injection	4 μL
Following injections	8 μL
Injection spacing	300 s
Cell temperature	37°C
Reference power	14 μcal
Initial delay	900 s
Syringe concentration	50 μM (siRNA) - 100 μM (octanoate)
Cell concentration	5-10 μM
Stirring speed	310 rpm

Binding experiments were carried out with albumin concentrations of 5 μM for siRNA
10 experiments and 10 μM for octanoate experiments. Between experiments the cell was flushed manually with 3x2 mL Contrad-70, 5 %, 3x2 mL methanol and 3x2 mL water.

Immunocytochemistry (ICC)

The following procedure was used to prepare the samples:

- 15 1. Remove media and replace with PBS (If cells are cultured on permeable membranes, leave cells in inserts and carry out step 1-7 by adding 0.5 mL to apical side and 1.5 mL to basolateral side)
2. The emptying can be done by tilting the slide upside-down and carefully tapping it to release most fluid
- 20 3. Wash 3x5min with PBS (add 1mL PBS, shake gently, leave 5 min, tilt to remove PBS)
4. Fix the cells by adding 1 mL 4% paraformaldehyde to the wells for 15 min at RT
5. Remove the paraformaldehyde solution and wash 3x5min in PBS
6. Remove the PBS and wash 10 min while shaking gently with 1 mL 1 % Triton-
25 X100 in PBS to permeabilize the cells
7. Remove the triton and wash in PBS for 3x5min

8. If cells are cultured on permeable membranes, remove the membrane from insert with scalpel before proceeding to step 9
9. Step 10-12 can be carried out on Para-film, where 100 μ L AB solution will be enough to cover sample
- 5 10. Antibody staining (if nothing is stained move to step 11)
 - 10.1. Remove the PBS from the wells
 - 10.2. Add the primary antibody, 1:1000 in 0.5 % Triton X-100
 - 10.3. Incubate for 30-60 min
 - 10.4. Remove antibody and wash 3x5 min with 1 mL PBS
- 10 10.5. Add secondary antibody diluted 1:300 in Triton-X100
 - 10.6. Incubate for 60 min at RT, or overnight at 4 °C
 - 10.7. Remove antibody and wash 3x5 min with 1 mL PBS
11. Rhodamine staining (if nothing is stained move to step 12)
 - 11.1. Dilute Rhodamine-Phalloidin 1:50 in PBS, enough to cover the samples
 - 15 11.2. Add Rhodamine-Phalloidin, covering the sample, and incubate for 30 min
 - 11.3. Remove Rhodamine-Phalloidin and wash 3x5 min with 1 mL PBS
12. Hoechst staining (if nothing is stained move to step 13)
 - 12.1. Dilute stock Hoechst solution 1:10000 in PBS
 - 12.2. Add Hoechst to samples, incubate for 15 min
 - 20 12.3. Remove Hoechst and wash 3x5 min in 1mL PBS
13. Seal the samples in mounting media using nail polish

Further Examples

Materials and methods

25 *Gel mobility shift binding assay*

Materials

Albumin siRNA

Protogel 30% (National diagnostics)

Ammonium Persulfate, APS (Sigma)

30 TEMED (Sigma)

10X TBE Buffer

Glycerol 80% (Sigma)

SYBR-Gold (Invitrogen)

35 Method

Binding experiments were done using 17-well 8% polyacrylamide gels made from Protogel 30%. For a 100 mL gel, 26.6 mL protogel, 10 mL 10X TBE buffer and 63.3mL water were mixed. Before pouring gel, 1mL of 10% w/v ammonium persulfate (APS) and 0.1mL TEMED were added to initiate polymerization.

5

In the binding setup, the siRNA amount was kept constant (3.75pmol) in each well and the albumin amount was varied from 86.6 to 1.6pmol, using a dilution series of 15 points, where each sample was diluted 1:1.33 compared to the previous.

To each well, 3.75µL of 1µM siRNA was added, together with 5µL of albumin solution in
 10 PBS, 7.25 µL PBS, and 4µL loading buffer (30% glycerol, 0.02% bromophenol blue and xylene cyanol or orange G, gels where scanning for both SYBR-Gold and Cy7 was needed were run with glycerol only). The gels were run at 400V for 45 mins or until loading buffer had migrated 70% of the gel. Gels were stained with SYBR Gold using a 1:10000 dilution according to standard protocol, and scanned using Typhoon Trio using
 15 Blue (488) excitation laser, Alexa Fluor 532 emission filter and PMT=600.

Biodistribution

Albumin (Alexa 680 labeled)

The fluorescence labeled Albumins were synthesized by conventional conjugation
 20 chemistry, in which the Cys34 of rAlbumin is coupled to a maleimide group using Alexa Fluor® 680 C₂ maleimide (Molecular Probes®, Invitrogen, Life Technologies Europe BV), Alexa Fluor® 488 C₅ Maleimide (Molecular Probes®, Invitrogen, Life Technologies Europe BV), Fluorescein isothiocyanate (FITC, Thermo Scientific Inc.) and Fluorescein-5-Maleimide (F5M, Thermo Scientific Inc.).

25 Multivalent labeling of rAlb was conducted using Fluorescein isothiocyanate (FITC), which couples to thiol and amine groups.

siRNA (Cy7 labeled, Robotask)

NMRI Mice (Taconic)

Diet, D10001 AIN-076 (Research diets Inc.)

30 Microvette 300 CB (MCB-16440) Sarstedt)

Dulbecco's PBS (pH 7.4, Sigma)

Animal lancet (3 or 4 mm, Goldenrod)

BD Microlance Needles 27Gx½"

Terumo Syringe 1mL

35 Disposable capillaries, 20µL (Hirschmann Laborgerate)

IsoFlo Vet Isofluran (Abbott)

Mice

All experiments were done on 8-9 week old NMRI female mice bought from Taconic. The animals were kept a minimum of 10 days on special feed, with lowered

- 5 autofluorescence (D10001 AIN-076, irradiated, Research Diets). Feed and water was given *ad libitum*. The animals were kept and monitored according to guidelines by the Danish Council of Animal Research. Experiments were done under license 2013-15-2934-00789.
- 10 On the first day of the experiment, the animals were administered with 1nmol of Alexa 680 labeled albumin or 1nmol Cy7 labeled siRNA. For the dual fluorophore experiment, 1nmol siRNA and 6nmol albumin were complexed and co-administered. The samples were prepared in 400μL PBS and administered IV in the tail vein at <40μL/s.
- 15 Blood was drawn:
method 1) from the chin vein using the Goldenrod lancet, or
method 2) from tail by tail nicking at intermediary time points, or
method 3) retro-orbitally under anesthesia (3.5% isofluran) terminally. The blood sample were collected in the Microvette tubes (method 1 and 3), left to coagulate for
20 15 mins at RT, centrifuged for 10 mins at 1500g to isolate serum, or collected directly in a capillary tube and scanned (method 2). Serum samples were kept frozen until use. Animals were sacrificed by cervical dislocation, dissected and organs collected for scanning. Organs were flushed carefully with DPBS prior to scanning and intestines were thoroughly cleaned. Serum samples were scanned in 5μL or 20μL glass capillary
25 tubes in the IVIS.
- For all scans, an IVIS Spectrum Live Imager was used.

The following excitation/emission wavelength settings were used for the scanning:
Alexa 680:

- 30 Table 14: Excitation and emission filters used when recording spectra for Alexa 680 labeled albumin

	Excitation wavelength		
Emission wavelength	605	640	670
660	X		
680	X	X	
700		X	

720		X	X
740		X	X
760			X
780			X
800			X
820			X

Cy7:

Table 15: Excitation and emission filters used when recording spectra for Cy7 labeled siRNA

	Excitation wavelength		
Emission wavelength	675	710	745
720	X		
740	X		
760		X	
780		X	
800		X	X
820		X	X
820			X

5

A reference library was created for the spectral unmixing was recorded by injecting the fluorophores (0.2nmol) subcutaneously in a NMRI mouse and scanning at the same wavelength settings as used for the experiment. For organ quantification, spectral unmixing was used. For serum quantification of Alexa 680 labeled albumin, the excitation/emission pair 645/740nm was used, which has the highest signal intensity. The images were quantified using Living Image 4.3.1 software (PerkinElmen).

10

TNF-α ELISA assay

The human peripheral blood mononuclear cells PBMCs was isolated from the human blood buffy coats using Ficoll-Paque™ PLUS density gradient centrifugation medium (GE Healthcare) according to the following protocol:

15

1. Transfer the buffy coat from the bag to the sterile 250 mL bottle in the laminar flow hood.
2. Dilute the buffy coat 1:1 with 1x PBS containing 2mM Natrium Citrate
3. Transfer 40 mL of buffy coat dilution to 50 mL falcon tube and underlay this carefully with 10 mL of FICOLL.

20

4. Spin at 1800 rpm for 25 minutes
5. Resulting layers are from top to bottom: Plasma – platelets -- PBMC – Ficoll – red blood cells (with granulocytes).
6. Remove and discard plasma.
- 5 7. Carefully aspirate the buffy coat with PBMCs from 3 large (50 mL) tubes using syringe with blunt end needle and transfer to one new 50 mL tube. Discard the remaining Ficoll and red blood cells in closed tubes.
8. Add PBS to the PBMC fraction to make up 50 mL. Spin at 1200 rpm for 10 minutes
- 10 9. Discard the supernatant. Resuspend each pellet in 2 mL ACK lysing buffer (Invitrogen) to lyse remaining erythrocytes. Incubate 2 min at room temperature. Adjust the volume to 50 mL with PBS and spin down, 10 min/1200 rpm.
- 15 10. Discard the supernatant, loosen pellet, and combine the pellets from the same buffy coat in 10 mL PBS. Rinse the tubes with another 10 ml 1x PBS and add to the rest.
11. Count the cells.
12. If not used immediately, freeze in -140 °C in freezing media (20% RPMI, 70% HI-FBS, 10% DMSO).

20

After isolation cells were frozen in freezing medium (20% RPMI, 70% HI-FBS, 10% DMSO) in -140 °C. For each experimental set up a new frozen stock from the same isolated batch was used.

- 25 The PBMCs were seeded in 96-well round-bottom microtiter plates at 200.000 cells/well. For the experiment, each well was incubated with siRNA or siRNA:albumin complexes containing 20pmol (100nM in 200 µL total volume) siRNA and 200pmol albumin (1µM). The cells were incubated with the complexes for 18 hours. The supernatant from PBMC cells treated with albumin-siRNA complexes was assayed on
- 30 human TNF- α ELISA Max™ Deluxe Set (Biolegend, #430205). ELISA assay was performed according to the manufacturer's protocol, as follows. Each incubation step was followed by sealing and shaking the microtiter plate on the rotating table at 150-200 rpm, except the overnight incubation with the Capture Antibody, where plates were not shaken. One day prior to carrying out ELISA the 96-well assay plates were coated
- 35 with the Capture Antibody, diluted 1:200 in 1x Coating Buffer (5x Coating Buffer diluted in ddH₂O). 100 µL of this Capture Antibody solution was added into all wells, sealed and incubated overnight (16-18 hours) at 4°C. The next day all reagents from the set were brought to room temperature (RT) before use. The plate was washed 4 times with 300

5 μ L Wash Buffer (1x PBS, 0.05% Tween 20) per well. The residual buffer in the following washing was removed by blotting the plates against absorbent paper. Next 200 μ L of the 1x Assay Diluent A (5x Assay Diluent A diluted in PBS pH = 7.4) was added for 1 hour to block non-specific binding. While the plate was being blocked, all samples and standards (mandatory for each plate) were prepared. Standards and samples were analysed in triplicate. 1 mL of the top standard 250 pg/mL was prepared in 1x Assay Diluent A (1x AD) from the TNF- α stock solution (55 ng/ mL). The six two-fold serial dilutions of the 250 pg/mL top standard were performed, with the human TNF- α standard concentration: 250 pg/mL, 125 pg/mL, 62.5 pg/mL, 31.2 pg/mL, 15.6 pg/mL, 7.8 pg/mL and 3.9 pg/mL, respectively. 1x Assay Diluent A serves as the zero standard (0 pg/mL). After blocking the plate, washing was performed and 100 μ L standards and samples were assayed in triplicate and incubated for 2 hours in RT. Samples were not diluted, the whole supernatant from the PBMC cells was assayed. After washing, 100 μ L of the Detection Antibody was applied to each well, diluted 1:200 in 1x AD, and

15 incubated for 1 hour. The plate was washed and followed by 30 minutes incubation with 100 μ L of Avidin-HRP solution per well, diluted 1:1000 in 1x AD. The final washing was performed 5 times with at least 30 seconds interval between the washings. Next 100 μ L of the freshly mixed TMB Substrate Solution (10 mL per plate, 5 mL of each from 2 substrates provided in the set) was applied and left in the dark for 15 min. During this

20 step the positive wells turn blue, and must be observed to avoid oversaturation. After incubation in the dark the reaction was stopped with 100 μ L of 2N H₂SO₄ per well. After adding H₂SO₄ the positive wells turn yellow. Absorbance was read at 450 nm and 570 nm (background) with a spectrophotometer within 30 minutes.

25 Albumin annealing of siRNA

For the dual modified (cholesteryl and Cy7) siRNA, the standard annealing procedure previously described resulted in precipitation of the hydrophobically modified siRNA, resulting in very low concentration yields. Concentration measurements for the Cy7 modified siRNA revealed the following concentrations in solution (Table 3).

30 Concentration of siRNA was quantified by diluting 1:20 in RNase free water (Qiagen) and measuring A260 using a Nanodrop 3300 in triplicate of triplicate dilutions.

Table 16: Concentration of siRNA following annealing using standard procedure

	Concentration/ μ M (Theoretical)	Concentration/ μ M (Measured)	Yield %
30.IV	19.88889	19.88889	99.44445

31.IV	7.32043	7.32043	36.60215
32.IV	2.345833	2.345833	11.72917

Due to the very low yield of these annealings a new annealing protocol was developed, where the siRNA was annealed in the presence of albumin.

In short:

- 5 1. Mix 10 μ L the 2 single stranded oligonucleotides 1:1 from 200 μ M stocks in a prebaked (2 hours at 200°C), sterile glass tube, 2mL
2. Add 13.33 μ L 1500 μ M albumin for a final concentration of 500 μ M (1:10 complex) or
- Add 6.67 μ L 1500 μ M albumin for a final concentration of 250 μ M (1:5 complex)
- 10 3. Add 5 μ L 800mM KOAc buffer for final concentration of 100 μ M
4. Add RNase free water for total volume of 40 μ L
5. If more siRNA is needed the volumes can be scaled up linearly
6. Incubate the siRNA:albumin protected from light at 25°C for 24 hours.
7. Keep at 4°C for 24 hours or at -20°C if stored for longer.

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Biodistribution studies of Albumin variants in NMRI mice

Aims

- Establish experimental procedures for half-life and biodistribution experiments by IVIS using new Alexa 680 labeled albumin.
- 20 - Determine the half-life and biodistribution of LB, WT and HB in NMRI mice.

Experimental set-up

For each albumin, LB (HSA with mutation K500A and a lower binding affinity, than wild-type HSA, to FcRn), WT (wild-type) and HB (HSA with mutation K573P and a higher binding affinity, than wild-type HSA, to FcRn), a separate experiment was carried out, using 8-9 week old female NMRI mice divided into 3 groups of: 4 treated and 1 untreated animal. Each treated animal received 400 μ L of WT or LB or HB in PBS, or PBS only, by tail vein injection. The amounts of injected albumin were adjusted so that each animal received 65 μ g of labeled material. One minute post-injection, blood was taken from each animal by chin-vein puncture. Blood samples of <50 μ L were taken from the animals according to the following table:

Table 17: Representation of the time points in the experiments

Group	N	0h	4h	8h	24h	48h	72h	96h	120h
1	5	Blood	Blood	-	Term	-	-	-	-
2	5	-	-	-	-	Blood	Term	-	-
3	5	-	-	Blood	-	-	-	Blood	Term

(Term = termination)

At termination, larger blood samples ~ 500µL were collected before the animals were sacrificed. The blood samples were left to coagulate for 30mins at RT before centrifugation and collection of serum. Serum was frozen and stored at -80°C for later

5 scanning. The serum samples were scanned in quartz capillaries of different sizes: Larger 20µL capillaries could only be used for the terminal blood samples; whereas 5µL capillaries were used for the intermediary blood samples (larger capillaries give a stronger signal).

10 After sacrifice, organs were collected from the animals, carefully cleaned of residual blood, and scanned for presence of fluorescent signal. Further, it was confirmed using native gel electrophoresis, that intact, non-denatured and non-degraded labeled albumin was present in the blood samples.

The setup was the same for all 3 experiments.

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Results

Albumin levels in blood at 24-120 hours

The terminal blood samples were analysed using the larger, 20µL capillary tubes and
20 the signals were obtained (see Figure 36).

At 24 hours, the highest signal was observed with the HB, and lowest with the LB. At 72 and 120 hours, only small differences were detected.

Albumin levels in blood at 0-48 hours

25 This data (Figure 37) was performed using the thin, 5µL capillaries.

This data (Figure 37) has been normalized to the blood samples at T0, which were all collected 1 min after injection of the labeled material. Spectral un-mixing was not used, since the background in serum samples at 675nm is negligible. The analysis was carried out at the excitation/emission filter set 675/740, where the signal/noise ratio was

30 largest.

A very large initial loss of signal was observed, with two thirds lost in the first 4 h. This is possibly due to translocation of albumin from the blood into the extravascular tissue and loss by excretion. Following the initial phase, an exponential removal of albumin was observed.

From these time-points, the following half-life can be calculated:

Table 18: Half-life calculated on the basis of Figure 2, and initial loss of signal over 4 hours.

	T _{1/2} /hours	Initial loss / 4 hours
LB	12.6	66%
WT	14.1	63%
HB	15.8	59%

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The half-life is dependent on the timeframe used to calculate, and it seems from these results that there are at least two phases of removal, an initial (up to 4 hours after injection) and a more steady-state phase in the latter time-points. The calculated half-life values, therefore, vary depending on the timeframe of the calculation. The current

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setup does not allow for a detailed analysis of the initial phase, however, it is possible that there are differences in the behavior between variants in the first hours of the experiment.

A trend, however, was observed, where the half-life is seemingly dependent on the

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- There seem to be at least 2 mechanisms of albumin removal:
- An initial rapid clearance from the blood of two thirds of the material within the first 4 h
- A slower clearance that remains stable over a longer period of time (up to 48 h)
- It is, therefore, difficult to fit the data to a simple exponential model, and the calculated half-life depends on the timeframe chosen.
- The differences in calculated half-life over 48 hours show a trend with FcRn binding capability correlated to longer half-life. The largest difference between the intensity of the three variants was observed at 24 hours.
- The initial amount of signal lost is also dependent on the binding, for the LB material is mostly lost in the initial phase of the experiment.
- The effects of the FcRn binding on half-life modulation are not very pronounced, but this could be due to very low expression of FcRn in adult mice FcRn FcRn.

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Albumin levels in carcass and organs

Following sacrifice of the animals, heart, lungs, kidneys, spleen, liver and bladder were collected, and scanned in the IVIS. The data (Figure 38) was un-mixed and background successfully removed, followed by organ albumin level quantification.

The data suggests a difference in albumin levels between the kidneys and the liver, however, there is no clear decrease/ or increase correlated with the FcRn binding affinity.

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After removal of the internal organs, the remaining carcass was scanned in the IVIS. After performing spectral un-mixing and quantification, the albumin levels were measured (Figure 39).

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At 24 hours, there are differences between the observed signals between the albumin variants. From 24 hours to 120 hours the HB and WT follow an exponential removal. The LB shows a faster removal until 72 hours, leading to an 8-fold difference at 72 hours between the LB and the other variants. At 120 hours, 5-fold less LB was observed in the tissue than HB. It, therefore, seems that there is a difference in the behavior of these albumins over the time course of 120 hours in the peripheral tissue.

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The WT and HB can be fitted to an exponential function with $R^2 > 0.99$, whereas the LB behaves differently with $R^2 < 0.9$.

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Conclusions

- Blood analysis shows an initial difference between the clearance rates of the materials in the first 48 hours. A loss of 66%, 63% and 59%, for the LB, WT and HB, respectively, was observed indicating a more rapid removal of the LB.
- Half-lives for the first 48 hours are calculated to be 12.6 hours, 14.1 hours and 15.8 hours, for the LB, WT and HB respectively. At 120 h the trend is no longer observable, with very small variations between the variants.
- These observations indicate that a possible FcRn binding effect is more pronounced in the initial phase after injection.
- No clear differences for the variants were observed in the isolated organs, however, less HB was observed at 24 hours and this may reflect greater recycling.
- Differences were observed between the variants in the carcass, representing the peripheral tissue. The HB and WT show exponential removal, where the LB signal was removed much faster at 24-72 hours. The largest difference between the variants was observed at 24 hours in the blood, and at 72 h in the peripheral tissue.

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Biodistribution Studies of Albumin Variants in Wistar Rats

Aims

- Establish experimental procedures for half-life and biodistribution experiments in rats using IVIS bioimaging and new Alexa 680 labeled albumin.
- Determine the half-life and biodistribution of LB and HB in Wistar rats.

Rat albumin biodistribution

650µg of either LB or HB was injected into the tail vein of female Wistar rats. The animals were split into two groups of 5 animals: 1 PBS, 2 low-binder (LB), 2 high binder (HB). Blood samples were taken according to the following time schedule (table 19):

	T0	T1	T4	T8	T24	T32	T48	T56	T72
Group 1	X	X	X	X	X				
Group 2	X					X	X	X	X

Serum was isolated from all blood samples and fluorescence was measured using the IVIS. At the terminal time points 24 hours and 72 hours, animals were sacrificed and organs were taken for scanning.

The following organs and tissues were taken:

Internal organs: Kidney, lungs, liver, heart, spleen, bladder.

Peripheral tissues: Abdominal fat, external fat (from hip), skin (from back), stomach lining and muscle (hind leg).

Blood

Fluorescence was quantified in all blood samples (Figure 40).

A difference between the two variants was observed at all time points, with the difference becoming larger at the later time points. There was no fast, initial loss of signal, as was observed with the mice. In this experiment, all the time points can be fitted to an exponential function. The calculated half-lives are (Table 3):

Table 20: Half-lives calculated from the blood fluorescence at time intervals 0-24 hours and 32-72 hours respectively.

	0-24h	32-72h
LB	6.6 h	10.7 h
HB	9.8 h	13.6 h

The calculated half-lives are similar to what has been previously reported on human albumin in rats (Renal clearance of human and rat albumins in the rat, Gaizutis M, Pesce AJ, Pollak VE 1975) of 15.8 hours (calculated over 5 days).

5 Organs and Tissues

At 24 and 72 hours, respectively, the organs were scanned and the data quantified (Figures 41-44).

There was more High Binder present in most of the tissues after both 24 and 72 hours.

- 10 The largest differences were observed in the kidneys and skin, where a 2-fold difference was observed. The trend of the biodistribution was, however, the same, and there was no indication that the HB or LB preferentially accumulates in any particular tissue. In conclusion, a difference in blood concentration was observed between HB and LB. The largest difference was again, as in the mice, observed at 24 hours. At the
- 15 subsequent time points both albumin variants behaved similarly. A difference in concentration was also observed in the organs and the general biodistribution was very similar for both the albumin variants.

Immunohistochemistry on rat intestines

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- The biodistribution data from the IVIS has been supplemented with immunohistochemistry on rat small and large intestine from the animals sacrificed 24 hours after injection (Figures 45-7). The organs were embedded in Tissue Tek OCT medium, frozen immediately in liquid nitrogen and stored at -20°C until sectioning. The
- 25 frozen tissue samples were cut using cryostat, in sections of 5µm for small and large intestine.

- Tissue sections from control, low-binder (LB) and high-binder (HB) treated rats were fixed in ice cold acetone and kept at -20°C for 10 minutes. DAPI (dark gray) and
- 30 ATTO488-phalloidine (light gray) co-staining was performed for nucleus and actin cytoskeleton, respectively. The sections were analyzed for Alexa Fluorophore 680-labeled albumin presence (gray).

Conclusion

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- Intravenous injected albumin can migrate to intestinal epithelium
 - HB may accumulate more than LB

- Albumin can potentially be utilised as a payload carrier for treatment of intestinal diseases such as Inflammatory Bowel Disease (IBD) due to accumulation in intestinal tissue

5 Biodistribution of siRNA/albumin complexes in NMRI mice I: 0, 1 and 2 cholesteryl siRNA

Aim

Determine the effect of 0, 1 and 2 cholesteryl modifications on the half-life and
10 biodistribution of siRNA.

Experimental setup

Animals were divided into 3 groups injected with siRNA according to Figure 48.

- 15 The siRNA was labeled on the AS strand with Cy7, and contains for increased stability LNA nucleotides in the sense strand.

The siRNA was pre-formulated 1:10 with HB. 1 nmol of siRNA and 10 nmol of albumin were injected in each animal in 400µL PBS *via* tail vein injection. After injection, blood
20 samples of 5µL were taken from the tail vein at 1, 15, 30, 60, 120 and 240 min, and analyzed in the IVIS scanner. At 360 mins the animals were terminated and the organs and bodies scanned for presence of fluorescent signal.

Results

25 Blood

Blood samples were collected in 5µL capillaries and scanned using IVIS. The normalized fluorescence intensity for each group is shown in Figure 49.

There was a clear difference in the serum half-lives of the different siRNA species. The
30 0 cholesteryl siRNA was rapidly cleared with only 10% left after 15 mins. The modified siRNA, however, exhibited a much slower clearance, with the double modification showing lower clearance than the single modification. Half-lives were estimated (Table 21 .

Table 21

siRNA	Half-life	Amount left after 6 hours
56	~12mins	<0.1%

57	45 mins	0.5%
58	71 mins	3%

As expected, increasing the affinity towards albumin increases the circulatory half-life. The number of cholesteryl modifications plays an important role, as expected, since more modifications increase the affinity towards albumin further.

- 5 At 6 hours after injection, the animals were sacrificed and organs were collected and scanned using the IVIS (Figure 50).

None of the siRNA variants appeared in the organs in significant quantities, but can be detected in the following:

- 10 siRNA 56 was only detected in trace amounts in the kidney, this suggests rapid excretion.

siRNA 57 was observed in the liver and kidney and was strongly present in the gallbladder.

siRNA 58 was observed in larger amounts than siRNA 57 in kidney, liver, gallbladder.

- 15 Trace amounts were also seen in spleen, lungs and stomach.

Conclusion

Blood analysis shows that by modifying siRNA with cholesteryl its serum half-life can be improved. Further it shows that the number of cholesteryl modifications plays a role,

- 20 with two modification performing better than one.

Modifications can change the biodistribution of the siRNA.

Oral delivery of Albumin Variants to healthy mice NMRI mice

- 25 Aim

Visualization of LB, WT and HB after oral administrations in healthy NMRI mice.

Characterization of the effects of non-fluorescent feed on the background fluorescence of the animals and the effect of this on the IVIS live bioimaging.

- 30 Experimental setup

The animals were kept on a special, non-fluorescent diet ("phytoestrogen-free" mouse chow D10001 AIN-76A, Research Diets, Inc., NJ, USA) for 10 days prior to experimental onset. 1 animal received normal feed for comparison (Figure 51).

At T=0, 16 animals were dosed with either PBS, LB, WT or HB, with 4 animals in each group. 200µg of labeled material was dosed in 300µL of PBS by oral gavage. One animal from each group was scanned alive at ½, 2, 4, and 24 hours after dosing, followed by blood sampling, organ harvest and scanning.

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Results - Scanning of live animals

Figure 51 represents the live animal scan at the 4 time points, the data is quantified in Table 15. Very little signal was observed at 4 and 24 hours. At T=½ a strong signal was observed in the HB-administered mouse, and a very weak signal was observed in the remaining animals. At T=2 a strong signal was observed in the WT-administered and HB-administered mice.

Table 22: Quantified data from Figure 51

Time	0.5	2	4	24
PBS	-	-	-	-
LB	1.2e8	1.9e9	2.5e8	-
WT	2.0e8	1.1e10	1.7e8	-
HB	1.4e10	4.2e10	-	-

15 Organs

At T=1, 2, 4 and 24 hours, animals were sacrificed and the following organs harvested: Stomach, small intestine (divided into two pieces), cecum, colon, bladder, liver, kidneys, spleen, heart, lungs and fat tissue. The remaining carcass was also scanned. All organs were carefully flushed and cleaned before scanning, to ensure removal of residual blood, feed and feces. Blood samples were taken before sacrifice (Figure 52).

A signal in the stomach and the intestine was observed at 1 hour for all three dosed animals. Most material was localized in the HB stomach and upper small intestine. Two hours after dosing, a strong signal was observed only in the WT animal. After 4 hours, the signal was still present in the WT and HB, but at a much lower intensity than at 1 and 2 hours. Small amounts of signal were detected in the liver and fat for all three albumins at T=1.

It seems from these results that the variation between animals is quite large. The trend observed, however, is that the WT and HB albumin was present for a longer time in the stomach and intestine than the LB. The time frame of 24 hours was too long for these experiments, since very weak signal were observed as early as 4 hours. Higher doses of

albumin may be needed to observe systemic translocation and immunohistochemistry to investigate cellular uptake.

Accumulation of albumin in DSS induced IDB mice

Aim

- 5 Study the influence of inflammation on the accumulation of HB in the intestines.

Experimental setup

Inflammation was induced for 5 days prior to dosing by 3% DSS in the drinking water of the animals (n=2). Control animals got normal water (n=2). Weight loss was

- 10 observed in the DSS induced mice indicative of disease induction.

After the 5 day induction of inflammation the animals were dosed IV with 65µg of Alexa 680 labeled HB albumin or PBS buffer. 24 hours after the injection, the animals sacrificed and organs were scanned in the IVIS. The following organs were scanned

- 15 Stomach

Upper small intestine

Lower small intestine

Cecum

Colon

- 20 The colon length was reduced, and the intestinal walls very thin and fragile, corresponding to an inflamed state of the tissue. Darkening and enlargement of the gall-bladder was also observed in the DSS animals.

Results

- 25 The organs of the animals were scanned using the IVIS live imager, and analysed by spectral unmixing using a library previously created by subcutaneous injection of Alexa 680 albumin (See Figure 53).

- 30 There are large differences between the accumulation between the animals (See Figure 54). In the healthy animal, strong signal was observed in the upper part of the GI including stomach and small intestine, whereas the DSS induced IBD animal shows large accumulation in the cecum and colon that are the inflammatory sites of this disease.

Design and characterization of siRNA

A range of cholesteryl modified siRNA and single strand (antisense) RNA – see Figure 55.

The binding experiments for Phase II are summarized in Figure 56. The K_d values are
5 acquired by quantification of bands from mobility shift assay experiments.

It was observed that increasing the number of cholesteryl modifications increases the binding strength. Generally, a single stranded nucleotide has higher affinity than a double strand with the same type of modification, compared 31 to 34 and 30 to 33.

10 This is likely caused by increased steric hindrance in the duplex, making the single stranded nucleotide more flexible. For the duplex, we observe increasing binding with increasing number of cholesteryl modification.

The data also indicate that the 3' modifications provide more efficient binding than the
15 mid-sequence modifications, compare 33 to 36. Again this is likely caused by steric hindrance of the mid-sequence modification, which is less accessible for binding than the 3'.

Conclusion

20 Increasing the number of cholesteryl modifications increases binding efficiency further
Terminal modifications (3') provide stronger binding than mid-sequence modifications
Increasing the number of cholesteryl modifications promotes formation of albumin
larger complexes.

25 LNA modified siRNA

LNA (locked nucleic acid) can be incorporated into siRNA to improve the efficiency of annealing and to reduce degradation and deannealing in vitro and in vivo. LNA modified siRNA allow the characterization of the cholesteryl:albumin binding in serum and in vivo where non-modified siRNA would be rapidly degraded.

30 The following LNA oligoes were included in the binding and serum degradation experiments to see whether the cholesteryl:siRNA interaction could still be utilized in the presence of chemical modifications. See Figure 57.

Non-cholesteryl LNA siRNA 52 does not interact, as expected (See Figure 58)

The modified siRNA 54 binds with similar affinity as non-modified. Very little dimer formation is observed, however, possible due to increased steric hindrance because of the more rigid oligo.

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Cy7 labeled siRNA

For biodistribution experiments, Cy7 modified siRNA was included (See Figure 59). The Cy7 was combined with cholesteryl and LNA, to enable studies on the effect of

10 cholesteryl modifications in vitro.

Albumin dimers

Increasing the number of modifications promotes formation of albumin complexes,
15 which correspond on the gel to albumin dimers and trimmers (See Figure 60).

The formation is likely caused by one siRNA molecule being able to interact with several albumin molecules, and can potentially influence significantly the serum half-life of such complex.

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The dimer formation is also observed for the single stranded RNA with 2 cholesteryl modifications (see Figure 61).

Evaluation of siRNA efficiency

25 The aim of these experiments is to determine whether cholesteryl modified siRNA is still biologically active (see Figure 62).

This indicates that siRNA 33 and 34 which are only modified on the SS are still highly active (>80% KD), whereas modification of both strands somewhat reduces the
30 efficiency (~40% KD). This data also indicates that the current system does not induce knockdown with albumin as the only transfectant.

Even extensively modified siRNA is still active, which means that the modifications suggested here can be used in a therapeutic context.

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Cross species affinity screen

The following recombinant albumins have been used for the binding experiments, all supplied by NZ:

Name	Gene
Rat Serum Albumin	NCBI BC085359
Mouse Serum Albumin	NCBI BC024643
Macaque Serum Albumin	NP_001182578
Pig Serum Albumin	CAA30970.1
Rabbit Serum Albumin	NCBI U18344
Bovine Serum Albumin	NP_851335.1

5 Results

All the binding gels have been run in a similar setup, where the siRNA amount is constant and the albumin amount decreases from right to left in Figure 63. The more clear bands present in the top left, the higher the affinity. Due to the solubility issues with the cholesteryl siRNA, the free siRNA is less visible in the gel than the albumin-bound.

The following order of affinities has been observed:

Lowest

Rabbit<<Pig<<Bovine<<Rat<<Macaque<<Mouse<<Human

15 Highest

The data indicates that the interaction of the cholesteryl siRNA varies with the species, with preferred binding to human. This supports a specific binding site for the interaction.

20 Binding of siRNA to albumin variants

Binding

Aim was to determine the effect of mutations on the FcRn binding region of albumin on the albumin:siRNA interaction of cholesteryl modified siRNA 34. In the study (see Figure 64), three variants were included: Low binder (LB), wild type (WT) and high binder (HB). Further, a rat serum albumin was included.

