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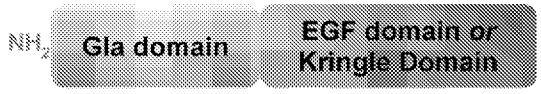
(54) Title: METHOD OF TARGETING EXOSOMES

(57) Abstract: The present disclosure relates to a method of targeting extracellular vesicles employing a molecule comprising a GLA domain and extracellular vesicles obtained or obtainable from a method disclosed herein.

Figure 1E An embodiment of a GLA-component



METHOD OF TARGETING EXOSOMES

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 62/554,530 filed September 5, 2017, U.S. Provisional Application No. 62/554,533 filed September 5, 2017, U.S. Provisional Application No. 62/569,403 filed October 6, 2017, U.S. Provisional Application No. 62/569,411 filed October 6, 2017, U.S. Provisional Application No. 62/584,565 filed November 10, 2017, and U.S. Provisional Application No. 62/593,014 filed November 30, 2017, each of which applications is herein incorporated by reference in its entirety.

INCORPORATION OF SEQUENCE LISTING

This application contains a sequence listing submitted electronically via EFS-web, which serves as both the paper copy and the computer readable form (CRF) and consists of a file entitled "ST-CT7-PCT_sequence.txt", which was created on September 5, 2018, which is 9,823 bytes in size, and which is herein incorporated by reference in its entirety.

[0001] The present disclosure relates to a method of targeting extracellular vesicles employing a molecule comprising a GLA domain and extracellular vesicles obtained or obtainable from a method disclosed herein.

TECHNICAL BACKGROUND

[0002] Extracellular vesicles (also known as microvesicles or microparticles) were historically thought to be vehicles for cells to eject waste material. However, in recent times it has been established that in fact extracellular vesicles are involved in vitally important cell to cell communication.

[0003] It has been demonstrated that extracellular vesicles can be derived from almost all mammalian cells, including healthy cells, stem cells, and diseased cells, such as cancer cells. The extracellular vesicles are extremely stable and can exist in almost all body fluids including: blood plasma, salvia, urine, bile, synovial fluid, semen and breast milk.

[0004] Diseased cells such as cancer cells, are thought to employ extracellular vesicles, such as exosomes, to prepare and seed sites for metastasis. Pathogen infected cells may employ extracellular vesicles to spread infection. In addition, bacterial cells are known to release extracellular vesicles.

[0005] There is great interest in studying, understanding and harnessing these extracellular vesicles, especially those involved in pathogenic processes. It has been suggested that these vesicles can be employed in therapy as an alternative to stem cells.

[0006] However, there are certain practical difficulties because the vesicles are minute and are present at low concentrations *in vivo*. In addition, there are few markers to distinguish disease-cell-derived extracellular vesicles from normal-cell-derived extracellular vesicles. A further complication is that *in vivo* these tiny entities are in a complex environment comprising a melange of biological molecules, factors, ions,

minerals, etc, etc. Therefore, even isolating and/or monitoring these extracellular vesicle is a challenge.

[0007] Seven or more steps may be required to isolate the vesicles and the main parameter employed is usually size. Since the diameter of extracellular vesicles may be below 300 nm and because they have a low refractive index, extracellular vesicles are below the detection range of many currently used techniques. A number of miniaturized systems, exploiting nanotechnology and microfluidics, have been developed to expedite extracellular vesicle analysis. These new systems include a microNMR device, a nanoplasmonic chip, and an magneto-electrochemical sensor for protein profiling; and an integrated fluidic cartridge for RNA detection. Flow cytometry is an optical method to detect extracellular vesicles in suspension. Nevertheless, the applicability of flow cytometry to detect single extracellular vesicles is still inadequate due to limited sensitivity and potential measurement artifacts such as swarm detection. Other methods to detect single extracellular vesicles are atomic force microscopy, nanoparticle tracking analysis, Raman microspectroscopy, tunable resistive pulse sensing, and transmission electron microscopy.

[0008] Beyond the opportunity to isolate the extracellular vesicle for study and/or diagnostic purposes, it is also considered that these extracellular vesicles may be suitable for use as natural vehicles to load with payloads, target and/or treat an array of maladies. However, significant challenges currently exist to realizing the potential to load cargos and targeting moieties:

- into these extracellular vesicles -
  - such as proteins, nucleic acids – both natural and non-natural oligonucleotides – small molecules, enzymes, probes to define the content within the extracellular vesicles for treatment, diagnostic/prognostic purposes etc;
- and onto these extracellular vesicles -
  - to develop, alter or enhance targeting, to display therapeutic proteins, to label and for example collect extracellular vesicles for analysis or manipulation,
- some microRNAs have been shown to enter vesicles but to date no robust mechanism exists for getting material inside the vesicle,
  - e.g. damaged via electroporation, integration of transfection reagents may compromise the commercial utility of the extracellular vesicle,
  - inefficient transduction by viral vectors, loading or isolating via means that alter the extracellular vesicles may also compromise the diagnostic or therapeutic potential, etc. (rev. in Vader et al., Adv Drug Delivery Rev 106: 148-156, 2016, Sutaria et al., Pharm Res 34: 1053- 1066, 2017, Lu et al., Eur J Pharm and Biopharm 119: 381-395, 2017),
  - saponins have also been suggested as reagents for increasing the permeability of the extracellular vesicles. However, saponins have complicated biological activity including being hemolytic. Fractions of saponins Quil A and QS-21 are used as vaccine adjuvants to increase immune responses to antigen. Therefore the use of saponins is not straightforward.

[0009] Thirdly, it may be useful to target pathogenic material and signals in the extracellular vesicles to reduce the spread of pathogenesis.

[0010] The disclosure herein addresses the above issues.

[0011] Surprisingly, those extracellular vesicles released from pathogenic cells, for example cancerous cells, bacterial cells etc have surface exposed phosphatidylserine. The exposed phosphatidylserine may, for example, act to downregulate immune responses to the "pathogenic" vesicles. Whilst not wishing to be bound by theory it may be that the "pathogenic extracellular vesicles" are characterized by the presence of exposed phosphatidyl serine as opposed to normal-healthy extracellular vesicles, which do not have surface exposed phosphatidylserine.

[0012] Phosphatidylserine can be targeted by GLA-components comprising a GLA-domain without the presence of a catalytic domain. These molecules can be employed to target vesicles derived from apoptotic cells, for example abnormal, diseased/infected cells.

[0013] Even more surprisingly the present inventors have also shown that the GLA-components also bind stems cells (for example healthy stem cells). Whilst not wishing to be bound by theory these cells may present phosphatidylserine on their surface, which may contribute to the immune suppressive effects an inflammatory effects of stem cells.

[0014] Extracellular vesicles derived from cells with surface exposed phosphatidyl serine also have surface exposed phosphatidyl serine on their outer surface. Thus, the GLA-components can also used to target extracellular vesicles from stem cells.

[0015] The GLA-domain can be linked or fused to a payload, for example a label, bead, a diagnostic molecule, a targeting motif and/or therapeutic. This allows, isolation, identification, tracking, and/or therapeutic intervention of or via these extracellular vesicles.

[0016] Alternatively, or additionally the payload can be a therapeutic, for example a drug, a biological therapeutic, a polymer or a toxin, such as a therapeutic virus, an oncolytic virus, a viral vector, an anti-viral drug, anti-bacterial drug, anti-parasitic agent, anti-cancer drug, an anti-cancer therapy or a chemotherapeutic agent, a virus or viral vector (such as an oncolytic virus).

[0017] Thus, the GLA-components can be employed to anchor payloads to the surface of the extracellular vesicles via the GLA-domain binding surface exposed phosphatidyl serine.

[0018] In addition, in the present inventors have data to suggest that the GLA-component employed in the present disclosure may be able to transport payloads attached thereto inside the extracellular vesicle. Thus, the GLA-component may be employed to deliver payloads to the interior of the extracellular vesicle.

[0019] This has important implications for therapeutic and/or diagnostic uses because known and existing techniques and effector/reporter molecules can be refocused and employed to monitor, isolate and therapeutically intervene with the vesicles.

[0020] Once labelled, the vesicles can be monitored, for example *in vivo*, or isolated using known techniques, such as flow cytometry, magnetic sorting and the like.

[0021] Furthermore, it is starting to emerge that the presence of the particular types of vesicles may be used as a non-invasive diagnostic for certain pathologies.

[0022] In addition, given the hypothesis that cancers use the vesicles to seed and promote metastasis, then destroying, removing or targeting these vesicles with therapy may be a method to prevent or reduce metastasis, for example nucleotides, such as RNAi can be transported into the extracellular vesicle to knock out active microRNAs carried in the vesicle.

[0023] The vesicles shed from infected cells, such as virally infected cells, contain cellular material, for example RNA, protein, lipids and carbohydrates, from the infected cell and also nucleic acids of viral origin. These vesicles may have a part to play in the infection of healthy cells. Altan-Bonnet N. 2016. Extracellular vesicles are the Trojan horses of viral infection. Curr Opin Microbiol 32:77-81; Schorey JS, Cheng Y, Singh PP, Smith VL. 2015, Exosomes and other extracellular vesicles in host-pathogen interactions; EMBO Rep 16:24-43, Schorey JS, Harding CV. 2016; and Extracellular vesicles and infectious diseases: new complexity to an old story. J Clin Invest 126:1181-9.

[0024] Vesicles, such as exosomes have several properties which make them ideal for delivering material into cells, which includes their small size (e.g. able to cross the blood brain barrier), natural ability to fuse with the plasma membrane of cells to deliver their contents, stable internal environment and their ability to deliver functional molecules to the recipient cell which include: nucleic acids (DNA, mRNA and miRNA), lipids and proteins.

[0025] Recent approaches have been aimed at engineering extracellular vesicles for therapeutic applications. These approaches include altering the vesicles content or manipulating their migratory pathways. In particular, it has been demonstrated that extracellular vesicles may serve a role as therapeutic vesicles for the treatment of cancer by decreasing tumour cell invasion, migration and proliferation, increase sensitivity to chemotherapy and may trigger enhanced immune responses and cell death.

[0026] It is also emerging that the vesicles may be suitable for use as a vaccine. Vesicles released from virally infected cells represent a unique source of correctly folded and processed viral material, which are ideal for use as antigen in a vaccination. However, vaccines usually require the presence of adjuvant to boost the immune response to the antigen component.

[0027] Previous attempts to manipulate extracellular vesicle content using the classical approaches of incubation, electroporation and transfection have suffered limitations due to poor efficiency of transfer, limited size of payload, the presence of residual excipient in the membrane and restrictions on what type of payload can be used. Thus, there are still some challenges to realizing the potential of these vesicles. Therefore, there is a need for novel methods which can effectively deliver content onto and into extracellular vesicles.

[0028] The present disclosure facilitates harnessing the potential of extracellular vesicles by enabling them to be: isolated, used for diagnostic purposes, targets for therapeutic intervention and to be employed to deliver therapeutics, for example through

attachment or genetic fusion including molecules such as nucleotides, including RNA and DNA to cells.

[0029] What is more the expression of phosphatidyl serine on the surface of the vesicle downregulates immune responses to the vesicle. A GLA-component (without a payload attached) binding phospatidylserine on the surface of extracellular vesicle decloaks the vesicle to the immune system. Thus, the GLA-component of the present disclosure may be employed to increase the visibility of the extracellular vesicles to the immune system.

SUMMARY OF THE DISCLOSURE

[0030] The present disclosure will now be summarised in the paragraphs below:

1a. A method for targeting extracellular vesicles with surface exposed phosphatidylserine said method comprising the step of introducing a molecule comprising:
a gamma-carboxyglutamic acid component (GLA-component) said GLA-component comprises a GLA domain or an active fragment thereof, and which does not comprise an active catalytic domain from a GLA protein,
into a fluid which may comprise the extracellular vesicle, for example microvesicles, apoptotic bodies and exosomes.

1b. A molecule comprising a payload linked to a gamma-carboxyglutamic acid component (GLA-component),
wherein said GLA-component comprises a GLA domain or an active fragment thereof, and does not comprise an active catalytic domain from a GLA protein for use in treatment or diagnosis of an extracellular vesicle.

1c. A molecule comprising a payload linked to a gamma-carboxyglutamic acid component (GLA-component),
wherein said GLA-component comprises a GLA domain or an active fragment thereof, and does not comprise an active catalytic domain from a GLA protein for use in the manufacture of a medicament for treatment or diagnosis of an extracellular vesicle

2. A method or molecule for use according to paragraph 1a, 1b or 1c, wherein the extracellular vesicle has a diameter in the range 10nm to 1000nm.

3. A method or a molecule for use according to paragraph 1a, 1b, 1c or 2, wherein the extracellular vesicle is an exosome.

4. A method or a molecule for use according to any one of paragraphs 1 to 3, wherein the extracellular vesicle (such as an exosome) has a diameter of 1000nm or less, for example 1nm to 10 μm, such as 10nm to 5μm, in particular 10nm to 1μm, more specifically 20nm to 100nm, more particularly 30, 40, 50, 60, 70, 80, 90 or 100nm

5. A method or a molecule for use according to any one of paragraphs 1a, 1b or 1c to 4, wherein the vesicle has a density in the range 1 to 1.5g/ml, such as 1 to 1.2g/ml, in particular 1.13 to 1.19g/ml.

6. A method or a molecule for use according to any one of paragraphs 1a, 1b, 1c to 5, wherein the vesicle comprises one or more transmembrane proteins independently

selected from Lamp- 1, Lamp-2, CD 13, CD86, Flotillin, Syntaxin-3, CD2, CD36, CD40, CD40L, CD41a, CD44, CD45, ICAM-1, Integrin alpha4, LiCAM, LFA-1, Mac-1 alpha and beta, Vti-IA and B, CD3 epsilon and zeta, CD9, CD18, CD37, CD53, CD63, CD81, CD82, CXCR4, FcR, GluR2/3, HLA-DM (MHC II), immunoglobulins, MHC-I or MHC-II components, TCR beta, tetraspanins and combinations of two or more of the same.

7. A method or a molecule for use according to any one of paragraphs 1a, 1b, 1c to 6, wherein the vesicle was released from an unhealthy cell, for example an apoptotic cell, a necrotic cell, a cancer cell, a pathogen infected cell (such as a virus infected cell, a bacteria infected cell or a parasite infected cells).
8. A method or a molecule for use according to paragraph 7, wherein the cell is a cancer cell.
9. A method or a molecule for use according to paragraph 8, wherein the cell is a cancer stem cell.
10. A method or a molecule for use according to any one of paragraphs 1 to 6, wherein the extracellular vesicle is from a healthy stem cell.
11. A method or a molecule for use according to any one of paragraphs 1a, 1b, 1c to 10 wherein the fluid comprises an *ex vivo* patient sample, for example a blood sample, fluid drawn from a cyst or tumor, homogenised biopsy or similar.
12. A method or a molecule for use according to any one of claims 1 to 10, wherein the GLA-component is administered *in vivo*.
13. A method or a molecule for use according to any one of paragraphs 1a, 1b or 1c to 12, wherein GLA domain or active fragment thereof is independently selected from thrombin, factor VII, factor IX, factor X, protein C, protein S, protein Z, Osteocalcin, Matrix GLA protein, GAS6, Transthretin, Periostin, Proline rich GLA 1, Proline rich GLA 2, Proline rich GLA 3 and Proline rich GLA 4.
14. A method or a molecule for use according to any one of paragraphs 1a, 1b or 1c to 13, wherein GLA domain or active fragment thereof is independently selected from thrombin, factor VII, factor IX, factor X, protein C, protein S, protein Z and GAS6, for example protein S, in particular comprising a sequence shown in SEQ ID NO: 1
15. A method or a molecule for use according to any one of paragraphs 1 to 11, wherein the GLA-domain-component further comprises an EGF domain, for example a calcium binding EGF domain.
16. A method or a molecule for use according to paragraph 15, wherein the construct comprises an EGF domain selected from thrombin, factor VII, factor IX, factor X, protein C, protein S, protein Z, Osteocalcin, Matrix GLA protein, GAS6, Transthretin, Periostin, Proline rich GLA 1, Proline rich GLA 2, Proline rich GLA 3 and Proline rich GLA 4.
17. A method or a molecule for use according to paragraph 16, wherein the construct comprises an EGF domain selected from independently selected from thrombin, factor VII, factor IX, factor X, protein C, protein S, protein Z and GAS6, for example the EGF domain from protein S.

18. A method or a molecule for use according to an one of paragraphs 1a, 1b or 1c to 17, wherein the GLA-component comprises a sequence shown in SEQ ID NO: 6 or a derivative thereof wherein the His-tag is absent, in particular a sequence shown in SEQ ID NO: 6.
19. A method or a molecule for use according to any of paragraphs 1a, 1b or 1c to 14, wherein the GLA-domain component further comprises a Kringle domain.
20. A method or a molecule for use according to paragraph 19, wherein the Kringle domain is from a protein selected from the group comprising Activating transcription factor 2 (ATF); Factor XII (F12); thrombin (F2); Hyaluronan-binding protein 2 (HABP2); Hepatocyte growth factor (HGF); Hepatocyte growth factor activator (HGFAC); Kremen protein 1 (KREMEN1); KREMEN2; Lipoprotein(a) (LPA); LPAL2; Macrophage-stimulating protein (MSP or MST1); Phosphoinositide-3-kinase-interacting protein 1 (PIK3IP1); Tissue plasminogen activator (PLAT); Urokinase (PLAU); Plasmin (PLG); PRSS12; Tyrosine-protein kinase transmembrane receptor ROR1 (ROR1); and Tyrosine-protein kinase transmembrane receptor ROR2 (ROR2).
21. A method or a molecule for use according to any one of paragraphs 1a, 1b or 1c to 20, wherein the GLA-component is linked to a payload.
22. A method or a molecule for use according to paragraph 21, wherein the GLA-component is conjugated to the payload.
23. A method or a molecule for use according to any one of paragraphs 1a, 1b or 1c to 22, wherein the GLA-component is part of a fusion protein.
24. A method or a molecule for use according to paragraph 23, wherein payload comprises a detectable label.
25. A method or a molecule for use according to paragraph 24, wherein the label is selected from a fluorescent molecule (including a fluorescent probe (such as rhodamine dye, FITC, FAM, CY5), biotin, an enzyme, a tag (for example a HIS-tag, FLAG-tag, myc-tag), a radionuclides (particularly radioiodide, radioisotopes, such as ^{99m}Tc), luminescent labels or compounds which may be detected by NMR or ESR spectroscopy including wherein the detectable label is in a Molecular Beacon.
26. A method or a molecule for use according to any one of paragraphs 1a, 1b or 1c to 25, wherein the payload is a bead, plate or a tag, for example an isolatable bead, such as a magnetic bead.
27. A method or a molecule for use according to paragraph 21 to 26, wherein the payload is linked to the GLA-component via a linker.
28. A method or a molecule for use according to paragraph 27, wherein the linker is cleavable.
29. A method or a molecule for use according to any one of paragraphs 21 to 28, wherein the GLA-component and payload are a diagnostic.
30. A method or a molecule for use according to any one of paragraphs 1a, 1b or 1c to 29, wherein the method comprises a further step of providing an enriched population of the extracellular vesicles.