Competition

To further assess the binding of siRNA 34 to the variants, a competition study as described previously was done with the LB, WT and HB with siRNA to verify the binding mechanism.

The same pattern was observed for all three ligands, indicating that the siRNA binds via the mechanism and binding site to all three variants. See Figure 65.

Serum degradation of siRNA

Non-modified

The aim is to determine whether the preformulation of cholesteryl siRNA with albumin can influence the rate of serum degradation of the siRNA.

The initial experiment was done using non-LNA siRNA to look at degradation (see Figure 66).

This data indicates that albumin has a protective effect on siRNA, which is present even after 24 hours in serum when cholesteryl modifications are present. The non-modified siRNA is degraded quickly.

LNA

The serum degradation was repeated using the LNA modified siRNA 52, 53 and 54, to determine whether the siRNA: albumin complex stays together over time when incubated in serum, See Figure 67.

Reduction in immune response

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The aim of this experiment was to determine the TNF- α response of Primary Blood Monocytic Cells (PBMC) towards the siRNA, to assess the therapeutic potential of the system.

This data (see Figure 68) indicates that albumin preformulation of cholesteryl siRNA can lower the TNF- α production. Therefore, formulation of these siRNA with albumin can potentially lower the immuneresponse towards modified nucleic acids.

Albumin annealing of siRNA

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Due to the very hydrophobic modifications, there have been issues with proper preparation of siRNA 57 and 58, where cholesteryl and Cy7 modifications are combined (see Figures 69).

- 5 Concentration assessment of the oligonucleotides and gels revealed that the annealing is efficient, however, the product siRNA is not soluble and therefore precipitates out as soon as is anneals. Temperature cycling experiment revealed an annealing temperature of approximately 68 °C, below which the oligonucleotides become insoluble when mixed. Each of the oligonucleotides alone is readily soluble during temperature cycling, suggesting that indeed it is the double stranded siRNA which causes the issues.

Concentration measurement of the oligonucleotides reveal that about 40% of siRNA 57 and 90% of siRNA 58 precipitates out during annealing. Several annealing vessels were tried to see whether the hydrophobic wall of the normal eppendorf tube influenced the precipitation:

- 15 Normal Eppendorf

LoBind tube from Eppendorf

Low Binder tube from Sarstedt

Glass tube

- Concentration assessment after annealing revealed that the siRNA precipitates out in all
20 tubes.

Following this, several different annealing procedures were tried (using siRNA 58).

Below is a summary of the tested parameters, as well as a gel example of the assessment. Gel data was supplemented by concentration measurements on all

- 25 oligonucleotides. See also Figure 69.

Procedure	Result
RT annealing 1:1 plastic	Annealing OK, precipitates
RT annealing 1:3 plastic	Annealing better, more product, 30% of the total concentration
RT annealing 1:1 glass	Annealing OK, large aggregation
RT annealing 1:3 glass	Aggregation visible on gel, lower yield
Addition of TWEEN 0.1%	Aggregation
Addition of Ethanol 15%	Low efficiency annealing
Addition of DMSO 1%	Low efficiency annealing
Addition of acetonitril 1%	Low efficiency annealing
Slower cooling (2 and 3 hours instead	Annealing OK, still precipitated below 70 degrees

of 1)	
Lower concentrations (20, 10 and 5 μ M instead of standard 50 μ M)	Does not anneal well
Longer heating (2, 5 minutes instead of 1)	Annealing OK, still precipitated below 70 degrees
Annealing at lower temperature (80 degrees)	Annealing OK, still precipitated below 70 degrees
Annealing with albumin at high temperature	Albumin denatures and forms aggregates
Annealing with albumin at RT	Efficient annealing with no single strands visible

Due to the success of the albumin annealing competition assays were carried out to determinewhether the resulting complex behaves as the normally annealed siRNA.

First, siRNA 56 (no cholesteryl, Cy7), 57 and 58 were annealed with WT albumin, and siRNA 58 was annealed with LB, WT and HB albumin. The oligonucleotides were run on

5 a gel and scanned for both Cy7 and SYBR Gold. See Figure 70.

The gel suggest a homogeneous product with no left over single strands, and is bound to albumin. siRNA 58 forms some dimer, as expected, whereas siRNA 56 shows no binding.

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Following this a series of experiments were done to determine the behavior of siRNA 57, which can be annealed with and without albumin. Competition ligand screen where a series of ligands were used as competition ligand. See Figure 71.

15 The following ligands were used:

1	Albumin
2	Stearic acid+warfarin
3	Sodium octanoate
4	Fusidic acid
5	Sodium myristate
6	Lithocholic acid
7	Cholesterol
8	Myristic acid
9	Stearic acid

These gels indicate that stearic (and some by myristic acid) can displace the siRNA from the albumin, as was observed previously in all previous competition experiments. There is no significant difference between the annealing with/without albumin.

Following this, a stearic acid titration was carried out on both annealings to determine any difference in binding strength. See Figure 72. The amount of stearic acid decreases from left to right. Again there are no significant differences between annealing with/without albumin, and the annealing product look homogenous in both cases.

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To follow this up, a competition experiment with siRNA 58 annealed with albumin was done. The control experiment without albumin was not included because the siRNA 58 cannot be annealed efficiently without. See Figure 73.

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This complex, again, behaves were similarly to the single cholesteryl siRNA 57, but is more difficult to remove with stearic acid, as expected when increasing the number of cholesteryls.

15 In conclusion, the albumin annealed siRNA complex seems to behave similarly to the normally annealed siRNA we have previously used.

The data indicate that albumin can help efficiently anneal and solubilize siRNA which is very hydrophobic.

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Serum degradation of albumin annealed siRNA

To further characterize the siRNA annealing with albumin, a serum degradation experiment was done on siRNA 58 annealed with albumin. A non-albumin control could not be included because this siRNA cannot readily be solubilized, and therefore cannot be annealed, without albumin.

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Because the siRNA is Cy7 labeled, dual detection was done, were siRNA was detected via SYBR-Gold, and the Cy7 was detected directly. See Figure 74. This data again indicates that some the siRNA:albumin complex is still present after 72 hours serum

30 incubation.

Binding of siRNA to labeled albumin

The aim was to determine the effect of Alexa 680 labeling of albumin on the Cys34 on cholesteryl interactions. The LB, WT and HB previously described have been assessed for this, all labeled with Alexa 680 using standard maleimide chemistry and protocols supplied by the manufacturer. See Figure 75.

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There are issues with the staining of the siRNA with SYBR-gold when the labeled albumins are present, this can possibly be due to traces of solvent left from the labeling which interfere with the labeling. More staining of the free siRNA was also observed,
5 this may be due to solvent.

The experiments show that there is still strong interaction between cholesteryl siRNA and albumin when the albumin has been labeled with Alexa 680.

10 Covalent conjugation

Covalent conjugation of siRNA to albumin is mentioned in the patent. We have therefore set up a method of covalent conjugation of a DNA 22-mer to albumin using SMCC chemistry and targeting the Cys34 of albumin. This procedure can be directly
15 transferred to RNA. See Figure 76.

Protocol for SMCC activation

Dissolve SMCC in DMSO to 100mM concentration

Dilute Hepes 10X with Milli-Q water and adjust pH to 8.0

20 Mix in an eppendorf the oligonucleotide, SMCC, DMSO and Hepes according to the following scheme.

	DNA AS	SMCC	DMSO	Hepes pH 8,0	→ Overnight RT shaker
Equivalent	1	50	-	-	
Concentration	2mM	100mM	-	0,1M	
Volume	10μL	10μL	10μL	30μL	
N	20nmol	1μmol			
Order of addition	II	IV	I	III	

Follow the order of addition in the table:

DMSO 10μL

Oligo 10μL 2mM

25 Hepes 30μL

SMCC 10μL

Leave sample to incubate under 650rpm shaking at 25 degrees overnight.

The following day, take 3μL of sample diluted to 20μL with RNase free water for
30 analysis in the HPLC.

Run on a kinetex 2.6u XB-C18 100A column at a standard program for oligonucleotides,. As control, an unmodified oligonucleotide in the same buffer and DMSO amount should be used.

The HPLC should reveal >90% SMCC modified material.

5

After the SMCC reaction, the oligonucleotide is purified by precipitation to get rid of DMSO:

To the complete oligo sample (ca 57µL if prepared according to the above), add 60 µL RNase free water

10 Add 20µL 3M NaAc

Add 500µL EtOH:Acetone 1:1 purest grade

Leave 30mins at RT

Put for 30 mins in -20

Centrifuge at 12000g in 4 degrees for 60 min The oligo should be present in the pellet

15 along with salts

Remove the supernatant, wash 1x with 1mL 100% EtOH, pipet up and down very carefully. The pellet will get smaller, as salt is washed out, but should not dislodge. If it does, spin again for 10 min

Wash 2X with 70% EtOH

20 After final wash, remove supernatant, and leave the pellet to dry in the open tube for 15 mins at 40 degrees.

Dissolve the oligo in RNase free water to the desired concentration

Measure concentration on nanodrop to calculate yield of precipitation >60% is acceptable

25

Now the oligonucleotide can be coupled to albumin with the following protocol

For the conjugation, 100pmol of oligo and 1000pmol of albumin was mixed in volumes of 25µL.

30 100mM HEPES buffers were prepared by diluting a 1 M (Sigma H3537) 1:10. Three buffers were prepared with pH 6.5, 7.0 and 7.5, respectively, by adjusting pH with 1M NaOH.

Three reaction tubes were prepared with 20µL of the respective HEPES buffer

Three 1:10 (40µM) oligo dilutions were prepared in the three HEPES buffers

35 Three 1:3.75 dilutions of albumin (albacult, final: 400µM) were prepared in the three buffers

To each reaction tube, 2.5 μ L albumin and 2.5 μ L oligo solution was added for a total volume of 25 μ L

The reaction was left shaking at RT overnight (20h).

- 5 The resulting conjugates have been run on gels to determine whether the conjugation was successful. In this case the conjugation was done at three different pH to optimize the protocol.

- SYBR-Gold staining of the gel reveals presence of oligo in the albumin band, which
- 10 indicates successful conjugation of the oligo to the albumin. It is not possible to determine the reaction yield based on this gel, but it indicates that pH6.5 is beneficial for the reaction compared to pH7.0 and 7.5. See Figure 77.

Claims

1. A composition comprising a payload molecule and albumin or variant albumin or fragment thereof, or fusion polypeptide comprising albumin or variant albumin or fragment thereof.

5

2. A composition according to claim 1, wherein the payload molecule is selected from the group consisting of a small molecule, a synthetic small molecule, plasmid DNA, a peptide and a siNA.

10 3. The composition according to claim 1 or 2, wherein the payload molecule is a siNA.

4. The composition according to any of claims 1-3, wherein the siNA is conjugated with one or more cholesteryls.

15 5. The composition according to any of claims 1-4, wherein the siNA is conjugated with two or more cholesteryls.

6. The composition according to claims 1-5, wherein the siNA comprises a sense strand and an antisense strand, and wherein said sense strand is conjugated with said one or
20 more cholesteryl.

7. The composition according to claims 1-6, wherein the siNA comprises at least one terminal nucleic acid conjugation to said one or more cholesteryl.

25 8. The composition according to claims 1-7, wherein the siNA comprises at least two terminal nucleic acid conjugations to said one or more cholesteryl.

9. The composition according to any of claims 1-8 wherein the siNA comprises at least one mid-sequence conjugation to said one or more cholesteryls.

30

10. The composition according to claims 1-9 wherein the siNA comprises at least two mid-sequence conjugations to said one or more.

11. The composition according to claims 1-10 wherein the siNA comprises a
35 combination of at least one mid-sequence conjugation and at least one terminal nucleic acid conjugation to said one or more cholesteryl.

12. The composition according to claims 1-12 wherein the siNA has a nucleic acid length selected from the group consisting of 8-250, 8-120 , 16-50, 18-35, 18-25, 18-21, 18, 21, and 27.

5

13. The composition according to claims 1-12, wherein the siNA is a dicer substrate.

14. The composition according to claims 1-13, wherein the siNA further has a linker between the cholesteryl and the siNA.

10

15. The composition according to claims 1-14, wherein the linker is selected from the group consisting of disulfide moiety, amide, phosphate, phosphate ester, phosphoramidate, polyethylene glycol (PEG) or thiophosphate ester linkers.

15 16. The composition according to claims 1-15, wherein the siNA comprises further modifications selected from the group consisting of a dye label, a radioactive label, 3'OMe.

17. The composition according to claim 1-15, wherein the albumin is wildtype Human Serum Albumin (HSA), SEQ ID No. 2 or an albumin with at least 70%sequence identity to SEQ ID No. 2.

18. The composition according to any of claims 1-17, wherein the albumin, albumin fragment or fusion polypeptide comprises an alteration at one or more (several) positions corresponding to positions 417, 440, 464, 490, 492, 493, 494, 495, 496, 499, 500, 501, 503, 504, 505, 506, 510, 535, 536, 537, 538, 540, 541, 542, 550, 573, 574, 575, 577, 578, 579, 580, 581, 582 and 584 of the mature polypeptide of SEQ ID NO: 2 or as described in EP12191856.9.

19. The composition according to claim 18 wherein the variant is not the variant consisting of SEQ ID NO: 2 with the substitution H464A, D494N, E501K, E503K, E505K, H510A, H535A, H536A, K536E, I537N, K541E, D550G,A, K573E, K574N or K584E.

20. The composition according to any of claims 1-19, wherein the albumin variant has an alteration at position 573 or 500, particularly K573P, K573Y, K573W, K500A, K500D or K500G.

21. The composition according to any of claims 1-20, wherein the siNA and albumin is a siNA albumin conjugate.

5 22. A payload molecule modified by one or more cholesteryls.

23. A payload molecule modified by two or more cholesteryls.

24. The payload molecule according to anyone of claims 22 or 23, wherein the payload
10 molecule is selected from the group consisting of a siNA, a peptide and a small molecule.

25. The payload molecule according to anyone of claims 22-24 wherein the payload molecule is a siNA.

15

26. The composition according to any of claims 1-21 or the payload molecule of claim 22-25 being a pharmaceutical composition comprising one or more excipients, diluents and/or carriers.

20 27. The composition according to any of claims 1-21, the pharmaceutical composition of claim 26 or the payload molecule of claim 22-25 for use as a medicament.

28. The composition according to any of claims 1-21, the pharmaceutical composition of claim 26 or the payload molecule of claim 22-25 formulated for oral or mucosal
25 administration.

29. Use of albumin for the formation of a conjugate comprising the albumin and a payload molecule modified by at least two cholesterol.

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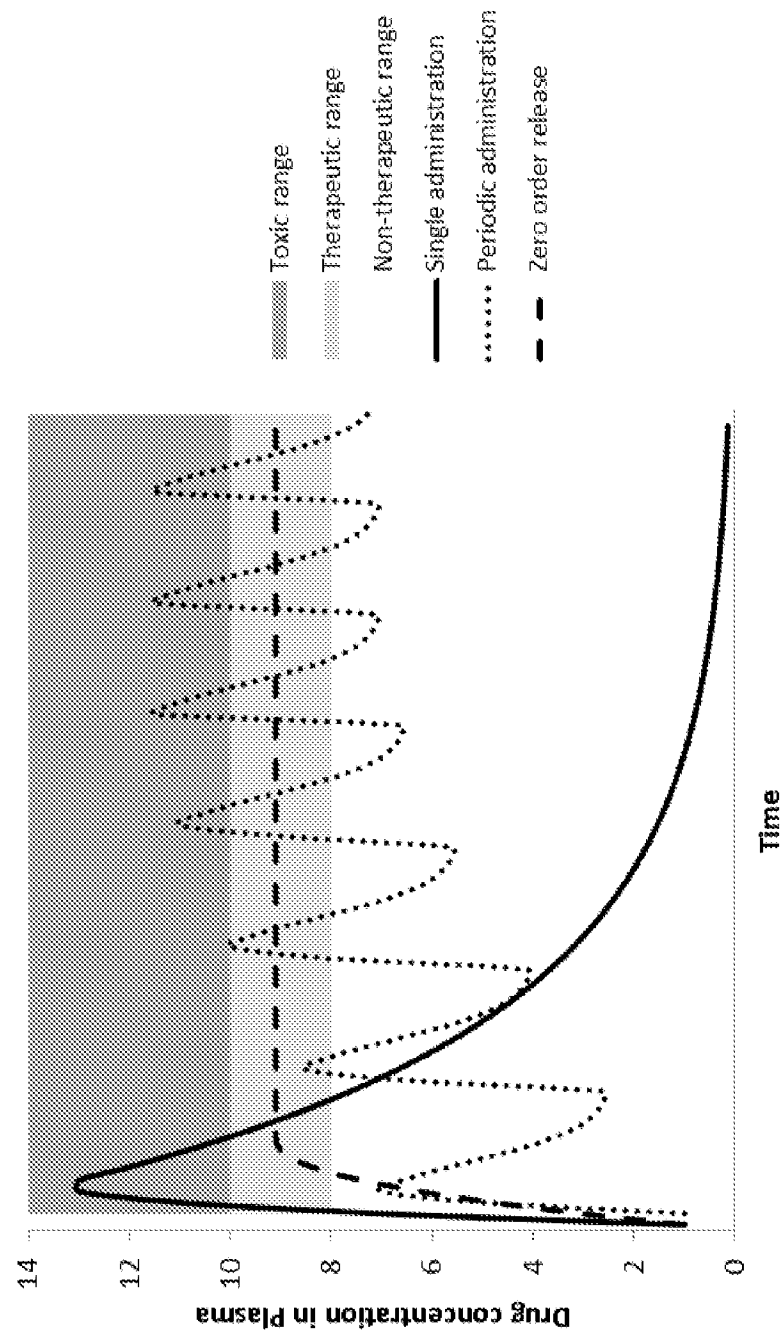


Fig. 1

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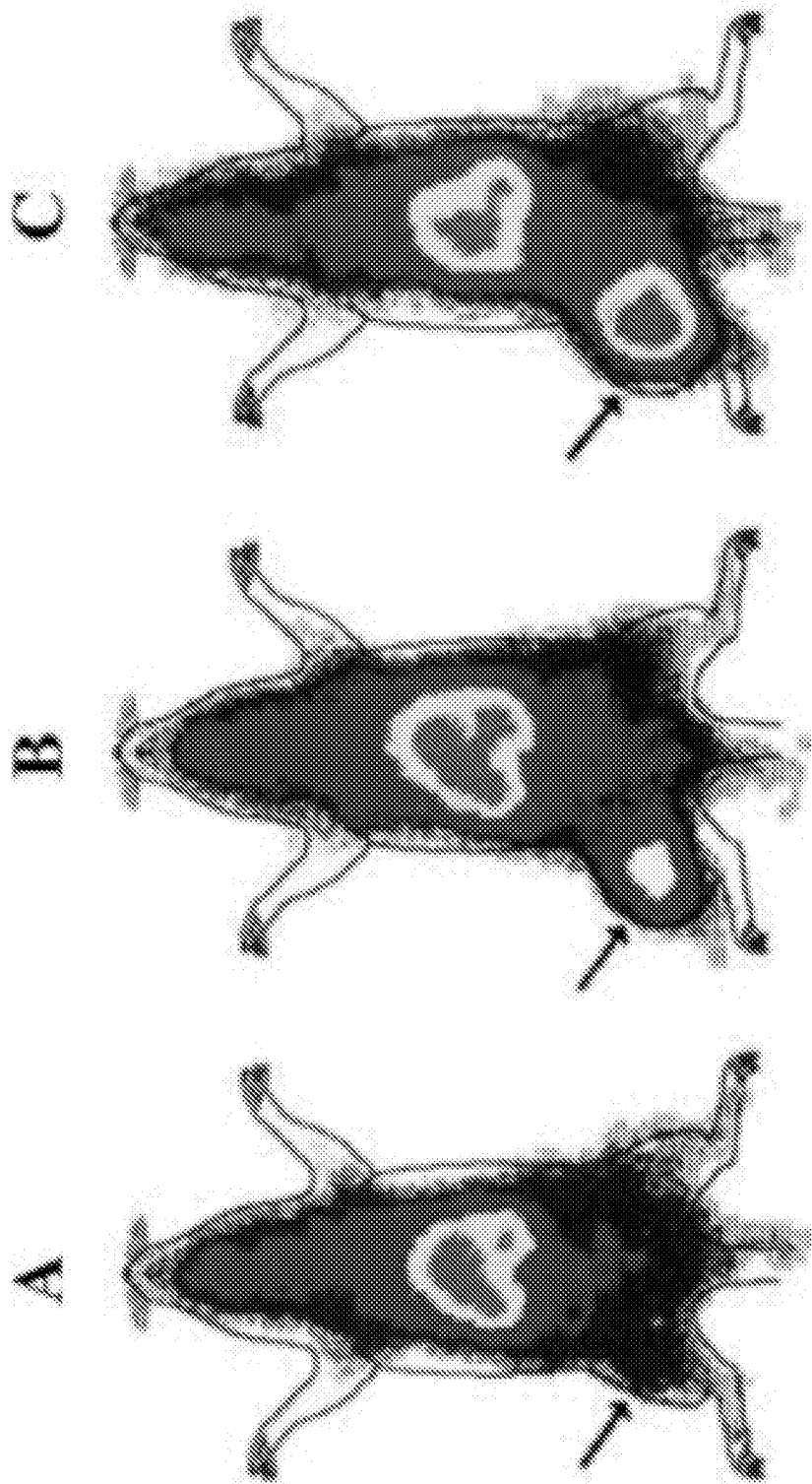


Fig. 2

3/78

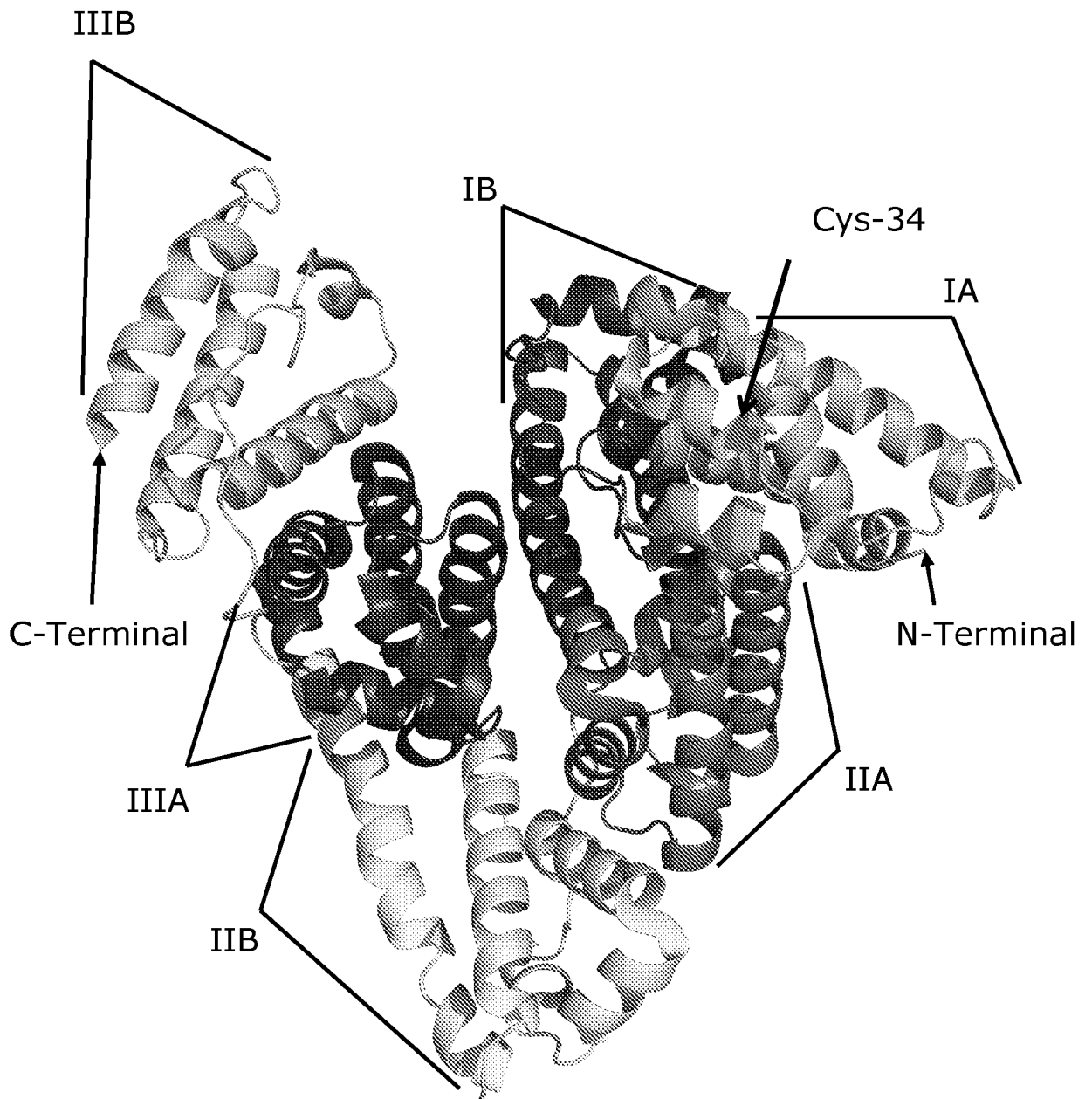


Fig. 3

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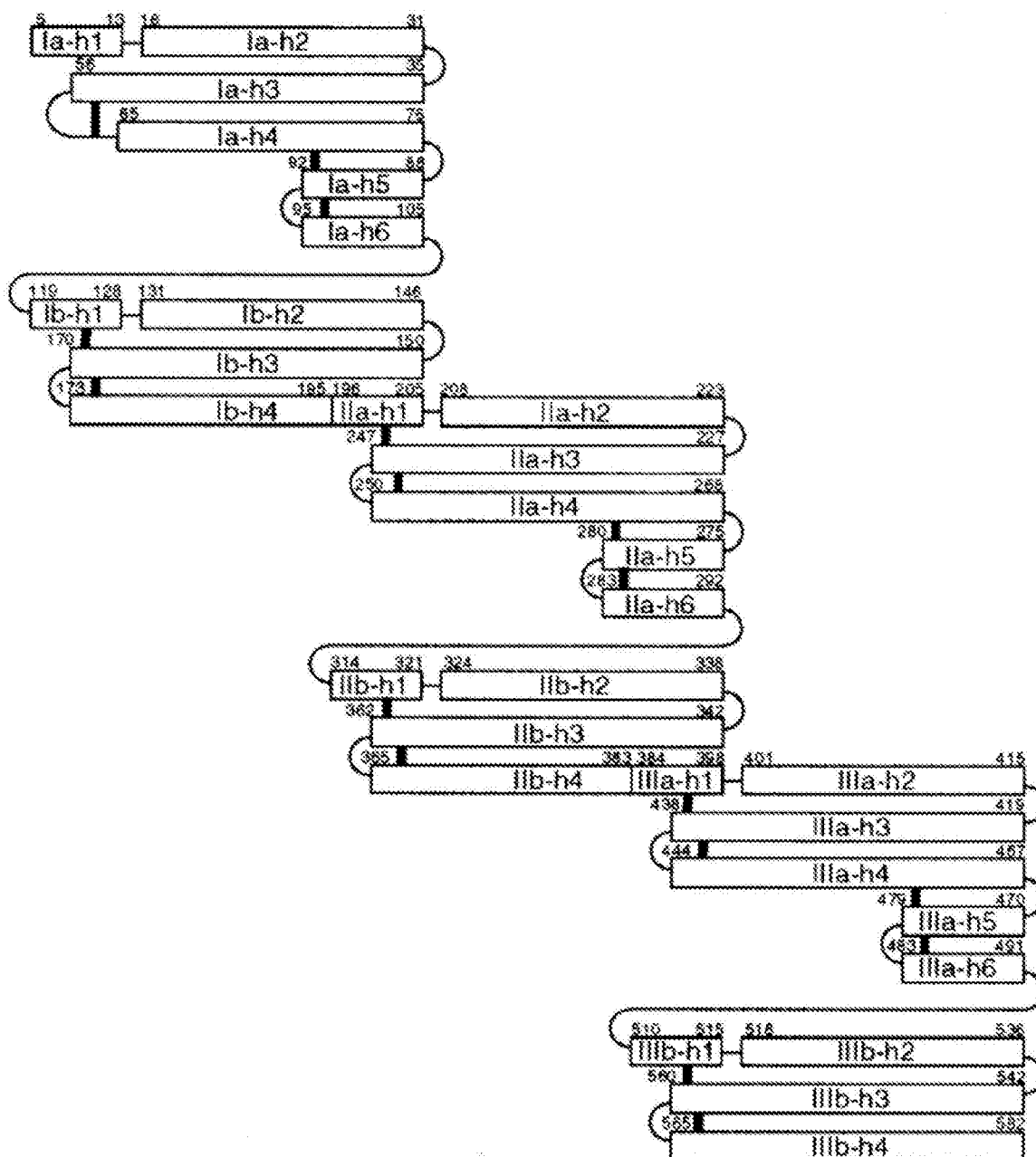


Fig. 4

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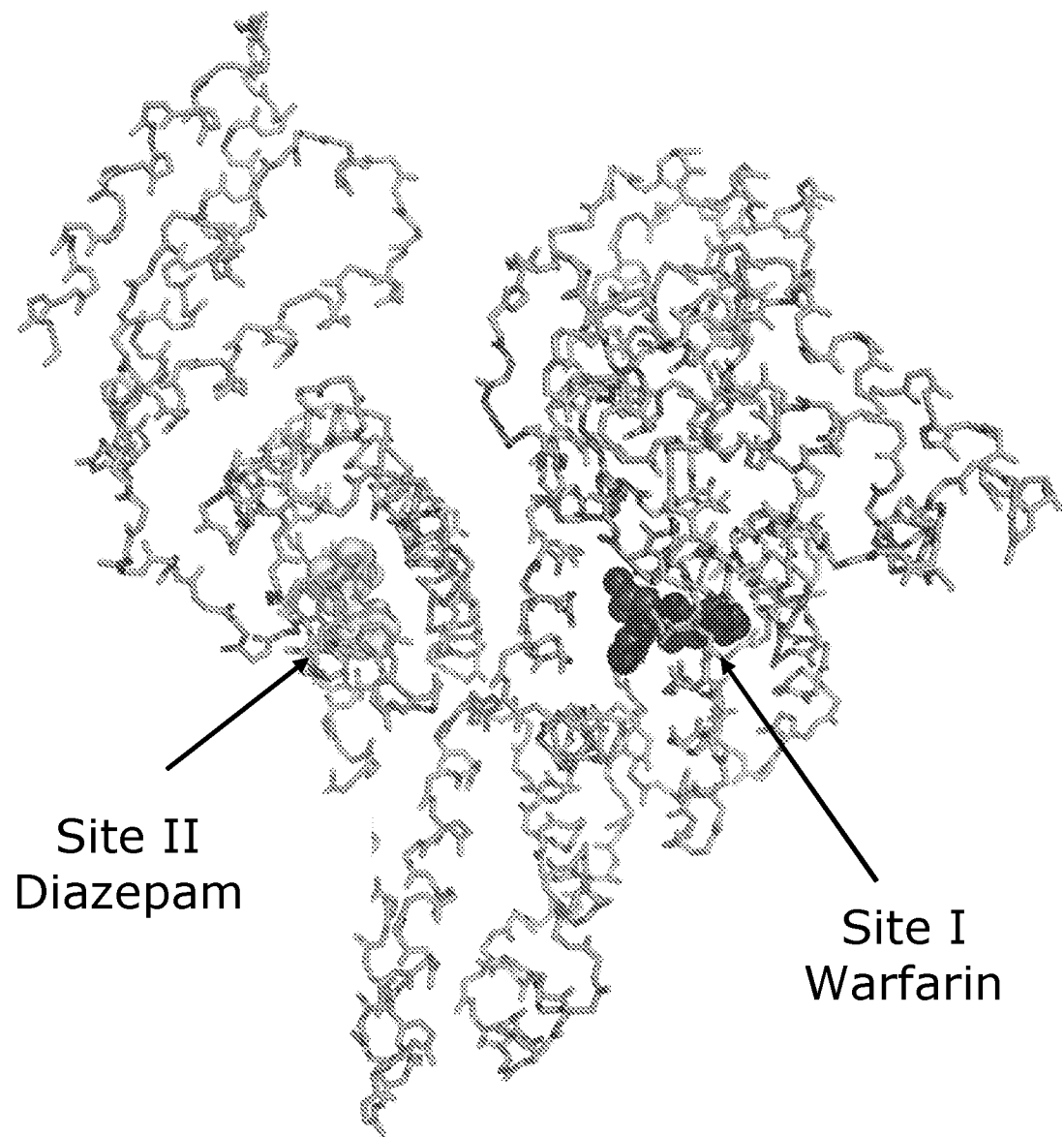


Fig. 5

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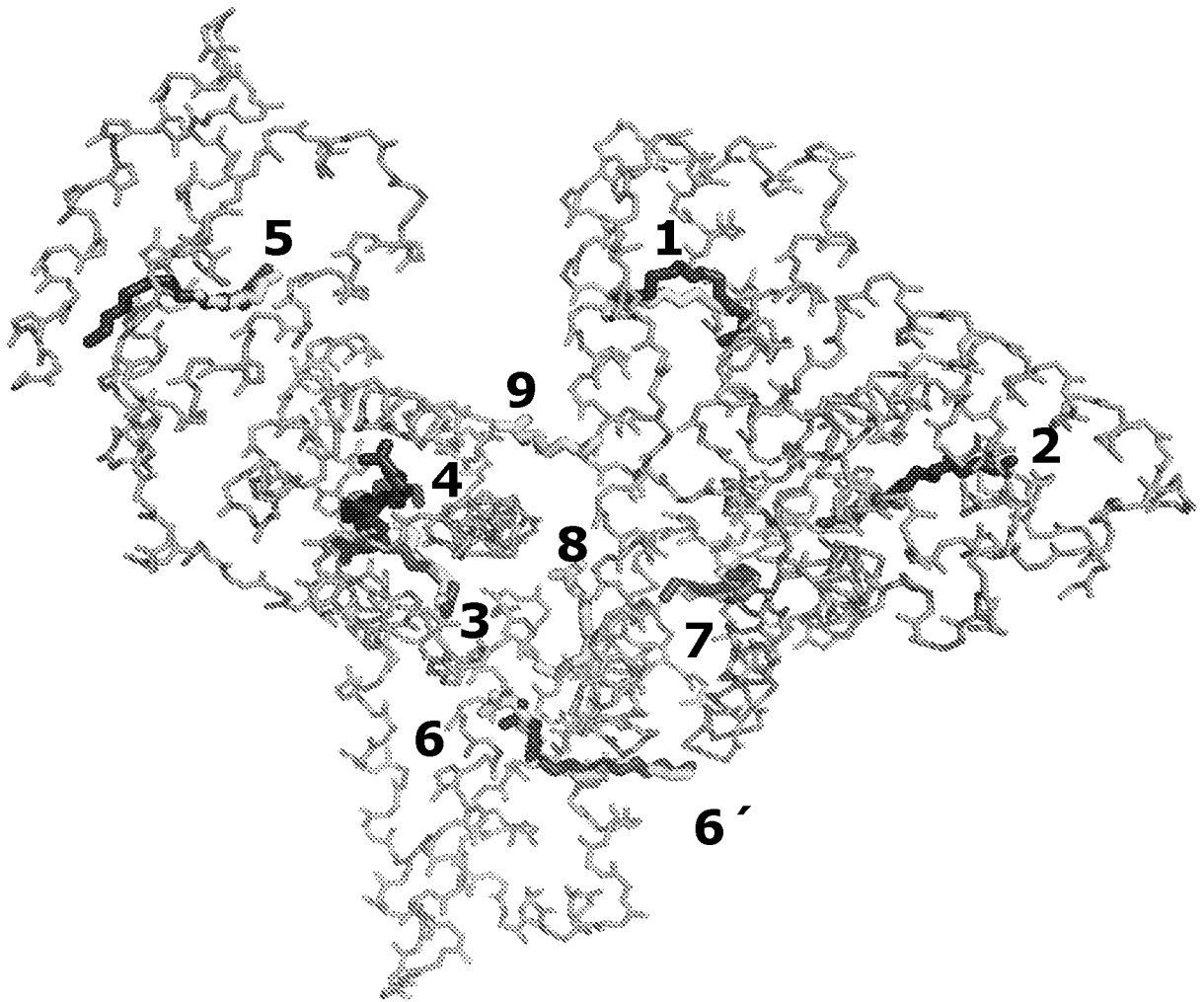


Fig. 6

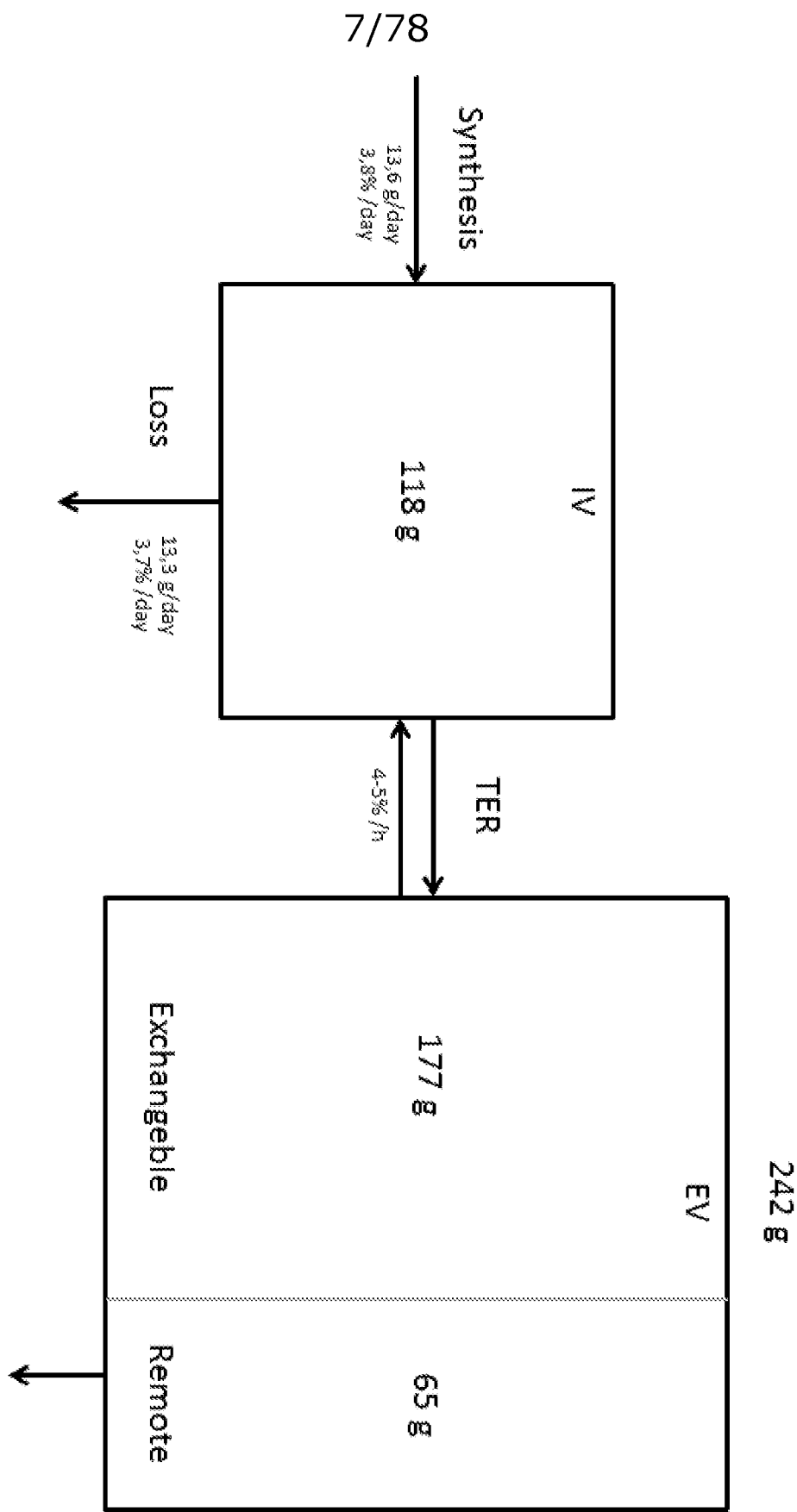
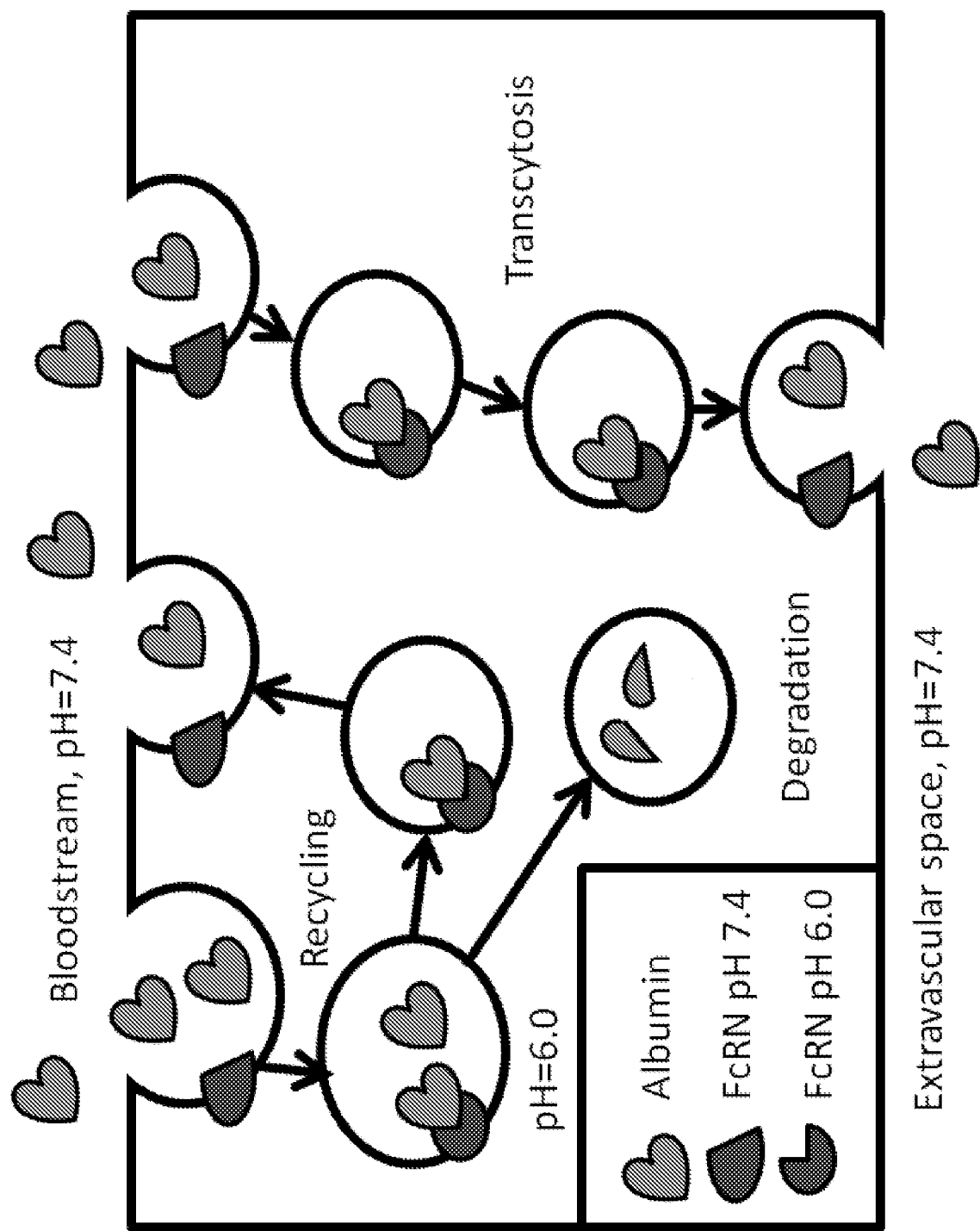


Fig. 7

Fig. 8



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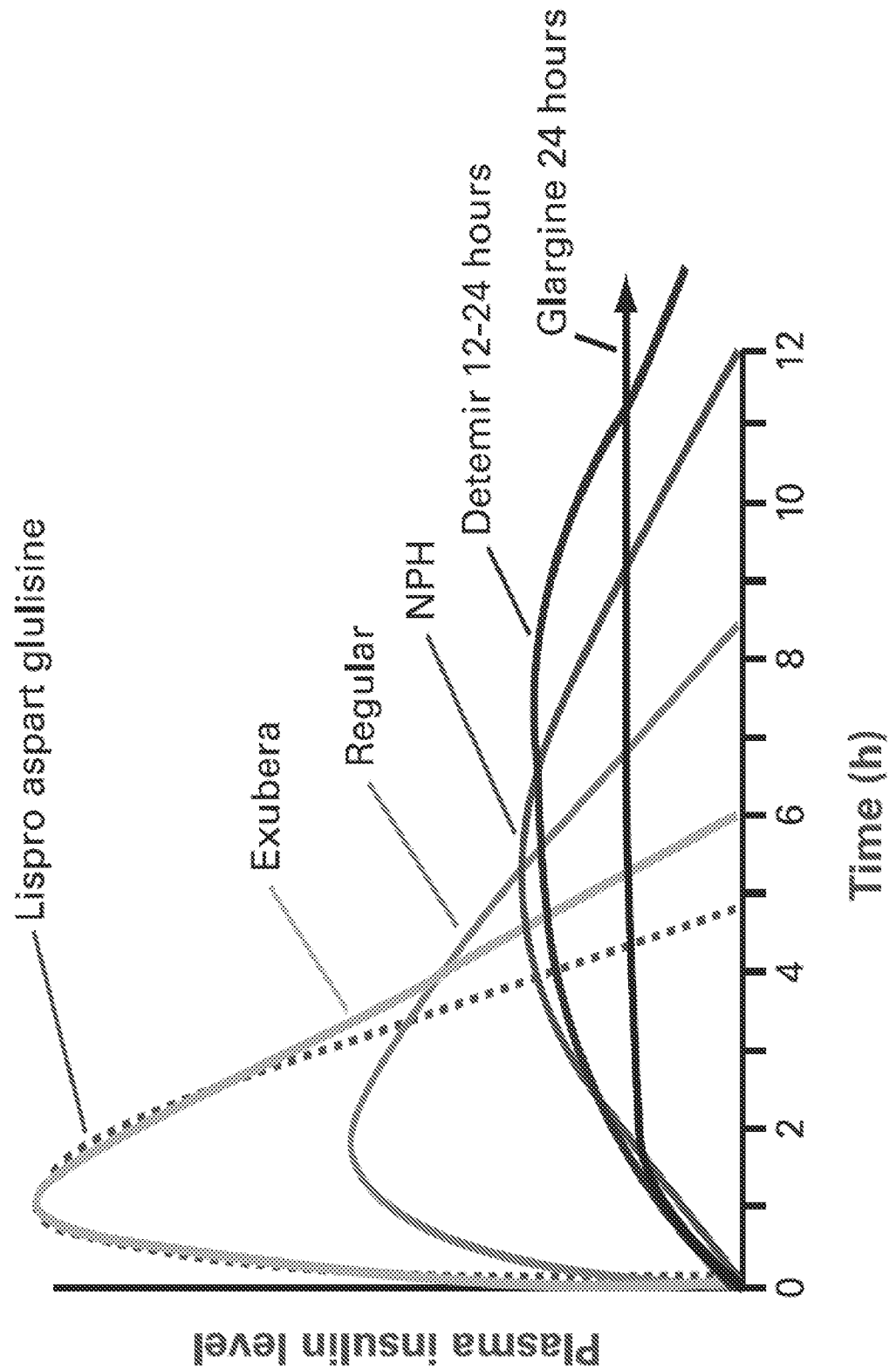


Fig. 9

10/78

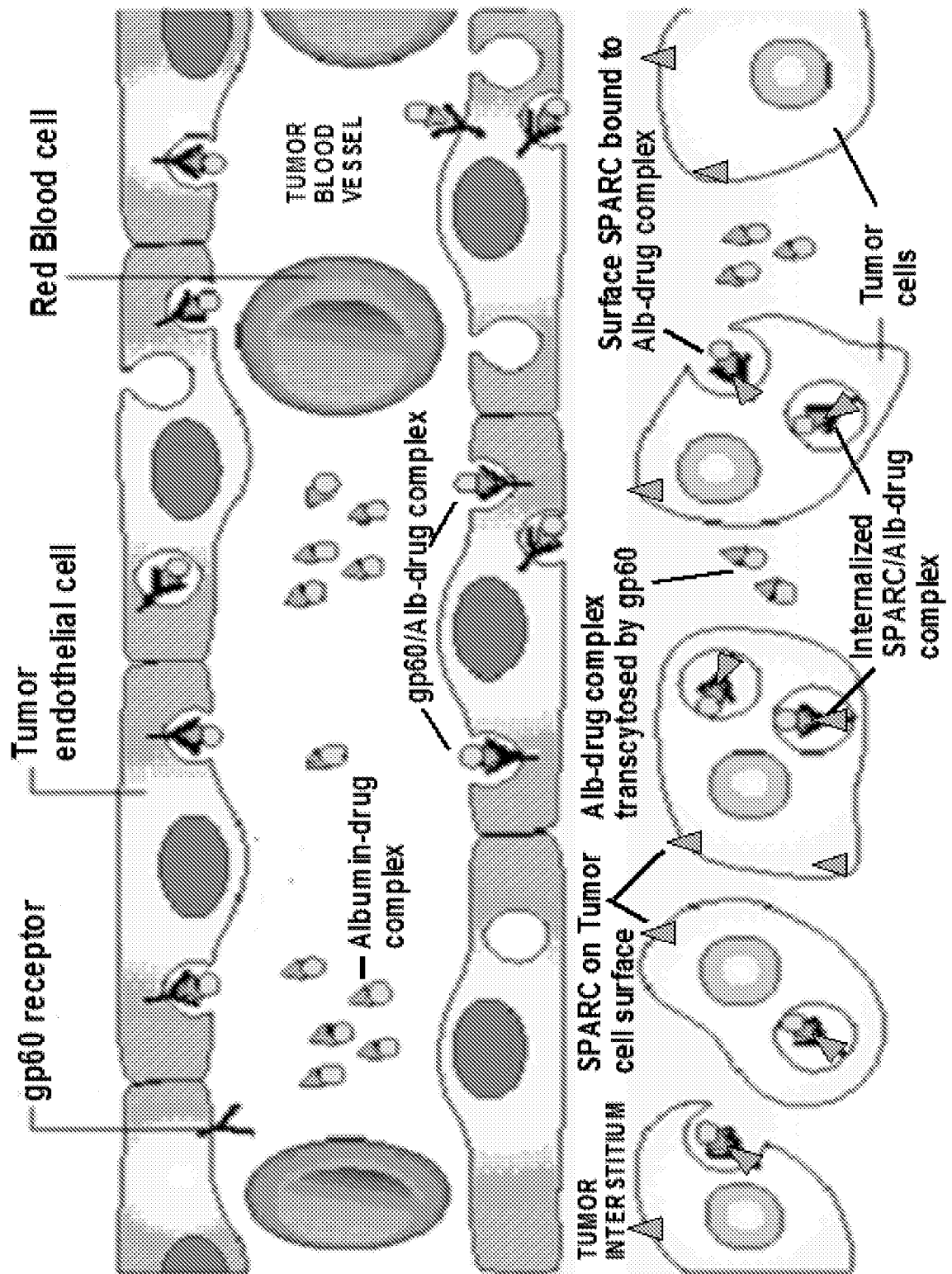


Fig. 10

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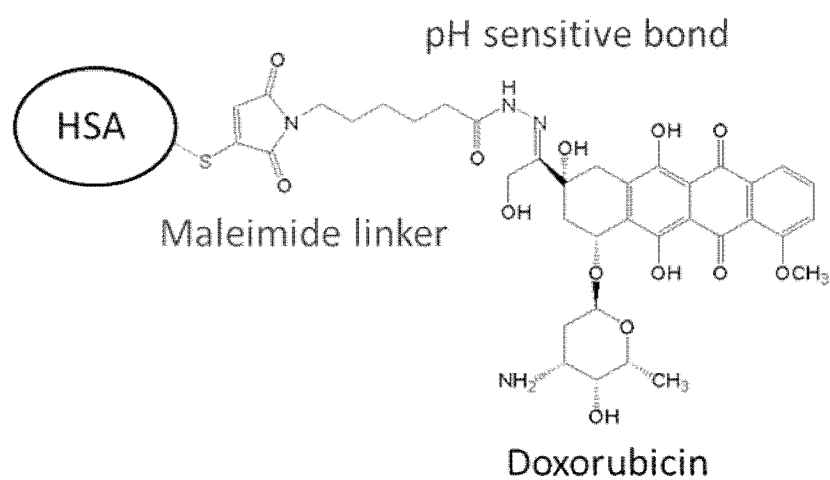


Fig. 11

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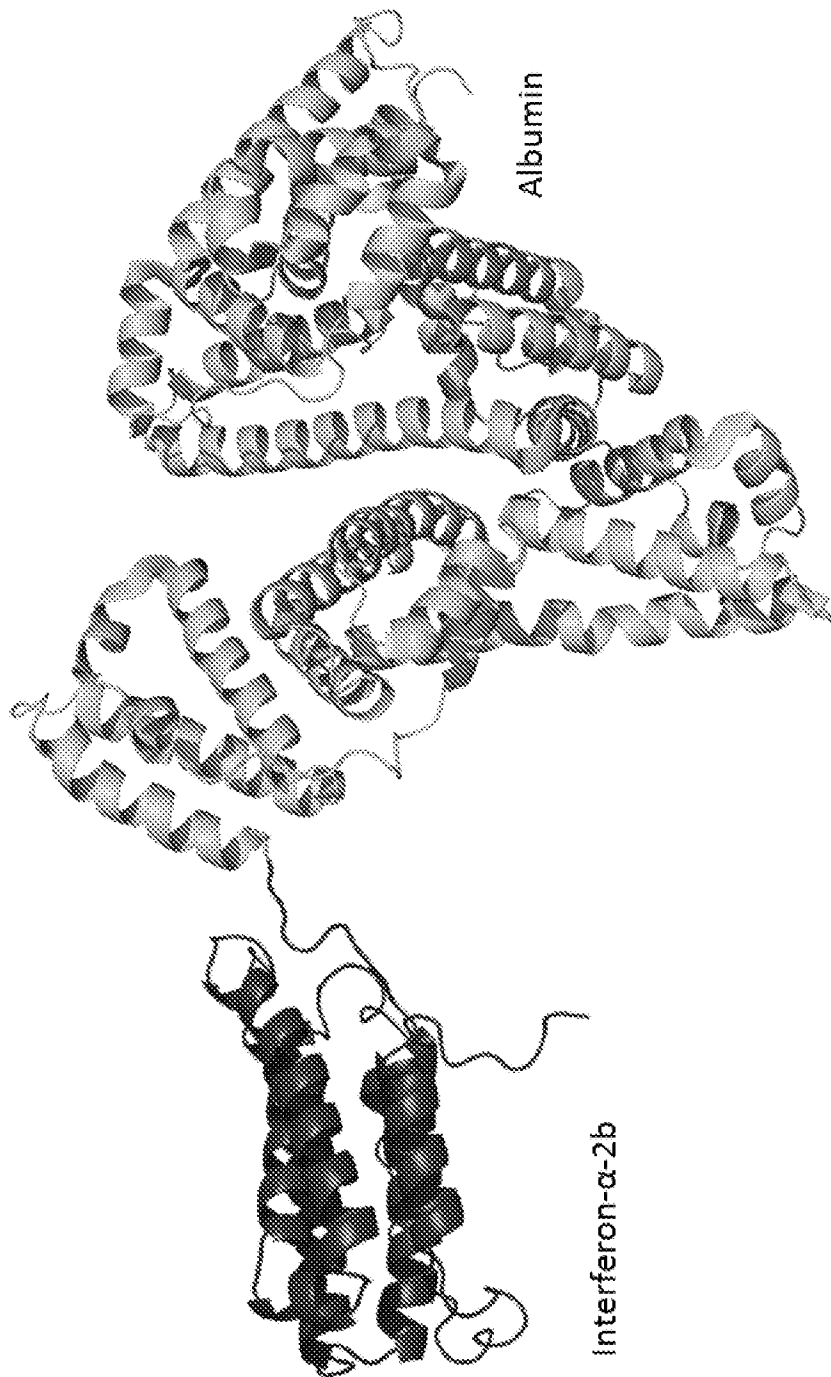


Fig. 12

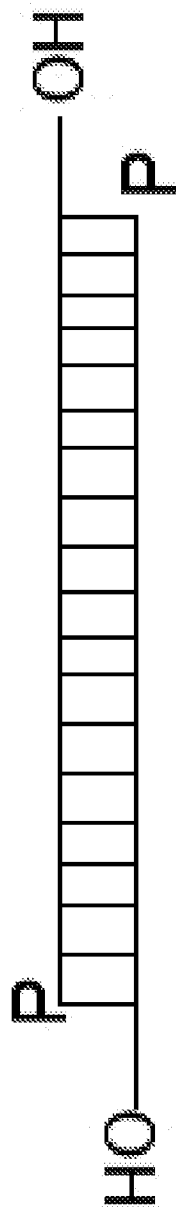


Fig. 13

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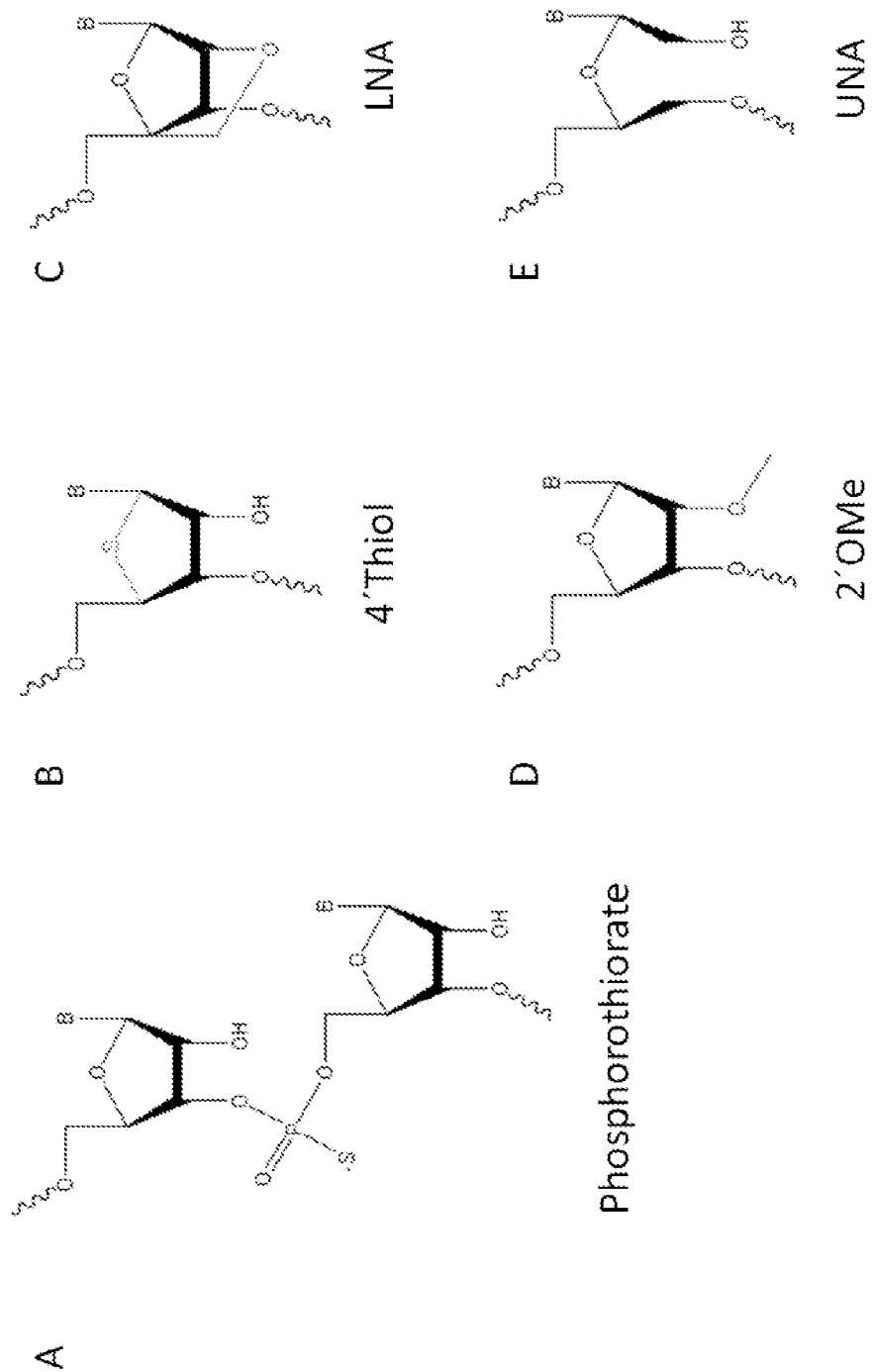


Fig. 14

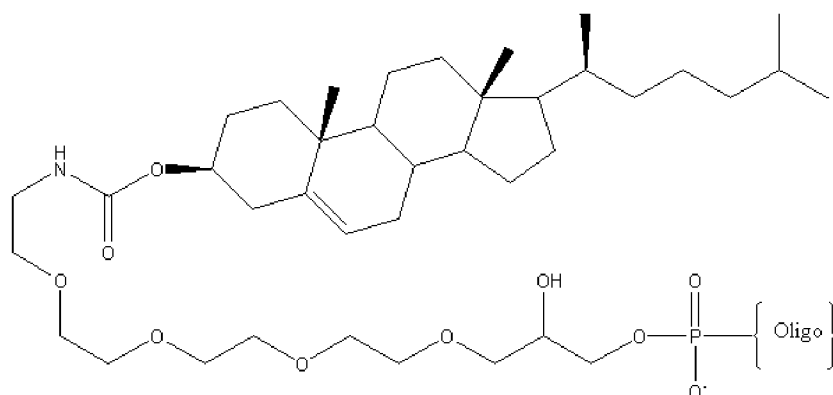
15/78

Number	Schematic representation	Modification
ss7		SS: Non modified
ss8		SS: Palmitoyl mid-sequence
ss9		SS: Palmitoyl mid-sequence and 3'
ss10		AS: Cholesteryl 3'
ss12		AS: Palmitoyl 3'
ss13		SS: Palmitoyl 3'
ss14		AS: Non modified
15		AS: Non modified SS: Palmitoyl 3'
16		AS: Non modified SS: Palmitoyl mid-sequence
17		AS: Non modified SS: Palmitoyl mid-sequence and 3'
18		AS: Non modified SS: Non modified
19		AS: Palmitoyl 3' SS: Palmitoyl 3'
20		AS: Palmitoyl 3' SS: Palmitoyl mid-sequence
21		AS: Palmitoyl 3' SS: Palmitoyl mid-sequence and 3'
22		AS: Palmitoyl 3' SS: Non modified
23		AS: Cholesteryl at 3' SS: Palmitoyl 3'
24		AS: Cholesteryl at 3' SS: Palmitoyl mid-sequence
25		AS: Cholesteryl at 3' SS: Palmitoyl mid-sequence and 3'
26		AS: Cholesteryl at 3' SS: Non modified

Fig. 15

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A)



B)

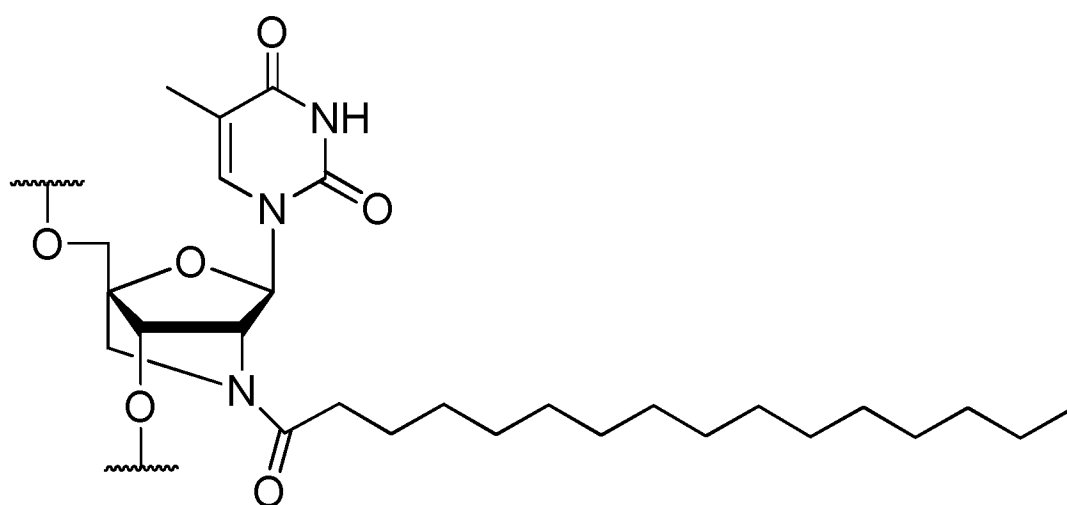


Fig. 16

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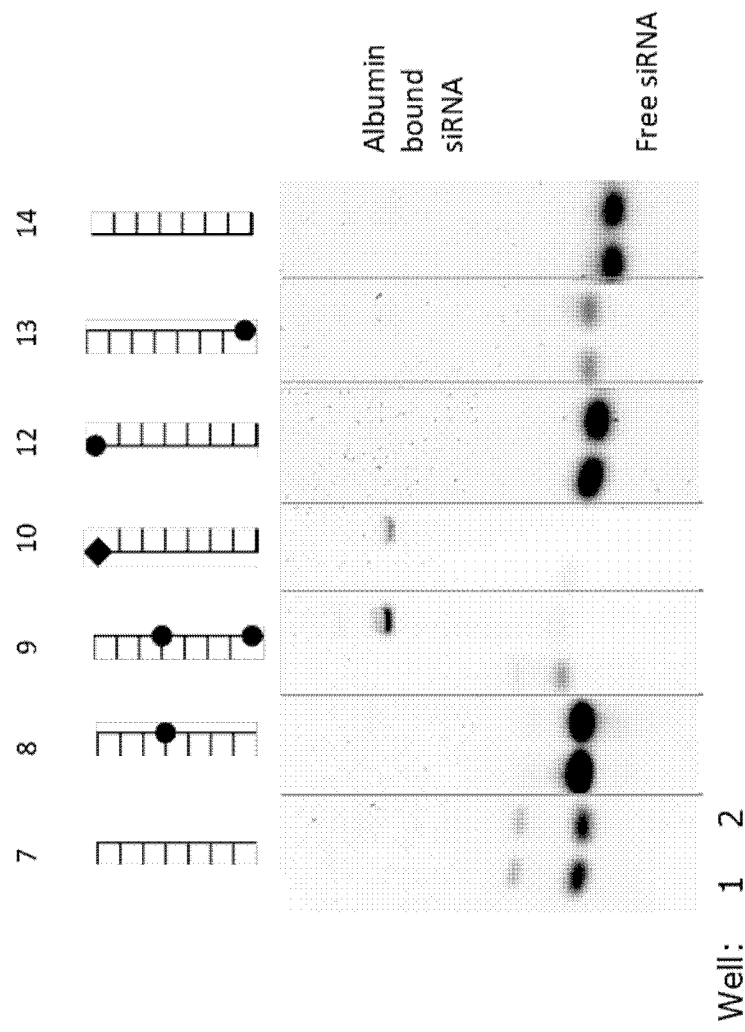