31. A method or a molecule for use according to any one of paragraphs 1a, 1b or 1c to 30, wherein the method comprises the step of isolating the extracellular vesicles.
32. A method or a molecule for use according to any one of paragraphs 1a, 1b or 1c to 31, wherein the method is performed *in vitro*.
33. A method or a molecule for use according to any one of paragraph 1a, 1b or 1c to 32, wherein the molecule comprising the GLA-component is a therapeutic.
34. A method or a molecule for use according to any one of paragraphs 21 to 25 and 33, wherein the payload comprises a drug , a chemotherapeutic agent, a peptide (including stapled peptides) or biological therapeutic, for example: an anti-viral drug, anti-bacterial drug, anti-parasitic agent, anti-cancer drug, an anti-cancer therapy.
35. A method or a molecule for use according to any one of paragraphs 21 to 25, 33 and 34, wherein the payload comprises a toxin, a polymer (for example synthetic or naturally occurring polymers), biologically active proteins (for example enzymes, other antibody or antibody fragments e.g. intrabodies), a drug (small molecule (chemical entity), c, nucleic acids and fragments thereof (for example DNA, RNA and fragments thereof) a metal chelating agent, nanoparticles or a combination of two or more of the same.
36. A method or a molecule for use according to paragraph 35, wherein the toxin is selected from an auristatin (for example MMAE (monomethyl auristatin E), MMAF (monomethyl auristatin F)), pyrrolobenzodiazepine (PBD), doxorubicin, duocarmycin, a maytansinoid (for example N 2'-deacetyl-N 2'-(3-mercapto-1-oxopropyl)-maytansine (DM1), N 2'-deacetyl-N2'-(4-mercapto-1-oxopentyl)-maytansine (DM3) and N 2'-deacetyl-N 2'(4-methyl-4-mercapto-1-oxopentyl)-maytansine (DM4)), calocheamicin, dolastatin, maytansine, α-amanitin, Pseudomonas exotoxin (PE38), ricin A chain, diphtheria toxin, Pokeweed antiviral protein (PAP), saporin, gelonin and a tubulysin.
37. A method or a molecule for use according to any one of paragraphs 34 to 36, wherein the chemotherapeutic is selected from temozolomide, epothilones, melphalan, carmustine, busulfan, lomustine, cyclophosphamide, dacarbazine, polifeprosan, ifosfamide, chlorambucil, mechlorethamine, busulfan, cyclophosphamide, carboplatin, cisplatin, thiotepa, capecitabine, streptozocin, bicalutamide, flutamide, nilutamide, leuprolide acetate, doxorubicin hydrochloride, bleomycin sulfate, daunorubicin hydrochloride, dactinomycin, liposomal daunorubicin citrate, liposomal doxorubicin hydrochloride, epirubicin hydrochloride, idarubicin hydrochloride, mitomycin, doxorubicin, valrubicin, anastrozole, toremifene citrate, cytarabine, fluorouracil, fludarabine, floxuridine, interferon α-2b, plicamycin, mercaptopurine, methotrexate, interferon α-2a, medroxyprogersterone acetate, estramustine phosphate sodium, estradiol, leuprolide acetate, megestrol acetate, octreotide acetate, deithylstilbestrol diphosphate, testolactone, goserelin acetate, etoposide phosphate, vincristine sulfate, etoposide, vinblastine, etoposide, vincristine sulfate, teniposide, trastuzumab, gemtuzumab ozogamicin, rituximab, exemestane, irinotecan hydrocholride, asparaginase, gemcitabine hydrochloride,

altretamine, topotecan hydrochloride, hydroxyurea, cladribine, mitotane, procarbazine hydrochloride, vinorelbine tartrate, pentrostatin sodium, mitoxantrone, pegaspargase, denileukin diftitix, altretinoin, porfimer, bexarotene, paclitaxel, docetaxel, arsenic trioxide, tretinoin and combinations of two or more of the same.

38. A method or a molecule for use according to any one of paragraphs 34 to 37, wherein the chemotherapeutic is selected from an alkylating agent, an antimetabolite including thymidylate synthase inhibitors, a taxane, an anthracycline, an anti-microtubule agent including plant alkaloids, and combinations of two or more of the same.
39. A method or a molecule for use according to paragraph 38, wherein the chemotherapeutic is selected from paclitaxel, docetaxel, abraxane, carbazitaxel, derivatives of any one of the same, and combinations of two or more of any of the aforementioned.
40. A method or a molecule for use according to claim 38 or 39, wherein the alkylating agent is selected from a nitrogen mustard, a nitrosourea, a tetrazine, a aziridine, a platin and derivatives thereof, a non-classical alkylating agent and a combination of two or more of the same.
41. A method or a molecule for use according to paragraph 40, where the platin is selected from cisplatin, carboplatin, oxaliplatin, satraplatin, picoplatin, nedaplatin, triplatin, lipoplatin and a combination of two or more of the same.
42. A method or a molecule for use according to any one of paragraphs 38 to 41, wherein the alkylating is an antimetabolite selected from anti-folates (for example methotrexate and pemetrexed), purine analogues (for example thiopurines, such as azathiopurine, mercaptopurine, thiopurine, fludarabine (including the phosphate form), pentostatin and cladribine), pyrimidine analogues (for example fluoropyrimidines, such as 5-fluorouracil and prodrugs thereof such as capecitabine [Xeloda®]), floxuridine, gemcitabine, cytarabine, decitabine, raltitrexed(tomudex) hydrochloride, cladribine and 6-azauracil and combination of two or more thereof.
43. A method or a molecule for use according to any one of paragraphs 40 to 42, wherein the anthracycline is selected from daunorubicin (Daunomycin), daunorubicin (liposomal), doxorubicin (Adriamycin), doxorubicin (liposomal), epirubicin, idarubicin, mitoxantrone and a combination of two or more thereof, in particular doxorubicin.
44. A method or a molecule for use according to any one of paragraphs 34 to 43, wherein the drug is an anti-cancer drug, for example selected from a topoisomerase inhibitor, a PARP inhibitor and a combination of or more of the same.
45. A method or a molecule for use according to any one of paragraphs 34 to 44, wherein the anticancer therapy is a radionuclide, for example selected from Y-90, P-32, I-131, In-111, Sr-89, Re-186, Sm-153, Sn-117m and a combination of two or more of the same.

46. A method or a molecule for use according to any one of claims 1a, 1b or 1c to 25 and 27 to 45, which comprises administering the molecule comprising the GLA component and payload to a cancer patient.
47. A method or a molecule for use according to paragraph 46, wherein the cancer is an epithelial cancer, for example colorectal cancer, testicular cancer, liver cancer, biliary tract cancer, prostate cancer, pancreatic cancer, breast cancer, ovarian cancer, cervical cancer, uterine cancer, gastric cancer, oesophageal cancer, thyroid cancer, renal cancer, bladder cancer, brain cancer, head and neck cancer or lung cancer or alternatively the cancer may be a haematological cancer, for example leukaemia, lymphoma, myeloma and chronic myeloproliferative diseases, such as AML.

In one embodiment the method of the present disclosure does not target an apoptotic body.

[0031] Thus, in one embodiment the molecules according to the present disclosure are employed in the treatment of a pathogen, for example viral, bacterial, protozoan, parasitic infections, including intracellular forms thereof.

[0032] The present disclosure also extends to the use of a GLA-component comprises a GLA domain or an active fragment thereof, wherein said GLA-component does not comprise an active catalytic domain from a GLA protein, for intravesicle targeting and delivery (including intravesicle delivery of the payload).

[0033] The present disclosure also extends to the use of a GLA-component comprises a GLA domain or an active fragment thereof, wherein said GLA-component does not comprise an active catalytic domain from a GLA protein, for the manufacture of a medicament for intracellular targeting and delivery (including intravesicle delivery of the payload, in particular where the payload comprises a therapeutic entity/molecule).

[0034] Thus, in one aspect there is provided an *in vitro* method of generating/isolating vesicles from pathogen infected human cell-lines employing method disclosed herein, for example HEp-2 cells, A549 cells, Calu-3 cells, HEK and Madin Darby Kidney Cells (MDCK), for example for use in a vaccine.

[0035] In one embodiment the *in vitro* generated/isolated vesicle is loaded with a payload, for example an oligonucleotide or polynucleotide, such as an RNA or DNA, such CPG, employing a a gamma-carboxyglutamic acid component (GLA-component) comprising a GLA domain or an active fragment thereof, and which does not comprise an active catalytic domain from a GLA protein.

[0036] In one embodiment the *in vitro* generated/isolated vesicle is loaded an immunostimulator molecule, employing a gamma-carboxyglutamic acid component (GLA-component) comprising a GLA domain or an active fragment thereof, and which does not comprise an active catalytic domain from a GLA protein.

[0037] Extracellular vesicles mimetic can be generated in vitro by breaking down cells through serial extrusion, see for example Jang et al Nano 2013, 7, 7698. These mimetic will have phosphatidylserine on their surface if they are generated from apoptotic cells or stem cells.

[0038] In one embodiment the oligonucleotide or polynucleotide is conjugated to the GLA component.

[0039] The present disclosure also extends to a vaccine composition comprising extracellular vesicles from a pathogen infected cell and loaded with: an exogenous immunostimulatory molecule, for example selected from an adjuvant, for example TLR9 agonist, such as a CPG, C3b, ICAM-1; and a GLA component according to the present disclosure.

[0040] The vesicles in the vaccine may be have been generated *in vivo* or *in vitro.* Vesicles may be generated *in vitro* in a pathogen infected human cell line, for example selected from HEp-2 cells, A549 cells, Calu-3 cells, HEK and Madin Darby Kidney Cells (MDCK).

[0041] The exogenous immunostimulatory molecule may be loaded: on the exterior, in the interior of the "pathogen-derived" vesicle or may be located in both locations.

[0042] The GLA component may be loaded: on the exterior, in the interior of the "pathogen-derived" vesicle or may be located in both locations.

[0043] In one embodiment the immunostimulatory molecule is not linked to the GLA component i.e. they are loaded separately, for example the immunostimulatory molecule may be provided as a plasmid, which may be transfected into the vesicle and the GLA component is provide as a protein.

[0044] Loading the vesicle with both a GLA component and the immunostimulatory molecules provides two separate mechanisms of action, in that the GLA component binds the phosphatidylserine on the surface of the vesicle thereby revealing the presence of the vesicle to the immune system. The immunostimulatory molecule then boosts the immune systems response to the pathogenic material in the vesicle.

[0045] The exogenous immunostimulatory molecule may be linked to the GLA domain, for example conjugated to the GLA component or may be a fusion (expression construct) with the GLA component.

[0046] It is advantageous to provided immunostimulatory molecule linked to the GLA-component because the GLA-component can bind phosphatidylserine on the surface of the vesicle thereby loading itself and also the immunostimulatory molecule on the vesicle. In addition, in some instances the GLA-component may internalised on the vesicle and pull with it the immunostimulatory molecule.

[0047] In one embodiment the pathogen is bacterial or viral, for example as listed herein, in particular influenza virus, for example influenza A, B, C or D. Influenza A has hemagglutinin subtypes H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, H16, H17, H18 and neuraminidase subtypes N1, N2, N3, N4, N5, N6, N7, N8, N9, N10, N11, such as a strain selected from influenza A: (H1N1) H1N2, H2N1, H911,H3N1, H3N2, and H2N3.a new influenza A H1N1 virus (CDC 2009 H1N1 Flu website).

[0048] The technology of the present disclosure may be suitable for preparing a universal flu vaccine.

[0049] However, even when used to the prepare a seasonal flu vaccine the present technology is likely to be more efficient and convenient that growing the currently available vaccines on eggs.

[0050] The present disclosure also provides a "pathogen-derived" vesicle according to the present disclosure, for treatment, in particular for use as a vaccine.

[0051] Also provided is use of a "pathogen-derived" vesicle according to the present disclosure for the manufacture of a medicament, in particular for the manufacture of a vaccine.

[0052] As discussed above the GLA-component can be employed to carry a payload, such as a therapeutic payload into the interior of the extracellular vesicle. The therapeutic payload may act on the vesicle itself or may be delivered to a cell by the vesicle and act on the cell, or a combination of these two scenarios.

DETAILED DISCLOSURE

[0053] In one embodiment 1, 2, 3, 4 or 5 payloads are linked per GLA-component.

[0054] GLA-component (also referred to herein as a gamma-carboxyglutamic acid component) refers to a polypeptide comprising a GLA-domain in the absence of catalytic domain from a GLA protein, such as protein S. The polypeptide may further comprise an EGF domain and/kringle domain, for example from protein S. In one embodiment the GLA-component comprises 30 to 300 amino acid residues, for example 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, ,170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290 or 300 residues. In one embodiment the GLA component is in the range of 4.5 to 30kDa. In one embodiment the GLA-component comprises the sequence shown in SEQ ID NO: 1. In one embodiment the GLA-component comprises a sequence shown in SEQ ID NO: 6 or a derivative thereof excluding the His-tag.

[0055] GLA domains (Vitamin K-dependent carboxylation/gamma-carboxyglutamic) as employed herein are protein domains which have been modified by vitamin K dependent post-translational carboxylation of glutamate residues in the amino sequence to provide gamma-carboxyglutamate (Gla). In one embodiment the GLA domain employed in the molecules of the present disclosure comprises 30 to 45 consecutive residues from a native (wild-type) GLA domain. In one embodiment the GLA domain comprises 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 GLA residues.

[0056] In one embodiment 30% or less of the GLA-component is GLA residues.

[0057] In one embodiment the GLA-component comprises 1 to 5 disulfide bonds, for example 1, 2, 3, 4 or 5 disulfide bonds.

[0058] The GLA domain binds calcium ions by chelating them between two carboxylic acid residues. These residues are part of a region that starts at the N-terminal extremity of the mature form of Gla proteins, and that ends with a conserved aromatic residue. This results in a conserved Gla-x(3)-Gla-x-Cys motif that is found in the middle of the domain, and which seems to be important for substrate recognition by the carboxylase.

[0059] GLA domains are contained in a number of proteins, such as Thrombin, Factor VII, Factor IX, Factor X, Protein C, Protein S (PrS), Protein Z, Osteocalcin, Matrix

GLA protein, GAS6, Transthretin, Periostin, Proline rich GLA 1, Proline rich GLA 2, Proline rich GLA 3, and Proline rich GLA 4.

[0060] GLA domain as employed herein also extends to proteins where 1 to 10 percent (such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10%) of the amino acids in the native GLA domain may be replaced and/or deleted, provided that modified domain retains at least 70% (such as 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%) of the native activity of the native (unmodified GLA domain) in a suitable *in vitro* assay. In one embodiment the domain is the full-length native domain.

[0061] EGF domain as employed herein refers is a conserved protein domain. It comprises about 30 to 40 amino-acid residues and has been found in a large number of mostly animal proteins. Most occurrences of the EGF-like domain are found in the extracellular domain of membrane-bound proteins or in proteins known to be secreted. The EGF-like domain includes 6 cysteine residues. The main structure of EGF-like domains is a two-stranded β-sheet followed by a loop to a short C-terminal, two-stranded β-sheet. These two β-sheets are usually denoted as the major (N-terminal) and minor (C-terminal) sheets. EGF-like domains frequently occur in numerous tandem copies in proteins: these repeats typically fold together to form a single, linear solenoid domain block as a functional unit. In one embodiment the domain employed is the full-length native domain.

[0062] EGF domain as employed herein also extends to proteins where 1 to 10 percent (such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10%) of the amino acids in the native EGF domain may be replaced and/or deleted, provided that modified domain retains at least 70% (such as 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%) of the native activity of the native (unmodified EGF domain) in a suitable *in vitro* assay. In one embodiment the domain is the full-length native domain.

[0063] Kringle domain as employed herein refers to autonomous protein domains that fold into large loops stabilized by 3 disulfide bonds. They are characterized by a triple loop, 3-disulfide bridge structure, whose conformation is defined by a number of hydrogen bonds and small pieces of anti-parallel beta-sheet. They are found throughout the blood clotting and fibrinolytic proteins, in a varying number of copies, in some plasma proteins including prothrombin and urokinase-type plasminogen activator, which are serine proteases belonging to MEROPS peptidase family S1A.

[0064] Kringle domain as employed herein also extends to proteins where 1 to 10 percent (such as 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10%) of the amino acids in the native kringle domain may be replaced and/or deleted, provided that modified domain retains at least 70% (such as 75, 80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%) of the native activity of the native (unmodified Kringle domain) in a suitable *in vitro* assay. In one embodiment the domain employed is the full-length native domain.

[0065] An active fragment of a protein as employed herein is a less than the whole native protein (or relevant domain), which retains at least 50% (such as 60, 70,

80, 85, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99 or 100%) of the activity of the native full-length domain or protein in a relevant *in vitro* assay.

[0066] Catalytic domain as employed herein is a domain (or fragment) downstream of the EGF domain in the C-terminal direction, for example as illustrated in Fig 1A.

[0067] *In vitro* as employed herein refers to laboratory work not performed in a human or animal body.

[0068] *In vivo* as employed herein refer to work/testing/treatment in a living organism, in particular a human or animal, in particular human.

[0069] Extracellular vesicles (EVs) are important mediators of long distance intercellular communication and are involved in a diverse range of biological processes across both prokaryotic and eukaryotic organisms (rev. in Arenaccio and Federico, Adv Exp Med Biol 998: 3-19, 2017, Lu et al., Eur J Pharm Biopharm 119: 381-195, 2017, Vader et al., Adv Drug Delivery Rev 106:148-156, 2016, Lefebvre and Lecuyer, Front Microbiol 8: 377, 2017).

[0070] Extracellular vesicles are shed from infected cells (viral, bacterial or parasitic) and contain material from these infectious agents (rev. in Schorey et al., EMBO Rep. 16: 24-43, 2015, Schorey and Harding, J. Clin Invest. 126: 1181-1189, 2016). This material can vary from toxins to virulence factors to infectious virus (both enveloped and non-enveloped;rev. in Altan-Bonnet, N, Curr Opin Microbiol 32: 77-81, 2016) and may be a novel means to more effectively deliver viral populations rather than single viral particles (Chen YH et al., Cell 160: 619-630, 2015). Consequently, their isolation could provide rapid diagnostic insight into disease.

[0071] Extracellular vesicles is a broad term to describe all secreted membrane vesicles. As employed herein the term includes exosomes, microvesicles (also referred to microparticles), ectosomes, matrix vesicles, calcifying vesicles, prostasomes, oncosomes, retrovirus-like particles, bacterial extracellular vesicles, intralluminal vesicles and apoptotic bodies.

[0072] In addition, the extracellular vesicles, which contain proteins, RNA, andcarbohydrates specific to the infectious agent and cell may also potentially be systems for *in situ* vaccination. In this setting, it is envisioned that the engagement and neutralization of the immune dampening phosphatidylserine molecules due to the GLA domain enables immune detection and clearance and serve as a novel and more effective method of specific vaccination for the infectious disease.