Fig. 17

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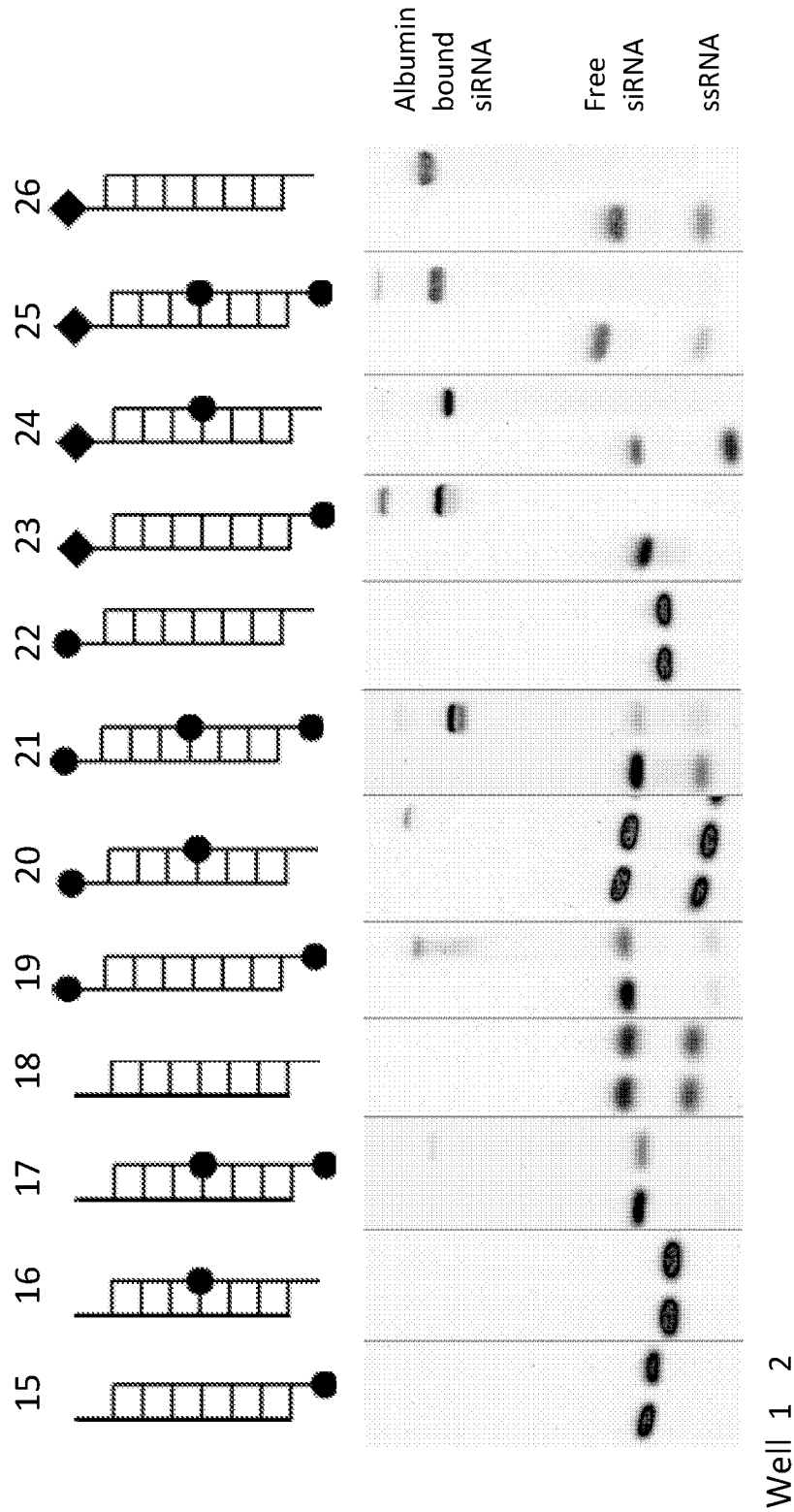


Fig. 18

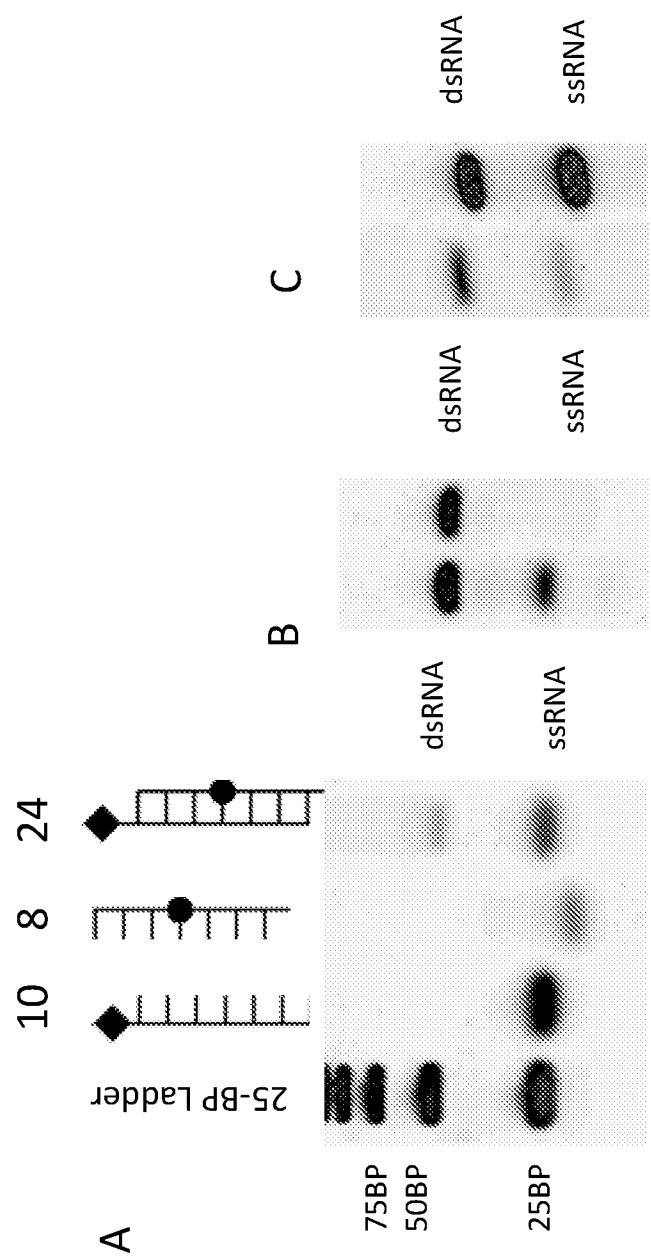


Fig. 19

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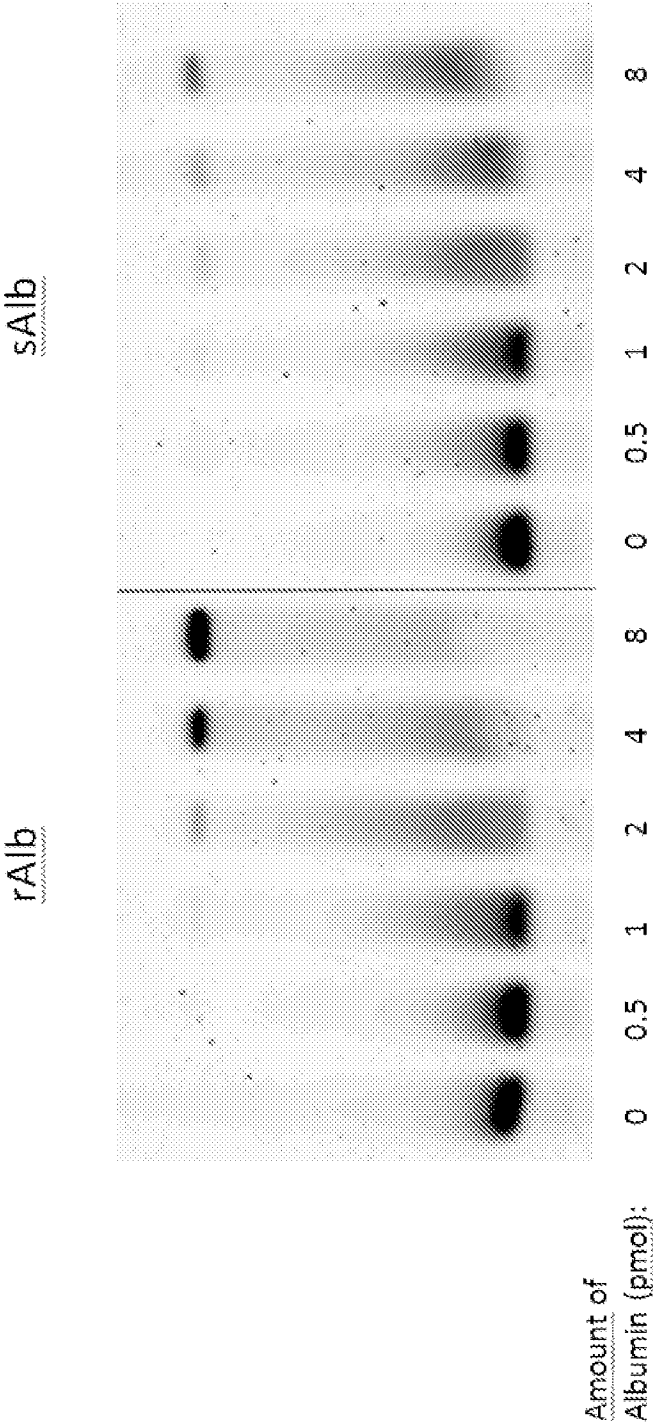


Fig. 20

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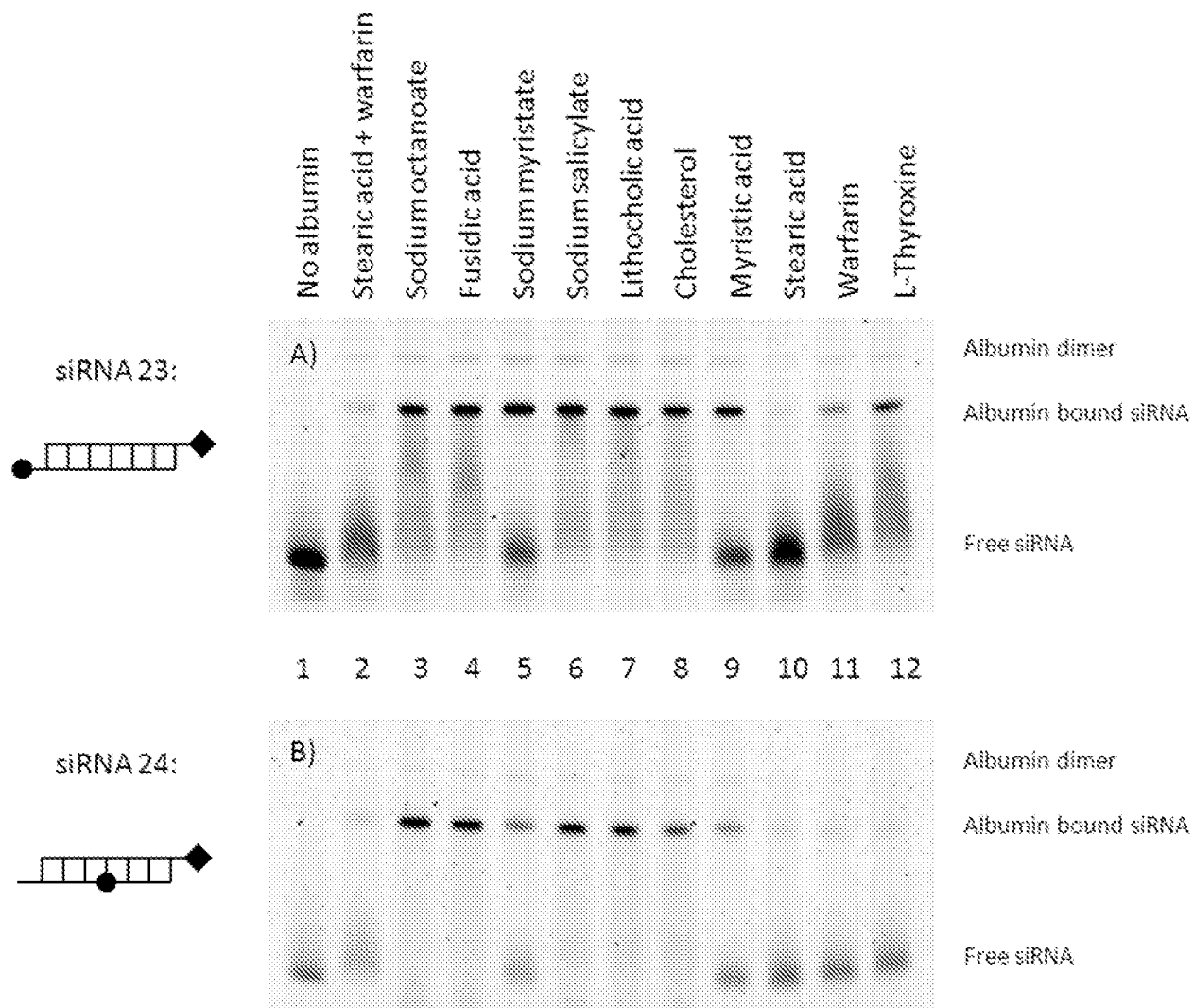


Fig. 21

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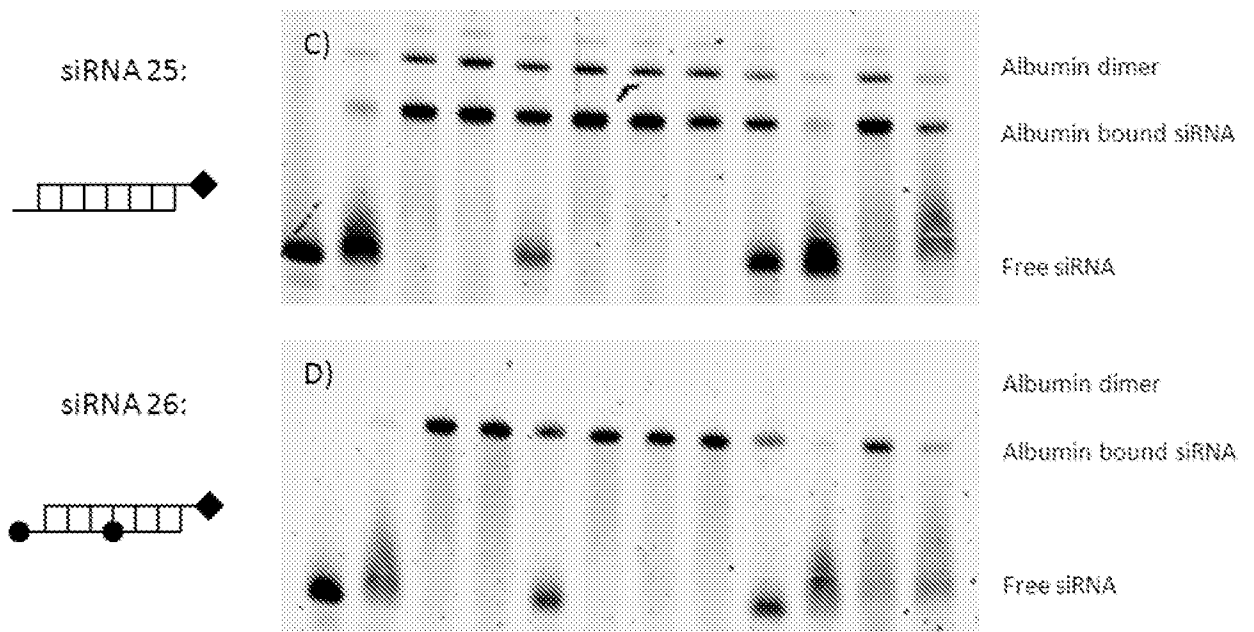


Fig. 21(continued)

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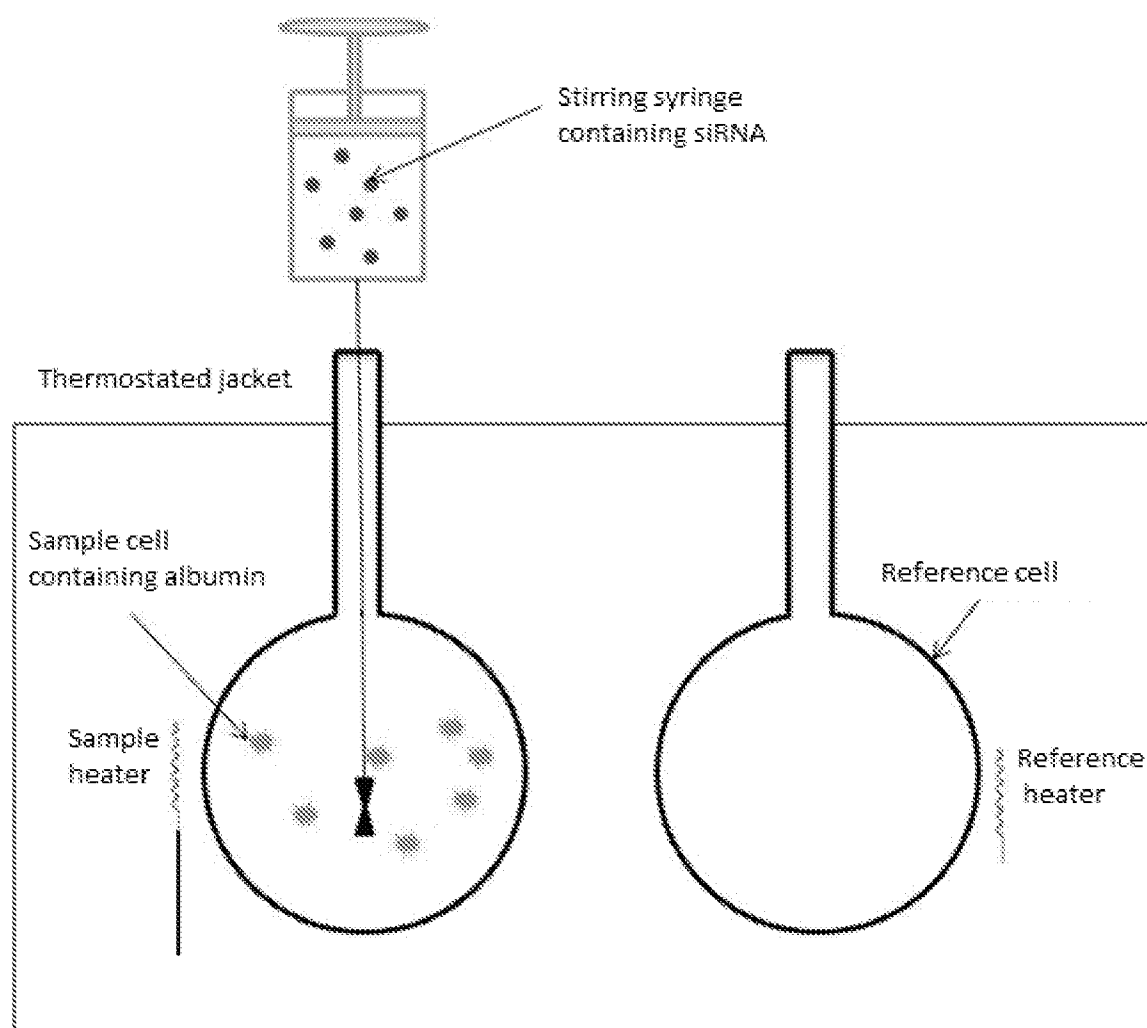


Fig. 22

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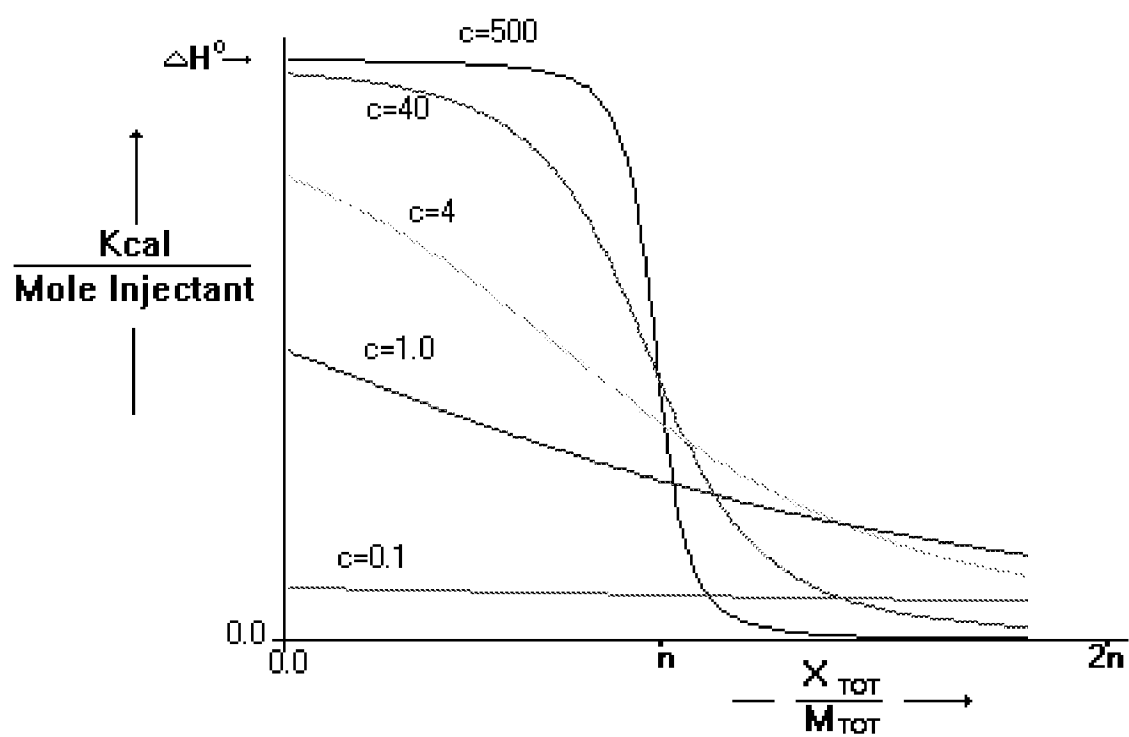


Fig. 23

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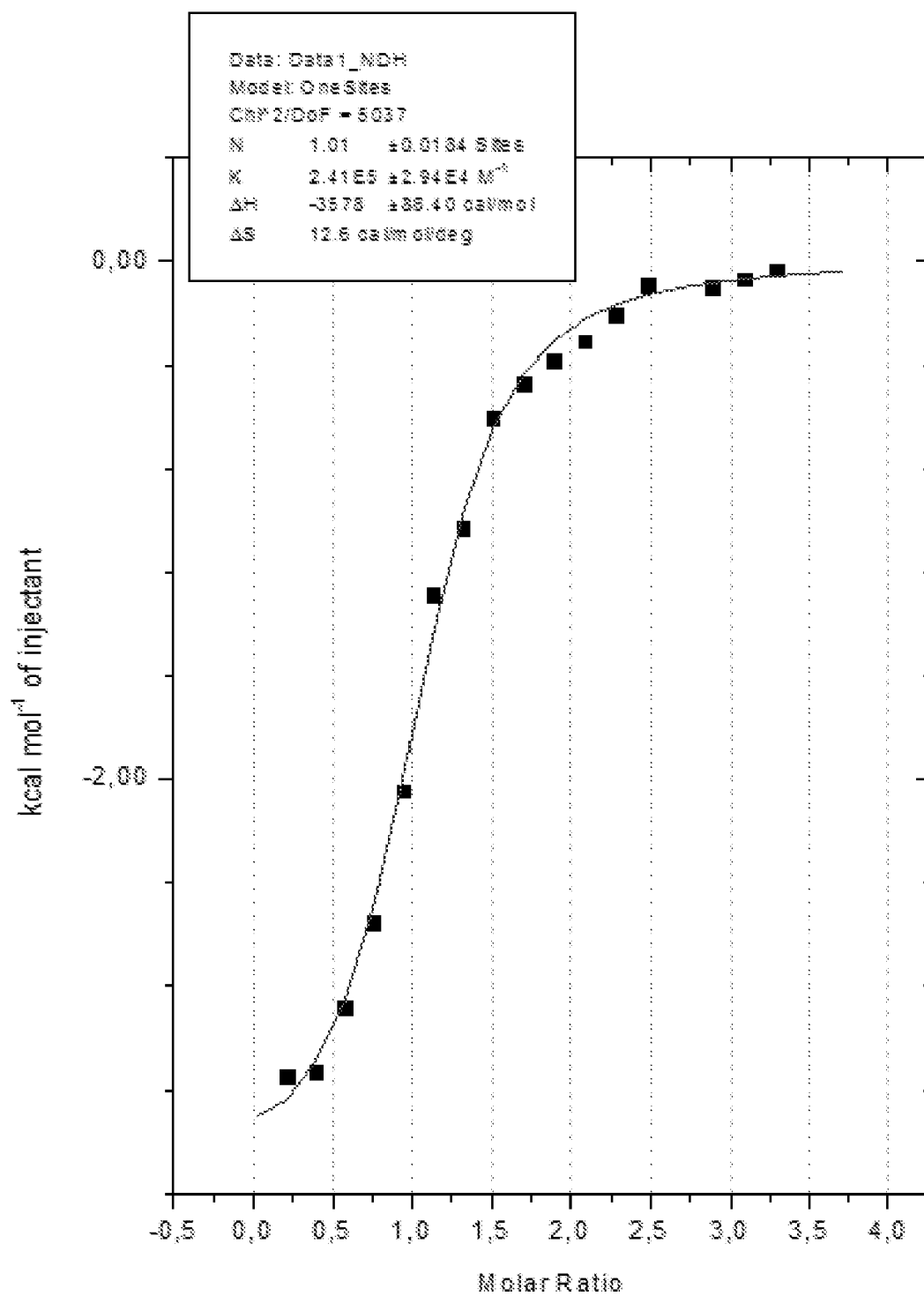


Fig. 24

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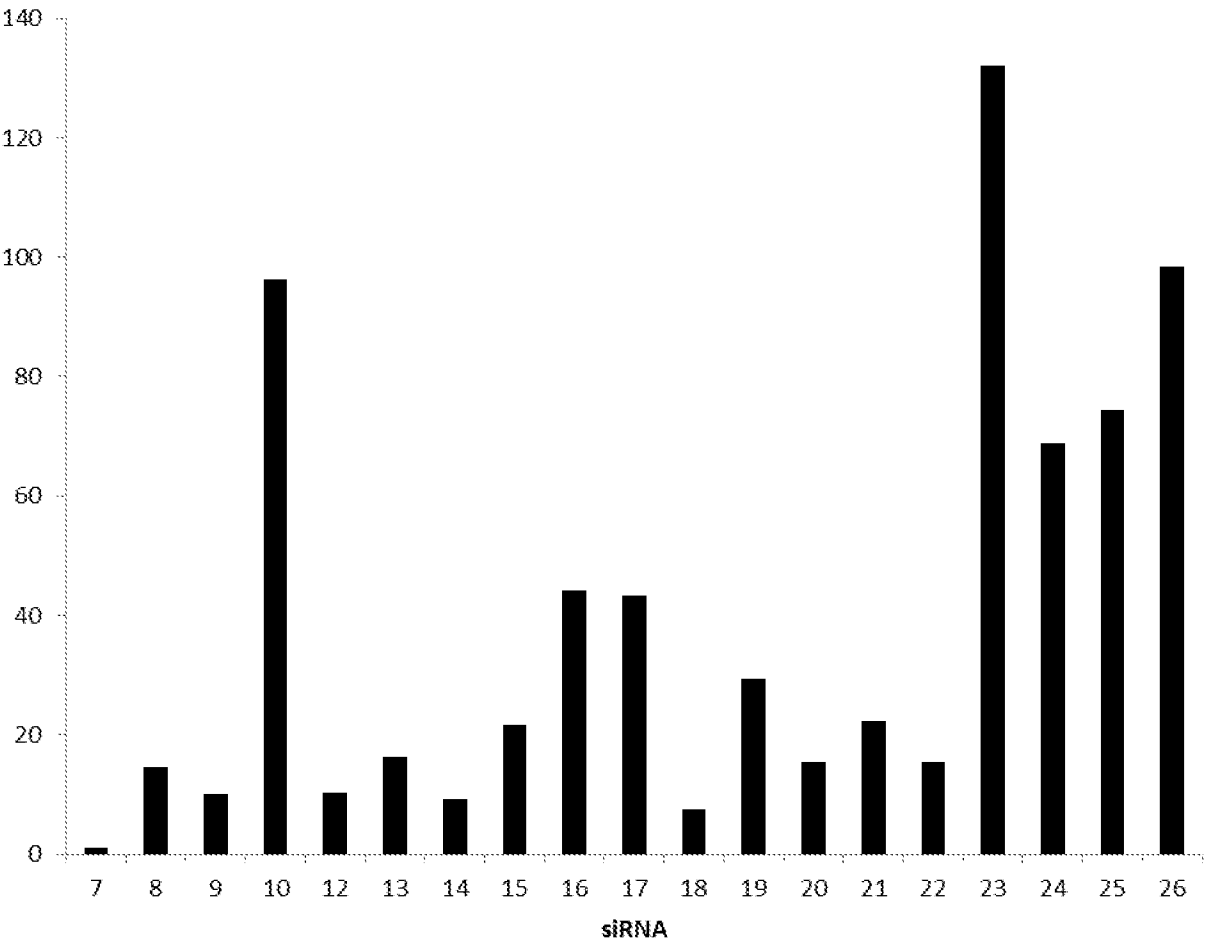


Fig. 25

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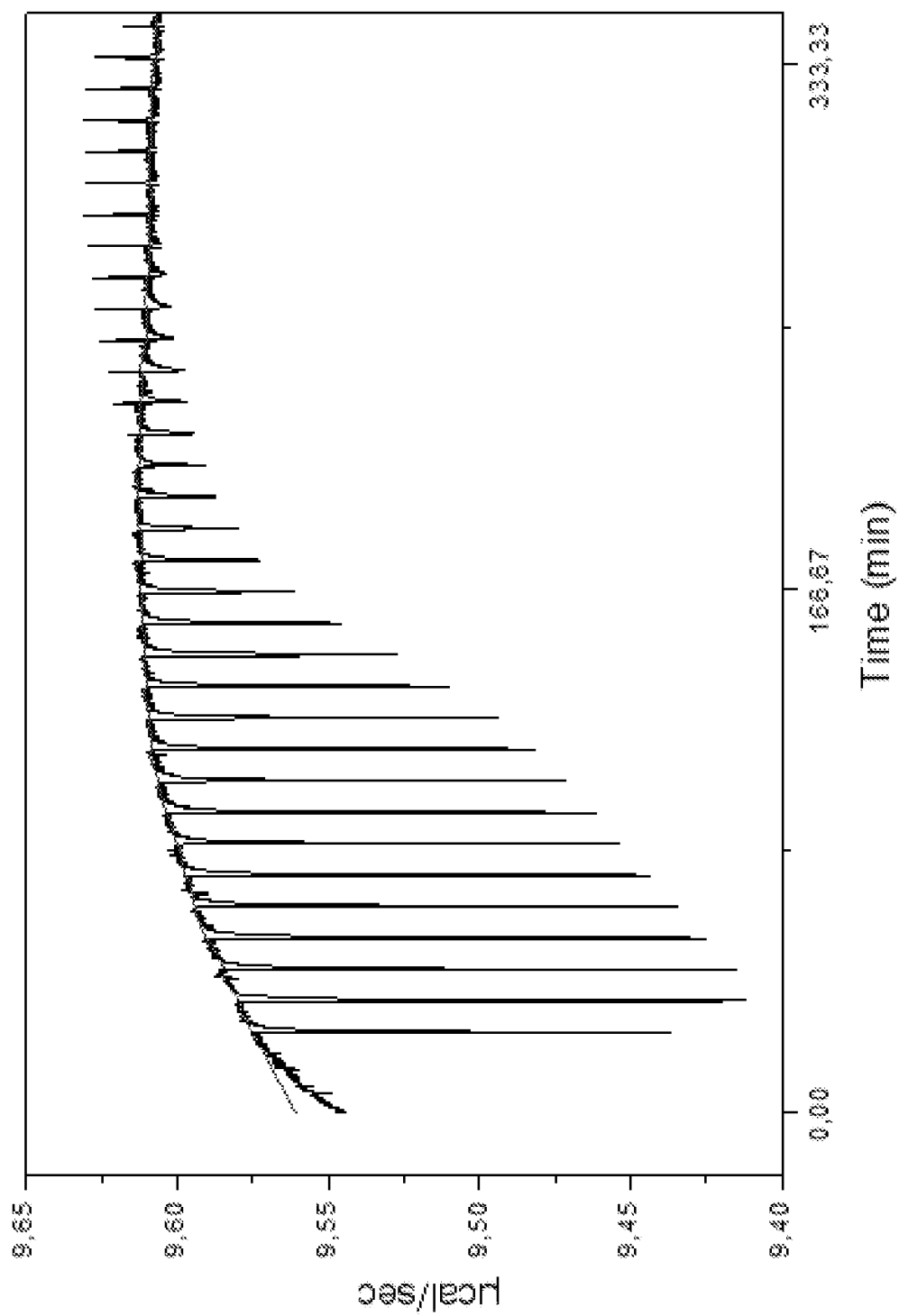


Fig. 26

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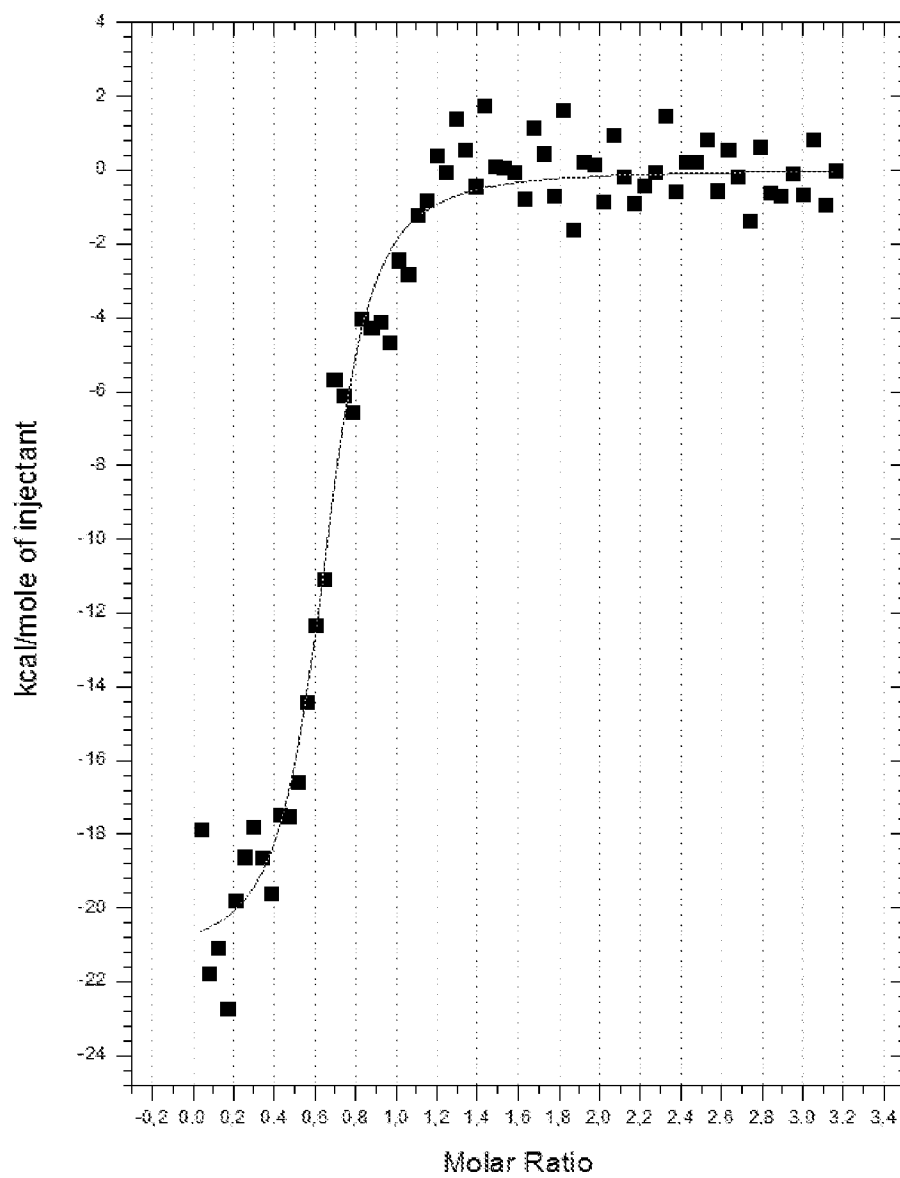
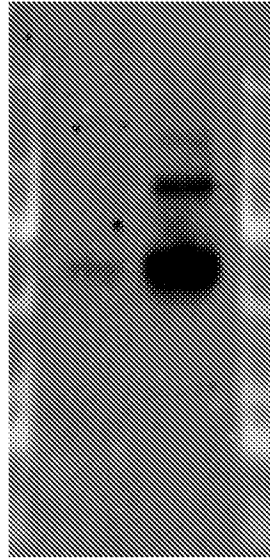


Fig. 27

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A



1 2

B



1 2

Fig. 28

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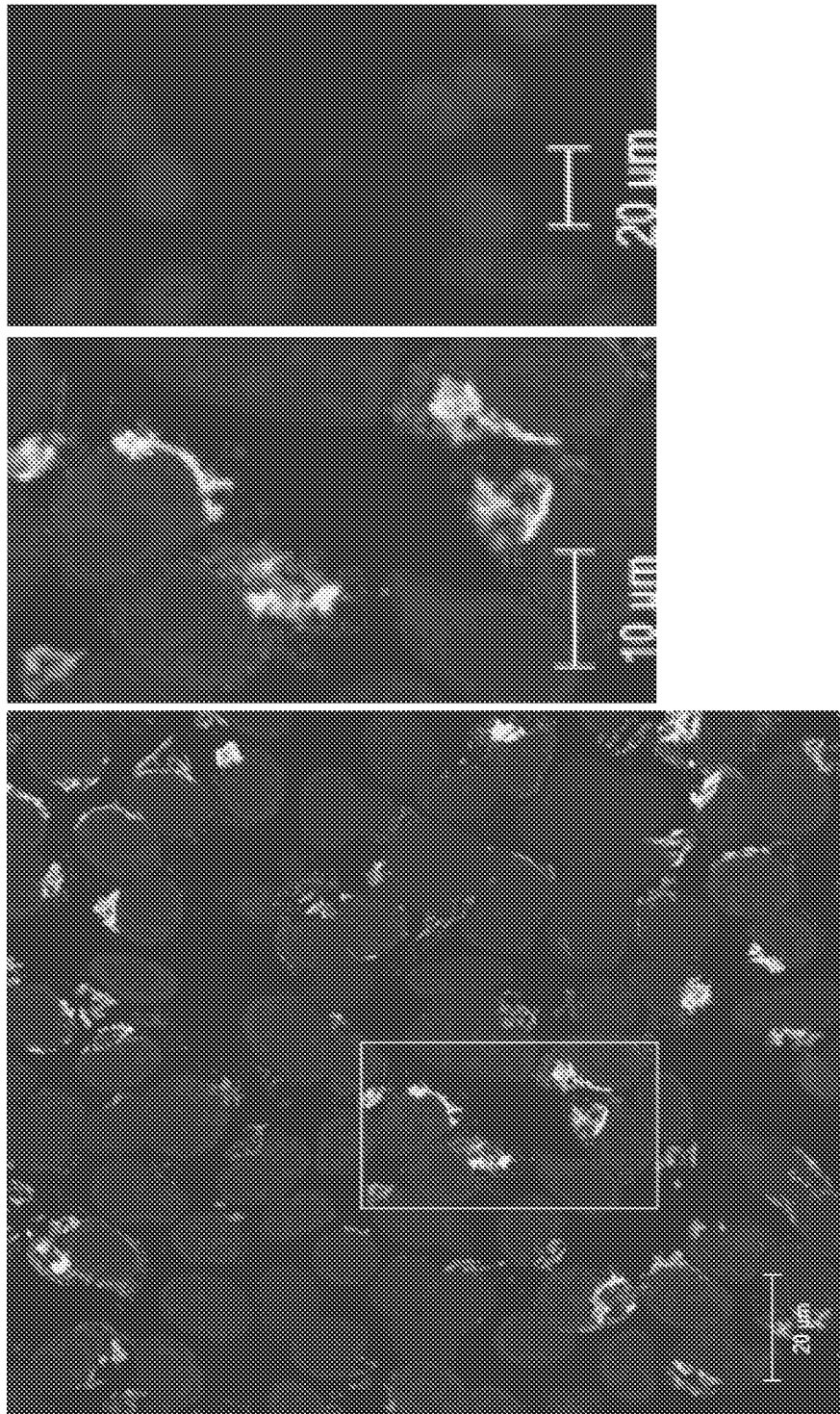
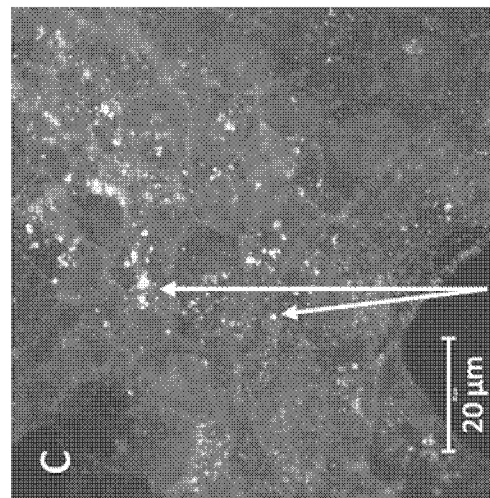
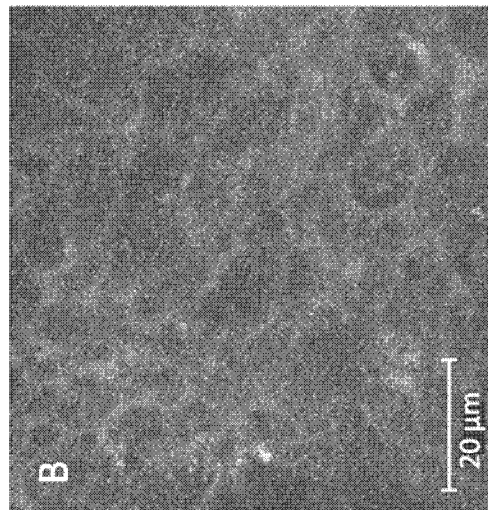


Fig. 29

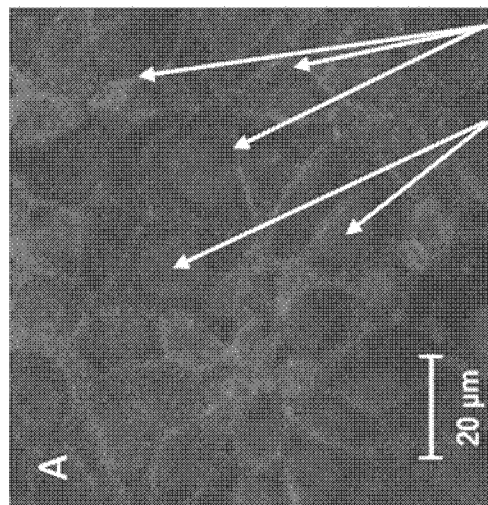
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Light (dots):
Albumin



Medium (lines):
Rhodamine-
Phalloidin



Dark (round
shapes): Hoechst

Fig. 30

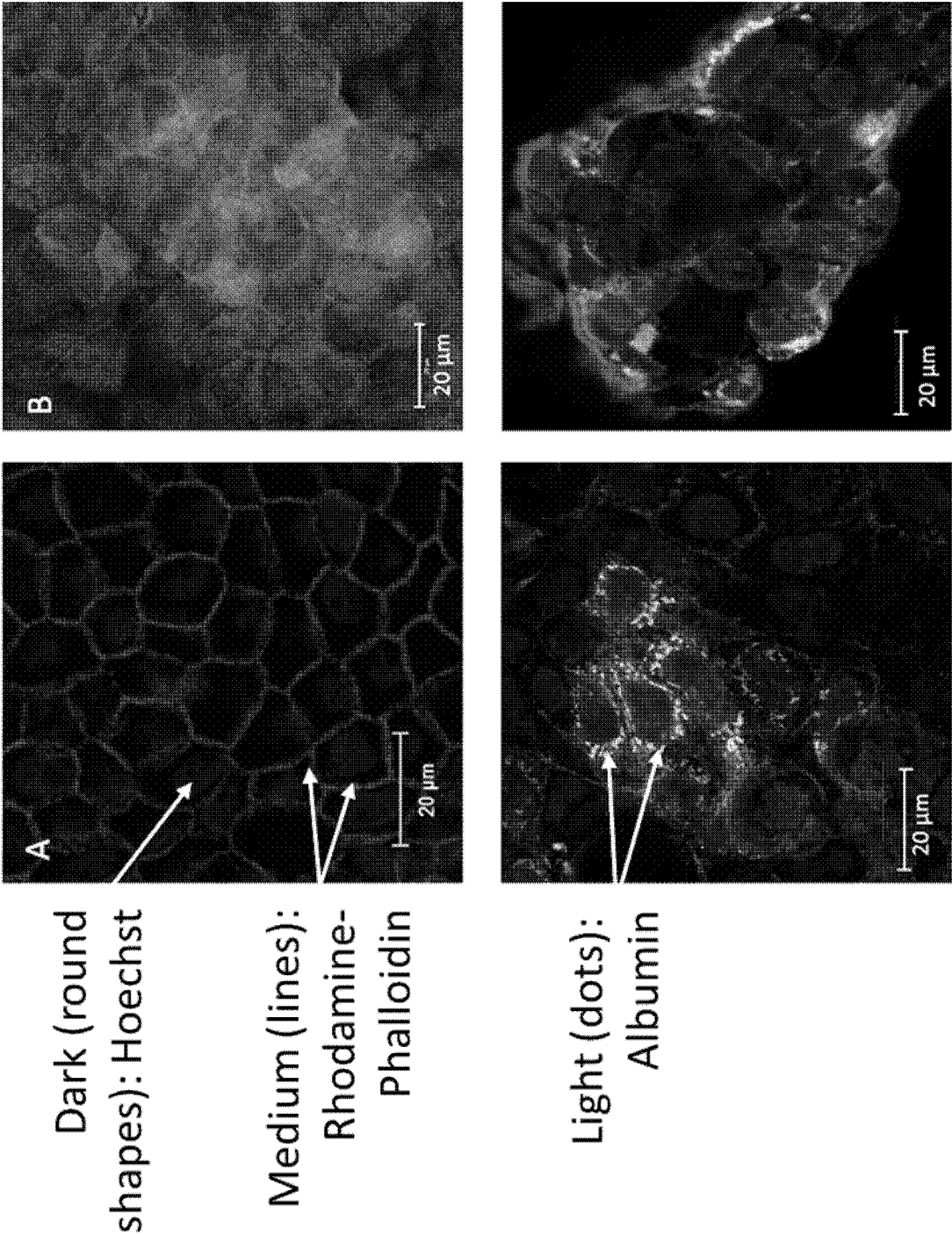


Fig. 31

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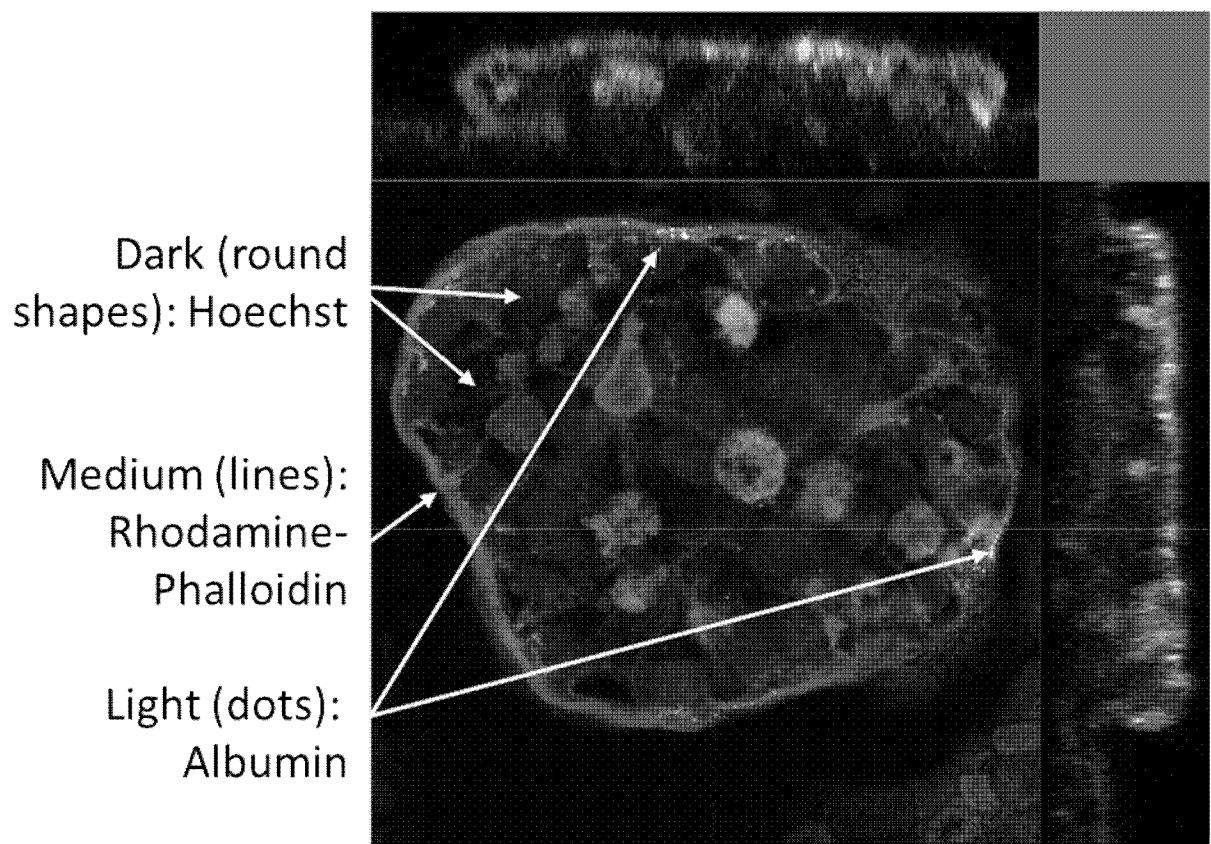


Fig. 32

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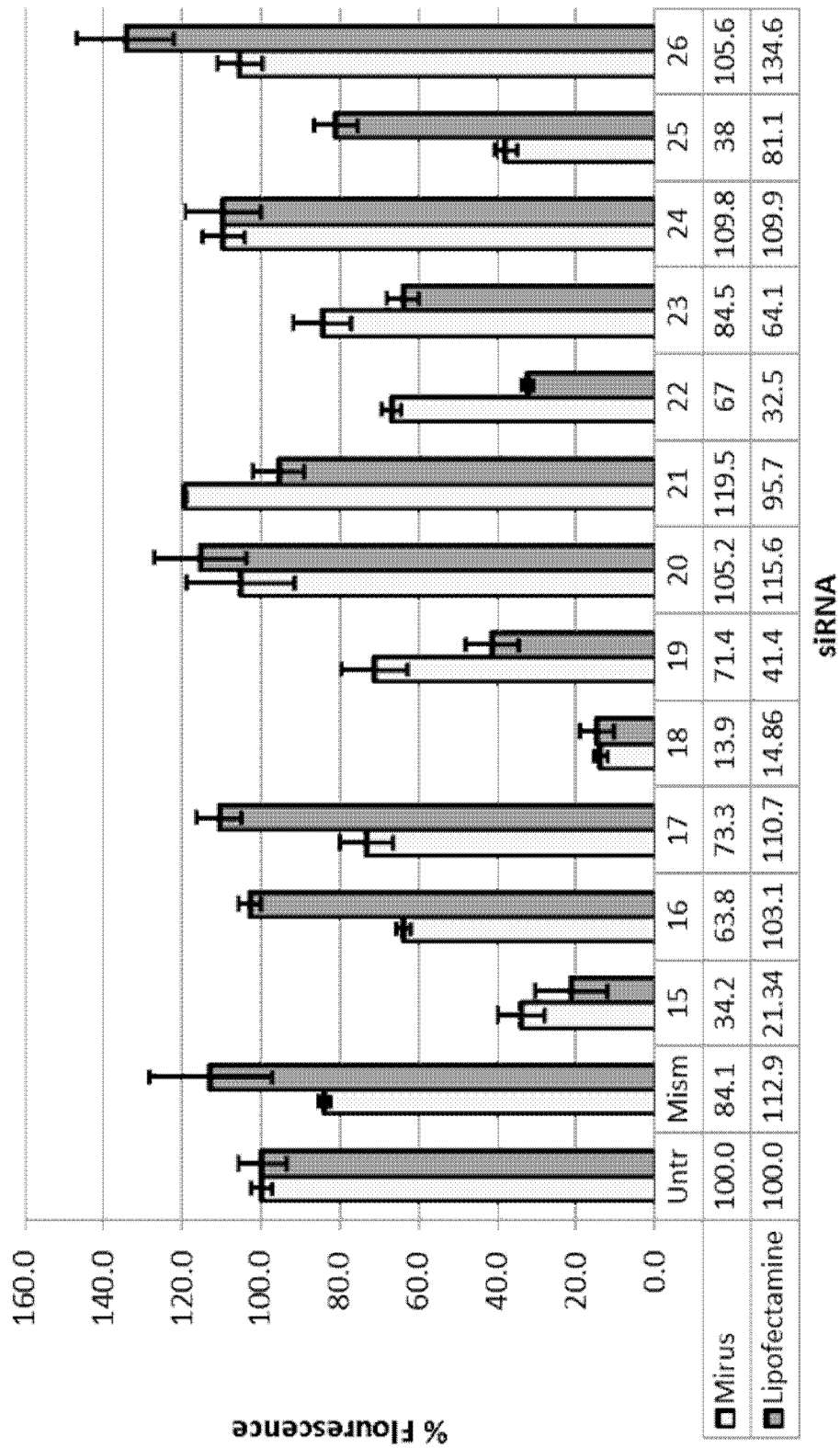


Fig. 33

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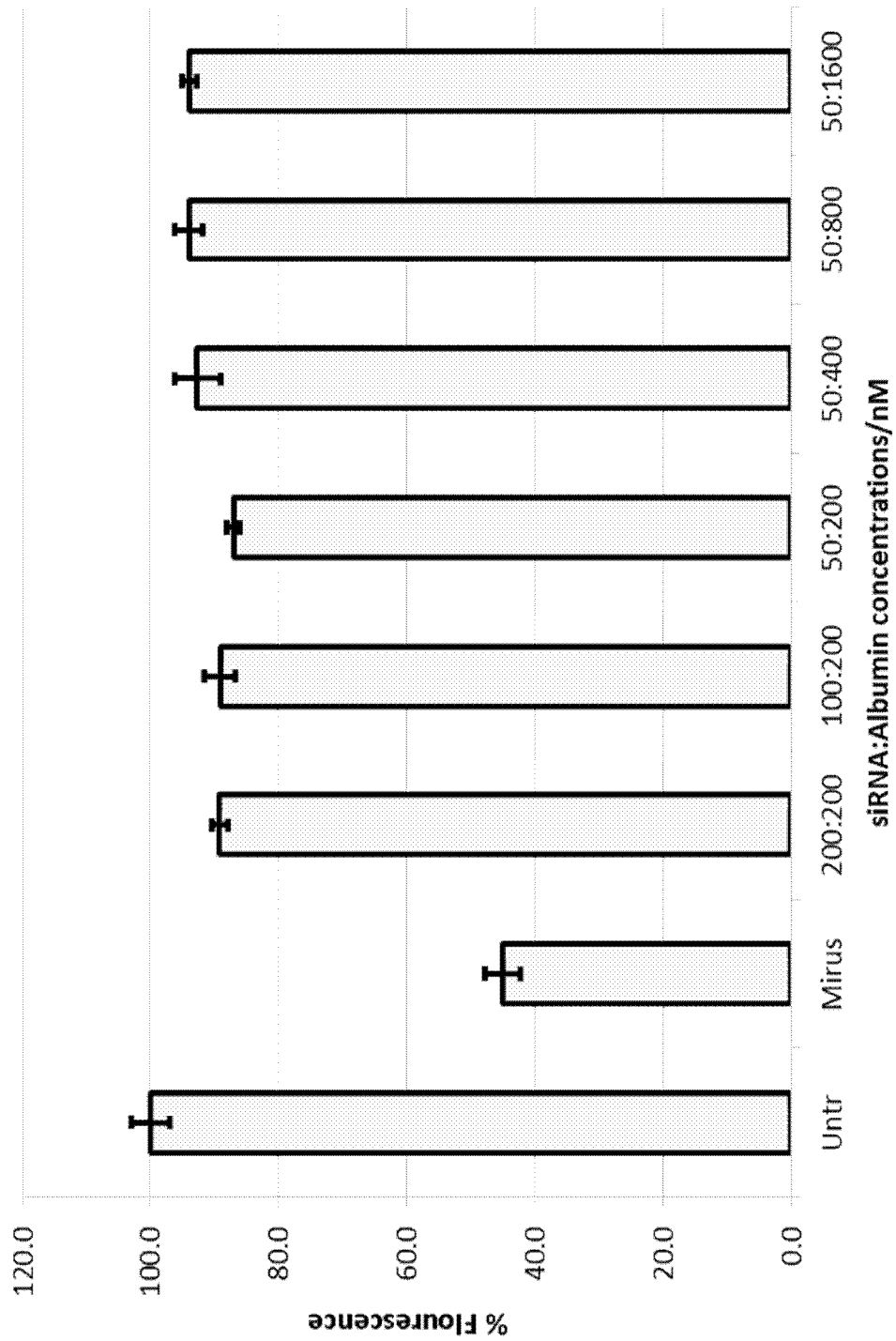


Fig. 34

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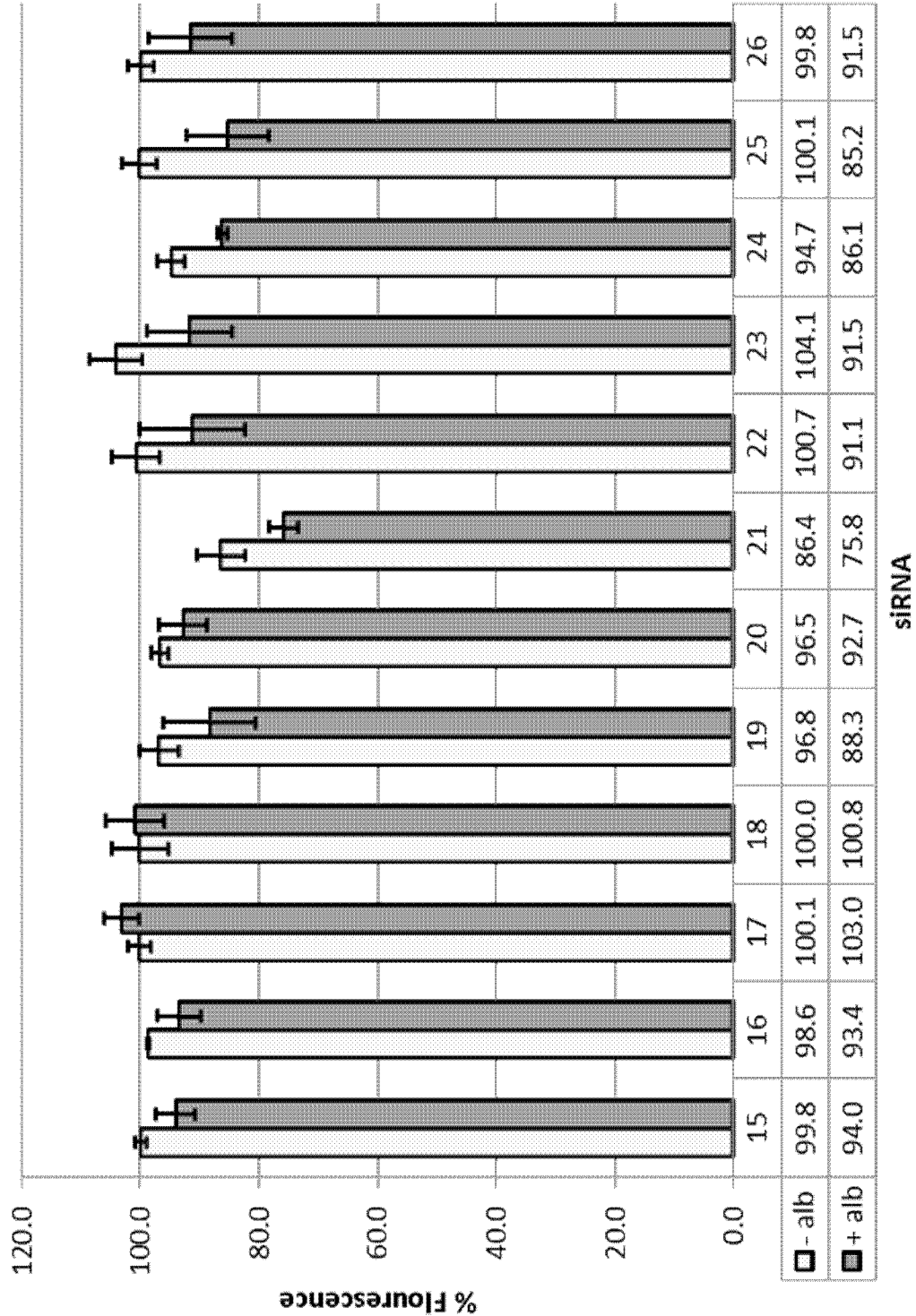


Fig. 35

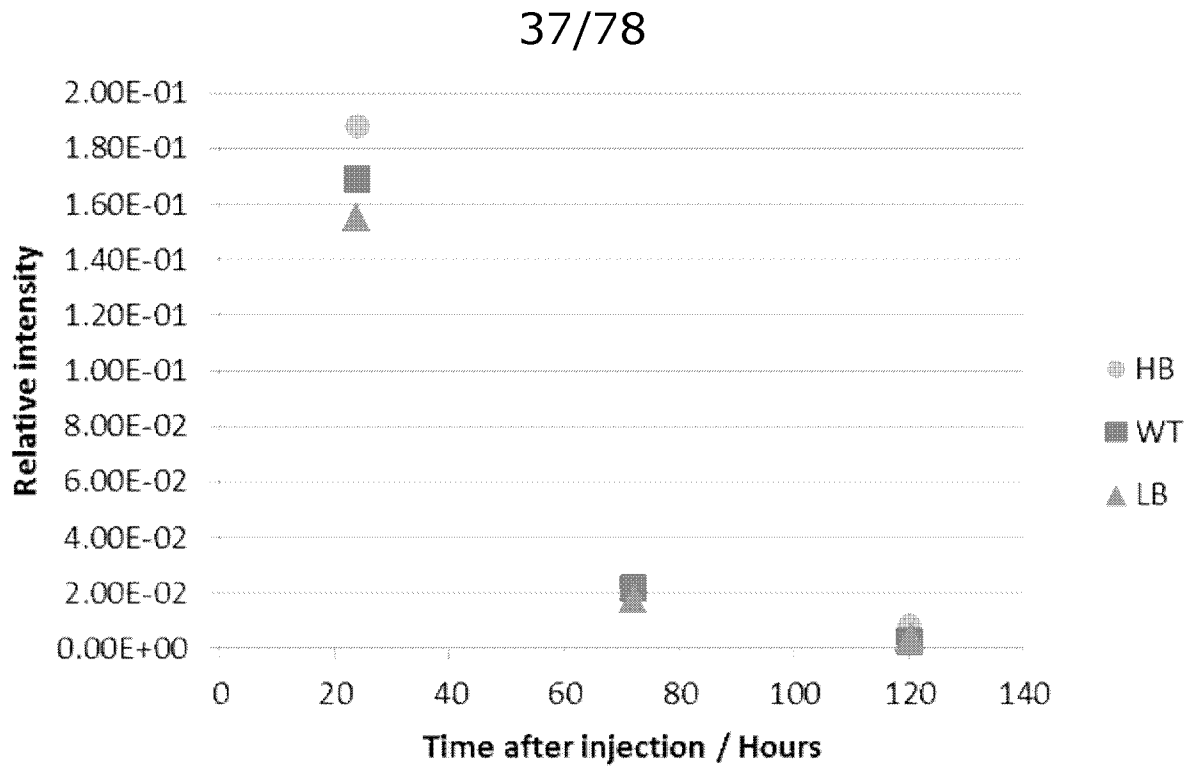


Fig. 36

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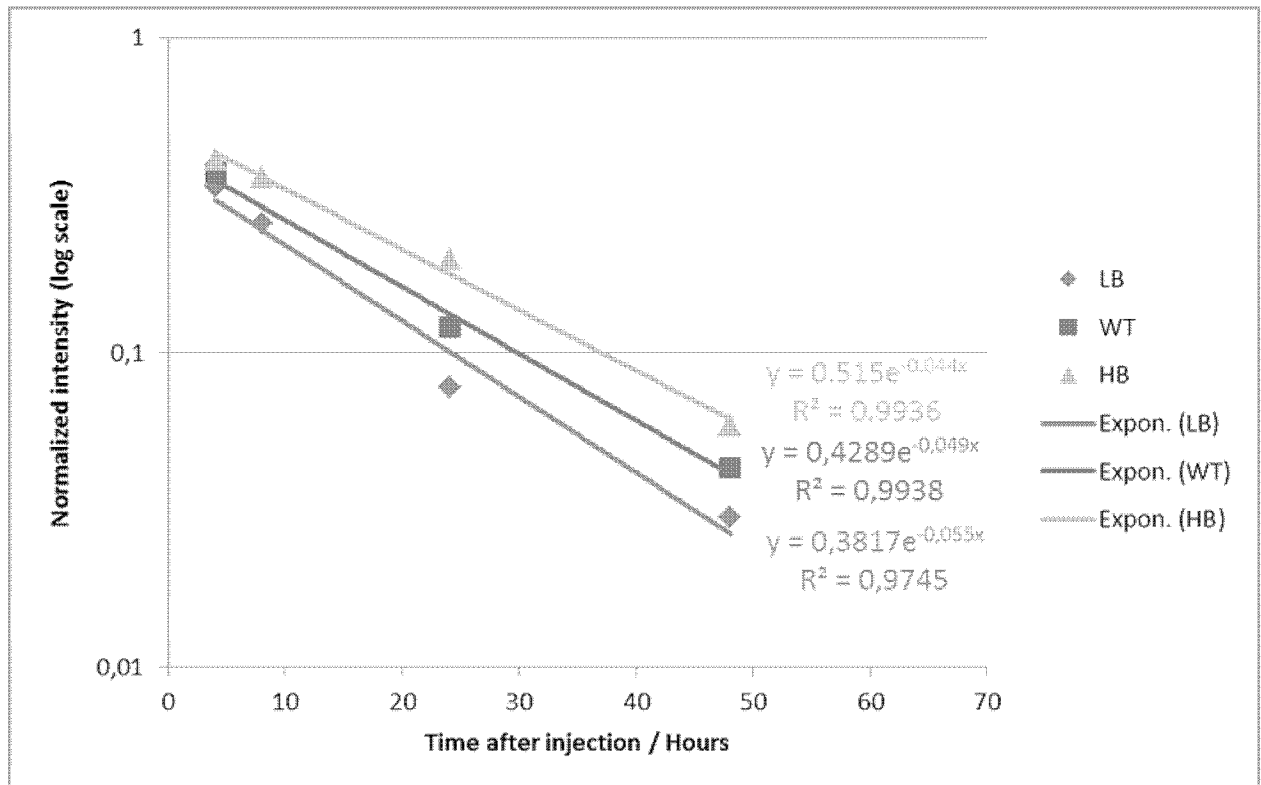


Fig. 37

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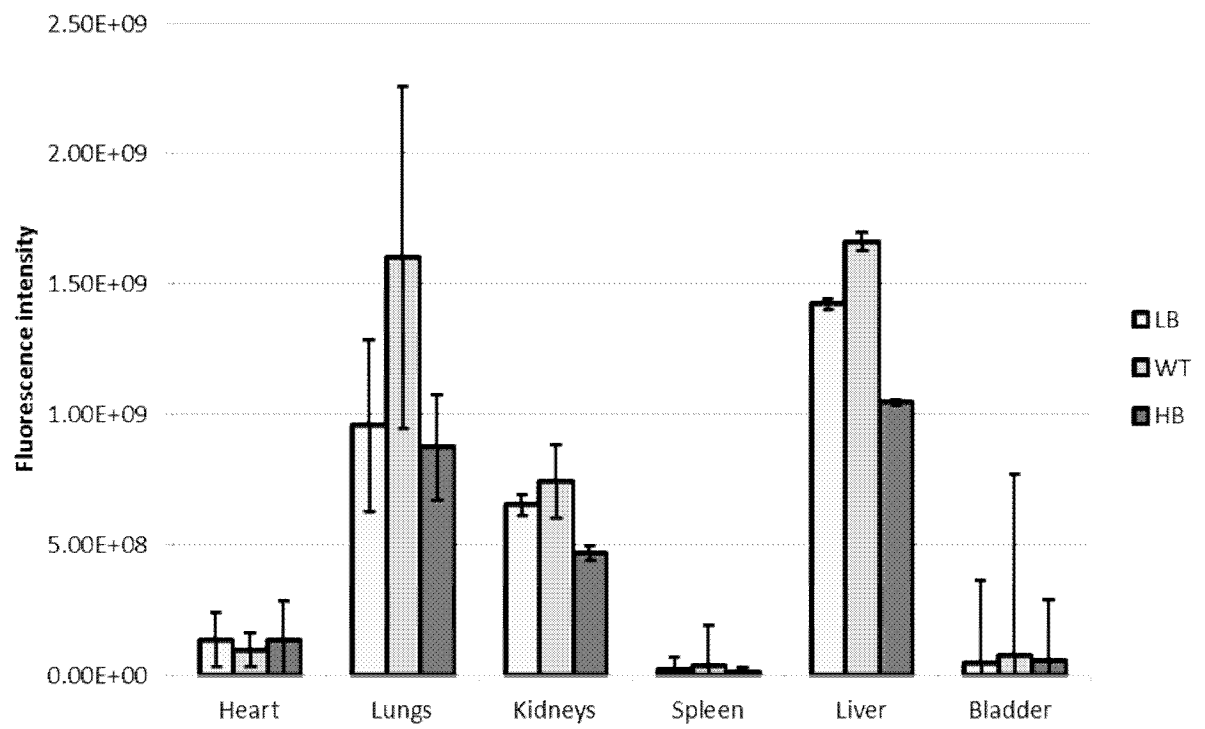