[0073] Extracellular vesicles as employed herein includes microvesicles, apoptotic bodies, and exosomes. Extracellular vesicles generally have a diameter in the range 10nm to 5000nm.

[0074] Microvesicles as employed herein refers to vesicles released after formation by budding form the cytomembrane, and for example generally have a diameter in the range 100nm to 1000nm.

[0075] Exosomes are produced inside multivesicular bodies and are released after fusion of the multivesicular body with the cytomembrane. Generally, exosomes have a diameter in the range 30 to 100nm.

[0076] Apoptotic bodies as employed herein refer to vesicles shed into the extracellular environment by apoptotic cells. Apoptotic bodies may not be involved in intracellular communication. Generally, the diameter of apoptotic bodies is in the range 800nm to 5000nm.

[0077] See for example Modularized Extracellular Vesicles: The Dawn of Prospective Personalized and Precision Medicine, *Tao et al* Adv. Sci. 2018, 5, 1700449; Mesenchymal Stem Cell-derived Extracellular Vesicles: Towards Cell-free Therapeutic Applications *Rani et al*, www.moleculartherapy.org vol. 23 no. 5, 812-823 May 2015; and Achieving the Promise of Therapeutic Extracelluar Vesicles: The Devil is in the Detail of Therapeutic Loading *Sutaria et al*, Pharma Res. 2017 May; 34(5) 1053-1066, incorporated herein by reference.

[0078] Thus, the present disclosure provides use of extracellular vesicles for the diagnosis, for example of infection with a pathogen, for example a viral infection, bacterial infection and/or parasitic infection.

[0079] Thus, the present disclosure provides use of extracellular vesicles for the treatment, for example of infection with a pathogen, for example a viral infection, bacterial infection and/or parasitic infection, in particular where the extracellular vesicle is treated to neutralize or eliminate pathogenic material and/or the extracellular vesicle is loaded with therapeutic material for delivery to a target cell.

[0080] Thus, the present disclosure provides use of extracellular vesicles for the diagnosis of infection with a pathogen, for example a viral infection, bacterial infection and/or parasitic infection.

[0081] Thus, the present disclosure provides use of extracellular vesicles for the treatment, for example of infection with a pathogen, for example a viral infection, bacterial infection and/or parasitic infection, in particular where the extracellular vesicle is treated to neutralize or eliminate pathogenic material and/or the extracellular vesicle is loaded with therapeutic material for delivery to a cell.

[0082] Thus, the present disclosure provides use of extracellular vesicles for the diagnosis of cancer.

[0083] Thus, the present disclosure provides use of extracellular vesicles for the treatment of a cancer, for example where the extracellular vesicle is treated to neutralize or eliminate pathogenic material and/or the extracellular vesicle is loaded with therapeutic material for delivery to a cell.

[0084] Extracellular vesicles and methods according to the present disclosure may also be useful in the diagnosis and/or treatment of autoimmune disease, especially extracellular vesicles from stem cells.

[0085] In one embodiment the extracellular vesicles of the disclosure are from a human cell.

[0086] In one embodiment the extracellular vesicles are from stem cells (in particular healthy stem cells). These vesicles can be used in a similar way to stem cell therapy.

[0087] In one embodiment the extracellular vesicles of the disclosure are from a pathogenic cell, such as a bacterial cell.

[0088] Cells can secrete different types of EVs and these have been classified according to their sub-cellular origin (Colombo *et al.*, Annu Rev Cell Dev Biol 30: 255-289, 2014). Despite differences in origin and size, no uniform EV nomenclature exists due to the overlap in vesicle sizes and in the absence of subtype-specific markers. As a result, it remains difficult to purify and thereby distinguish between vesicle types. For example, the most popular exosome purification protocols used historically in the literature (differential ultracentrifugation, 220 nm filtration (Thery et al., Curr Protoc Cell Bioil Chapter 3, Unit 3.22, 2006) – and recently released in commercial kits – co-isolates different types of EVs (rev. Tkach and Thiery, Cell 164: 1226-1232, 2016). Thus, terms like "exosome" are generally referred to as a mixed population of small EVs and hence, for this invention, we have chosen to use the generic term EVs so as to refer to all vesicle subtypes.

[0089] Generally, extracellular vesicles comprise a lipid bilayer comprising ceramides, cholesterols, phosphoglycerides and sphingolipids (Subra *et al* 2010, Trajkovic *et al* 2008, Vlassov *et al* 2012)

[0090] All extracellular vesicles bear surface molecules that allow them to be targeted to recipient cells where they signal and/or deliver their content into its cytosol through an array of means (e.g. endocytosis and/or phagocytosis, fusion etc.), thereby modifying the physiological state of the recipient cell. Since they are natural delivery vehicles for protein, lipids and genetic material, they represent a unique bio-vector that is actively being explored across an array of disease indications for imaging, diagnostics, and/or for use as therapeutic carriers (rev. in Rufino-Ramos et al., J. Control Release 262: 247-258, 2017, Vader et al., Adv Drug Deliv. Rev 106: 148-156, 2016, Sutaria et al., Pharm Res. 34: 1053-1066, 2017, Ingato *et al.*, J. Control Release 241: 174-185, 2016).

[0091] In one embodiment the extracellular vesicle is an exosome. Exosomes are generally in the range 30 to 150nm, such as 30 to 100nm in diameter, and for example may bear one or more surface markers selected from transferrin, CD9, CD63, CD61, CD81, TSG101, LAMPS and Alix.

[0092] In one the extracellular vesicle is a microvesicle (also referred to a microparticle) and include endothelial microparticles. Generally, microvesicles have a diameter in the range 50 to 2000nm and, for example may bear one or more surface markers, selected from VCAMP3 and ARF6. Although, circulating endothelial microparticles can be found in the blood of normal individuals, increased numbers of circulating endothelial microparticles have been identified in individuals with certain diseases, including hypertension and cardiovascular disorders, and pre-eclampsia and various forms of vasculitis. The endothelial microparticles in some of these disease

states have been shown to have arrays of cell surface molecules reflecting a state of endothelial dysfunction. Therefore, endothelial microparticles may be useful as an indicator or index of the functional state of the endothelium in disease, and may potentially play key roles in the pathogenesis of certain diseases, including rheumatoid arthritis.

[0093] In one embodiment the extracellular vesicle is an ectosome. Generally, ectosomes have a diameter in the range 100 to 1000nm, such as 350 to 400nm and may, for example bear one or more surface markers selected from TyA nd C1a.

[0094] In one embodiment the vesicle is a calcifying extracellular vesicle. These vesicles are released from cells within atherosclerotic plaques. Recently, calcifying EVs derived from macrophages and smooth muscle cells (SMCs) have received increased attention for their role in vascular calcification. These vesicles are thought to have a role in mediating vascular calcification, a major predictor of cardiovascular morbidity and mortality.

[0095] In one embodiment the extracellular vesicle is a matrix vesicle. Matrix vesicles as referred to herein are involved in bone development, wherein osteoblast-derived vesicles nucleate hydroxyapatite crystals along collagen fibres in the developing bone. They also serve as nucleating foci for the formation of microcalcifications within atherosclerotic plaque fibrous caps, which leads to plaque instability, rupture and subsequent myocardial infarction and stroke.

[0096] In one embodiment the extracellular vesicle is a prostasome. Prostasome as employed herein refers to vesicles secreted by the prostate gland epithelial cells into seminal fluid. They generally have a diameter in the range 40 to 500nm. They possess an unusual lipid composition and a tight and highly ordered structure of their lipoprotein membranes resembling lipid raft. The physiological role of prostasomes implicates improvement of sperm motility and protection against attacks from the female immune defence during the passage to the egg. Investigations have shown that cancerous prostate cells and prostate cells with low differentiation continue to produce and secrete prostasomes. The high incidence of prostate cancer in elderly men could take advantage of the immune protective activities supported by the prostasomes. Immune regulating proteins found in prostasomes include: amino-peptidase N (CD13); dipeptidyl-peptidase IV (CD26); enkephalinase (neutral endopeptidase, CD10); angiotensin converting enzyme (ACE, CD143); tissue factor TF (CD142, thromboplastin); decay accelerating factor (CD55); protectin (CD59, inhibitor of MAC) and complement regulatory membrane cofactor protein (CD46). Prostasomes also contain high levels of the divalent cations: Zn^{2+}, Ca^{2+} and Mg^{2+}.

[0097] In one embodiment the extracellular vesicle is an oncosome. Oncosome as employed herein refers to large extracellular vesicles in the range 1μm to 10μm in diameter. In the context of brain tumors, the existence of EVs released from glioma cells and expressing EGFRvIII, a mutated form of the receptor. These vesicles were shown to be capable of transferring the oncoprotein EGFRvIII to the membrane of tumor cells

lacking this receptor, thus propagating tumor-promoting material and inducing transformation. Large oncosomes positive for Cav-1 have been shown to discriminate patients with locally confined prostate cancer from patients with castration resistant and metastatic disease.

[0098] In one embodiment the extracellular particles are retrovirus-like particles. These generally have a diameter in the range 75 to 100nm and may bear a surface marker Gag.

[0099] In one embodiment the extracellular vesicle is a bacterial extracellular vesicle. These vesicles generally have a diameter in the range 10 to 300nm and may have PAMPs on their surface.

[0100] In one embodiment the extracellular vesicle is a intralluminal vesicle. As employed herein this refers to a vesicle within a cell.

[0101] Back-fusion is the fusion of internal (intraluminal) vesicles within multivesicular bodies or late endosomes with the endosome's limiting membrane. The process is believed to be mediated by lysobiphosphatidic acid (LBPA), phosphatidylinositol-3-phosphate, Alix, and an apparent dependence on an acidic pH. MHC class 2 and other proteins (CD63 and MPR) utilize such a process to effectively transport to locations in the cytosol and back to the plasma membrane. However, pathogens also exploit this mechanism to efficiently enter the cytosol of the cell (e.g. VSV, anthrax). Unlike regular fusion in the cell between endosomes and organelles, back-fusion requires the exoplasmic leaflets of the internal vesicles and outer membrane to fuse - similar to sperm-egg fusion.

[0102] In one embodiment the extracellular vesicle is an apoptotic body. Apoptotic bodies as employed herein generally have a diameter in the range 500 to 5,000nm, such as 500 to 4000nm, and are released by cells undergoing programmed cell death.

Stem Cells and Markers

[0103] In one embodiment the extracellular vesicle according to the present disclosure is from a stem cell.

[0104] In one embodiment the stem cells are embryonic stem cells. In one embodiment the cell are not embryonic stem cells.

[0105] In one the stem cell is an adult stems cell, for example including progenitor cells, and haemotopoietic stem cells, myogenic stem cells, osteoprogenitor stem cells, neural stem cells, mesenchymal stem cell, such as satellite cells, radial glial cells, bone marrow stromal cells, periosteum, pancreatic progenitor cells, endothelial progenitor cells, blast cells and trophoblast stem cells.

[0106] In one embodiment the stem cell is a cancer stem cell.

[0107] In one embodiment the method relates to mammalian stem cells, for example human stem cells. The stem cell discussed herein are primarily human stem cells. However, the skilled person is able to identify the relevant or corresponding stem cell population for other mammals, as required. For example **SSEA-1** is a marker for

murine embryonic stem cells, human germline cells and embryonal carcinoma cells; **SSEA-3** is a marker for primate embryonic stem cells, human embryonic germline cells, human embryonic stem cells and embryonal carcinoma cells; **SSEA-4** is a marker for primate embryonic stem cells, human embryonic germ cells, human stem cells, embryonal carcinoma cells; **CD324** is a marker for human & murine embryonic stem cells, embryonal cancer cells; **CD90** is a marker for human & murine embryonic stems cells, hematopoietic stem cells, embryonal carcinoma cells; **CD117** is a marker for human & murine embryonic stem cells, hematopoietic stem progenitor cells, neural crest-derived melanocytes, primordial germ cells, embryonal carcinoma cells; **CD326** is a marker for human & murine embryonic stem cells, embryonal carcinoma cells; **CD9** is a marker for human & murine embryonic stems; **CD24** is a marker for human & murine embryonic stems; **CD29** is a marker for human & murine embryonic stems; **CD59** is a marker for human & murine embryonic stems; **CD133** is a marker for human & murine embryonic stems, embryonal carcinoma cells, hematopoietic stem cells; **CD31** is a marker for human & murine embryonic stems; **TRA-1-60** is a marker for human embryonic stem cells, teracarcinoma, embryonic germ cells, embryonal carcinoma cells; **TRA-1-81** is a marker for human embryonic stem cells, teracarcinoma, embryonic germ cells, embryonal carcinoma cells; **Frizzled5** is a marker for human & murine embryonic stem cells; **Stem cell factor** (SCF) is a marker for human & embryonic stem cells, hematopoietic stem cells, mesenchymal stem cells, embryonal carcinoma cells; and **Cripto** is a marker for human & murine embryonic stem cells, cardiomyocytes and embryonal carcinoma cells.

[0108] Hematopoietic stem cells (HSCs) or hemocytoblasts are the stem cells that give rise to all the other blood cells through the process of haematopoiesis. They are derived from mesoderm and located in the red bone marrow, which is contained in the core of most bones.

[0109] Cancer stem cell as employed herein refers to tumorigenic cells (i.e. cancer cells found within tumors or hematological cancers) that possess characteristics associated with normal stem cells, specifically the ability to give rise to all cell types found in a particular cancer sample. See, for example Identification and Targeting of Cancer Stem Cells, BioessayS 2009 Oct; 31 (10) 1038-1049, incorporated herein by reference. Cancer stem cells are defined by three distinct properties: i) a selective capacity to initiate tumour and drive neoplastic proliferation: ii) an ability to create endless copies of themselves through self-renewal, and iii) the potential to give rise to more mature non-stem cell cancer progeny though differentiation. Cancer stem cells are not necessarily derived from a healthy stem cell but may originate from a differentiated cell.

[0110] CD34 is also known as hematopoietic progenitor cells antigen CD34, has a function as cell-cell adhesion factor. It can be employed as a marker to enrich stem populations.

[0111] Stem cells are generally negative for lineage positive surface markers (i.e. is Lin -ve), for example the stem cell is Lin -ve, CD34 +ve, CD38 -ve, CD45RA -ve, CD90 positive and CD49f _+ve.

[0112] Haemotopoietic stem cells may express a surface marker from CD48, CD150, CD244, CD34, CD38, SCA-1, Thy1.1, C-kit, lin, CD135, slam1/CD150, Mac-1 (CD11b), CD4, stem cell factor (SCF) and combinations of two or more of the same.

[0113] Osteoprogenitor cells may express a surface marker selected from Gremlin-1, TGF-beta, bFGF, BMP-2, ALPP, MCAM, Collagen I, Collagen 1 alpha 1, Collagen II, RUNX2, Decorin, and combinations of two or more of the same (such as all said markers).

[0114] Osteoblasts or a progenitor thereof may express a surface marker selected from Runx2, alkaline phosphatase/ALPP/ALPI, osteocalcin, BAP1, OPN, BAP31, Collagen I, SCUBE3, Fibronectin, SPARC, IGFBP-3, and combinations of two or more of the same.

[0115] Osteocyte or progenitor thereof may express a surface marker selected from:

i) TGF beta, RANKL, MCSF, Sclerostin, DKK, and combinations of two or more of the same (such as all said markers) and/or

ii) Osterix +ve, CD90 +ve, osteocalcin +ve, collagen I +ve, bone sialoprotein +ve and combinations of two or more of the same (such as all said markers), and/or

iii) alkaline phosphatase/ALPP(alkaline phosphatase placental)/ALPI +ve, collagen I +ve, collagen II +ve, decorin +ve, MCAM/CD146 +ve, MEPE/OF45 +ve, osterix +ve, CD90 +ve, osterix/Sp7 +ve, RUNX2/CBFA1 +ve, thrombopoietin/Tpo +ve, and combinations of two or more of the same (such as all said markers)

Myogenic stem cells may express a marker selected from CD56, CD146, VE-cadherin, alpha-smooth muscle actin, FABP3, integrin alpha 7, desmin, myosin heavy chain, UEA-1 receptor, and combinations of two or more of the same (such as all said markers).

[0116] Neural stem cells may express a marker selected from:

i) CD133, CD15, CD24 low or -ve, GCTM-2, CD45, CD34, Nestin, Sox-2, ABCG2, FGF R4, Frizzled-9, and combinations of two or more of the same (such as all said markers), and/or

ii) CD24 marker is low or -ve, and/or

iii) a marker combination of CD133 +ve, 5E12 +ve, CD34 -ve, CD45 -ve, and CD24 low or -ve

[0117] Mesenchymal stem cells may express a surface marker, selected from CD10, CD13, CD73, CD105, CD271, CD140b, CD240, frizzled-9, CD29, CD90, CD146, oct4, SSEA4, STRO-1, stem cell factor (SCF) and combinations of two or more of the same.

A stem cell that is adipose-derived may express a surface marker selected from:

i) K15, CD34, Nestlin, follistatin, p63, integrin alpha 6, teacin C, EGFR, IGFR, frizzled factors, and combinations of two or more of the same, and/or

i) iCD44, ICAM/CD54, CD34, integrin family members and combinations of two or more of the same.

[0118] A stem cell from an ovary and tubal epithelial stem cell may express a surface marker selected from Gremlin 1, Lrig1, Lgr5, Bmi1, Tert, HopX and combinations of two or more of the same.

[0119] Embryonic stem cells may one or more a surface marker selected from CD24, CD29, CD31, CD59, CD90, CD117, CD133, CD324, CD326, SSEA-3, SSEA-4, TRA-1-60, TRA-1-81, frizzled5, stem cell factor, crypto (TDGF-1).

[0120] Extracellular vesicles from stems cells may be identified on the basis of the markers from the cell from which they were derived.

Other Definitions

[0121] Payload as employed herein refers to a molecule which is linked to the GLA domain, in particular for delivery to the vesicle. Payloads may comprise a drug, a toxin, a chemotherapeutic, a polymer, a biologically active protein, a peptide (such as stable peptide), a polynucleotide (such as DNA and RNA including microRNA, shRNA, RNAi and the like, including molecular beacons) radionuclides, a metal chelating agent, oncolytic viruses, viral vectors, labels and/or a reporter group. Linked as employed herein refers to any means of the associating the payload to the GLA-domain, including fusion protein (for example employing an amide bond) or a chemical conjugation (for example maleimide chemistry, click chemistry or the like). Payloads may also be used to refer to material delivered to the interior of the extracellular vesicle, wherein the material is not linked to the GLA-component.

[0122] The GLA domain is a unique detection and delivery platform that takes advantage of the exposure of phosphatidylserine (PS), to target and access selected cells. Phosphatidylserine is a principal signal for recognition and engulfment of apoptotic cells. Apoptosis is an evolutionarily conserved and tightly regulated cell death modality (Poon IK et al., Nat Rev Immunol 14: 166-180, 2014). The engulfment and ingestion of apoptotic cells is known as efferocytosis, which serves the immediate and effective removal of apoptotic cells before loss of membrane integrity and release of inflammatory contents and thus counterbalances the harmful inflammatory effects of apoptosis. PS expression acts to inhibit TLR-induced and cytokine-induced signaling cascades and immunogenic DC maturation (Poon IK et al, Nat Rev Immunol 14: 166-180, 2014, Birge RB Celll Death Differ 23: 962-978, 2016). Consequently, under physiological conditions, externalized phosphatidylserine functions as a dominant and evolutionarily conserved immunosuppressive signal that promotes tolerance and prevents local and systemic immune activation. Pathologically, the innate immunosuppressive effect of phosphatidylserine has been hijacked by numerous viruses, other microorganisms, and parasites to facilitate infection and in many cases, establish latency (Birge RB et al., Cell Death Differ 23: 962-978, 2016, Amara A and

Mercer J, Nat Rev Microbiol 13: 461-469, 2015, Moller-Tank, S and Maury W Virology 468-470: 565-580, 2014). Phosphatidylserine is dysregulated in the tumor microenvironment and antagonizes the development of tumor immunity and phosphatidylserine expressing exosomes are now being explored as early diagnostics in the battle with cancer (Birge RB et al., Cell Death Diff. 23:962-978, 2016, Lea et al., Oncotarget 8: 14395-14407, 2017, Li X and Wang X, Mol Cancer 16: 92, 2017). Whilst not wishing to be bound by theory the present inventors believe that not all phosphatidylserine is equivalent from a biological perspective. The inventors believe that the phosphatidylserine exposes by the enzyme TMEM16F is involved in immune suppression and is the one "seen" by the molecules of the present disclosure.

[0123] Oncolytic virus as employed herein refer to a virus that:

- preferentially infects and kills cancer cells, or
- selectively replicates in the cancer cells (for example because their replication is dependent on a gene that is upregulated in the cancer cells, such as p53.

[0124] Viral vector as employed herein refers to a replication deficient virus, generally encoding a transgene.

[0125] *In vitro* as employed herein refers to laboratory work not performed in a human or animal body.

[0126] *In vivo* as employed herein refer to work/testing/treatment in a living organism, in particular a human or animal.

[0127] Molecule as employed herein is used in the broadest sense and includes a synthetic chemical molecule but also macromolecules such as proteins, polymers (natural or otherwise), ribonucleic acid molecules, labels etc.

[0128] A molecular beacon is an oligonucleotide hybridization probes that can report the presence of specific nucleic acids in homogenous solutions. They are hairpin shaped molecules with an internally quenched fluorophore whose fluorescence is restored when they bind to a target nucleic acid sequence.

[0129] Stapled peptide as employed herein refers to multiple tandem peptides, held by a synthetic brace, which is intended to enhance the pharmacological performance of the peptides.

[0130] A drug as employed herein, unless the context indicates otherwise, is intended to refer to a small chemical entity, for example which has been synthesised by organic chemistry methods, in particular a molecule approved or licensed or in the process of being licensed for therapeutic use, especially in humans. Drug as employed herein includes an anti-viral compound, an antibiotic, and an anti-cancer therapy.

[0131] An antiviral compound (antiviral agent) as employed herein refers to the class of medicaments used specifically for treating viral infections, including broad spectrum anti-viral agents and also "narrow" spectrum specific to a particular virus or particular family of viruses.

[0132] Antibiotic as employed herein refers to medicine or agent that inhibits the growth of bacteria or destroys bacteria. Anti-bacterial and antibiotic are used interchangeable here unless the context indicates otherwise.

[0133] Anti-parasitic as employed herein in refers to a medicine or agent that inhibits the growth of parasite, destroys parasite or removes parasites from the host.

[0134] Anti-cancer therapy is a broad term which includes anti-cancer drugs, chemotherapy, radiotherapy, immune-oncology therapies, etc.

[0135] Anti-cancer drug as employed herein generally refers to a small molecule cancer therapy.

[0136] Chemotherapy as herein generally refers to a cytotoxic agent and includes antineoplastics.

[0137] A biological therapeutic (also referred to as a biopharmaceutical, biological or biologic) is a therapeutic product "derived" from a biological source, for example a recombinant proteins and fragments, including antibodies molecules, including antibodies, antibody binding fragments and multispecific antibody molecules, polynucleotides, therapeutic viruses, oncolytic viruses, viral vectors and complex combinations of such materials. A biologically active protein is a subgroup of a biological therapeutic and includes recombinant proteins and active fragments thereof (including antibody molecules).

[0138] Antibody molecules as employed herein include a complete antibody having full length heavy and light chains or a fragment thereof and a molecule comprising any one of the same for example a Fab, modified Fab, Fab', modified Fab', F(ab')2, Fv, Fab-Fv, Fab-dsFv, single domain antibodies (e.g. VH or VL or VHH), scFv, intrabodies, bi, tri or tetra-valent antibodies, Bis-scFv, diabodies, triabodies, tetrabodies and epitope-binding fragments of any of the above (see for example Holliger and Hudson, 2005, Nature Biotech. 23(9):1126-1136; Adair and Lawson, 2005, Drug Design Reviews - Online 2(3), 209-217). The methods for creating and manufacturing these antibody fragments are well known in the art (see for example Verma *et al.*, 1998, Journal of Immunological Methods, 216, 165-181). Other antibody fragments for use in the present invention include the Fab and Fab' fragments described in WO2005/003169, WO2005/003170 and WO2005/003171. Multi-valent antibodies may comprise multiple specificities e.g bispecific or may be monospecific (see for example WO 92/22853 and WO05/113605). Bispecific and multispecific antibody variants are especially considered in this example since the aim is to neutralise two independent target proteins. Variable regions from antibodies disclosed herein may be configured in such a way as to produce a single antibody variant which is capable of binding to and neutralising two target antigens.

[0139] Antibody and binding fragments thereof, in particular small antibody fragments such as domain antibodies, VHHs, single chain Fvs (scFvs), ds-scFvs, dsFv, and intrabodies may be delivered intracellularly using the present technology.

[0140] In one embodiment the antibody molecule is human or humanised.

[0141] A toxin is a poisonous substance, especially derived from a natural source, in particular a protein. Many toxins, such as calicheamicin are used in cancer therapy. In addition, chemotherapeutic agents can be considered toxic (or toxins). Thus the definition of toxin overlaps with other definitions herein. However, neurotoxins like snake venom are toxin but not a chemotherapeutic. However, those skilled in the art are familiar with these technical definitions and are capable of understanding the meaning the context of the present disclosure.

[0142] Diagnostic as employed herein is agent used in analysis or imaging to diagnose, or monitor or understand a disease status. A diagnostic will generally comprise a reporter molecule, such as a label or similar that can visualized, measured or monitored in some way.

[0143] Radionuclides suitable for use the present disclosure include thallium-201, technetium-99m, Iodine-123, Iodine 131, Iodine-125, Fluorine-18 and Oxygen-15.

[0144] Abnormal cell or pathogenic cell as employed herein relates to a cell that has differences to a normal healthy cell, in particular mutations or upregulation of a marker or markers, for example an abnormality linked to a predisposition to or development of a condition or diseases; linked with a condition or disease, such as pre-cancerous cell, cancer, a pathogen infected cell, sickle-cell anemia or similar.

[0145] Apoptosis as employed herein is cell death pathway which occurs as normal and controlled part an organism growth. Cell death by apoptosis is less damaging to surrounding tissue than cell death mechanisms, such as necrosis.

[0146] Necrosis as employed herein is cell death from disease or injury. It releases cytokines and factors into the surrounding tissue that may damage surrounding cells. Gangrene is an example of necrotic cell death.

Chemotherapeutic Agents

[0147] Chemotherapeutic agent and chemotherapy or cytotoxic agent are employed interchangeably herein unless the context indicates otherwise.

[0148] Chemotherapy as employed herein is intended to refer to specific antineoplastic chemical agents or drugs that are “selectively” destructive to malignant cells and tissues, for example alkylating agents, antimetabolites including thymidylate synthase inhibitors, anthracyclines, anti-microtubule agents including plant alkaloids, topoisomerase inhibitors, parp inhibitors and other antitumour agents. Selectively in this context is used loosely because of course many of these agents have serious side effects.

[0149] The preferred dose may be chosen by the practitioner, based on the nature of the cancer being treated.

[0150] Examples of alkylating agents, which may be employed in the method of the present disclosure include an alkylating agent nitrogen mustards, nitrosoureas, tetrazines, aziridines, platins and derivatives, and non-classical alkylating agents.

[0151] Example a platinum containing chemotherapeutic agent (also referred to as platins), such as cisplatin, carboplatin, oxaliplatin, satraplatin, picoplatin, nedaplatin,

triplatin and lipoplatin (a liposomal version of cisplatin), in particular cisplatin, carboplatin and oxaliplatin.

[0152] The dose for cisplatin ranges from about 20 to about 270 mg/m^2 depending on the exact cancer. Often the dose is in the range about 70 to about 100mg/m^2.

[0153] Nitrogen mustards include mechlorethamine, cyclophosphamide, melphalan, chlorambucil, ifosfamide and busulfan.

[0154] Nitrosoureas include N-Nitroso-N-methylurea (MNU), carmustine (BCNU), lomustine (CCNU) and semustine (MeCCNU), fotemustine and streptozotocin. Tetrazines include dacarbazine, mitozolomide and temozolomide.

[0155] Aziridines include thiotepa, mytomycin and diaziquone (AZQ).

[0156] Examples of antimetabolites, which may be employed in the method of the present disclosure, include anti-folates (for example methotrexate and pemetrexed), purine analogues (for example thiopurines, such as azathiopurine, mercaptopurine, thiopurine, fludarabine (including the phosphate form), pentostatin and cladribine), pyrimidine analogues (for example fluoropyrimidines, such as 5-fluorouracil and prodrugs thereof such as capecitabine [Xeloda®]), floxuridine, gemcitabine, cytarabine, decitabine, raltitrexed(tomudex) hydrochloride, cladribine and 6-azauracil.

Examples of anthracyclines, which may be employed in the method of the present disclosure, include daunorubicin (Daunomycin), daunorubicin (liposomal), doxorubicin (Adriamycin), doxorubicin (liposomal), epirubicin, idarubicin, valrubicin currenlty used only to treat bladder cancer and mitoxantrone an anthracycline analog, in particular doxorubicin.

[0157] Examples of anti-microtubule agents, which may be employed in the method of the present disclosure, include include vinca alkaloids and taxanes.

[0158] Vinca alkaloids include completely natural chemicals for example vincristine and vinblastine and also semi-synthetic vinca alkaloids, for example vinorelbine, vindesine, and vinflunine

[0159] Taxanes include paclitaxel, docetaxel, abraxane, carbazitaxel and derivatives of thereof. Derivatives of taxanes as employed herein includes reformulations of taxanes like taxol, for example in a micelluar formulaitons, derivatives also include chemical derivatives wherein synthetic chemistry is employed to modify a starting material which is a taxane.

[0160] Topoisomerase inhibitors, which may be employed in a method of the present disclosure include type I topoisomerase inhibitors, type II topoisomerase inhibitors and type II topoisomerase poisons. Type I inhibitors include topotecan, irinotecan, indotecan and indimitecan. Type II inhibitors include genistein and ICRF 193 which has the following structure:

[0161] Type II poisons include amsacrine, etoposide, etoposide phosphate, teniposide and doxorubicin and fluoroquinolones.

[0162] In one embodiment the chemotherapeutic is a PARP inhibitor.

Viruses Suitable for Use as Payloads in the Present Disclosure

[0163] In one embodiment the virus employed in the present disclosure is an envelope virus, for example selected from a herpesvirus (such as Herpes simplex 1), a poxvirus (such as vaccina virus), a hepadnavirus, a flavivirus, a togavirus, a coronavirus, hepatitis D, orthomyxovirus, paramyxovirus (such as measles or Newcastle disease virus), rhabdovirus, bunyavirus, filovirus, and Rhabdoviridae (such as vesicular stomatitis Indiana virus (VSV).

[0164] In one embodiment the virus employed in the present disclosure is a non-envelope virus, for example selected from adenoviridae (such as an adenovirus), papilomaviridae, picornaviridae (such as coxsackie virus or Seneca Valley virus (eg Senecavirus)), reovirus.

[0165] In one embodiment the virus is an adenovirus, for example a human adenovirus, such as selected from a group B virus (in particular Ad3, Ad7, Ad11, Ad14, Ad16, Ad21, Ad34, Ad35, Ad51 or a chimeria thereof, such as Enadenotucirev), a group C virus (in particular Ad1, 2, 5, 6 or a chimeria thereof), a group D virus (in particular Ad8, Ad10, Ad13, Ad15, Ad17, Ad19, Ad20, Ad22, Ad30, Ad32, Ad33, Ad36, Ad37, Ad38, Ad39, Ad42, Ad43, Ad44, Ad45, A46, Ad47, Ad48, Ad49, Ad50 or a chimeria thereof), a group E virus (in particular Ad4), a group F virus (in particular Ad40, Ad41 or a chimeria thereof) and a chimeria of two or more of group B, C, D, E or F viruses.

[0166] The vast majority of viruses have well described proteins associated with target cell recognition and uptake. Modification of their tropism to re-direct or enable more selective tumor targeting into oncolytic viruses may be introduced using methods described in rev. in Verheije and Rottier, Adv. Virology 2012: 798526, 2012.

[0167] Additional viral cell surface proteins not involved in native viral targeting can have targeting motifs engineered onto them (e.g. Ad virion minor coat protein IX Salisch et al., PLoS One 12: e0174728, 2017).

[0168] Envelope viruses have an outer membrane (envelope) covering the virus capsid. The envelope is typically derived from the portions of the host cell membranes (phospholipids and proteins) but also include some viral proteins. Glycoproteins on the surface of the envelope serve to identify and bind to receptor sites on the host's membrane. The viral envelope then fuses with the host's membrane, allowing the capsid and viral genome to enter and infect the host.

[0169] Various oncolytic viruses are disclosed in WO2014/13834, incorporated herein by reference.

[0170] Herpes simplex virus (HSV) enters cells by means of four essential glycoproteins - gD, gH/gL, gB, activated in a cascade fashion by gD binding to one of its receptors, nectin1 and HVEM. Retargeting of HSV has been achieved by the insertion of ligands and scFvs into the gC and/or gD protein or gH (Campadelli-Fiume, G et al., Rev in Med Virol 21: 213-226, 2011, Gatta, V PLoS Pathog 11: e1004907, 2015). Oncolytic herpes simplex virus type 1 vectors have been developed for clinical use. These viruses are replication competent and have mutations in the genes that affect viral replication,

neuropathogenicity, and immune evasiveness, and for example include first generation viruses such as NV1020 (R7020), *dls*ptk, d18.36tk, hrR3, R3616, 1716, second generation viruses such as G207 (MGH-1), 3616UB, SUP, NV1023, third generation viruses such as G47Δ, transcriptional expressing vectors such as G92A, *d*12.CALP, Myb34.5, transgene expressing vectors such as rRP450, and other viruses such as Talimogene laherparepvec (T-Vec). The HSV-1 vectors are the thought to be useful in the treatment of a wide of solid tumors, for example including glioma, melanoma, breast, prostate, colon, ovarian, and pancreatic cancers. The HSV-1 virus infects a broad range of cells types and species, it is cytolytic by nature, the replicative life cycle of the virus results in host cell destruction, it has a well characterised and large genome (152K) but contains many non-essential genes providing up to 30K of space for the insertion of therapeutic genes. Generally, HSV viruses are not mutated in the thymidine kinase gene for safety reasons. Talimogene laherparepvec is an oncolytic herpes virus, which is approved for use in the treatment of melanoma. Other herpes bases viruses include G207, SEPREHVIR (HSV-1716), by Virttu Biologics, HSV-1 R3616 mutant, HSV-1 1716 mutant, NV1020 (R7020), R3616 mutant (deleted *RL1*), KM100 mutant has insertions in *UL48* (encodes the transactivator tegument protein pUL48 [VP16]) and *RL2* genes, G92A, mutants, Myb34.5 and rQNestin34.5.

[0171] **Poxvirus** -Vaccina virus, such as Modified Vaccinia Ankara (MVA) may be employed (Galmiche MC et al., J Gen Virol 78: 3019-3027, 1997), MVA may be replaced with a p14 fusion molecule carrying an inserted scFv directed against the tumor associate antigen MUC-1 (Paul, S et al., Viral Immunol 20: 664-671, 2007) See also rev. in Liang L et al., Viruses 6: 3787-3808, 2014, Hsiao JC et al., J Virol 73: 8750-8761, 1999, rev. in Chen TL and Roffler S, Med Res. Rev. 28: 885-928, 2008 and Kinoshita T et al., J Biochem 144: 287-294, 2008. JX-594, by Jennerex, is a thymidine kinase-deleted Vaccinia virus plus GM-CSF. GL-ONC1 is an attenuated vaccinia virus (Lister strain) that causes regression and elimination of a wide range of solid tumors in preclincal mouse models.

[0172] **Paramyxovirus** (such as measles or Newcastle disease virus),

[0173] Measles virus (MeV) is a single-stranded, negative-sense, enveloped (non-segmented) RNA virus of the genus Morbillivirus within the family Paramyxoviridae.Measles virus has two envelope glycoproteins: the hemagglutinin (H) attachment protein and the fusion (F) protein. Attachment, entry and subsequent cell-cell fusion is mediated via 2 measles receptors, CD46 and the signaling lymphocyte activation molecule (SLAM). See for example rev. in Msaouel P et al., Methods Mol Biol 797: 141-162, 2012, Robinson S. and Galanis, E. Expert Opin Biol Ther. 17: 353-363, 2017, Aref S et al., Viruses 8. Pii:E294, 2016); (rev. in Chen TL and Roffler S, Med Res. Rev. 28: 885-928, 2008 and Kinoshita T et al., J Biochem 144: 287-294, 2008), and (Russell SJ and Peng KW, Curr Topic Microbiol. Immunol 330: 213-241, 2009, Robinson S and Galanis, E Expert Opin Biol. Ther 17: 353-363, 2017, Aref S et al., Viruses 8. Pii: E294, 2016). Measles virus encoding the human thyroidal sodium iodide symporter or

MV-NIS is an attenuated oncolytic Edmonston (Ed) strain of measles virus. Radioactive Iodine imaging provides a novel technique for NIS gene expression monitoring.

[0174] Newcastle disease virus may also be employed.

[0175] **Adenoviridae** Adenoviruses are among the most extensively studied viruses being used as oncolytic agents. An array of peptides and proteins have been engineered into virion associated viral proteins to alter the native tropism of the virus (rev. in Verheije MH and Rottier PJM Adv Virol 2012: 798526, 2012). However, all of these are dependent upon viral assembly in the nucleus which presents significant challenges.