Fig. 38

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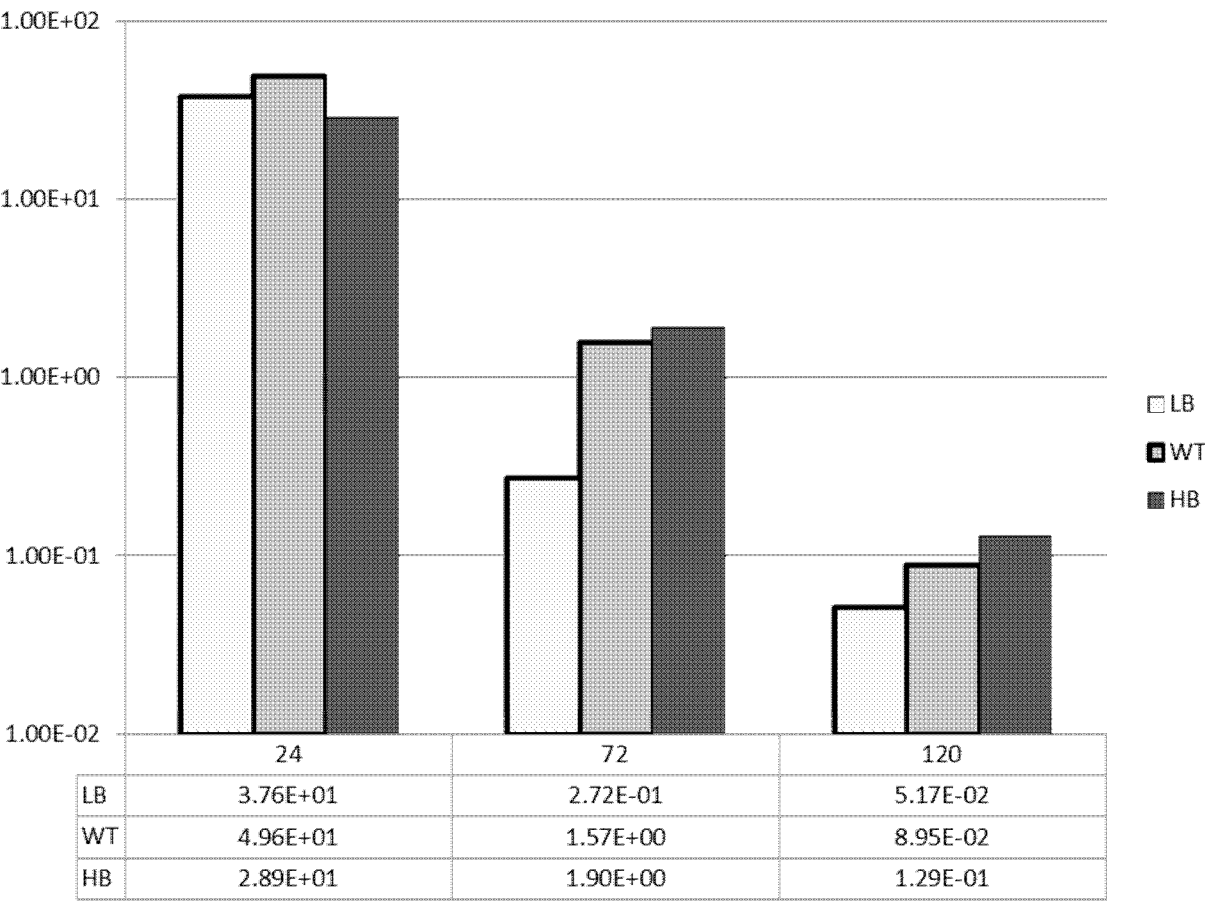


Fig. 39

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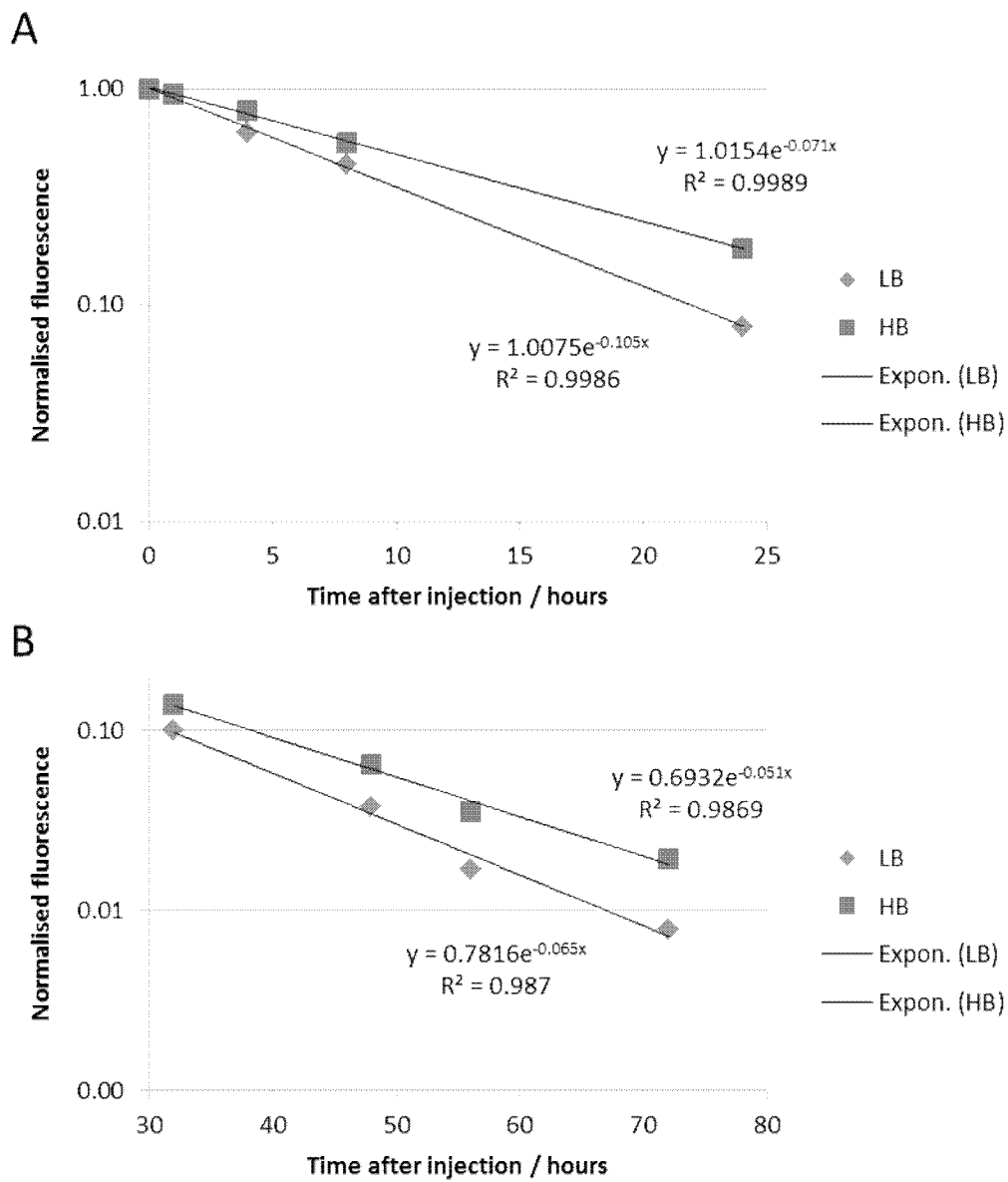


Fig. 40

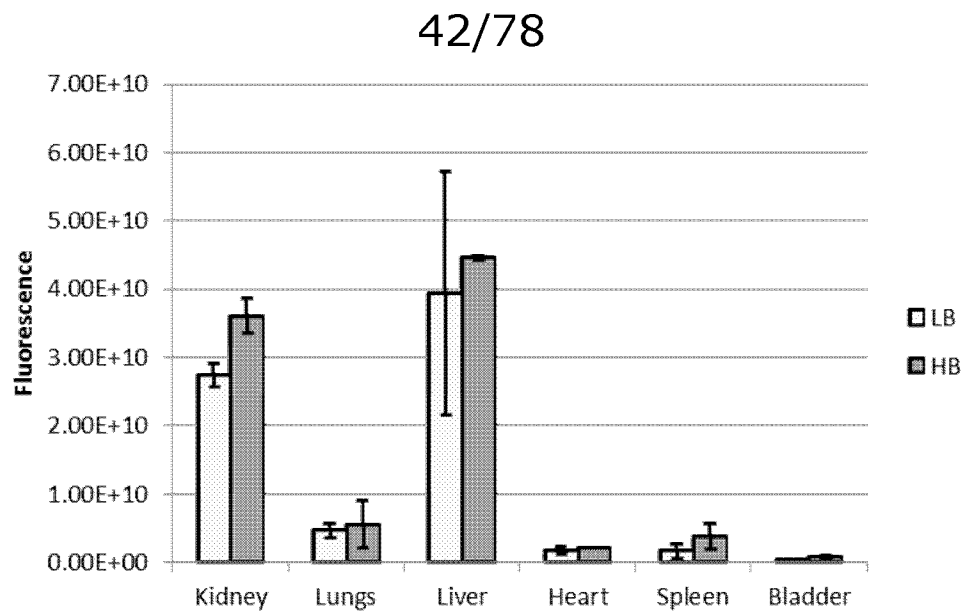


Fig. 41

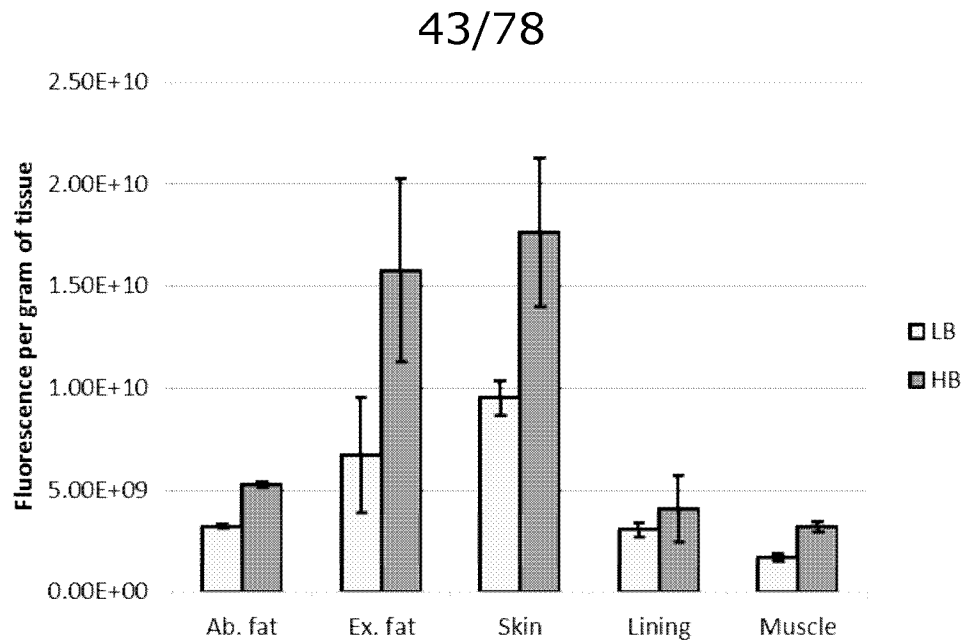


Fig. 42

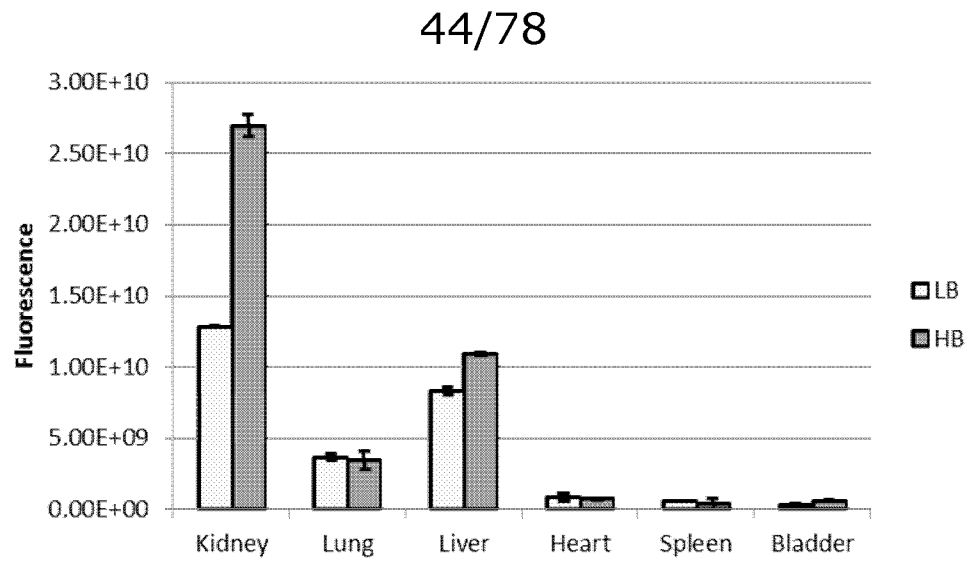


Fig. 43

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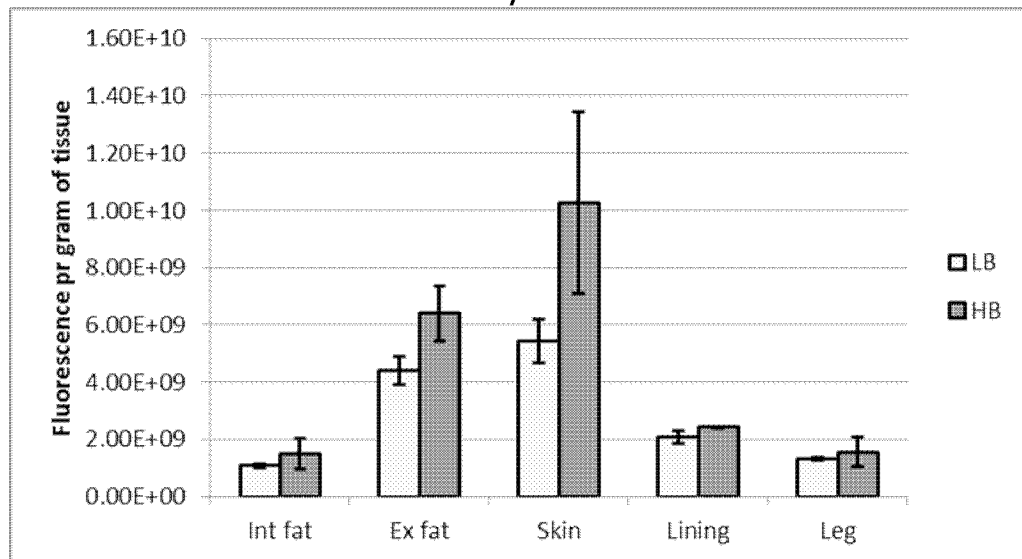


Fig. 44

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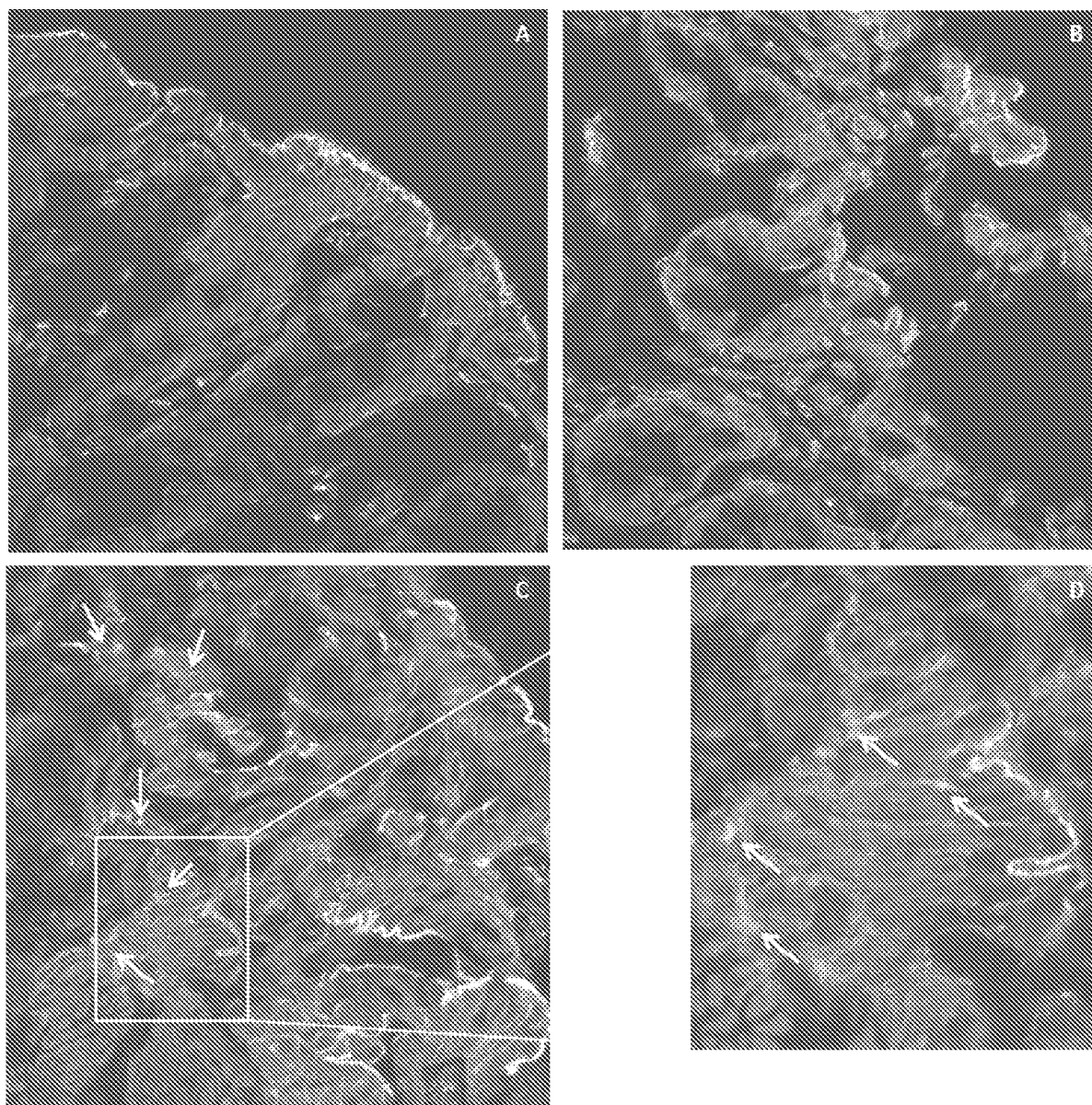


Fig. 45

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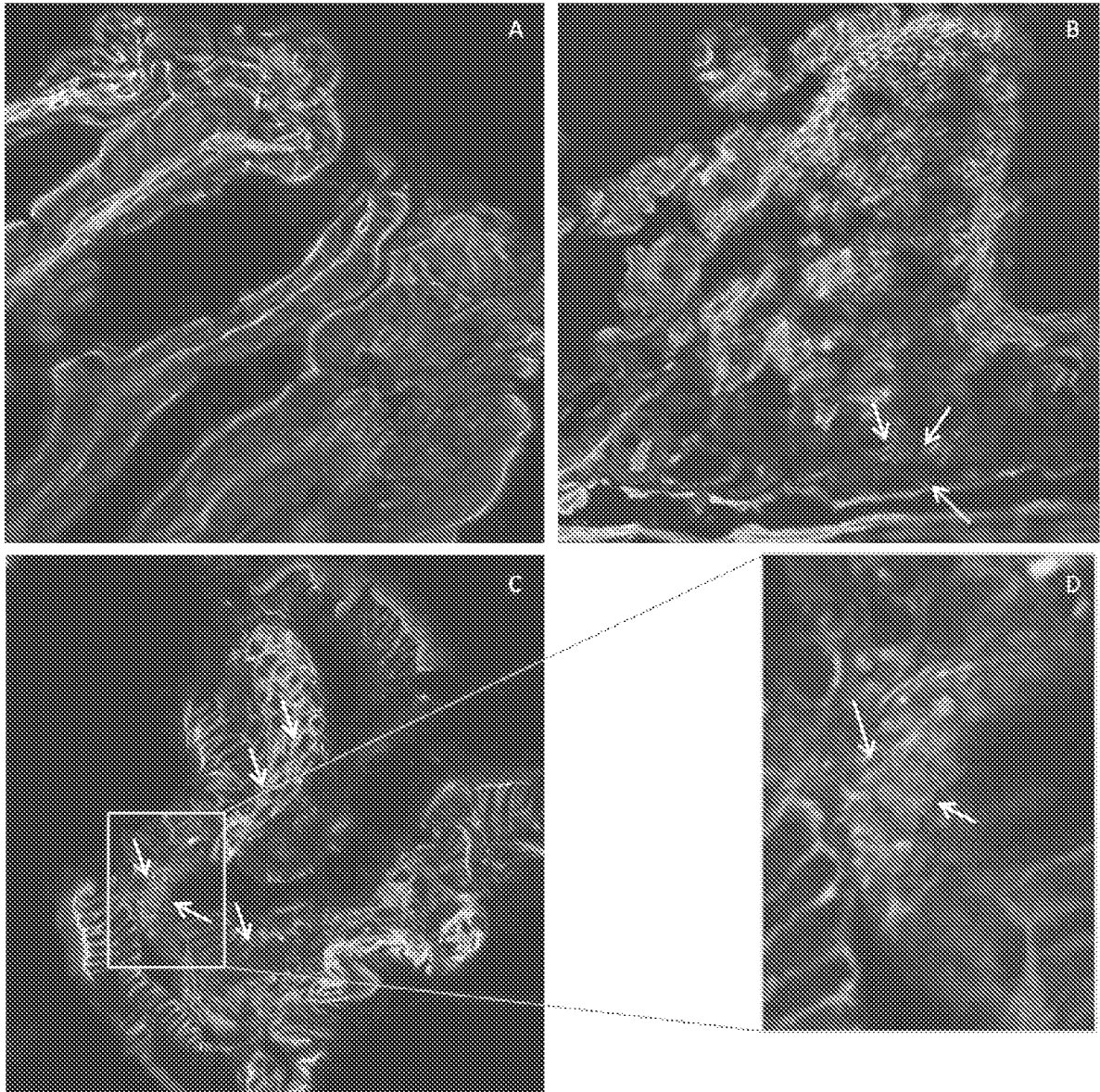


Fig. 46

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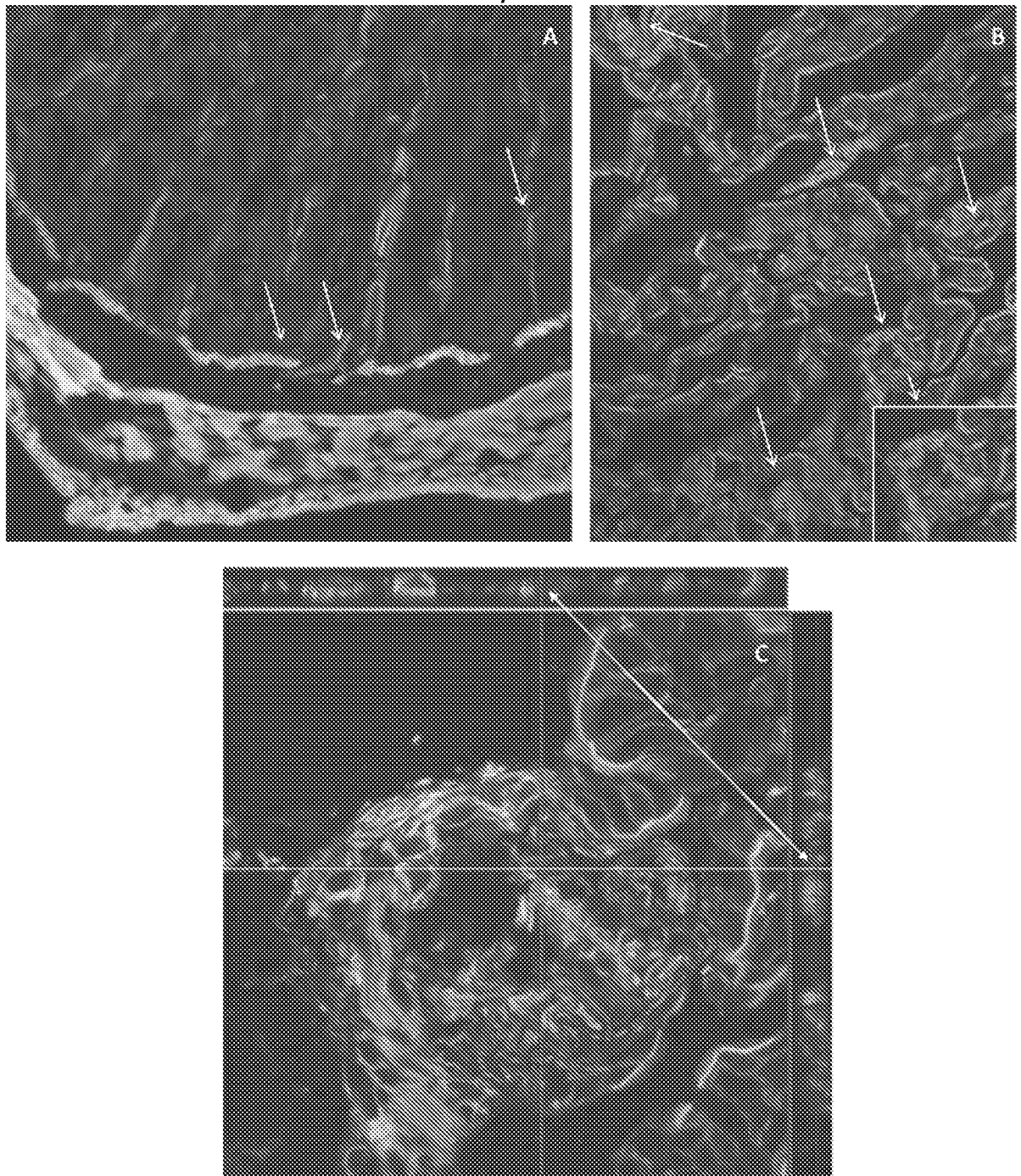


Fig. 47

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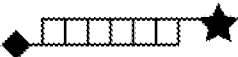
Group	n	Treatment	Treatment
1	4	siRNA 56 0 cholesteryl	
2	4	siRNA 57 1 cholesteryl	
3	4	siRNA 58 2 cholesteryl	

Fig. 48

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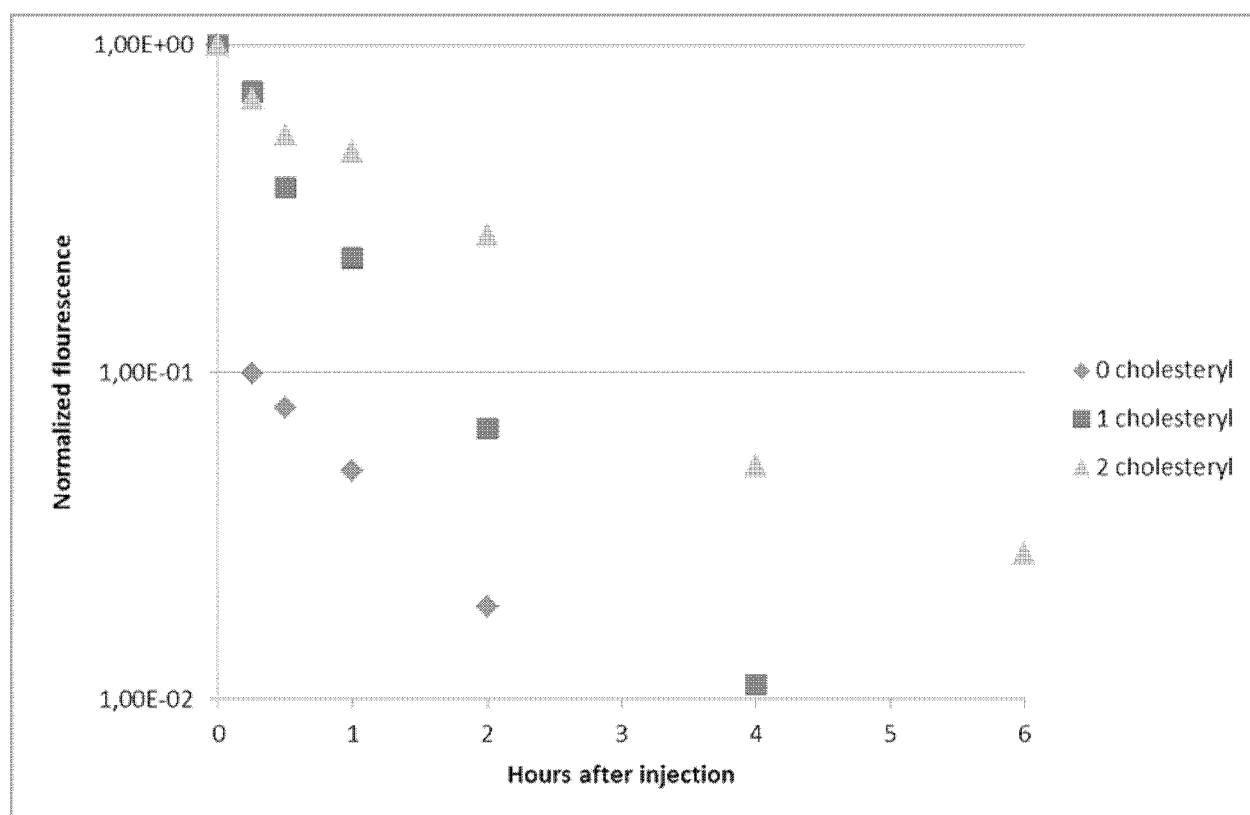
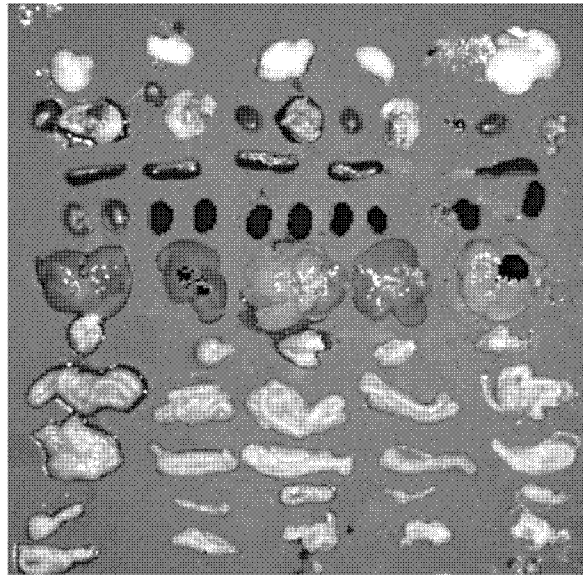


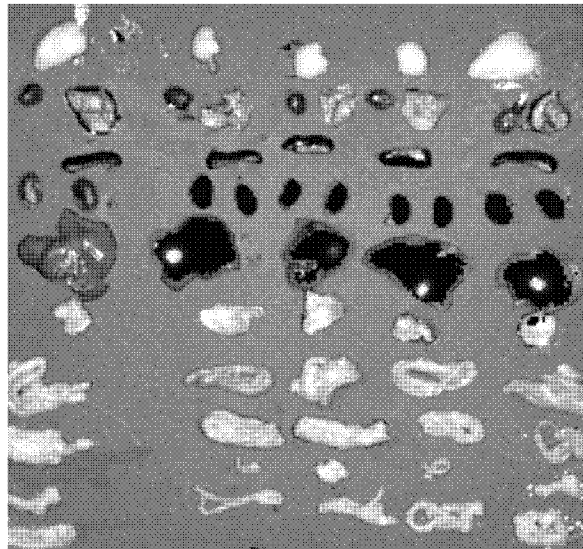
Fig. 49

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A



B



C

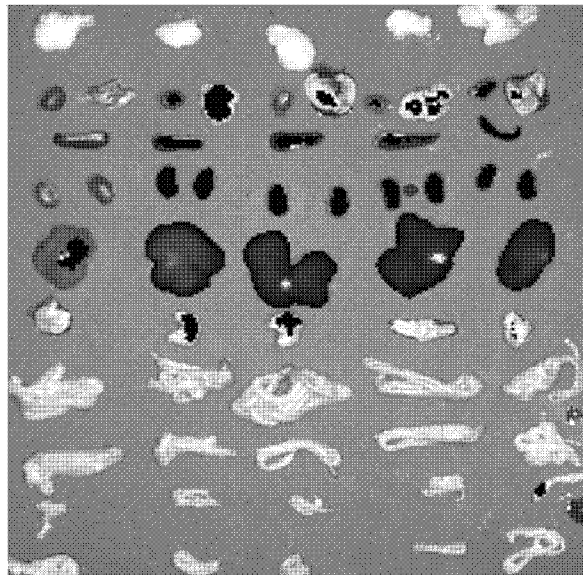


Fig. 50

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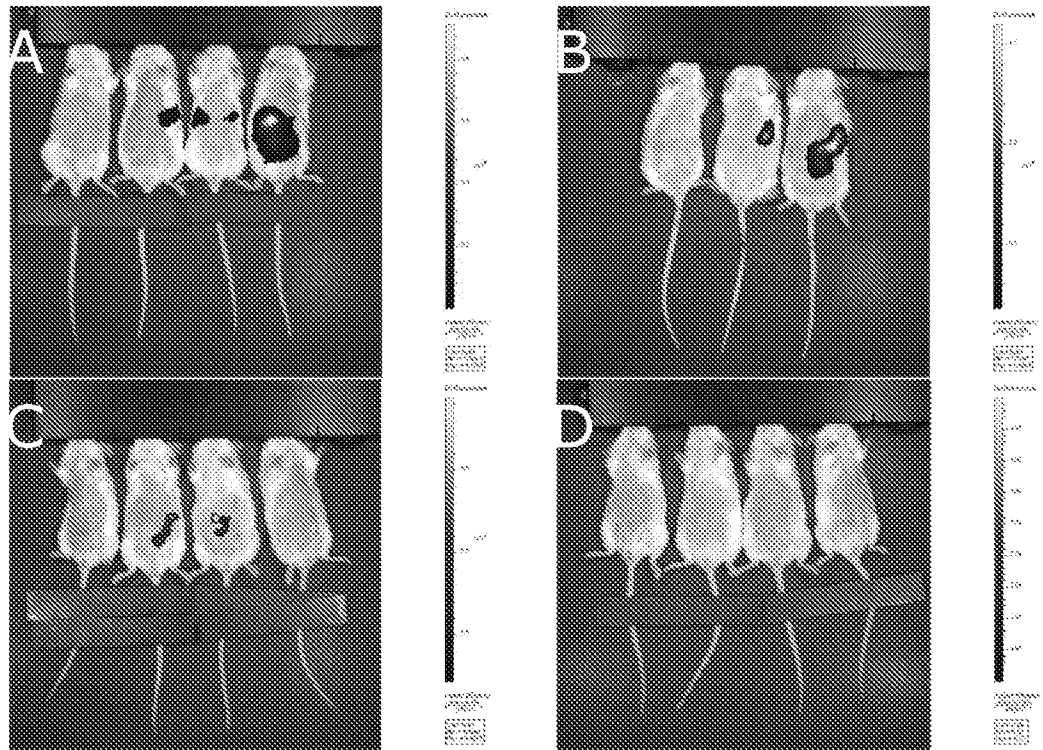