[0176] Other non-enveloped viruses include Coxsackievirus, Poliovirus and Reovirus. See for example rev. in Altan-Bonnet, N, Curr Opin Microbiol 32: 77-81, 2016 and Chen YH et al., Cell 160: 619-630, 2015, rev. in Chen TL and Roffler S, Med Res. Rev. 28: 885-928, 2008 and Kinoshita T et al., J Biochem 144: 287-294, 2008 and rev. in Verheije MH and Rottier PJM Adv Virol 2012: 798526, 2012).

[0177] There are a numerous adenoviruses for example Ad5-yCD/mutTKSR39rep-hIL12, such as for the treatment of prostate cancer was initiated, CGTG-102 (Ad5/3-D24-GMCSF), by Oncos Therapeutics, for example for the treatment soft tissue sarcomas, Oncorine (H101), CG0070, Enadenotucirev (EnAd) WO2005/118825, OvAd1 and OvAd2 disclosed in WO2008/080003, ONCOS-102, for example for Unresectable Malignant Pleural Mesothelioma, and DNX-2401 for example for glioma.

[0178] Cavatak is the trade name for a preparation of wild-type Coxsackievirus A21, useful in the treatment of malignant melanoma. Seneca Valley virus (NTX-010) and (SVV-001), for example for small cell lung cancer and neuroblastoma

[0179] **Reovirus**-Reolysin® (pelareorep; Wild-Type Reovirus; Serotype 3 Dearing; Oncolytics Biotech), for example for the treatment of various cancers and cell proliferative disorders.

[0180] **Vesicular Stomatitis Virus (VSV)** VSV is another enveloped virus being explored as on oncolytic agent. See for example Betancourt D et al., J Virol 89: 11786-11800, 2015) and rev. in Hastie E and Grdzelishvili VZ J Gen Virol 93: 2529-2545, 2012).

Proteins Encoded By A Virus

[0181] In one embodiment a virus or vector employed in the method of the present disclosure comprises a transgene, for example where the transgene is to replace defective genetic material in the cell, to provide a new or augmented function in the cell, to sensitize the cell to treatment, to block a function in the cell, or to express a therapeutic protein or peptide. In one embodiment the virus employed as the payload according to the present disclosures, comprises a transgene or transgenes, for example encoding an agent independently selected from an RNAi sequence, a protein, polypeptide or peptide (for example an antibody molecule or binding fragment thereof, a chemokine, a cytokine, an immunomodulator, a fluorescent tag or an enzyme).

[0182] This includes but is not limited to unique formats that have shown pre-clinical promise but have lacked an effective and economical means for delivery e.g. peptides, intrabodies and alternative scaffolds (rev. in Boldicke T, Protein Sci 26: 925-945, 2017, Marschall and Dubel, Comput Struct Biotechnol J 14: 304-308, 2016, Miersch and Sidhu F1000Res 5.pii.F1000 Faculty Rev. 1947, 2016, Peptides, Tsomaia Eur J Med Chem 94:459-470, 2015, Marschall ALJ et al, Mabs 7: 1010-1035, 2015, AlDeghaither D et al., J Clin Pharmacol. 55: S4–S20, 2015))) and includes agents with therapeutic effects on the tumor cells tumor stem cells, tumor associated endothelium and tumor associated stroma. Of special interest are molecules that could serve multiple functions, for example as therapeutics, biomarkers and/or diagnostics. The herpes simplex virus thymidine kinase (HSV-TK) gene is a well-established pro-drug converting enzyme with a clinically approved pro-drug (ganciclovir- GCV) see for example Holder et al., Cancer Res. 53: 3475-3485, 1993, Touraine RL et al., Gene Therapy 5: 1705-1711, 1998),

[0183] In addition, the thymidine kinase protein expression can also be exploited to image and track the activity of the virotherapy during the course of treatment. Positron emission tomography and single photon emission computed tomography are both methods that are routinely used for the detection and monitoring of cancer and cancer therapies and are both viable means to detect the expression of the thymidine kinase protein when an appropriate thymidine kinase substrate is administered (Wang JQ et al., Bioorg Med Chem 13: 549-556, 2005, Tjuvajev JG et al, J Nucl Med 43: 1072-1083, 2002). Alternatively, the NIS gene may be used and has been explored as an agent for diagnostic and therapeutic purposes in oncolytic viruses, much like TK (Miller A and Russell S Expert Opin Biol Ther 16: 15-32, 2016, Ravera S et al., Annu Rev Physiol 79: 261-289, 2017, Portulano et al., Endocr Rev. 35: 106-149, 2014).

[0184] In one embodiment antibodies that interact and inhibit RAS or proteins in the RAS signaling pathway are encoded in the virus of the present disclosure, for example as as fusion protein with the GLA-component. *RAS* genes constitute a multigene family that includes *HRAS*, *NRAS*, and *KRAS*. See for example Bos JL, Cancer Res. 49: 4682-4689, 1989; and Cetin M et al., J Mol Biol. 429:562-573, 2017.

Labels

[0185] In one embodiment the payload comprises a fluorescent label, a chemi-lluminescent label, a radio label, an enzyme, a dye or a ligand.

[0186] A label in accordance with the present disclosure is defined as any moiety which may be detected using an assay. Non-limiting examples of reporter molecules include enzymes, radiolabels, haptens, fluorescent labels, phosphorescent molecules, chemiluminescent molecules, chromophores, photoaffinity molecules, colored particles or ligands, such as biotin. Label as employed herein also includes tags, for example His-tags, Flag-tags and the like. Labels include biotin, which is the substrate for avidin.

[0187] Labels can be linked to the GLA-components by conjugation or fusion. The label be the only payload or in addition to another entity, such as a therapeutic payload.

[0188] Label conjugates are suitable for use as diagnostic agents. Diagnostic agents generally fall within two classes, those for use in *in vitro* diagnostics, and those for use *in vivo* diagnostic protocols, generally known as "directed imaging." Many appropriate imaging agents are known in the art, as are methods for their attachment to peptides and polypeptides (see, for *e.g.*, U.S. Patents 5,021,236, 4,938,948, and 4,472,509). The imaging moieties used can be paramagnetic ions, radioactive isotopes, fluorochromes, NMR-detectable substances, and X-ray imaging agents.

[0189] In the case of paramagnetic ions, one might mention by way of example ions such as chromium (III), manganese (II), iron (III), iron (II), cobalt (II), nickel (II), copper (II), neodymium (III), samarium (III), ytterbium (III), gadolinium (III), vanadium (II), terbium (III), dysprosium (III), holmium (III) and/or erbium (III), with gadolinium being particularly preferred. Ions useful in other contexts, such as X-ray imaging, include but are not limited to lanthanum (III), gold (III), lead (II), and especially bismuth (III).

[0190] In the case of radioactive isotopes for therapeutic and/or diagnostic application, one might mention astatine^{211}, ^{14}carbon, ^{51}chromium, ^{36}chlorine, ^{57}cobalt, ^{58}cobalt, copper^{67}, ^{152}Eu, gallium^{67}, ^{3}hydrogen, iodine^{123}, iodine^{125}, iodine^{131}, indium^{111}, ^{59}iron, ^{32}phosphorus, rhenium^{186}, rhenium^{188}, ^{75}selenium, ^{35}sulphur, technicium^{99m} and/or yttrium^{90}. ^{125}I is suitable for use in certain embodiments, and technicium^{99m} and/or indium^{111} are particularly suitable due to their low energy and suitability for long range detection. Radioactively labeled peptides and polypeptides may be produced according to well-known methods in the art. For instance, peptides and polypeptides can be iodinated by contact with sodium and/or potassium iodide and a chemical oxidizing agent such as sodium hypochlorite, or an enzymatic oxidizing agent, such as lactoperoxidase. Petides may be labeled with technetium^{99m} by ligand exchange process, for example, by reducing pertechnate with stannous solution, chelating the reduced technetium onto a Sephadex column and applying the peptide to this column. Alternatively, direct labeling techniques may be used, *e.g.*, by incubating pertechnate, a reducing agent such as $SNCl_2$, a buffer solution such as sodium-potassium phthalate solution, and the peptide. Intermediary functional groups which are often used to bind radioisotopes which exist as metallic ions to peptide are diethylenetriaminepentaacetic acid (DTPA) or ethylene diaminetetracetic acid (EDTA).

[0191] Fluorescent labels suitable for use as payloads include Alexa 350, Alexa 430, AMCA, BODIPY 630/650, BODIPY 650/665, BODIPY-FL, BODIPY-R6G, BODIPY-TMR, BODIPY-TRX, Cascade Blue, Cy3, Cy5,6-FAM, Fluorescein Isothiocyanate, HEX, 6-JOE, Oregon Green 488, Oregon Green 500, Oregon Green 514, Pacific Blue, REG, Rhodamine Green, Rhodamine Red, Renographin, ROX, TAMRA, TET, Tetramethylrhodamine, and/or Texas Red.

[0192] Another type of payload is that suitable for use *in vitro*, is where a peptide is linked to a secondary binding ligand and/or to an enzyme (an enzyme tag) that will generate a colored product upon contact with a chromogenic substrate. Examples of suitable enzymes include urease, alkaline phosphatase, (horseradish) hydrogen peroxidase or glucose oxidase. Suitable secondary binding ligands are biotin and avidin and streptavidin compounds. The use of such labels is well known to those of skill in the art and is described, for example, in U.S. Patents 3,817,837, 3,850,752, 3,939,350, 3,996,345, 4,277,437, 4,275,149 and 4,366,241.

[0193] Other methods are known in the art for the attachment for linking a peptide to its "conjugate partner" Some attachment methods involve the use of a metal chelate complex employing, for example, an organic chelating agent such a diethylenetriaminepentaacetic acid anhydride (DTPA); ethylenetriaminetetraacetic acid; N-chloro-p-toluenesulfonamide; and/or tetrachloro-3α-6α-diphenylglycouril-3 attached to the antibody (U.S. Patents 4,472,509 and 4,938,948). Peptides or polypeptides may also be reacted with an enzyme in the presence of a coupling agent such as glutaraldehyde or periodate. Conjugates with fluorescein markers are prepared in the presence of these coupling agents or by reaction with an isothiocyanate.

[0194] In one embodiment the label is able to stain or label the nucleus of a stem cell.

Combination Therapy

[0195] In one embodiment a combination of chemotherapeutic agents employed is, for example a platin and 5-FU or a prodrug thereof, for example cisplatin or oxaplatin and capecitabine or gemcitabine, such as FOLFOX.

[0196] In one embodiment the chemotherapy comprises a combination of chemotherapy agents, in particular cytotoxic chemotherapeutic agents.

[0197] In one embodiment the chemotherapy combination comprises a platin, such as cisplatin and fluorouracil or capecitabine.

[0198] In one embodiment the chemotherapy combination in capecitabine and oxaliplatin (Xelox).

[0199] In one embodiment the chemotherapy is a combination of folinic acid and 5-FU, optionally in combination with oxaliplatin.

[0200] In one embodiment the chemotherapy is a combination of folinic acid, 5-FU and irinotecan (FOLFIRI), optionally in combination with oxaliplatin (FOLFIRINOX). The regimen consists of: irinotecan (180 mg/m^2 IV over 90 minutes) concurrently with folinic acid (400 mg/m^2 [or 2 x 250 mg/m^2] IV over 120 minutes); followed by fluorouracil (400–500 mg/m^2 IV bolus) then fluorouracil (2400–3000 mg/m^2 intravenous infusion over 46 hours). This cycle is typically repeated every two weeks. The dosages shown above may vary from cycle to cycle.

[0201] In one embodiment the chemotherapy combination employs a microtubule inhibitor, for example vincristine sulphate, epothilone A, N-[2-[(4-Hydroxyphenyl)amino]-3-pyridinyl]-4-methoxybenzenesulfonamide (ABT-751), a taxol

derived chemotherapeutic agent, for example paclitaxel, abraxane, or docetaxel or a combination thereof.

[0202] In one embodiment the chemotherapy combination employs an mTor inhibitor. Examples of mTor inhibitors include: everolimus (RAD001), WYE-354, KU-0063794, papamycin (Sirolimus), Temsirolimus, Deforolimus(MK-8669), AZD8055 and BEZ235(NVP-BEZ235).

[0203] In one embodiment the combination therapy employs a MEK inhibitor. Examples of MEK inhibitors include: AS703026, CI-1040 (PD184352), AZD6244 (Selumetinib), PD318088, PD0325901, AZD8330, PD98059, U0126-EtOH, BIX 02189 or BIX 02188.

[0204] In one embodiment the chemotherapy combination employs an AKT inhibitor. Examples of AKT inhibitors include: MK-2206 and AT7867.

[0205] In one embodiment the combination employs an aurora kinase inhibitor. Examples of aurora kinase inhibitors include: Aurora A Inhibitor I, VX-680, AZD1152-HQPA (Barasertib), SNS-314 Mesylate, PHA-680632, ZM-447439, CCT129202 and Hesperadin.

[0206] In one embodiment the combination therapy employs a p38 inhibitor, for example as disclosed in WO2010/038086, such as *N*-[4-({4-[3-(3-*tert*-Butyl-1-*p*-tolyl-1*H*-pyrazol-5-yl)ureido]naphthalen-1-yloxy}methyl)pyridin-2-yl]-2-methoxyacetamide.

[0207] In one embodiment the combination employs a Bcl-2 inhibitor. Examples of Bcl-2 inhibitors include: obatoclax mesylate, ABT-737, ABT-263(navitoclax) and TW-37.

[0208] In one embodiment the chemotherapy combination comprises an antimetabolite such as capecitabine (xeloda), fludarabine phosphate, fludarabine (fludara), decitabine, raltitrexed (tomudex), gemcitabine hydrochloride and cladribine.

[0209] In one embodiment the combination therapy comprises ganciclovir, which may assist in controlling immune responses and/or tumour vasculation.

[0210] In one embodiment the chemotherapy includes a PARP inhibitor.

[0211] In one embodiment the combination therapy includes an inhibitor of cancer metabolism with specific inhibition of the activity of the DHODH enzyme.

[0212] In one embodiment one or more therapies employed in the method herein are metronomic, that is a continuous or frequent treatment with low doses of anticancer drugs, often given concomitant with other methods of therapy.

[0213] In one embodiment, there is provided the use of multiple cycles of treatment (such as chemotherapy) for example 2, 3, 4, 5, 6, 7, 8.

[0214] In one embodiment the chemotherapy is employed in a 28 day cycle.

[0215] The GLA-components of the present disclosure can be adapted to treated one or more of the following infections by targeting extracellular vesicles derived from infected cells: Acinetobacter infections (*Acinetobacter baumannii*), Actinomycosis (*Actinomyces israelii, Actinomyces gerencseriae and Propionibacterium propionicus*),

African sleeping sickness also known as African trypanosomiasis (*Trypanosoma brucei*) AIDS-Acquired immunodeficiency syndrome (HIV (Human immunodeficiency virus)), Amebiasis (*Entamoeba histolytica*), Anaplasmosis (*Anaplasma species*), Angiostrongyliasis (*Angiostrongylus*), Anisakiasis (*Anisakis*), Anthrax (*Bacillus anthracis*), Arcanobacterium haemolyticum infection (*Arcanobacterium haemolyticum*), Argentine hemorrhagic fever (*Junin virus*), Ascariasis (*Ascaris lumbricoides*), Aspergillosis (*Aspergillus species*), Astrovirus infection (*Astroviridae family*), Babesiosis (*Babesia species*), Bacillus cereus infection (Bacillus cereus), Bacterial pneumonia (multiple bacteria), Bacterial vaginosis (bacterial vaginosis microbiota), Bacteroides infection (*Bacteroides*), Balantidiasis (*Balantidium coli*), Bartonellosis (*Bartonella*), Baylisascaris infection (*Baylisascaris*), BK virus infection (BK virus), Black piedra (*Piedraia hortae*), Blastocystosis (*Blastocystis*), Blastomycosis (*Blastomyces dermatitidis*), Bolivian hemorrhagic fever (Machupo virus), Botulism and Infant botulism (*Clostridium botulinum*; Note: Botulism is not an infection by Clostridium botulinum but caused by the intake of botulinum toxin), Brazilian hemorrhagic fever (Sabiá virus), Brucellosis (*Brucella*), Bubonic plague (*Enterobacteriaceae*), Burkholderia infection (Burkholderia), Buruli ulcer (*Mycobacterium ulcerans*), Calicivirus infection (*Caliciviridae* (Norovirus and Sapovirus)), Campylobacteriosis (*Campylobacter*), Candidiasis also known as Thrush (*Candida*), Capillariasis (Intestinal disease by *Capillaria philippinensis,* hepatic disease by *Capillaria hepatica* and pulmonary disease by *Capillaria aerophila*), Carrion's disease(Bartonella bacilliformis), Cat-scratch disease (*Bartonella henselae*), Cellulitis (usually Group A *Streptococcus* and *Staphylococcus*), Chagas Disease also known as American trypanosomiasis (*Trypanosoma cruzi*), Chancroid (Haemophilus ducreyi), Chickenpox (*Varicella zoster* virus), Chikungunya (Alphavirus), Chlamydia (*Chlamydia trachomatis*), Chlamydophila pneumoniae infection also known as TWAR (*Chlamydophila pneumoniae*), Cholera (*Vibrio cholerae*), Chromoblastomycosis (*Fonsecaea pedrosoi*), Chytridiomycosis (*Batrachochytrium dendrabatidis*), Clonorchiasis (Clonorchis sinensis), Clostridium difficile colitis (*Clostridium difficile*), Coccidioidomycosis (*Coccidioides immitis* and *Coccidioides posadasii*), Colorado tick fever (Colorado tick fever virus), Common cold/Acute viral rhinopharyngitis/Acute coryza (usually rhinoviruses and coronaviruses), Crimean-Congo hemorrhagic fever (Crimean-Congo hemorrhagic fever virus), Cryptococcosis (*Cryptococcus neoformans*), Cryptosporidiosis (*Cryptosporidium*), Cutaneous larva migrans (usually *Ancylostoma braziliense* and multiple other parasites), Cyclosporiasis (Cyclospora cayetanensis), Cysticercosis (Taenia solium), Cytomegalovirus infection (Cytomegalovirus), Dengue fever (Dengue viruses such as DEN-1, DEN-2, DEN-3 and DEN-4), Dientamoebiasis (Dientamoeba fragilis), Diphtheria (Corynebacterium diphtheriae), Diphyllobothriasis (Diphyllobothrium), Dracunculiasis (Dracunculus medinensis), Ebola hemorrhagic fever (Ebolavirus), Echinococcosis (Echinococcus), Ehrlichiosis (Ehrlichia), Enterobacteriaceae (Carbapenem-resistant *Enterobacteriaceae*), Enterobiasis (Enterobius vermicularis), Enterococcus infection

(*Enterococcus*), Enterovirus (Enterovirus), Epidemic typhus (Rickettsia prowazekii), Erythema infectiosum (Parvovirus B19), Exanthem subitum (Human herpesvirus 6 (HHV-6) and Human herpesvirus 7 (HHV-7)), Fasciolasis (*Fasciola hepatica* and *Fasciola gigantica*), Fasciolopsiasis (*Fasciolopsis buski*), Filariasis (Filarioidea), Food poisoning by Clostridium perfringens (*Clostridium perfringens*), Free-living amebic infection (various pathogens), Fusobacterium infection (Fusobacterium), Gas gangrene (usually Clostridium such as perfringens), Geotrichosis (Geotrichum candidum), Giardiasis (*Giardia lamblia*), Glanders (*Burkholderia mallei*), Gnathostomiasis (*Gnathostoma spinigerum* and *Gnathostoma hispidum*), Gonorrhea (*Neisseria gonorrhoeae*), Granuloma inguinale (Klebsiella granulomatis), Group A streptococcal infection (Streptococcus pyogenes), Group B streptococcal infection (*Streptococcus agalactiae*), Haemophilus influenzae infection (*Haemophilus influenzae*), Hand, foot and mouth disease (Enteroviruses, mainly Coxsackie A virus and Enterovirus 71 (EV71)), Hantavirus Pulmonary Syndrome (Sin Nombre virus), Heartland virus disease (Heartland virus), Helicobacter pylori infection (*Helicobacter pylori*), Hemolytic-uremic syndrome (*Escherichia coli* such as O157:H7, O111 and O104:H4), Hemorrhagic fever with renal syndrome (Bunyaviridae family), Hepatitis A (Hepatitis A virus), Hepatitis B (Hepatitis B virus), Hepatitis C (Hepatitis C virus), Hepatitis D (Hepatitis D Virus), Hepatitis E (Hepatitis E virus), Herpes simplex (Herpes simplex virus 1 and 2 (HSV-1 and HSV-2), Histoplasmosis (Histoplasma capsulatum), Hookworm infection (*Ancylostoma duodenale* and *Necator americanus*), Human bocavirus infection (Human bocavirus), Human ewingii ehrlichiosis (*Ehrlichia ewingii*), Human granulocytic anaplasmosis (*Anaplasma phagocytophilum*), Human metapneumovirus infection (Human metapneumovirus), Human monocytic ehrlichiosis (Ehrlichia chaffeensis), Human papillomavirus infection (Human papillomavirus), Human parainfluenza virus infection (Human parainfluenza viruses), Hymenolepiasis (*Hymenolepis nana* and *Hymenolepis diminuta*), Epstein–Barr virus infectious mononucleosis (Epstein–Barr virus), Influenza (*Orthomyxoviridae*), Isosporiasis (*Isospora belli*), Kawasaki disease, Keratitis (various pathogens), Kingella kingae infection (*Kingella kingae*), Lassa fever (Lassa virus), Legionellosis also known as Legionnaires' disease (Legionella pneumophila), Legionellosis also known asPontiac fever (Legionella pneumophila), Leishmaniasis (*Leishmania*), Leprosy (*Mycobacterium leprae* and *Mycobacterium lepromatosis*), Leptospirosis (*Leptospira*), Listeriosis (Listeria monocytogenes), Lyme disease (*Borrelia burgdorferi, Borrelia garinii*, and *Borrelia afzelii*), Lymphatic filariasis (*Wuchereria bancrofti* and *Brugia malayi*), Lymphocytic choriomeningitis (Lymphocytic choriomeningitis virus), Malaria (*Plasmodium*), Marburg hemorrhagic fever (Marburg virus), Measles (Measles virus), Middle East respiratory syndrome (Middle East respiratory syndrome coronavirus), Melioidosis (*Burkholderia pseudomallei*), Meningitis (various), Meningococcal disease (Neisseria meningitidis), Metagonimiasis (usually *Metagonimus yokagawai*), Microsporidiosis (*Microsporidia phylum*), Molluscum contagiosum (Molluscum contagiosum virus), Monkeypox (Monkeypox virus), Mumps

(Mumps virus), Murine typhus (Rickettsia typhi), Mycoplasma pneumonia (*Mycoplasma pneumoniae*), Mycetoma (Actinomycetoma) and fungi Eumycetoma), Myiasis (parasitic dipterous fly larvae), Neonatal conjunctivitis (most commonly *Chlamydia trachomatis* and *Neisseria gonorrhoeae*), Norovirus infection (Norovirus), Nocardiosis (*Nocardia* such as *N. asteroides*), Onchocerciasis (Onchocerca volvulus), Opisthorchiasis (*Opisthorchis viverrini* and *Opisthorchis felineus*), Paracoccidioidomycosis (*Paracoccidioides brasiliensis*), Paragonimiasis (*Paragonimus* such as *westermani*), Pasteurellosis (*Pasteurella*), Pelvic inflammatory disease (various pathogens), Pertussis (*Bordetella pertussis*), Plague (*Yersinia pestis*), Pneumococcal infection (*Streptococcus pneumoniae*), Pneumocystis pneumonia (*Pneumocystis jirovecii*), Pneumonia (various pathogens), Poliomyelitis (Poliovirus), Prevotella infection (Prevotella), Primary amoebic meningoencephalitis (usually *Naegleria fowleri*), Progressive multifocal leukoencephalopathy (JC virus), Psittacosis (*Chlamydophila psittaci*), Q fever (*Coxiella burnetii*), Rabies (Rabies virus), Relapsing fever (*Borrelia* such as *B. hermsii* and *B. recurrentis*), Respiratory syncytial virus infection (Respiratory syncytial virus), Rhinosporidiosis (*Rhinosporidium seeberi*), Rhinovirus infection (Rhinovirus), Rickettsial infection (*Rickettsia species*), Rickettsialpox (Rickettsia akari), Rift Valley fever (Rift Valley fever virus), Rocky Mountain spotted fever (*Rickettsia rickettsii*), Rotavirus infection (Rotavirus), Rubella (Rubella virus), Salmonellosis (*Salmonella*), Severe Acute Respiratory Syndrome (SARS coronavirus), Schistosomiasis (*Schistosoma*), Sepsis (various pathogens), Shigellosis (*Shigella*), Shingles (Varicella zoster virus), Smallpox (Variola major or Variola minor), Sporotrichosis (*Sporothrix schenckii*), Staphylococcal food poisoning (*Staphylococcus*), Staphylococcal infection (*Staphylococcus*), Strongyloidiasis (Strongyloides stercoralis), Subacute sclerosing panencephalitis (Measles virus), Syphilis (*Treponema pallidum*), Taeniasis (*Taenia*), Tetanus (*Clostridium tetani*), Tinea barbae (usually *Trichophyton*), Tinea capitis (*Trichophyton tonsurans*), Tinea corporis (usually *Trichophyton*), Tinea cruris (usually *Epidermophyton floccosum*, *Trichophyton rubrum*, and *Trichophyton mentagrophytes*), Tinea manum (*Trichophyton rubrum*), Tinea nigra (usually *Hortaea werneckii*), Tinea pedis (usually *Trichophyton*), Tinea unguium (usually *Trichophyton*), Tinea versicolor (Malassezia), Toxocariasis (*Toxocara canis* or *Toxocara cati*), Trachoma (*Chlamydia trachomatis*), Toxoplasmosis (*Toxoplasma gondii*), Trichinosis (*Trichinella spiralis*), Trichomoniasis (*Trichomonas vaginalis*), Trichuriasis (*Trichuris trichiura*), Tuberculosis (usually *Mycobacterium tuberculosis*), Tularemia (*Francisella tularensis*), Typhoid fever (*Salmonella enterica subsp. enterica, serovar typhi*), Typhus fever (Rickettsia), Ureaplasma urealyticum infection (*Ureaplasma urealyticum*), Valley fever (*Coccidioides immitis or Coccidioides posadasii*), Venezuelan equine encephalitis (Venezuelan equine encephalitis virus), Venezuelan hemorrhagic fever (Guanarito virus), Vibrio vulnificus infection (Vibrio vulnificus), Vibrio parahaemolyticus enteritis (*Vibrio parahaemolyticus*), Viral pneumonia (various viruses), West Nile Fever (West Nile virus), White piedra (Trichosporon beigelii), Yersinia pseudotuberculosis infection

(Yersinia pseudotuberculosis), Yersiniosis (*Yersinia enterocolitica*), Yellow fever (Yellow fever virus) and Zygomycosis (Zygomycetes).

Intracellular Pathogens

[0216] Intracellular cellular pathogens, can be some of the most difficult to treat because once the pathogen is inside the cell some level of protection may be provided to the pathogen by the cellular environment.

[0217] Pathogens, may be viral, bacterial, fungal, protozoan etc. The molecules are the present disclosure are particularly useful for treatment of intracellular pathogens, in particular those disclosed herein.

[0218] Notable intracellular bacteria include *Bartonella henselae, Francisella tularensis, Listeria monocytogenes, Salmonell typhi, Brucella, Legionella, Mycobacterium (such as Mycobacterium tuberculosis), Nocardia, Rhodococcus equi, Yersinia, Neisseria meninggitidis*.

[0219] One or more antibiotics selected from erythromycin, doxycycline, azithromycin, rifampin, streptomycin, gentamicin, doxycycline, ciprofloxacin, ampicillin, trimethoprim-sulfamethoxazole, chloramphenicol, TMP-SMZ (trimethoprim-sulfamethoxazole), levofloxacin, moxifloxacin, clarithromycin, tetracyclines, glycylcyclines, ethambutol, rifabutin, imipenem, cefotaxime, amikacin, vancomycin, minocycline, penicillin G, ampicillin, fluoroquinolone, aztreonam and combinations of two or more of the same.

[0220] *Mycobacterium tuberculosis* is notoriously difficult to treat. In one independent aspect the present disclosure provides a molecule for the treatment of latent and/or active TB where one or more TB drugs are conjugated as a payload to the GLA-component described herein.

[0221] The molecules of the present disclosure may be able to greatly increased the efficacy of current medicines by delivering them to inside the cell where the mycobacterium is located.

[0222] The treatment for TB depends on a number of factors, including whether the TB is latent or active, if the patient is an adult, is a child, is pregnant, is HIV positive or a combination of the above. The details below relate to all aspects of the invention, for example what molecules to prepare and how to use them to treat patients.

[0223] Treatment of Latent TB in HIV patients and children in the age range 2 to 11 is isoniazid daily for 6 to 9 months. Pregnant patients may be treated twice weekly as opposed to daily.

[0224] Thus, in one embodiment in the molecule of the present disclosure the GLA-component is linked to a payload comprising isoniazid or a an equivalent thereof. Treatment of Latent TB in patients 12 years or older without complicating factors may be given isoniazid and rifapentine one a week for 3 months. Alternatively, rifampin may be given daily for 4 months.

[0225] This in one embodiment the payload further comprises rifapentine.

[0226] Alternatively, a molecule may be provided where the GLA domain, as described herein, is linked to payload comprising rifapentine. A combination of molecules according to the present disclosure may be provided for use in treatment.

[0227] First line treatment for active TB is often selected from isoniazid, rifampin, ethambutol, pyrazinamide and combinations of two or more of the same.

[0228] Thus, in there is provide a molecule according to the present disclosure comprising a GLA-component, as described herein, linked to a payload comprising rifampin.

[0229] Also provided a molecule according to the present disclosure comprising a GLA-component, as described herein, linked to a payload comprising ethambutol.

[0230] In a further embodiment there is provided a molecule according to the present disclosure comprising a GLA-component, as described herein, linked to a payload comprising pyrazinamide.

[0231] As explicitly envisaged that the payloads used in the treatment of TB employing extracellular vesicles as disclosed herein may comprise two or more, such as three drugs or four drugs, such as: isoniazid and rifamycin, isoniazid and pyraxinamide, isoniazid and ethambutol, rifamycin and pyraxinamide, rifamycin and ethambutol, pyraxinamide and ethambutol, isoniazid and rifamycin and pyraxinamide, isoniazid and rifamycin and ethambutol, and isoniazid and rifamycin and pyraxinamide and ethambutol.

[0232] Viral pathogens include influenza, human immunodeficiency virus, dengue virus, West Nile virus, smallpox virus, respiratory syncytial virus, Korean hemorrhagic fever virus, chickenpox, varicella zoster virus, herpes simplex virus 1 or 2, Epstein-Barr virus, Marburg virus, hantavirus, yellow fever virus, hepatitis A, B, C or E, Ebola virus, human papilloma virus, rhinovirus, Coxsackie virus, polio virus, measles virus, rubella virus, rabies virus, Newcastle disease virus, rotavirus, HIV (such HTLV-1 and -2).

[0233] Antiviral drugs may be linked to the GLA-component may be independently be selected from one or more of the following: Abacavir, Aciclovir, Acyclovir, Adefovir, Amantadine, Amprenavir, Ampligen, Arbidol, Atazanavir, Atripla, Boceprevirertet, Cidofovir, Combivir, Darunavir, Delavirdine, Didanosine, Docosanol, Edoxudine, Efavirenz, Emtricitabine, Enfuvirtide, Entecavir, Entry inhibitors, Famciclovir, Fomivirsen, Fosamprenavir, Foscarnet, Fosfonet, Ganciclovir, Ibacitabine, Imunovir, Idoxuridine, Imiquimod, Indinavir, Inosine, Integrase inhibitor, Interferon type III, Interferon type II, Interferon type I, Interferon, Lamivudine, Lopinavir, Loviride, Maraviroc, Moroxydine, Methisazone, Nelfinavir, Nevirapine, Nexavir, Nucleoside analogues, Oseltamivir, Peginterferon alfa-2a, Penciclovir, Peramivir, Pleconaril, Podophyllotoxin, Protease inhibitor, Raltegravir, Reverse transcriptase inhibitor, Ribavirin, Rimantadine, Ritonavir, Pyramidine, Saquinavir, Stavudine, Synergistic enhancer (antiretroviral), Tea tree oil, Telaprevir, Tenofovir, Tenofovir disoproxil,

Tipranavir, Trifluridine, Trizivir, Tromantadine, Truvada, Valaciclovir, Valganciclovir, Vicriviroc, Vidarabine, Viramidine, Zalcitabine, Zanamivir and Zidovudine.

[0234] It is well known in the technical field that antiviral drugs may be used in combinations, for example to increase effectiveness.

[0235] Thus, in one embodiment the molecule of the present invention is provided with a payload to treat a protozoan disease, for example malaria, African sleeping sickness and the like.

[0236] Notable protozoan parasite include plasmodium type parasites, such as malaria. Drug used treat malaria, such as quinine and related agents, choloroquine, amodiaquine, pyrimethamine, proguanil, sulphonamides, mefloquine, atovaquone, primaquine, aremisinin and derivates thereof, halofantrine, doxycycline, clindamycin, sulfadiazine and combination of two or more of the same.

[0237] In one embodiment the molecules of the present disclosure are provided in a pharmaceutical composition comprising a excipient, diluent and/or carrier. In one embodiment the composition is as a parenteral formulation.

[0238] Parenteral formulation means a formulation designed not to be delivered through the GI tract. Typical parenteral delivery routes include injection, implantation or infusion.

[0239] In one embodiment the parenteral formulation is in the form of an injection. Injection includes intravenous, subcutaneous, intra-cranial, intrathecal, intra-tumoural or intramuscular injection. Injection as employed herein means the insertion of liquid into the body via a syringe.

[0240] In one embodiment the parenteral formulation is in the form of an infusion.

[0241] Infusion as employed herein means the administration of fluids at a slower rate by drip, infusion pump, syringe driver or equivalent device. In one embodiment, the infusion is administered over a period in the range of 1.5 minutes to 120 minutes, such as about 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 15, 16, 17, 18, 19 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 65, 80, 85, 90, 95, 100, 105, 110 or 115 minutes.

[0242] In one embodiment, the formulation is for intravenous (i.v.) administration. This route is particularly effective because it allows rapid access to the majority of the organs and tissue and is particular useful for the treatment of metastases, for example established metastases especially those located in highly vascularised regions such as the liver and lungs.

[0243] Therapeutic formulations typically will be sterile and stable under the conditions of manufacture and storage. The composition can be formulated as a solution, microemulsion, liposome, or other parenteral formulation suitable for administration to a human and may be formulated as a pre-filled device such as a syringe or vial, particular as a single dose.

[0244] As discussed above the formulation will generally comprise a pharmaceutically acceptable diluent or carrier, for example a non-toxic, isotonic carrier

that is compatible with the virus, and in which the virus is stable for the requisite period of time.

[0245] The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a dispersant or surfactant such as lecithin or a non-ionic surfactant such as polysorbate 80 or 40. In dispersions the maintenance of the required particle size may be assisted by the presence of a surfactant. Examples of isotonic agents include sugars, polyalcohols such as mannitol, sorbitol, or sodium chloride in the composition.

[0246] Thus, in embodiment there is provided a molecule according to the present disclosure where the GLA-component, described herein, is linked to a payload comprising one or more anti-malaria drugs.

[0247] "Comprising" in the context of the present specification is intended to mean "including".

[0248] Where technically appropriate, embodiments of the invention may be combined.

[0249] Embodiments are described herein as comprising certain features/elements. The disclosure also extends to separate embodiments consisting or consisting essentially of said features/elements.

[0250] Technical references such as patents and applications are incorporated herein by reference.

[0251] The technical backgrounds is part of the technical disclosure of the present specification and may be used as basis for amendments because the discussion therein is not limited to discussing the prior art as it also includes a discussion of the technical problems encountered in the field and the application of the present invention. a.

[0252] Any embodiments specifically and explicitly recited herein may form the basis of a disclaimer either alone or in combination with one or more further embodiments.

[0253] The present application claims priority from US serial numbers: 62/554530, 62/569,403, 62/554533, 62/569,411, 62/584,565 and 62/593,014. Each of these applications are incorporated by reference. These applications may be employed as the basis for a correction to the present specification.

[0254] The invention will now be described with reference to the following examples, which are merely illustrative and should not in any way be construed as limiting the scope of the present invention.

EXAMPLES

Figure 1A-D Shows various representations of GLA protein structures.

Figure 1E Shows an embodiment of a GLA-component according to the present disclosure.

Figure 2 Shows Protein S (PrS) and annexin staining of breast cancer cell lines treated with peroxide to induce apoptosis. **A**, human MDA-231 cells treated with peroxide and stained with FITC-PrS. **B**, untreated MDA-231 cells stained as in A. **C**, treated MDA-231 cells stained with annexin. **D**, human MCF-7 cells treated with peroxide and stained with PrS. **E**, murine MET-1 cells, as in D. **F**, murine 4T1 cells, as in D.

Figure 3 Shows overlapping, yet distinct, cellular localization of PrS and annexin. **A**, murine 4T1 cells treated with peroxide and stained with Cy5 PrS (**RED**) and FITC annexin (**GREEN**). Light arrow, co-localized signals; red arrows, cells staining with PrS and not annexin; green arrow, cell staining relatively brighter with annexin but less bright with PrS, indicating distinct binding patterns (insets show PrS and annexin staining separately). **B**, treated 4T1 cells stained with FITC PrS and Cy5 annexin. Green arrows, cells staining with PrS and not annexin. **C**, Cy5 annexin staining of treated 4T1 cells pre-incubated with 1,000-fold excess of cold annexin.