Fig. 51

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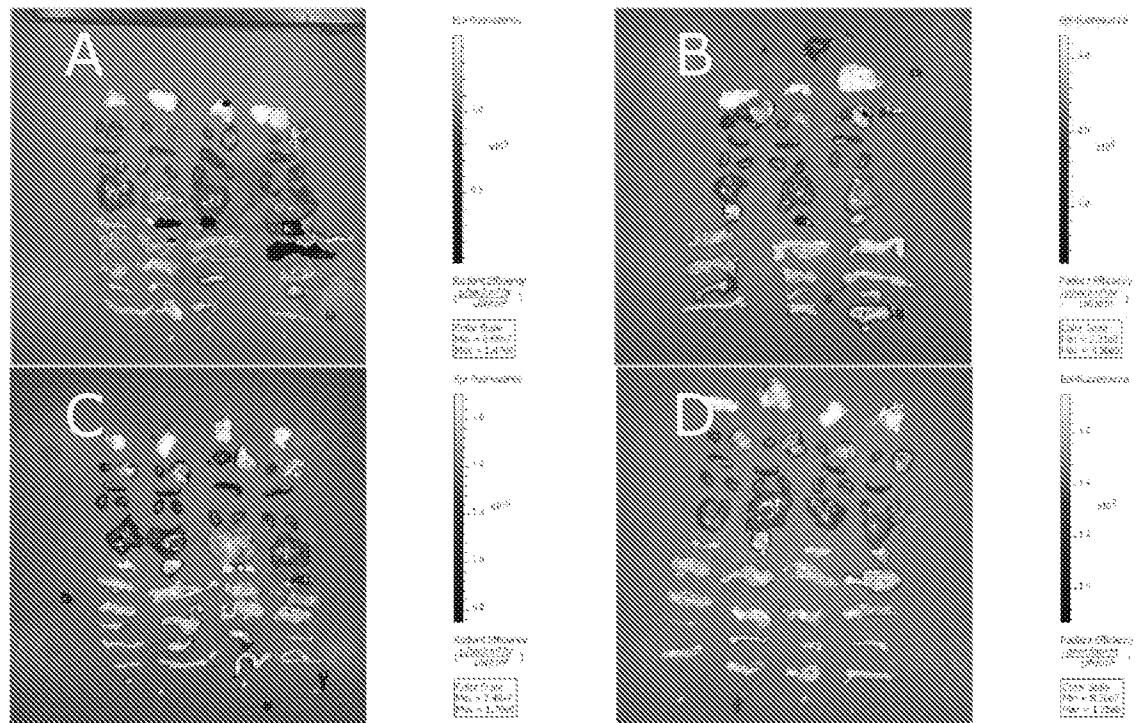


Fig. 52

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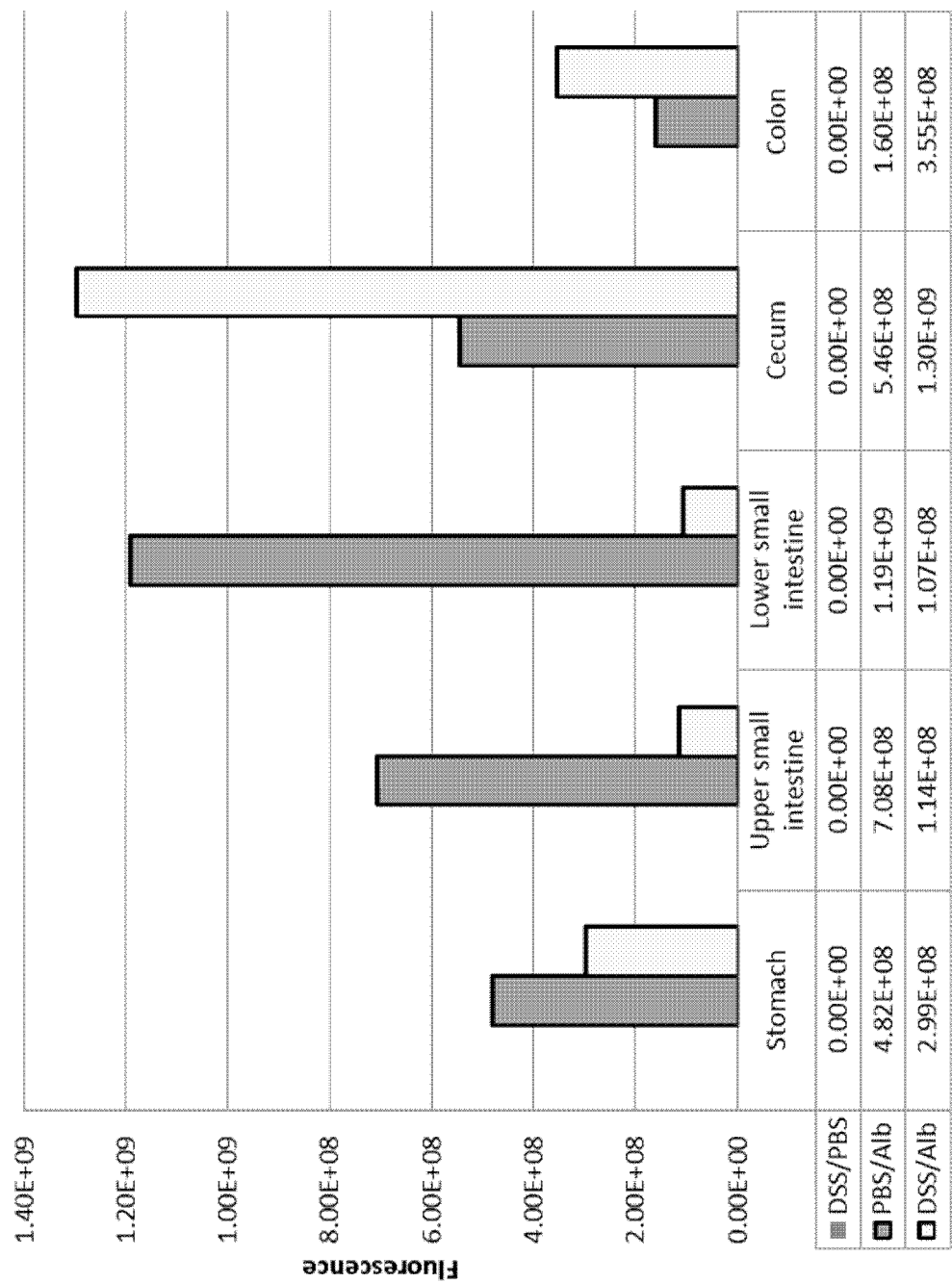


Fig. 53

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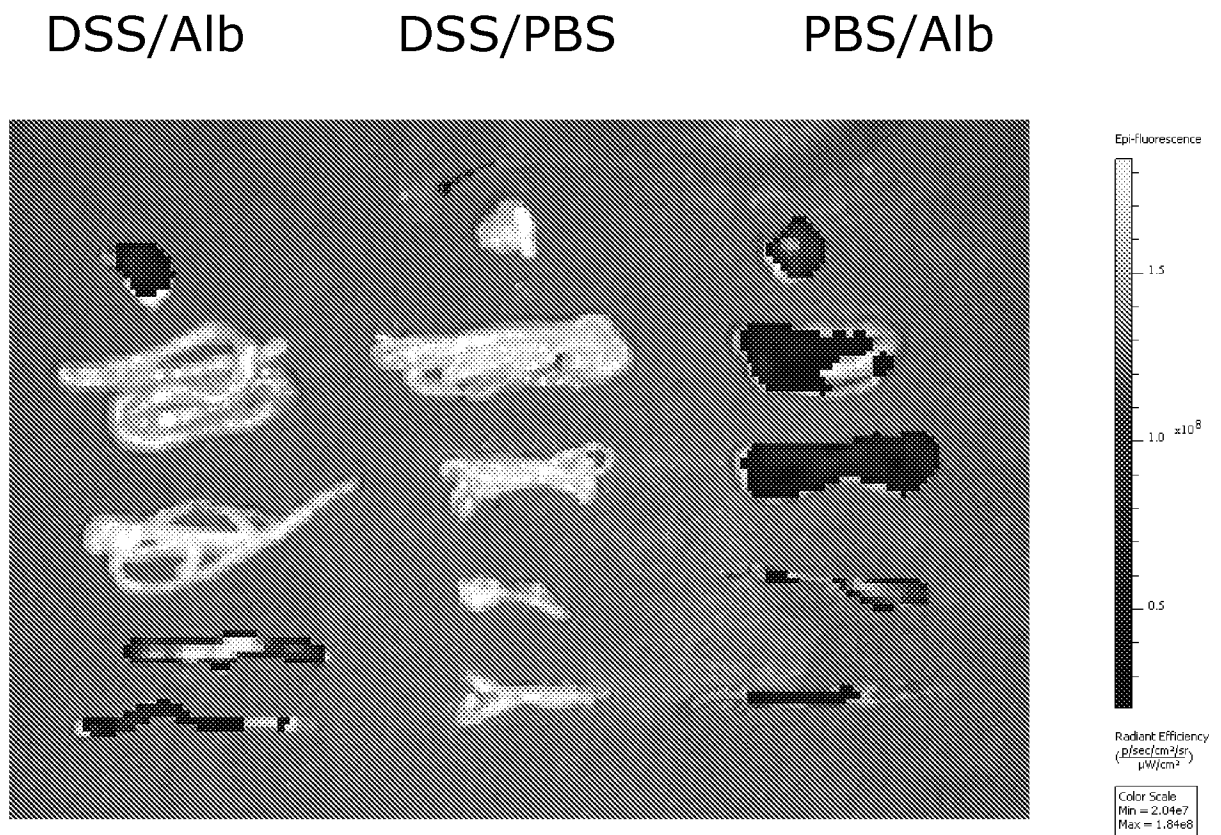


Fig. 54

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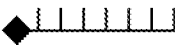
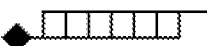
#	Phase	siRNA	Modification
30	II		2X Chol
31	II		1X Chol
33	II		2X Chol
34	II		1X Chol
35	II		3X Chol
36	II		2X Chol

Fig. 55

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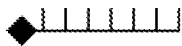
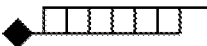
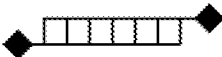
#	siRNA	Modification	Kd/M	Comment
30		2X Chol	$4.8 \cdot 10^{-7}$	At high protein concentration, the siRNA-albumin exists in several populations, localized in the gel as albumin dimer, trimmers and higher complexes
31		1X Chol	$7.3 \cdot 10^{-7}$	Only one siRNA-albumin population at high protein concentration
33		2X Chol	$7.5 \cdot 10^{-7}$	Albumin dimers visible at high concentration
34		1X Chol	$1.0 \cdot 10^{-6}$	Only one siRNA-albumin population at high protein concentration
35		3X Chol	$2.0 \cdot 10^{-7}$	Extensive formation of dimers and trimers, with the dimer population exceeding the monomer
36		2X Chol	$2.5 \cdot 10^{-7}$	Extensive formation of dimers and trimers, with the dimer population exceeding the monomer

Fig. 56

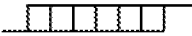
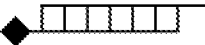
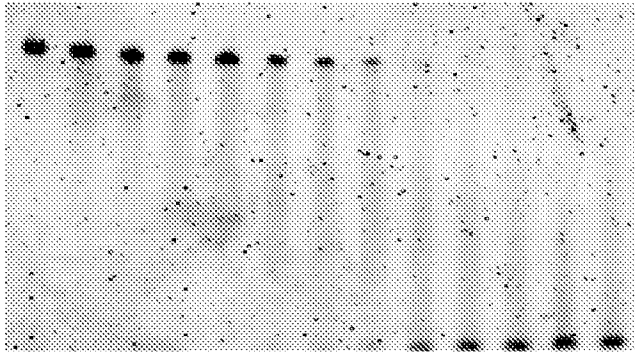
52	
53	
54	

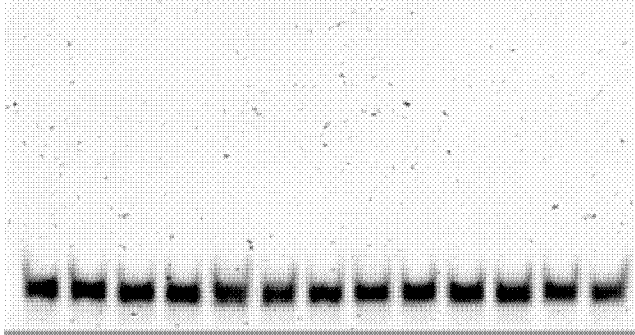
Fig. 57

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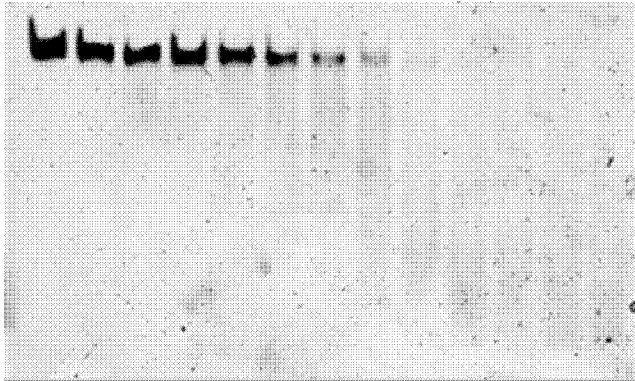
siRNA 34



siRNA 52



siRNA 54



siRNA 53

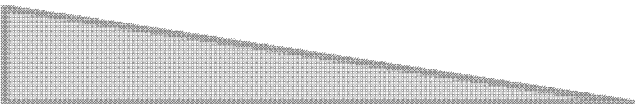
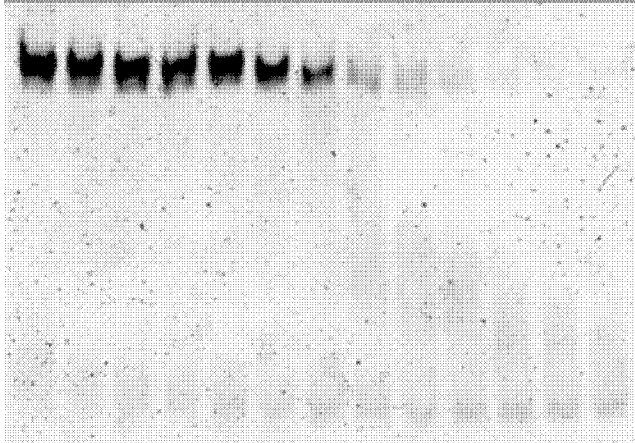
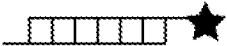
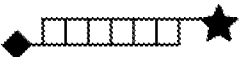


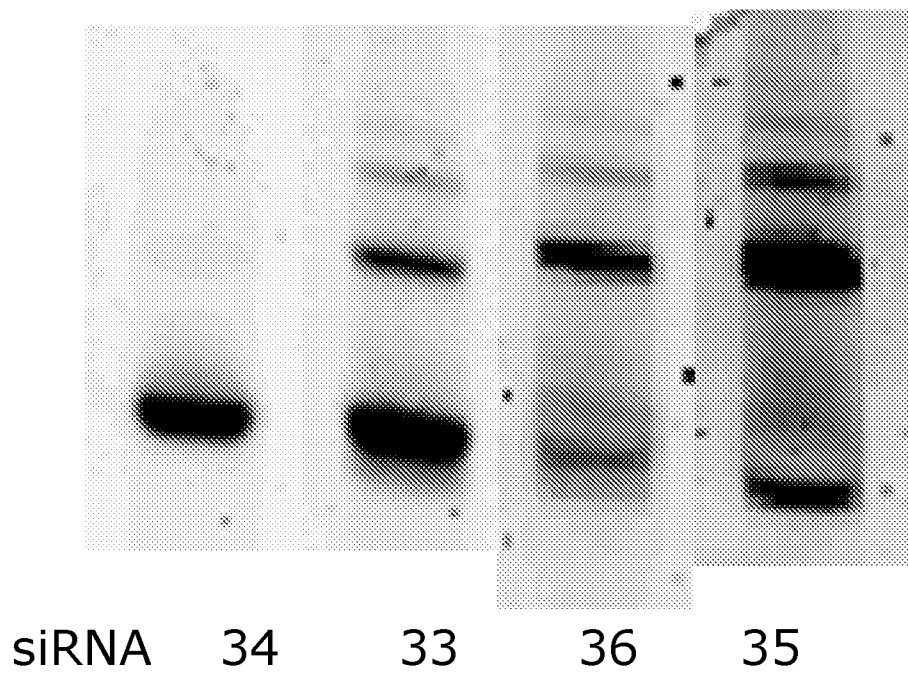
Fig. 58

Albumin

Fig. 59

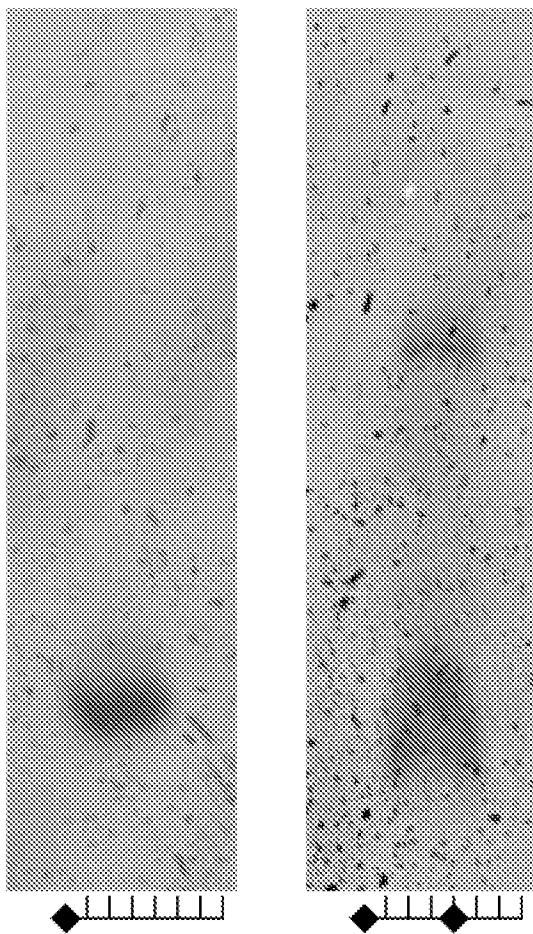
56	
57	
58	

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Fig. 60



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Fig. 61



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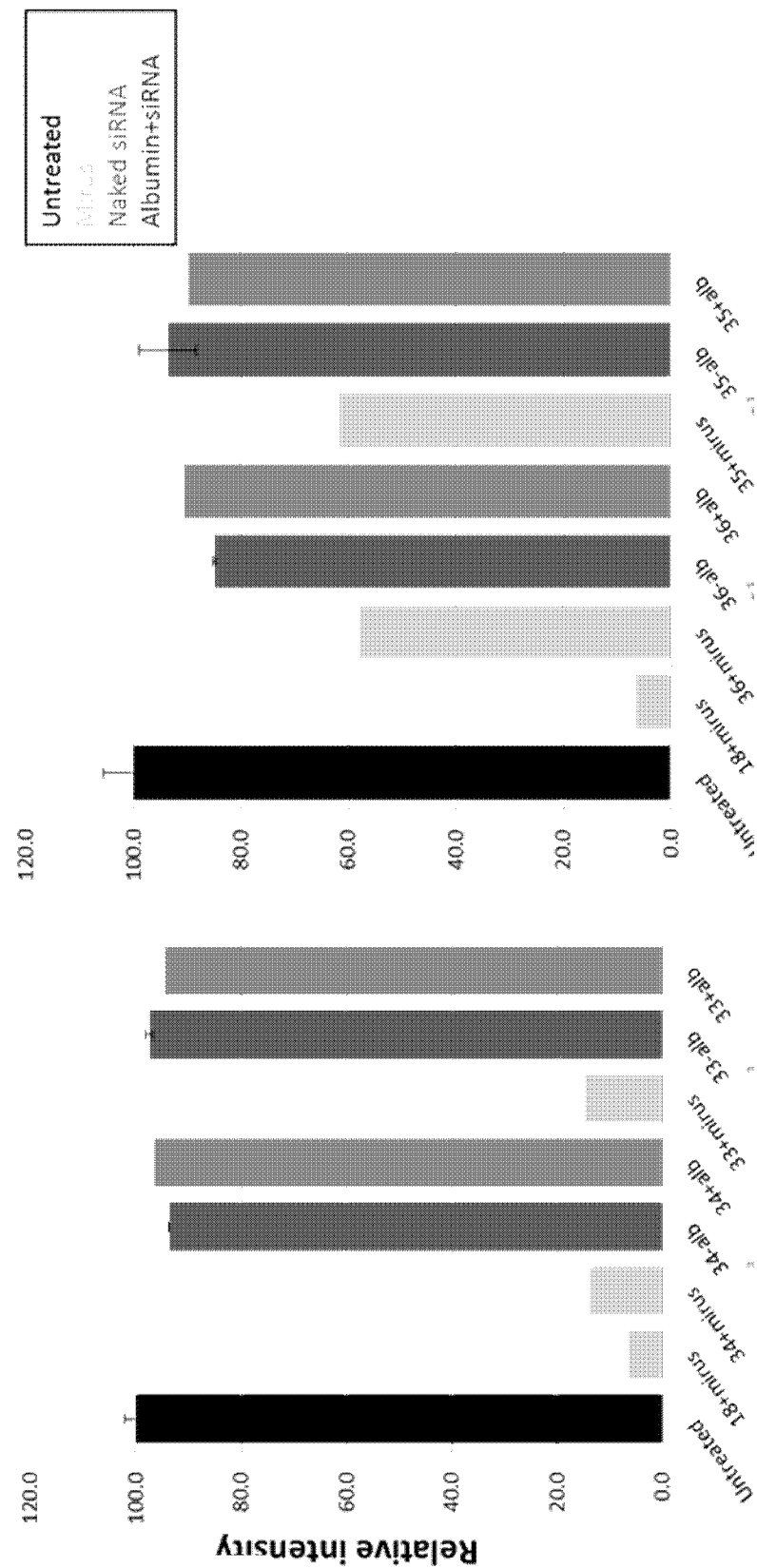
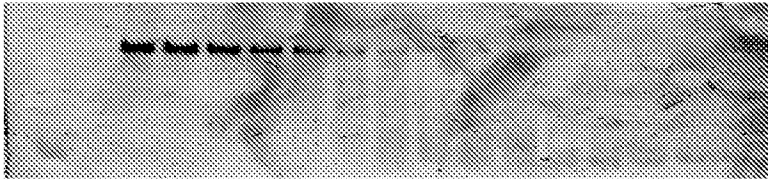


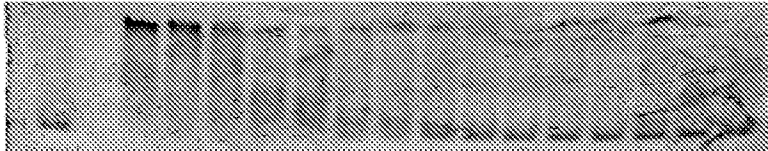
Fig. 62

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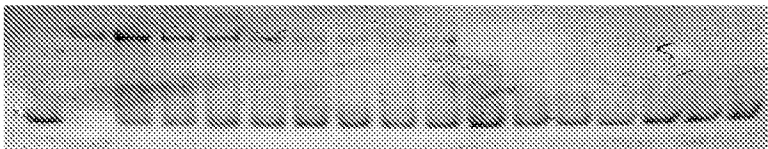
Macaque



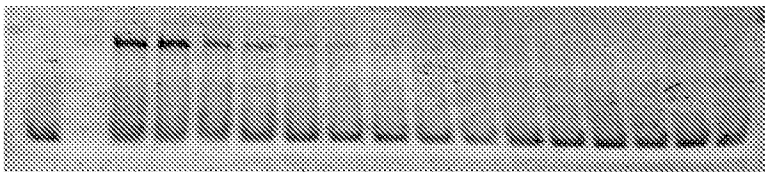
Pig



Rabbit



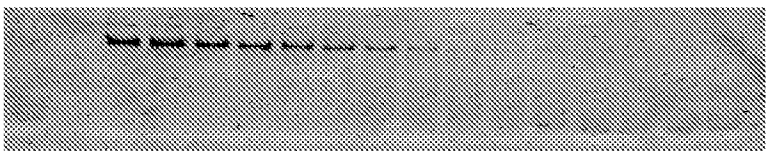
Bovine



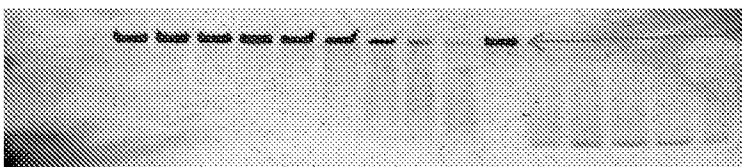
Rat



Mouse



Human

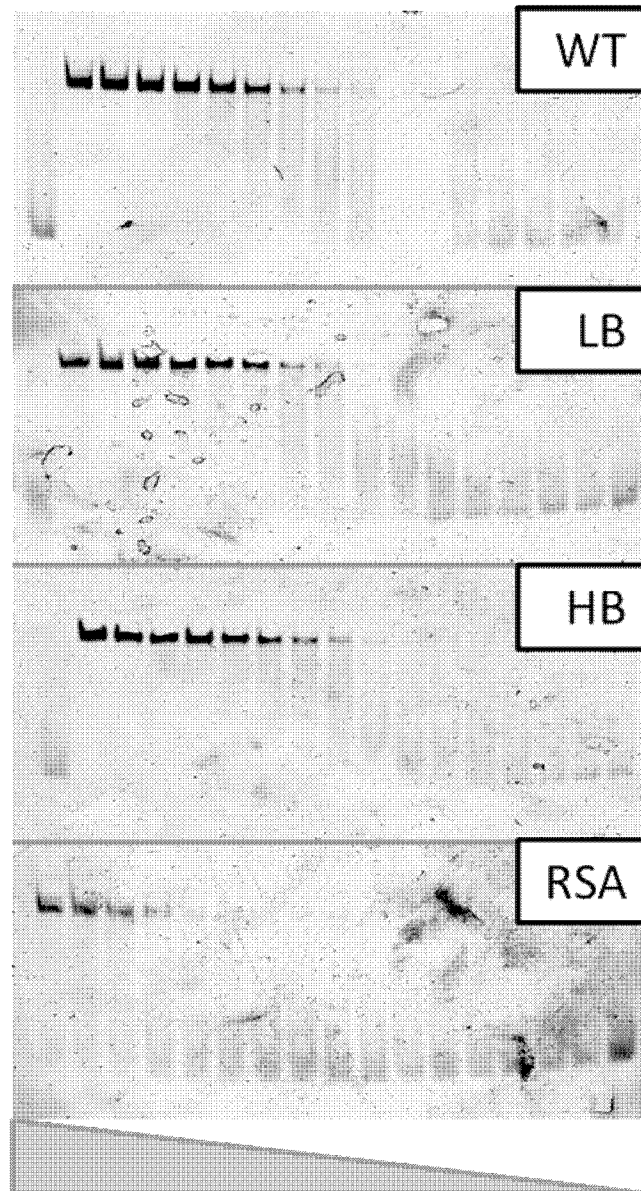


This lane is due to a loading error.

Fig. 63

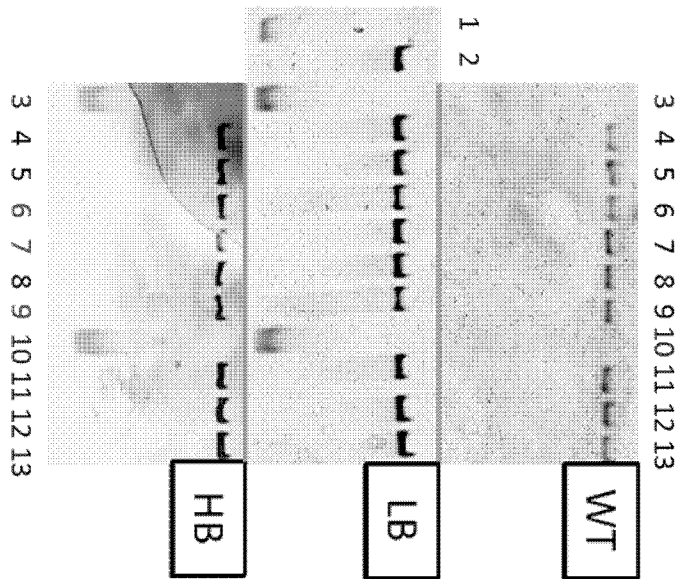
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Fig. 64



Albumin

Fig. 65

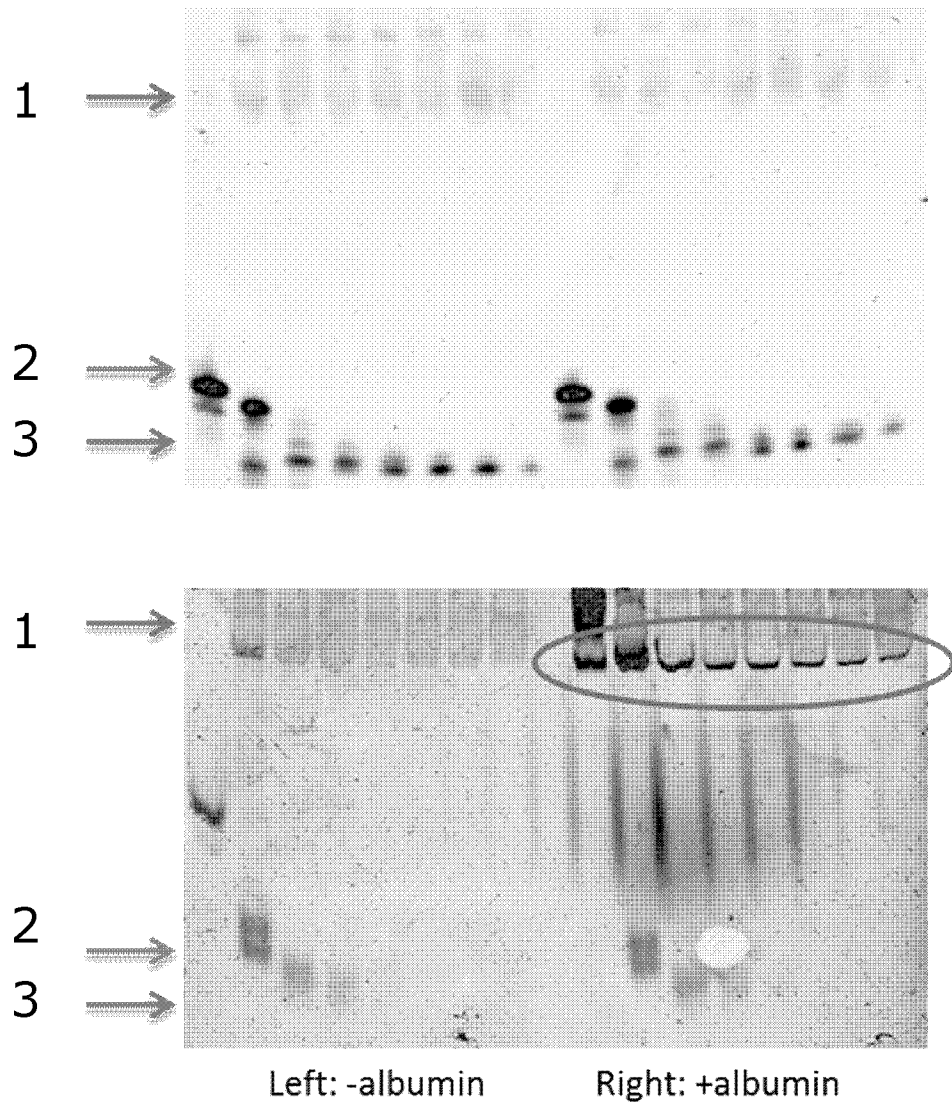


Ligands used

1. Free siRNA
2. siRNA+Albumin
3. Stearic acid+warfarin
4. Sodium octanoate
5. Fusidic acid
6. Sodium myristate
7. Lithocholic acid
8. Cholesterol
9. Myristic acid
10. Stearic acid
11. Warfarin
12. L-Thyroxine
13. L-Thyroxine+Sodium Octanoate