Figure 4 Shows staining of apoptotic COS-1 cells with PrS and annexin. Cells were treated with t-BHP as described and stained with FITC annexin (left) and Cy5 PrS (right). Arrows indicate subcellular structures presumed to be apoptotic bodies.

Figure 5 Shows differential staining of extracellular vesicles with PrS and annexin. Extracellular vesicles were prepared from 4T1 cells and stained with FITC PrS (GREED) and Cy5 annexin (RED). Arrows indicate vesicles staining with annexin only (RED arrow), PrS only (GREEN arrow) and both proteins (light arrow).

Figure 6 Shows subcellular localization of PrS and annexin. **A**, **B**, apoptotic 4T1 cells were stained with FITC PrS (GREEN arrows) and Cy5 annexin (RED arrows); light arrows, co-localization. **C**, Possible apoptotic bodies.

Figure 7 Shows internalization of PrS within 5 minutes. Apoptotic 4T1 cells were stained with FITC PrS (green) and Cy5 annexin (red) and imaged within 10 min of the addition of the proteins. **A**, Merged image. **B**, Hoescht nuclear stain alone.

Figure 8 Shows BLI images of 4T1 tumors in mice.

Figure 9 SPECT imaging of effect of doxorubicin on 4T1 tumors, using radiolabeled PrS and annexin. Mice with 4T1 breast cancer tumors were imaged with 99mTc PrS (A and B), or annexin (C and D), before (A and C) and 24 h after doxorubicin (B and D).

Figure 10 Shows SPECT imaging of cyclohexamide-treated mice. Five mice per panel are shown before (**A** and **C)** and 24 h after (**B** and **D**) treatment. The mice

were imaged with either ^{99m}Tc PrS (**A** and **B**), or annexin (**C** and **D**), Arrows indicate increased liver signal.

Figure 11 Shows localization of Cy5 PrS to infected spleen. CD1 mice were infected with bioluminescent *Listeria* and imaged on day 2 post infection. The mice were injected with Cy5 PrS 30 min before sacrifice, and the spleens removed and frozen. Modestly infected (**A**) and control uninfected (**C**) mice are shown. Sections of the infected (**B**) and uninfected (**D**) spleens of each mouse in the Cy5 channel are shown, merged with phase contrast images.

Figure 12 Shows localization of Cy5 PrS to tumors treated with doxorubicin. Mice implanted with 4T1 breast cancer tumors were treated with doxorubicin (right panels) or left untreated (left panels). 24 hours later the mice were injected intravenously with Cy5 PrS and sacrificed 30 min later. The tumors were removed, frozen, and sectioned for fluorescence microscopy. Merged Cy5/phase contrast images from four different mice are shown.

Figure 13 Shows differentiation of TSCs. TSCs were cultured in the presence (left) or absence (right) of growth factors. Arrows in the right panel indicate giant cells characteristic of differentiation.

Figure 14 Shows PrS staining of trophoblast stem cells and differentiated trophoblasts. Trophoblast stem cells (left) were differentiated into trophoblast giant cells (right) by withdrawal of growth factors. The cells were stained with Cy5 PrS and imaged.

Figure 15 Shows MSC differentiation. MSC were treated as described in the text, for differentiation into adipocytes (upper panels) or osteoblasts (lower panels). Differentiated cells exhibited the expected morphology in each case.

Figure 16 Shows MSCs stained with PrS (green), annexin (red), and Hoechst (blue). Cells were imaged within 10 min of addition of the stain mixture.

Figure 17 Shows TSCs stained with PrS (green, lightest area), annexin (red, light around the cell membrane), and Hoechst (blue). Cells were imaged within 5 min of addition of the stain mixture.

Figure 18 Shows differential staining of TSC vesicles. TSCs were stained as in **Figure 17**. The group of cells are secreting large vesicles that stain with annexin (red) and not PrS (green).

Figure 19 Shows PrS staining of C17.2 neural progenitor cells. The cells were stained with PrS-FITC and imaged with standard (non-confocal) microscopy.

Figure 20 Shows internalization of PrS into TSC at 4C. FITC PrS (green) and Cy5 annexin (red) were added to TSC at 4C and imaged with confocal microscopy.

Figure 21 Shows lineage-negative, SCA-1/c-kit staining cells from mouse bone marrow. The cells were not stained with either PI (propidium iodide; to detect dead cells) or PrS at this point in the analysis. Absence of staining for hematopoietic lineages (left panel) and staining of c-kit and SCA1 (right panel) defines the population of HSC, shown in green (lightest areas).

Figure 22 PrS staining of long-term HSC. HSC were isolated as in Figure 1, and stained with FITC PrS. SLAM pattern was determined with Cy7 (x-axis).

Figure 23 PrS staining of short-term HSC. HSC were isolated as in Figure 1, and stained with FITC PrS. SLAM pattern was determined with Cy7 (x-axis).

Figure 24 Shows internalization of PrS in long-term HSC. HSC were prepared as described, stained for PrS, and examined with confocal microscopy. Green (lightest areas), FITC PrS; blue, Hoescht nuclear stain; red, PI. Note that PI stain is excluded from the nucleus, indicating the cells are alive.

Figure 25 Shows an example of dead HSC exhibiting nuclear PI.

Figure 26 GLA-mediated delivery is non-toxic to cells

This specification also includes sequences 1 to 6, in the associated sequence listing.

This project initiated the testing of labeled recombinant PrS as an in vivo imaging agent for SPECT (Single Photon Computed Tomography). Surprisingly it was found that the molecule rapidly internalized into apoptotic cells. This unexpected finding led us to explore the phenomenon further, whereupon we found that PrS was also internalized into a subset of non-apoptotic stem cells of several types.

PrS is protein S GLA domain and protein S EGF domain as shown in SEQ ID NO: 6.

Methods

For fluorescence, conjugation of Cy5 and FITC was achieved using Amersham (GE Heathcare) and Molecular Probes (Invitrogen) labeling kits, respectively, according to the instructions of the manufacturers. Both kits provide columns for the removal of unconjugated fluorophore. Initially, 0.77 mg of PrS (Fraction 2) in 1 ml and 0.77 mg of annexin in 1 ml were labeled with FITC to test for specificity of binding to apoptotic cells. For co-localization and competition studies 0.68 mg of PrS (Fraction 3) in 1 ml and 0.68 mg of annexin were labeled with Cy5. For confocal microscopy, 0.76 mg of PrS from the second shipment was labeled with FITC and the previously labeled Cy5-conjugated annexin was used. It should be noted that the precise efficiency of labeling was not determined and the recovery from the columns was assumed to be 85%, according to the instructions of the manufacturers of the labeling kits. Thus, the relative staining intensity of the two proteins in any case may reflect these contingencies. The cells were stained for 30 min initially, but it was subsequently determined that less than 5 min was sufficient. To test PrS for apoptotic cell-specificity, four breast cancer cell lines were initially employed; human MDA-231 and MCF7 and murine 4T1 and MET-1. Subsequently, COS-1 monkey kidney cells were also used. Apoptosis was induced with hydrogen peroxide or tertiary-Butyl hydroperoxide (t-BHP). The cells were plated in 24-well plates at $6x10^4$ cells per

well or Eppendorf chamber slides at $1x10^4$ cells per well, and apoptosis was induced the next day, using 2mM H_2O_2, or t-BHP for time points from 30 min to 2 hrs. After induction, the wells were washed with Annexin Binding Buffer (AB; Santa Cruz Biotech), and stained with labeled protein. From past experience and the literature, 5.5 µg/ml of annexin protein was used for staining. This amount was adjusted for equimolar addition of PrS by assuming the molecular weights of annexin to be 36 kD and the recombinant PrS to be 30 kD, based on the gel images provided. The cells were stained for 15 min. Hoechst 33342 dye was used for visualizing nucleic acid. The wells were then washed with AB and observed using the EVOS fluorescence microscope while still viable. For confocal microscopy, the Leica SP8 microscope in the Stanford Cell Sciences Imaging Facility was employed. The wells were then washed with AB and observed using the Leica sp8 microscope. Hoechst 33342 dye was used for visualizing nuclei. For toxicity studies, PrS was added to trophoblast stem cells (TSCs) and the viability tested with trypan blue using a Nexcelom Cellometer.

To test the labeled proteins for the ability to detect tumors, $5x10^4$ 4T1-luc cells were implanted into groups of 5 male BALB/c mice, in the left axillary fat pad. The mice were imaged with in vivo bioluminescence imaging (BLI) each day to monitor tumor growth, starting at 1 week post implantation. The mice were then treated on day 11 post implantation with 13 mg/kg body weight of intraperitoneal (IP) doxorubicin, and BLI was performed the next day. Control mice bearing tumors were left untreated with doxorubicin. 48 hrs post treatment the mice were imaged 1 hr after intravenous tracer injection (anesthesia 1.3 g/kg of urethane IP), with single head A-SPECT gamma camera (Gamma Medica); 1 mm pin hole collimator, 128 steps into a 128 x 128 imaging matrix, 15 seconds per step, 2.7 cm ROR; FOV = upper chest/neck. The injected dose of each protiein was 160 µl (800 µCi). The animals were then sacrificed and biodistribution was performed. For the cyclohexamide treatment experiment, groups of 5 young (7 week old) male Swiss Webster mice were anesthetized (1.3 g/kg of urethane IP) and injected intravenously with 50 mg/kg cycloheximide. 1 hr 45 min after cycloheximide injection, tracer was injected (PrS = 180 ul / 1.2 mCi per dose; annexin V = 170 µl / 1.05 mCi per dose). 45 min after tracer injection, the mice were imaged with 10 min static whole body images using a single head parallel hole collimator (128 x 128 matrix) on the A-SPECT gamma camera.

To test for the specific localization of fluorescent PrS to apoptotic sites due to infection in live animals, CD1 mice were injected intravenously with bioluminescent *Listeria monocytogenes*. This bacterial pathogen infects many organs including the spleen, in which extensive apoptosis of monocytes and granulocytes occurs. At certain times post infection, spleen is the primary site of bacterial replication and so splenic BLI signals from the bacteria can be correlated with the localization of probes for apoptosis. Mice were infected and imaged each day. When splenic signals were evident (day 2 post infection for $2x10^5$ colony forming units of bacteria in 8 week old CD1 female mice), 300 mg/ kg body mass of Cy5 PrS was injected into mice, the animals were sacrificed 30 min later, and the

spleens removed, frozen in OCT, and sectioned for fluorescence microscopy. Uninfected control mice were employed.

Flow cytometry was performed. Freshly labeled FITC PrS, prepared as described above, was employed. Murine hematopoietic stem cells (HSCs) are routinely purified in this laboratory. The cells were isolated from normal mouse bone marrow by staining for c-Kit+, lineage-negative cells. To further characterize the cells, SLAM marker staining was also performed. These markers stain cells that self-renew and differentiate, whereas non-staining HSCs can only differentiate. Subsequent staining with FITC PrS revealed the percent positive in SLAM-staining cells, as shown in the Results. The cells were then sorted for FITC and examined with confocal microscopy, using Hoechst 33342 for nuclear visualization.

Results

To assess PrS binding specificity in the context of apoptosis in cell culture, we employed several human and murine breast cancer cell lines. Apoptosis was induced with peroxide as described above, and FITC PrS binding was assessed. Examples of these experiments are shown in **Figure 2**. Untreated cells exhibited minimal binding, such as shown in panel B of **Figure 2**. Concentrations of peroxide and incubation times were chosen such that only a minority of cells would be affected, because at higher concentrations and/or longer incubation times the cells detached and staining and microscopy was not possible. In addition, the presence of many unaffected cells served as an internal negative control within each field. FITC-annexin showed specificity for apoptosis similar to PrS, serving as an internal positive control. We then tested the two proteins for co-localization and competitive binding. For co-localization, both FITC and Cy5 labeled PrS and annexin were prepared. 4T1 cells were treated with peroxide and stained with Cy5 and FITC labeled PrS and annexin, using both combinations of fluorophores. The cells were then visualized in the EVOS fluorescence microscope. The results are shown in **Figure 3**. Under the conditions tested, all the brightly staining cells exhibited staining with both proteins. However, whether using Cy5 or FITC, PrS appeared to stain some cells that annexin did not, albeit weakly (**Fig. 3**). The relative staining intensity of different cells by each protein sometimes differed between the two probes, i.e., sometimes annexin stained two cells with equal intensity and PrS did not, and vice versa (**Fig. 3A**, green arrow and insert). Thus, while both probes generally stained the same cells, they appeared to exhibit subtle differences. In the competition assay, increasing excess amounts of unlabeled annexin were pre-incubated with apoptotic 4T1 cells for 15 min and the cells were then stained with Cy5 PrS. Surprisingly, the staining of PrS was not blocked by even 1,000 fold excess of annexin, the highest excess amount tested (**Fig. 3C**), although these proteins are thought to bind to the same target molecule, exposed PS. Co-staining of annexin and PrS was observed with many cell types. While the two proteins generally stained the same cells in each cell type, other differences became apparent. In particular, some objects smaller than cells were differentially stained (**Fig. 4**). These objects, which were present in increased numbers after peroxide treatment, were interpreted as apoptotic bodies; membrane-bound cell fragments produced during the

fragmentation of apoptotic cells. As shown in **Figure 4**, PrS stained these entities, whereas annexin did not, although some of these objects did stain with both proteins. This observation was unexpected. To further explore the differential staining of subcellular entities, extracellular vesicles (EVs) were prepared from 4T1 murine tumor cells using a standard centrifugation protocol. The two proteins also differentially stained these vesicles (**Fig. 5**), a result that may have biological and therapeutic implications.

EVs, specifically exosomes, microvesicles (MVs) and apoptotic bodies (ABs), are presumed to play key roles in cell-cell communication via transfer of biomolecules between cells. The biogenesis of these types of EVs differs, and they originate from either the endosomal (exosomes) or plasma membranes (MV) or are products of programmed cell death (ABs). All mammalian cells are thought to secrete EVs. Each type of EV can transfer molecular cargo to both neighboring and distant cells, affecting cellular behaviors such as those involved in tumor development and progression. In fact, EVs may play a role in nearly all the hallmarks of cancer, including sustaining proliferative signaling, evading growth suppression, resisting cell death, reprogramming energy metabolism, acquiring genomic instability, and developing the tumor microenvironment. They have also been implicated in the induction of angiogenesis, control of invasion, initiation of premetastatic niches, sustaining inflammation, and evading immune surveillance. Immune cells appear to also communicate through EVs and my recognize EVs as signals from tumor cells, infected tissues and wounds. A deeper understanding of the biology of EVs and their contribution to the hallmarks of cancer is leading to new possibilities for diagnosis and treatment of cancer. Development of additional EV surface markers is essential to advancing this field and PrS may be such a determinant.

Following these studies with fluorescence microscopy, the subcellular localization of the staining by PrS and annexin was then evaluated via confocal microscopy. Murine 4T1 cells (lacking the Luc-GFP reporters) were plated on 8-part chamber slides at 1 x 10^4 cells per chamber and apoptosis was induced with 2mM H_2O_2 or t-BHP (2 hr exposure) the next day. The cells were then washed and stained for 15 min with PrS and annexin. Hoechst 33342 dye was used to stain nucleic acid. In all cases, the most brightly staining cells were stained with both probes. However, in many cells labeled PrS was observed in the cytoplasm, whereas the labeled annexin was not (**Fig. 6**). Although annexin was internalized and appears in vesicles of a few cells, internalized annexin together with surface localized PrS in the same cell was not observed. These results were unexpected, because the two proteins are both presumed to bind PS.

Clearly however, the two proteins responded differently to the inhibitors tested. To further study the internalization of PrS, a time course experiment was performed. Apoptotic 4T1 cells were stained for 5 min with Cy5 annexin and FITC PrS, and observed within 5 min of the addition of the probes. PrS was observed in the cytoplasm of these cells immediately, indicating internalization within 5 min (**Fig. 7**). The time course images also showed that PrS and annexin did not always stain the same cells equally at early time points. The cells in **Figure 7** appear to be in different stages of apoptosis, as the cell on the left shows an uncondensed nucleus surrounded by an apparently intact nuclear

membrane, whereas the right cell exhibits the strong staining often characteristic of chromatin condensation that occurs later in the apoptotic process. Staining patterns such as these may indicate that PrS binds earlier in apoptosis than annexin. Although purely conjecture at this point, such a preference would explain many of the differences between these proteins that have been observed so far. For example, the staining of some cells by PrS and not annexin, such as in **Figure 3A** and **B** may be due to PrS binding earlier in the process of apoptosis. To examine PrS localization in live animals, several experiments were performed. These studies employed chemical and infectious induction of apoptosis in vivo, as well as the localization of PrS to tumors treated with doxorubicin, which is known to induce apoptosis. SPECT imaging using HYNIC-labeled PrS and annexin was performed in animals given 4T1luc breast tumors and treated with doxorubicin. Because the 4T1 tumors have been labeled with luciferase, they can be imaged in mice using in vivo bioluminescence imaging (BLI). One of the images from this experiment is shown in **Figure 8**. This method can be used to evaluate tumor implantation and to follow progression in individual animals over time. ^{99m}Tc labeled PrS and annexin were then employed for SPECT imaging of animals treated with doxorubicin and controls. An example of the results is shown in **Figure 9**. The images of the head and thorax of the two animals show non-specific accumulation of the PrS probe in the salivary gland, and a low signal to noise ratio using this probe. Therefore, the threshold of the display in the PrS images shown was lowered to reveal more background, resulting in the brighter false-color of the images. The low signal-to-noise ratio is likely due to HYNIC labeling of only 1 mg of protein, which is sub-optimal, and also due to the inability to perform controlled studies of HYNIC:protein labeling ratio.

SPECT imaging of mice treated with cyclohexamide, which induces apoptosis in the liver, was also performed (**Fig. 10**). In **Figure 10**, the whole-body images of 5 mice are shown in each panel. As with many radiolabeled probes, background is seen in the kidneys. Treatment of the mice with cyclohexamide increased the annexin SPECT signal in the liver. Again, the PrS showed low signal compared to annexin. Annexin was able to detect the apoptotic livers of cyclohexamide treated mice, whereas PrS showed only slight increase of signal in the liver due to treatment. To test the localization of PrS to apoptotic tissues and treated tumors independently of SPECT imaging and the concomitant complications of HYNIC labeling, mice infected with bacteria that induce apoptotic responses and tumor bearing mice were injected with Cy5 PrS. For infection, we employed *Listeria monocytogenes*, a bacterial pathogen labeled with luciferase and well characterized for BLI. Characteristic BLI signals from the spleen provide for excellent co-localization studies. CD1 mice were infected as described above and were imaged with BLI on day 2 post infection. The mice were then injected with Cy5 PrS and 30 min later sacrificed, and the spleens removed for sectioning and fluorescence microscopy (**Fig. 11**). In all cases, splenic sections from infected mice showed much greater Cy5 fluorescence signals than controls. In **Figure 11**, the infected mouse shown displayed low photon counts, indicating the infection had not yet progressed very far in this animal. Many mice exhibit 10 times this signal intensity from the spleen on this day. However, the Cy5

channel fluorescence was still very strong relative to the uninfected control shown. This result may reflect the ongoing innate immune response to infection, as granulocytes and macrophages have been shown to be the main source of annexin signal in such animals (these cells are programmed for apoptosis to limit tissue destruction).