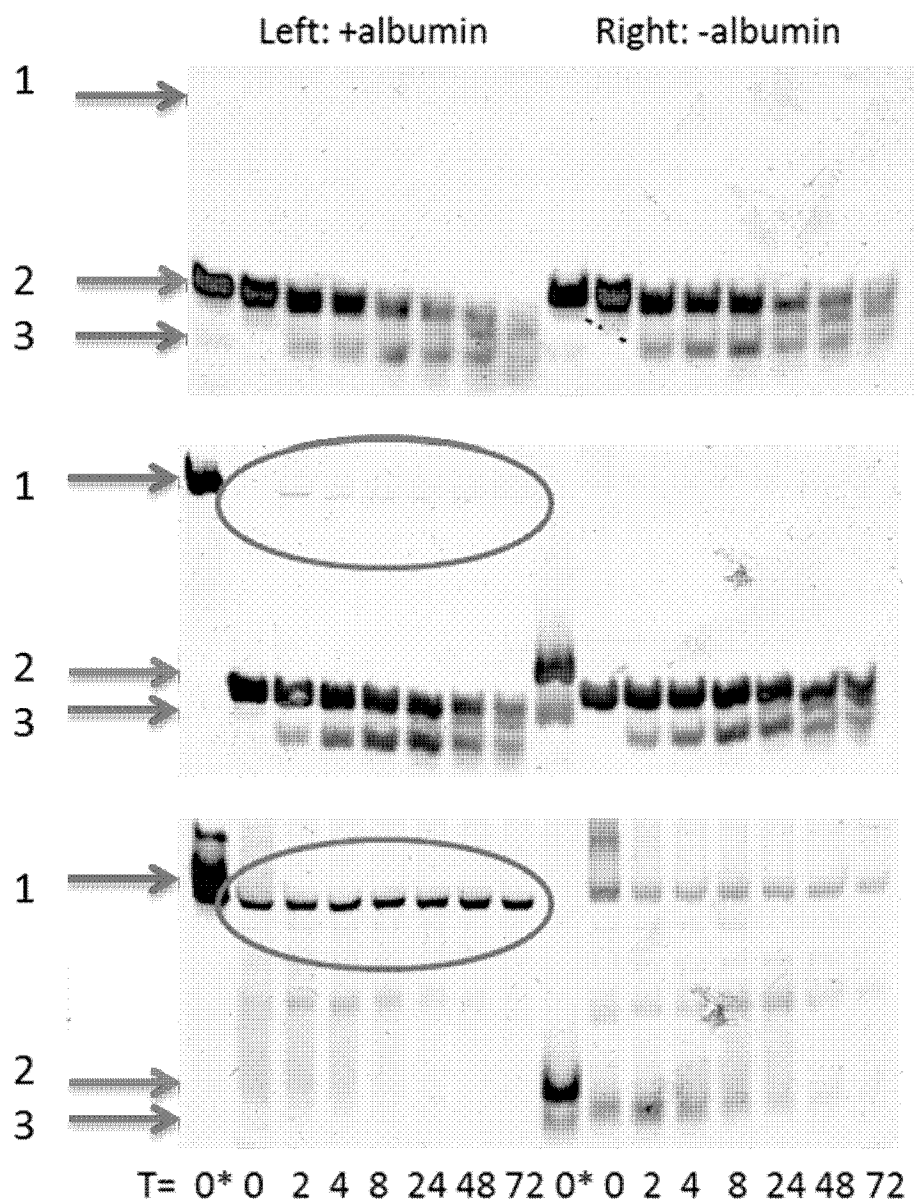
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Fig. 66



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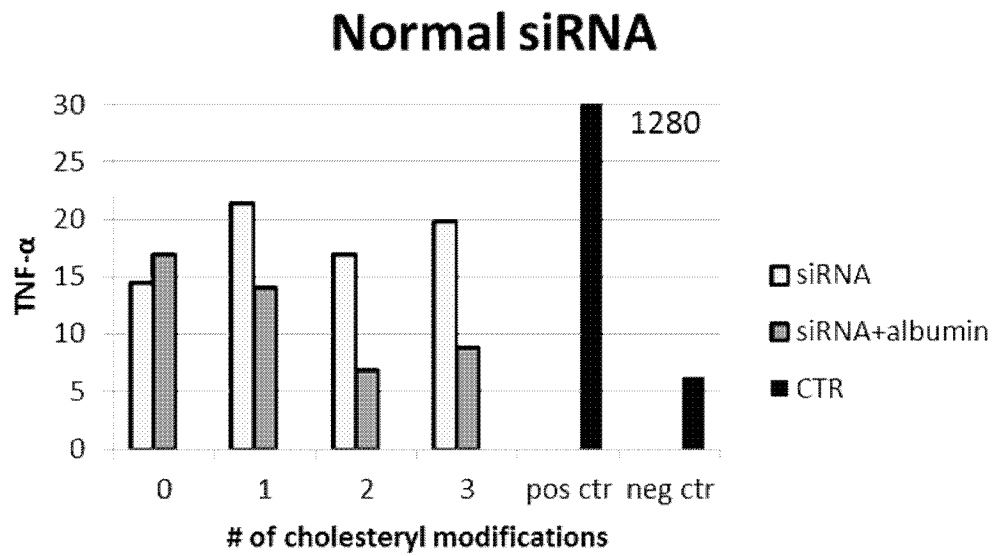
Fig. 67



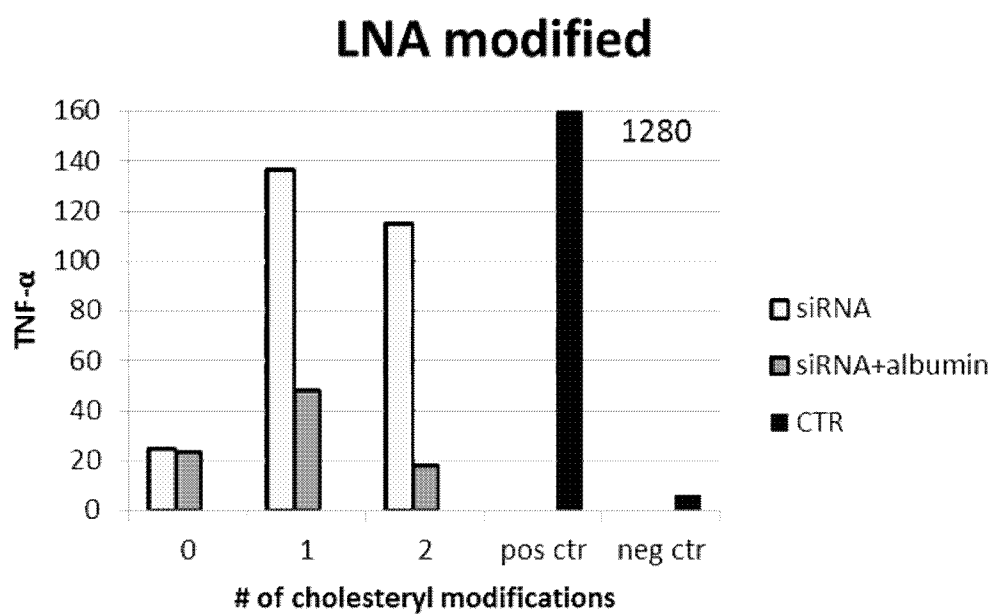
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Fig. 68

A



B



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Fig. 69

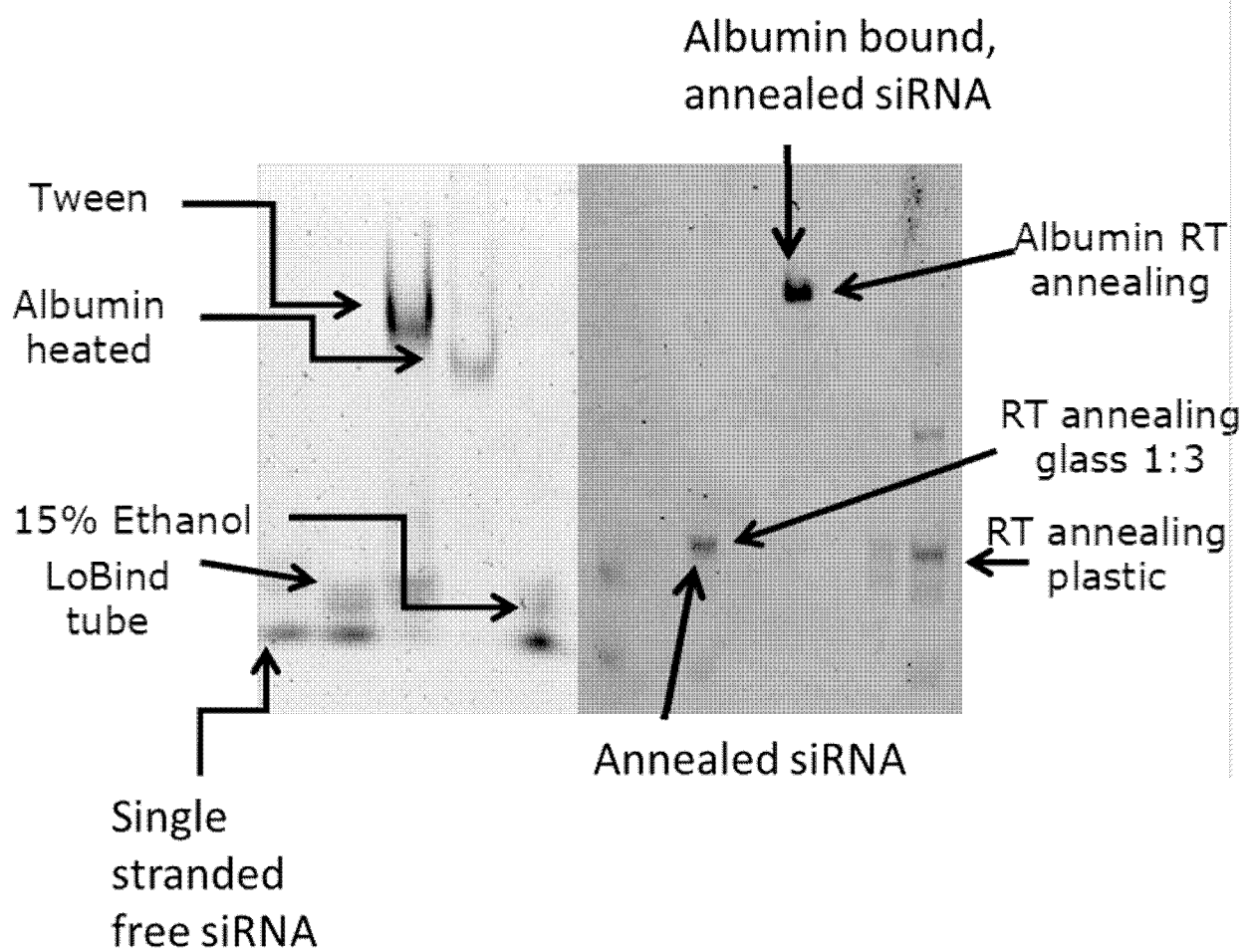
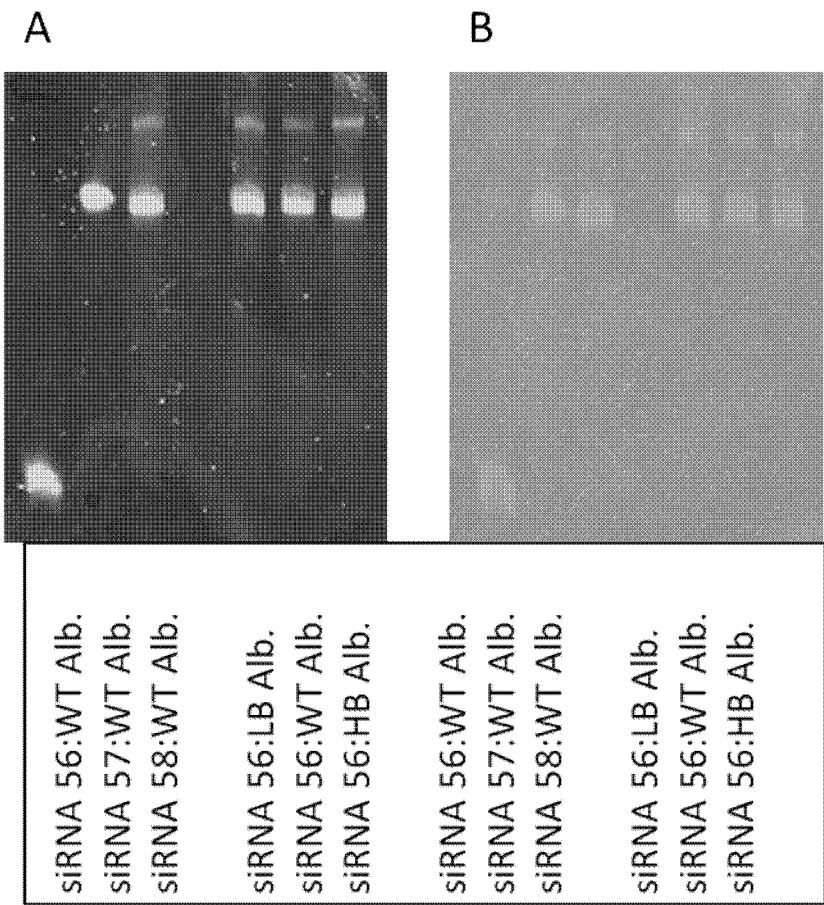
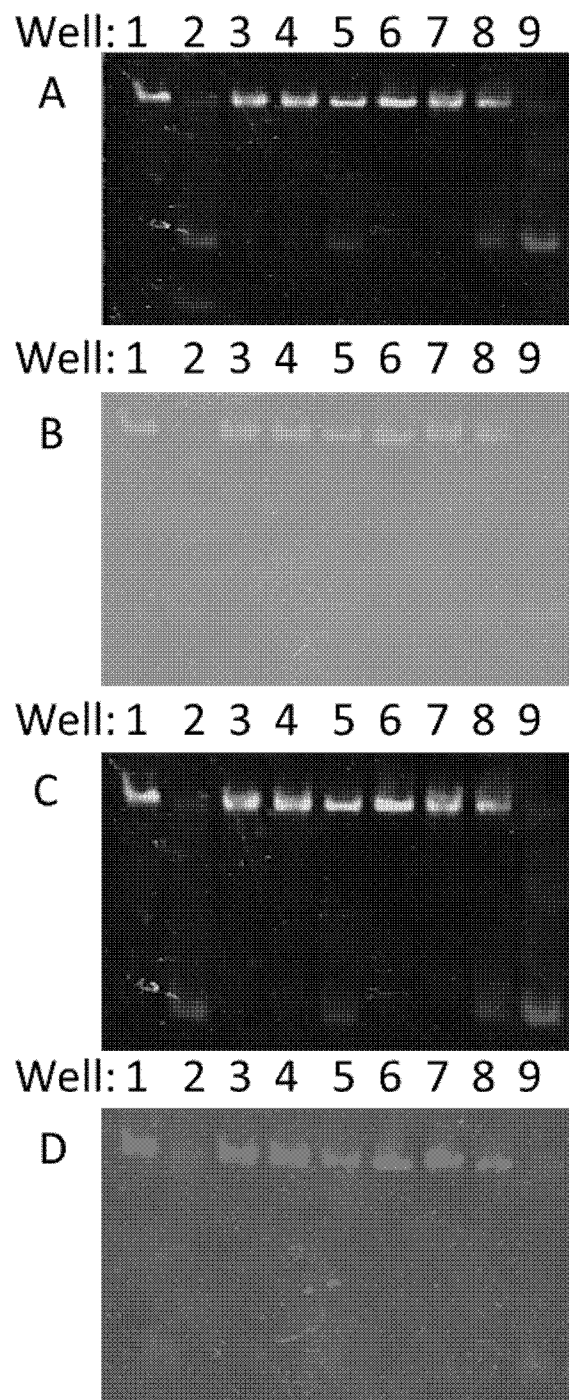


Fig. 70



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Fig. 71



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Fig. 72

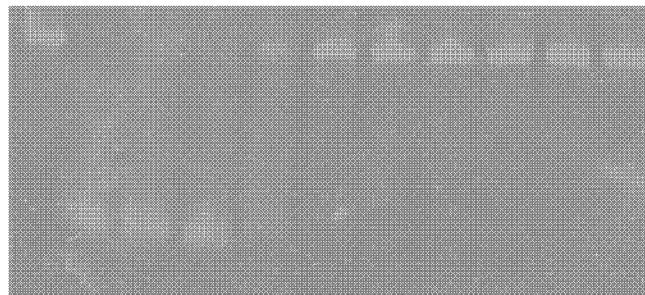
Well: 1 2 3 4 5 6 7 8 9 10 11

A



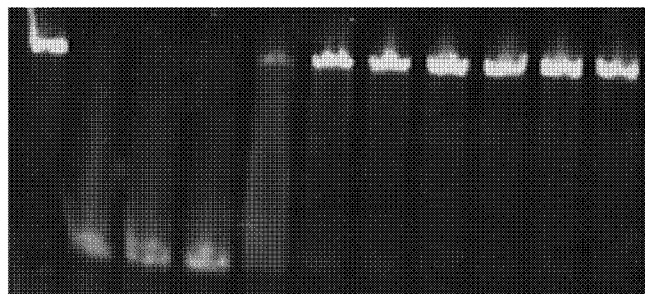
Well: 1 2 3 4 5 6 7 8 9 10 11

B



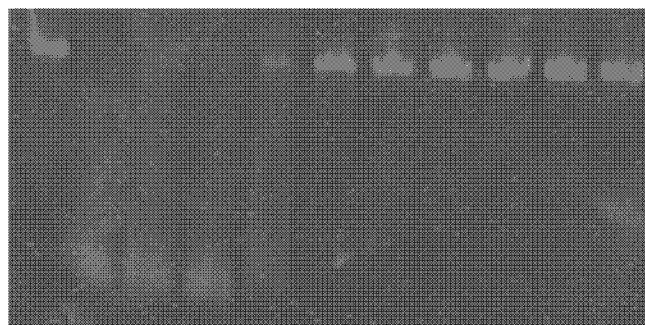
Well: 1 2 3 4 5 6 7 8 9 10 11

C



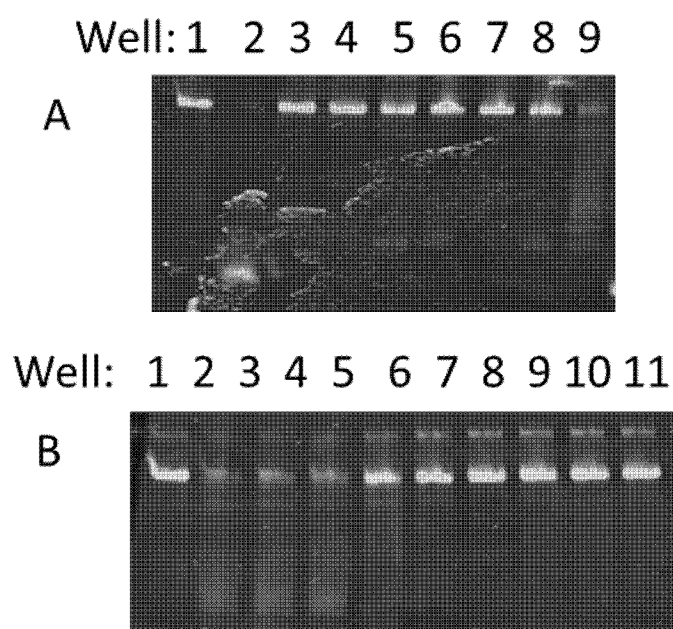
Well: 1 2 3 4 5 6 7 8 9 10 11

D



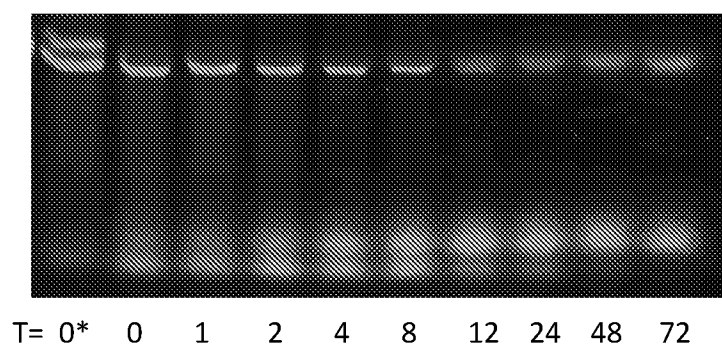
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Fig. 73



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Fig. 74



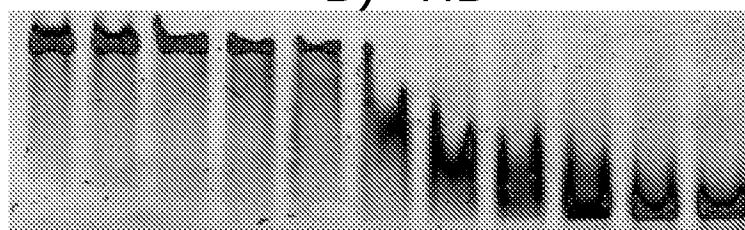
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Fig. 75

A) LB

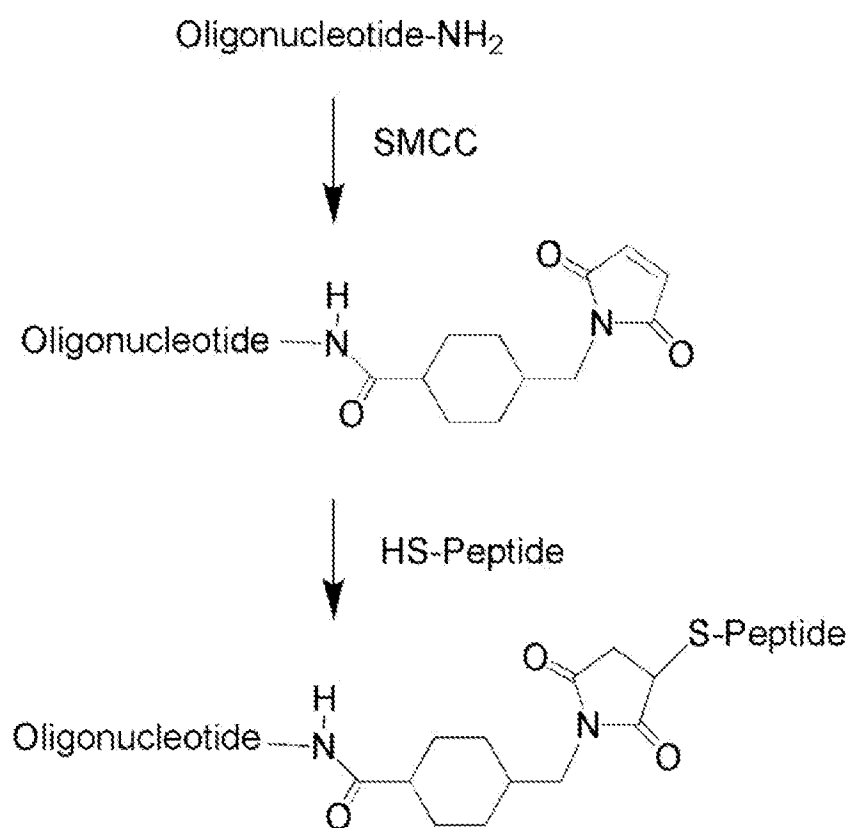


B) HB



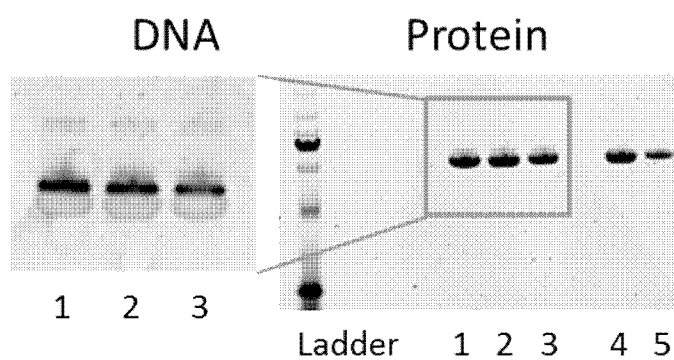
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Fig. 76



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Fig. 77



1: oligo pH 6.5

2: oligo pH 7.0

3: oligo pH 7.5

4: Albumin 100pmol

5: Albumin 10pmol

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2013/050223

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N15/11 C07K14/765 A61K31/7088 A61K47/48
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EP0-Internal, WPI Data, BIOSIS, EMBASE, Sequence Search, EMBL

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	BAKHEET ELSADEK ET AL: "Impact of albumin on drug delivery New applications on the horizon", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 157, no. 1, 16 September 2011 (2011-09-16), pages 4-28, XP028443365, ISSN: 0168-3659, DOI: 10.1016/J.JCONREL.2011.09.069 [retrieved on 2011-09-16] page 7 - page 10; figure 12 page 15 - page 16 ----- -/-	1,2, 17-20, 26-28



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

5 December 2013

Date of mailing of the international search report

13/12/2013

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

Bucka, Alexander

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2013/050223

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FIUME L ET AL: "Doxorubicin coupled to lactosaminated human albumin: a hepatocellular carcinoma targeted drug", DRUG DISCOVERY TODAY, ELSEVIER, RAHWAY, NJ, US, vol. 13, no. 21-22, November 2008 (2008-11), pages 1002-1009, XP025574305, ISSN: 1359-6446, DOI: 10.1016/J.DRUDIS.2008.07.009 [retrieved on 2008-09-11] figure 1	1,2, 17-20, 26-28
A	SMOLARCZYK R ET AL: "Negligible induction of IFN-gamma, IL-12 and TNF-alpha by DNA-PEI 750 kDa/albumin complexes", CYTOKINE, ACADEMIC PRESS LTD, PHILADELPHIA, PA, US, vol. 29, no. 6, 21 March 2005 (2005-03-21), pages 283-287, XP004769510, ISSN: 1043-4666, DOI: 10.1016/J.CYT0.2004.11.010 figure 1	1,2, 17-20, 26-28
A	US 2004/043079 A1 (D SOUZA MARTIN J [US]) 4 March 2004 (2004-03-04) claim 1	1,2, 17-20, 26-28
X	US 2007/172430 A1 (BRITO LUIS [US] ET AL) 26 July 2007 (2007-07-26)	1-3,12, 13,17, 26-28
Y	example 3	5,7-11, 15,16, 18-20
Y	LORENZ C ET AL: "Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells", BIOORGANIC & MEDICINAL CHEMISTRY LETTERS, PERGAMON, vol. 14, no. 19, 29 July 2004 (2004-07-29), pages 4975-4977, XP027213207, ISSN: 0960-894X [retrieved on 2004-09-01] page 4976; figure 3	5,7-11, 15,16, 18-20
	-/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2013/050223

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WOLFRUM CHRISTIAN ET AL: "Mechanisms and optimization of in vivo delivery of lipophilic siRNAs", NATURE BIOTECHNOLOGY, NATURE PUBLISHING GROUP, NEW YORK, NY, US, vol. 25, no. 10, 16 September 2007 (2007-09-16), pages 1149-1157, XP002493037, ISSN: 1087-0156, DOI: 10.1038/NBT1339 [retrieved on 2007-09-16]	1-4,6, 12-14, 17,26-28
Y	page 1150, right-hand column; figure 1 page 1156, right-hand column	5,7-11, 15,16, 18-20
A	----- K. K. LEE ET AL: "Capturing a Fusion Intermediate of Influenza Hemagglutinin with a Cholesterol-conjugated Peptide, a New Antiviral Strategy for Influenza Virus", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 286, no. 49, 9 December 2011 (2011-12-09), pages 42141-42149, XP55076229, ISSN: 0021-9258, DOI: 10.1074/jbc.M111.254243 figure 1	1,2, 17-20, 26-28
A	----- GOLDBERG: "Low Molecular Weight Opioid Peptide Esters Could be Developed as a New Class of Analgesics", PERSPECTIVES IN MEDICINAL CHEMISTRY, 25 July 2011 (2011-07-25), pages 19-26, XP55076228, ISSN: 1177-391X, DOI: 10.4137/PMC.S6803 tables 2,3	1,2, 17-20, 26-28
X	----- WO 2011/051489 A2 (NOVOZYMES BIOPHARMA DK AS [DK]; PLUMRIDGE ANDREW [GB]; SLEEP DARRELL []) 5 May 2011 (2011-05-05) claim 1; example 18	18-20
X	----- A. AHMED-OUAMEUR ET AL: "The Effects of Drug Complexation on the Stability and Conformation of Human Serum Albumin: Protein Unfolding", CELL BIOCHEMISTRY AND BIOPHYSICS, vol. 45, no. 2, 2006, pages 203-214, XP55076183, ISSN: 1085-9195, DOI: 10.1385/CBB:45:2:203 tables 1,2 ----- -/--	1,2, 17-20, 26-28

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2013/050223

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CREMERS H F M ET AL: "Albumin-heparin microspheres as carriers for cytostatic agents", JOURNAL OF CONTROLLED RELEASE, ELSEVIER, AMSTERDAM, NL, vol. 11, no. 1-3, January 1990 (1990-01), pages 167-179, XP023744017, ISSN: 0168-3659, DOI: 10.1016/0168-3659(90)90130-L [retrieved on 1990-01-01] page 169 - page 170; tables 2,3	1,2, 17-20, 26-28
X	MARIANGELA BELLUSCI ET AL: "Preparation of albumin-ferrite superparamagnetic nanoparticles using reverse micelles", POLYMER INTERNATIONAL, vol. 58, no. 10, 21 July 2009 (2009-07-21), pages 1142-1147, XP055076280, ISSN: 0959-8103, DOI: 10.1002/pi.2642 figures 1,2	1,2, 17-20, 26-28
X	US 2009/099071 A1 (NAKAJOU KEISUKE [JP] ET AL) 16 April 2009 (2009-04-16) paragraphs [0090], [0103] - paragraph [0106]; claim 17	1,2, 17-20, 26-28
X	US 2012/083455 A1 (KIM KWANGMEYUNG [KR] ET AL) 5 April 2012 (2012-04-05) examples 2,3	1-3,12, 13,17, 21,26-28
X	LAU SHANNEN ET AL: "Enhanced Extravasation, Stability and in Vivo Cardiac Gene Silencing via in Situ siRNA-Albumin Conjugation", MOLECULAR PHARMACEUTICS, AMERICAN CHEMICAL SOCIETY, US, vol. 9, no. 1, 5 December 2011 (2011-12-05), pages 71-80, XP008164358, ISSN: 1543-8384, DOI: 10.1021/MP200246B [retrieved on 2011-11-17] figures 2-5	1-3,12, 13,16, 17,21, 26-28
X	US 2011/159098 A1 (SLAGER JORAM [US]) 30 June 2011 (2011-06-30) claims 1-3; figure 1; example 1	1-3,12, 13,17, 26-28
	-/--	

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2013/050223

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SANA ABBASI ET AL: "Investigation of siRNA-Loaded Polyethylenimine-Coated Human Serum Albumin Nanoparticle Complexes for the Treatment of Breast Cancer", CELL BIOCHEMISTRY AND BIOPHYSICS, HUMANA PRESS INC, NEW YORK, vol. 61, no. 2, 10 May 2011 (2011-05-10), pages 277-287, XP019973946, ISSN: 1559-0283, DOI: 10.1007/S12013-011-9201-9 figures 1,8 -----	1-3,12, 13,17, 26-28
X	CUN D ET AL: "High loading efficiency and sustained release of siRNA encapsulated in PLGA nanoparticles: Quality by design optimization and characterization", EUROPEAN JOURNAL OF PHARMACEUTICS AND BIOPHARMACEUTICS, ELSEVIER SCIENCE PUBLISHERS B.V., AMSTERDAM, NL, vol. 77, no. 1, 18 November 2010 (2010-11-18), pages 26-35, XP027571341, ISSN: 0939-6411 [retrieved on 2010-12-22] page 27, right-hand column; table 4 -----	1-3,12, 13,17, 26-28
X	JENNIFER Y. KELLY ET AL: "Shape-Specific, Monodisperse Nano-Molding of Protein Particles", JOURNAL OF THE AMERICAN CHEMICAL SOCIETY, vol. 130, no. 16, 1 April 2008 (2008-04-01), pages 5438-5439, XP55085549, ISSN: 0002-7863, DOI: 10.1021/ja8014428 page 5438, left-hand column -----	1-3,12, 13,17, 26-28
A	WO 2006/019430 A2 (NASTECH PHARM CO [US]; AHMADIAN MOHAMMAD [US]; CUI KUNYUAN [US]; CHEN) 23 February 2006 (2006-02-23) the whole document -----	1-21, 26-28
A	TAKANORI KUBO ET AL: "Palmitic Acid-Conjugated 21-Nucleotide siRNA Enhances Gene-Silencing Activity", MOLECULAR PHARMACEUTICS, vol. 8, no. 6, 10 October 2011 (2011-10-10), pages 2193-2203, XP55087421, ISSN: 1543-8384, DOI: 10.1021/mp200250f table 1 ----- -/--	1-21, 26-28

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2013/050223

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	TAKESHI YAMADA ET AL: "Versatile Site-Specific Conjugation of Small Molecules to siRNA Using Click Chemistry", THE JOURNAL OF ORGANIC CHEMISTRY, vol. 76, no. 5, 7 February 2011 (2011-02-07), pages 1198-1211, XP055013736, ISSN: 0022-3263, DOI: 10.1021/jo101761g table 1 -----	1-21, 26-28

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK2013/050223

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
3-16, 21(completely); 1, 2, 17-20, 26-28(partially)
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No

PCT/DK2013/050223

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2004043079 A1	04-03-2004	AU 2003270030 A1 CA 2497142 A1 CN 1756535 A EP 1536773 A2 JP 4621022 B2 JP 2006511461 A US 2004043079 A1 US 2009081306 A1 WO 2004019906 A2	19-03-2004 11-03-2004 05-04-2006 08-06-2005 26-01-2011 06-04-2006 04-03-2004 26-03-2009 11-03-2004
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FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. claims: 1, 2, 17-20, 26-28(all partially)

A composition comprising a payload molecule and albumin or variant albumin or fragment thereof, or fusion polypeptide comprising albumin or variant albumin or fragment thereof, wherein the payload molecule is a small molecule.

2. claims: 1, 2, 17-20, 26-28(all partially)

A composition comprising a payload molecule and albumin or variant albumin or fragment thereof, or fusion polypeptide comprising albumin or variant albumin or fragment thereof, wherein the payload molecule is plasmid DNA.

3. claims: 1, 2, 17-20, 26-28(all partially)

A composition comprising a payload molecule and albumin or variant albumin or fragment thereof, or fusion polypeptide comprising albumin or variant albumin or fragment thereof, wherein the payload molecule is a peptide.

4. claims: 3-16, 21(completely); 1, 2, 17-20, 26-28(partially)

A composition comprising a payload molecule and albumin or variant albumin or fragment thereof, or fusion polypeptide comprising albumin or variant albumin or fragment thereof, wherein the payload molecule is a siNA.

5. claims: 25(completely); 22-24, 26-28(partially)

A payload molecule modified by one or more cholesteryls, wherein the payload molecule is a siNA.

6. claims: 22-24, 26-28(all partially)

A payload molecule modified by one or more cholesteryls, wherein the payload molecule is a peptide.

7. claims: 22-24, 26-28(all partially)

A payload molecule modified by one or more cholesteryls, wherein the payload molecule is a small molecule.

8. claim: 29

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Use of albumin for the formation of a conjugate comprising the albumin and a payload molecule modified by at least two cholesterols.