The localization of fluorescent PrS to 4T1 tumors treated with doxorubicin was then tested. Mice implanted with tumors were treated with doxorubicin as described above and Cy5 PrS was injected intravenously 30 min prior to sacrifice and removal of the tumors for sectioning and fluorescence microscopy. The results are shown in **Figure 12**. Areas of intense staining were observed in the treated animals, whereas more modest signal was observed from the untreated tumor sections. Although some untreated tumors did exhibit small areas of higher signal than background, no signals of similar intensity to the treated tumors were observed in any of the untreated sections.

Stem cells are distinct in phenotype from differentiated cells and may express PS non-apoptotically to avoid the induction of immune responses. Trophoblast stem cells (TSCs) differentiate into several types of trophoblasts in culture. TSCs are prepared from mouse uterine scrapings grown in the presence of fibroblast growth factor, activin, and heparin. TSCs spontaneously differentiate into giant cells when these factors are removed from the medium (**Fig. 13**). TSCs stained with PrS, whereas differentiated trophoblasts derived from these cells in culture did not stain (**Fig. 14**). We have also determined that PrS is internalized into stem cells without apoptotic induction. This result confirms observations made in tumor cell lines, in which apoptosis was induced. Without induction of apoptosis, minimal staining was observed in tumor cells. To test for internalization in stem cells, we employed mesenchymal stem cells (MSCs) and TSCs. MSCs were prepared from mouse bone marrow. The bone marrow was flushed from mice and cultured for 6 days in the absence of growth factors. During this incubation, MSCs and hematopoietic stem cells (HSCs) replicate, whereas fibroblasts adhere but do not multiply beyond a few generations. After 6 days, a monolayer is visible. Upon passage by trypsinization, the adherent MSCs are retained, whereas the HSCs, which grow in suspension, are lost. The fibroblasts do not persist due to absence of growth factors and are also not retained. Thus, this simple procedure results in a nearly homogeneous population of MSCs. To confirm the identity of these cells, we treated the cultures separately with dexamethasone and glycerol phosphate (to induce differentiation into osteoblasts) or dexamethasone and indomethacin (to induce differentiation into adipocytes). The results are shown in **Figure 15**. In response to the above treatments, differentiated cells showed the appearance of the respective cells. Adipocytes contained large fat vesicles and osteoblasts were dark with distinctive intracellular collagen and mineralization.

To assess subcellular staining pattern, undifferentiated MSC were stained with PrS and annexin, as well as Hoechst nuclear staining reagent, and observed with confocal microscopy. Results of the observations are shown in **Figure 16**. PrS was rapidly internalized. In the case of MSC, about 1 in 20 cells stained with PrS, consistent with previous data, however the precise percentage that stained was not determined. The morphology of MSCs is heterogeneous, and thee cells secrete abundant material into the

medium, some of which adheres to the surface of the chamber slide, making resulting in background in some of the images. Nonetheless, the data clearly show internalized PrS, within 5 minutes of addition and annexin on the surface. TSCs were also stained and imaged as was done with the MSCs. The observations confirm internalization into these cells as well, which also occurs within 5 minutes of addition of the protein. The results are shown in **Figure 17**. TSCs are morphologically quite variable, and can be multinucleate in the absence of differentiation, as can be seen in the figure. As with the MSCs, these primary cells shed abundant material into the medium, some of which we have established as extracellular vesicles (previous data). This material again makes the imaging difficult. Some EVs stain with annexin and not PrS, and this phenomenon can be seen in TSCs, in **Figure 18**. In this image of a cluster of TSCs, vesicles being released by the cells stain with annexin and not PrS, which is internalized. These patterns raise interesting questions regarding the specificity and binding targets of PrS and annexin. The two proteins are both reputed to bind PS. However, the differential binding to EVs as well as distinct subcellular localization patterns suggest that they are not binding in exactly the same manner. Further studies will be required to establish the basis of this distinction, which may prove to be significant. We have also observed PrS staining of the neural progenitor cell line C17.2 (**Figure 19**), which is a transformed cell line capable of differentiation in vitro into astrocytes and other neuronal cells. Approximately 5% of these transformed cells stained, although this percentage is an estimate. Remarkably, entry into TSCs occurred even when the cells were chilled to 4 °C (**Fig. 20**). However, it must be noted that the chamber could not be continually chilled once placed on the microscope. Nevertheless, the temperature could not have risen much within the 5 min time frame of the imaging procedure. This result, while provocative, must clearly be repeated under more controlled conditions. Should the finding be substantiated, the mechanism would have to be very interesting indeed.

We have succeeded in staining hematopoietic stem cells (HSC) with PrS. Using flow cytometry we determined that HSC stain with PrS, and have observed internalization of PrS in these cells with confocal microscopy. HSC were identified and isolated using fluorescence activated cell sorting (FACS). The cells were identified in bone marrow as lineage-negative, SCA/c-kit positive cells (**Fig. 21**). These were then stained with FITC-PrS. Two populations of HSC, short-term and long-term, can be identified with the pattern of SLAM marker staining. The SLAM (Signaling Lymphocyte Activation Molecule) markers CD48, CD150, CD229 and CD244 differentially stain HSC with distinct patterns such that SLAM pattern-positive staining is indicative of the ability to both self-renew and differentiate, whereas SLAM pattern-negative HSC can only differentiate. PrS stained a subset of long-term HSC (**Fig. 22**), and also short-term HSC (**Fig. 23**). The cells shown are propidium iodide (PI)-negative, meaning that they are all live cells. This result confirms previous experiments demonstrating that a subset of stem cells stains with PrS without the induction of apoptosis.

We then proceeded to test for internalization of PrS into HSC. This experiment was complicated by many factors. Perhaps the most difficult was the survival in culture of HSC,

which die in large numbers in medium overnight. We therefore had to time the experiment such that flow cytometry analysis and confocal microscopy occurred on the same day. Furthermore, the cells are not adherent, making microscopy less than optimal. To make microscopy more efficient, the cells were resuspended in a small drop of medium. Finally, we needed to make sure that the PrS-stained cells analyzed by microscopy were still alive. Many HSC died during the processes of analysis and isolation. Therefore, PI was added and scanned in addition to the Hoescht nuclear stain, and another channel was employed. The presence of PI-bright nuclei indicated dead cells. Despite these difficulties and the complexities of timing, we were able to perform the experiment, and confirmed internalization of PrS into live HSC (**Fig. 24**). The cells were confirmed as alive by lack of nuclear PI staining. However, some cells were dead or dying as shown in **Figure 25**. Despite the complexity and length of the experiment shown, the results show internalization.

Finally, in **Figure 26**, we have performed preliminary toxicity studies on TSC, and determined that at a concentration of 135 μg/ml, viability was reduced only by a very minimal extent after 30 min, from 78% to 74%, relative to PBS. Considering that, at this level, 10% of the culture volume was PrS-containing solution, this result confirmed our qualitative observations that PrS is basically non-toxic to stem cells, and the minor toxicity observed could well be due to contaminating contents of the preparation itself. Lower concentrations of PrS showed no effect on viability. The highest level of protein tested was more than 1000 times the concentration used for staining. While full toxicity studies, which were not formally part of this project, will require much more extensive tests, in our hands PrS exhibits very little toxicity.

Summary

[0275] The above results have shown that PrS is rapidly internalized into an array of cells expressing PrS, including stem cells of many types, which suggests that PrS possesses unique characteristics amenable to manipulation toward the goal of developing a therapeutic agent. In addition, the difference in specificity between PrS and annexin such as seen in **Figures 3** and **7** suggests that binding itself is different between these two proteins. The mere fact that annexin is a tetramer and PrS is a monomer cannot explain these differences and these data suggest that some other component on the cell surface may be involved in PrS binding. The mechanism of binding, specificity, and internalization of PrS, as well as the capability of modular manipulation provide a host of possibilities.

EXAMPLE 2

[0276] Stem cells are distinct in phenotype from differentiated cells and may express PS non-apoptotically to avoid the induction of immune responses. Stem cells were stained with a GLA domain molecule of the present disclosure comprising a payload of a fluorescent label, without the induction of apoptosis.

[0277] Trophoblast stem cells, (**Fig. 14**) which differentiate into several types of trophoblasts in the placenta, stained with Protein S, whereas differentiated trophoblasts derived from these cells in culture did not stain. The stain was able to distinguish between *in vivo* differentiated stems cells and cells differentiated i*n vitro*.

[0278] This data the molecules of the present disclosure may be employed to target cells *in vivo* or in *ex vivo* samples.

Conclusions

[0279] Extracellular vesicles represent an exciting opportunity for the diagnosis and treatment of an array of maladies. The present inventors have demonstrated that Gla domain recognizes the expression of phosphatidylserine. This property can be utilized for the: identification of extracellular vesicles expressing phophatidylserine, isolation of extracellular vesicles, targeting extracellular vesicles and/or loading the vesicles with, for example therapeutic materials. This is surprising because extracellular vesicles are minute sub-cellular entities.

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Claims

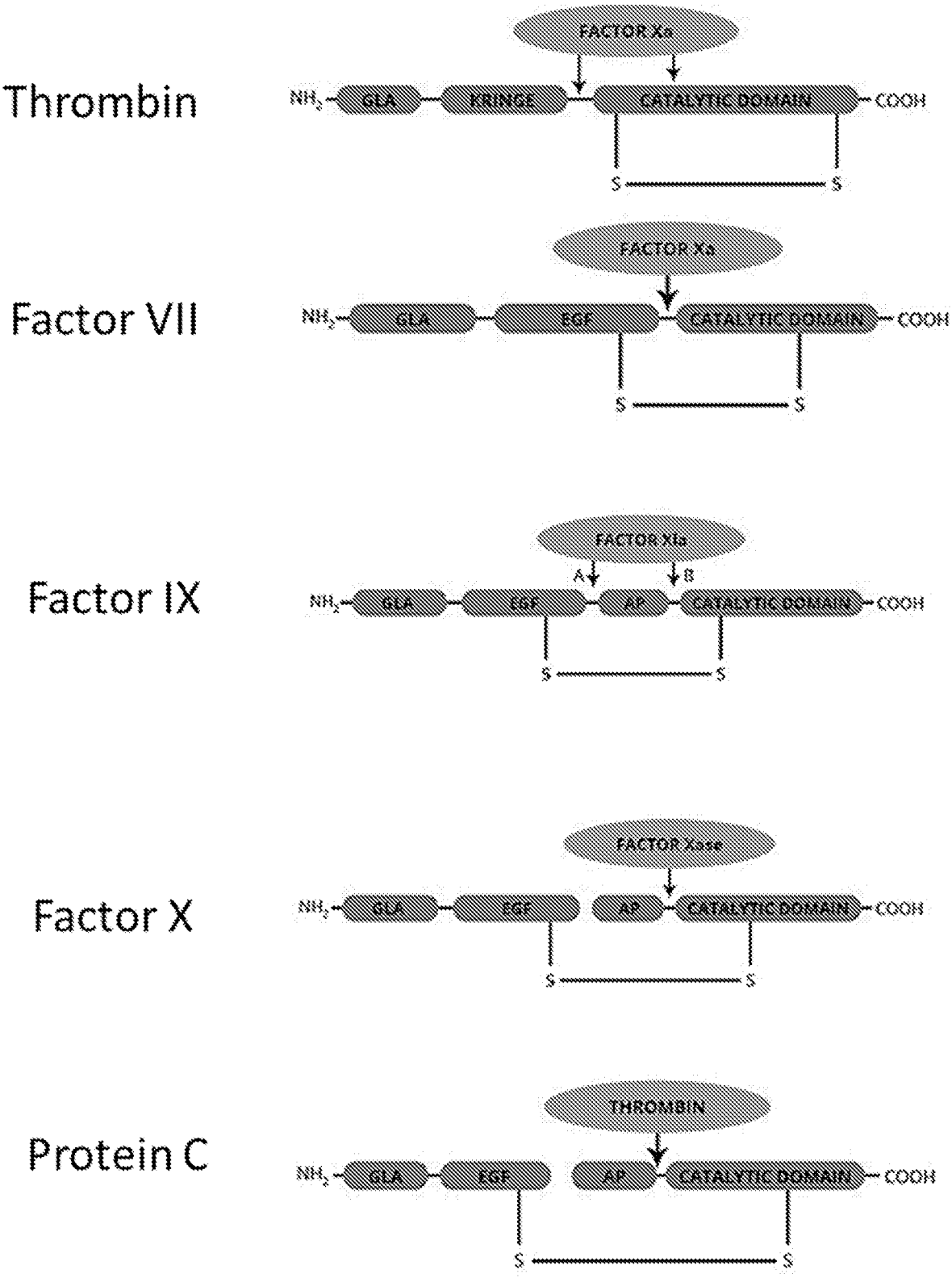
1. A method for targeting extracellular vesicles with surface exposed phosphatidyl serine said method comprising the step of introducing a molecule comprising:
   a gamma-carboxyglutamic acid component (GLA-component) said GLA-component comprises a GLA domain or an active fragment thereof, and which does not comprise an active catalytic domain from a GLA protein,
   into a fluid which may comprise the extracellular vesicle.
2. A method according to claim 1, wherein the extracellular vesicle is an exosome.
3. A method according to claim 2, wherein the exosome has a diameter in the range 30nm to 100nm.
4. A method according to any one of claims 1 to 3, wherein the vesicle has a density in the range 1 to 1.5g/ml, such as 1 to 1.2g/ml, in particular 1.13 to 1.19g/ml.
5. A method according to any one of claims 1 to 4, wherein the vesicle comprises one or more transmembrane proteins independently selected from Lamp- 1, Lamp-2, CD 13, CD86, Flotillin, Syntaxin-3, CD2, CD36, CD40, CD40L, CD41a, CD44, CD45, ICAM-1, Integrin alpha4, LiCAM, LFA-1, Mac-1 alpha and beta, Vti-IA and B, CD3 epsilon and zeta, CD9, CD18, CD37, CD53, CD63, CD81, CD82, CXCR4, FcR, GluR2/3, HLA-DM (MHC II), immunoglobulins, MHC-I or MHC-II components, TCR beta, tetraspanins and combinations of two or more of the same.
6. A method according to any one of claims 1 to 5, wherein the vesicle was released from an unhealthy cell, for example an apoptotic cell, a necrotic cell, a cancer cell, a pathogen infected cell (such as a virus infected cell, a bacteria infected cell or a parasite infected cells).
7. A method according to claim 6, wherein the cell is a cancer cell.
8. A method according to claim 7, wherein the cell is a cancer stem cell.
9. A method according to any one of claims 1 to 8, wherein the fluid comprises an *ex vivo* patient sample, for example a blood sample, fluid drawn from a cyst or tumor, homogenised biopsy or similar.
10. A method according to any one of claims 1 to 9, wherein GLA domain or active fragment thereof is independently selected from thrombin, factor VII, factor IX, factor X, protein C, protein S, protein Z, Osteocalcin, Matrix GLA protein, GAS6, Transthretin, Periostin, Proline rich GLA 1, Proline rich GLA 2, Proline rich GLA 3 and Proline rich GLA 4.
11. A method according to any one of claims 1 to 10, wherein GLA domain or active fragment thereof is from, protein S, for example comprises a sequence shown in SEQ ID NO: 1.
12. A method according to any one of claims 1 to 12, wherein the GLA-domain-component further comprises an EGF domain, for example a calcium binding EGF domain.
13. A method according to claim 12, wherein the construct comprises an EGF domain selected from thrombin, factor VII, factor IX, factor X, protein C, protein S, protein Z,

Osteocalcin, Matrix GLA protein, GAS6, Transthretin, Periostin, Proline rich GLA 1, Proline rich GLA 2, Proline rich GLA 3 and Proline rich GLA 4.

14. A method according to claim 13, wherein the construct comprises an EGF domain selected from protein S.
15. A method according to any one of claims 1 to 14, wherein the GLA-component comprises a sequence shown in SEQ ID NO: 6 or a derivative thereof wherein the His-tag is absent, in particular SEQ ID NO: 6.
16. A method according to any of claims 1 to 15, wherein the GLA-domain component further comprises a Kringle domain.
17. A method according to claim 16, wherein the Kringle domain is from a protein selected from the group comprising Activating transcription factor 2 (ATF); Factor XII (F12); thrombin (F2); Hyaluronan-binding protein 2 (HABP2); Hepatocyte growth factor (HGF); Hepatocyte growth factor activator (HGFAC); Kremen protein 1 (KREMEN1); KREMEN2; Lipoprotein(a) (LPA); LPAL2; Macrophage-stimulating protein (MSP or MST1); Phosphoinositide-3-kinase-interacting protein 1 (PIK3IP1); Tissue plasminogen activator (PLAT); Urokinase (PLAU); Plasmin (PLG); PRSS12; Tyrosine-protein kinase transmembrane receptor ROR1 (ROR1); and Tyrosine-protein kinase transmembrane receptor ROR2 (ROR2).
18. A method according to any one of claims 1 to 17, wherein the GLA-component is linked to a payload.
19. A method according to claim 18, wherein the GLA-component is conjugated to the payload.
20. A method according to any one of claims 1 to 19, wherein the GLA-component is part of a fusion protein.
21. A method according to claim 19, wherein payload comprises a detectable label.
22. A method according to claim 21, wherein the label is selected from a fluorescent protein (including where the, a fluorescent probe (such as rhodamine dye, FITC, FAM, CY5), biotin, an enzyme, a tag (for example a HIS-tag, FLAG-tag, myc-tag), a radionuclides (particularly radioiodide, radioisotopes, such as ^{99m}Tc), luminescent labels or compounds which may be detected by NMR or ESR spectroscopy including wherein the detectable label is in a Molecular Beacons.
23. A method according to any one of claims 1 to 22, wherein the payload a bead, plate or a tag, for example an isolatable bead, such as a magnetic bead.
24. A method according to claim 18 to 23, wherein the payload is linked to the GLA-component via a linker.
25. A method according to claim 24, wherein the linker is cleavable.
26. A method according to any one of claims 1 to 25, wherein the method comprises a further step of providing an enriched population of the vesicles.
27. A method according to any one of claims 1 to 26, wherein the method comprises the step of isolating the vesicles.
28. A method according to any one of claim 1 to 21, wherein the molecule comprising the GLA-component is a therapeutic.

29. A method according to any one of claims 17 to 21 and 29, wherein the payload comprises a drug , a chemotherapeutic agent, a peptide (including stapled peptides) or biological therapeutic, for example, an anti-viral drug, anti-bacterial drug, anti-parasitic agent, anti-cancer drug, an anti-cancer therapy or an oncolytic virus or viral vector.
30. A method according to any one of claims 1 to 21, 29 and 30, wherein the payload comprises a toxin, a polymer (for example synthetic or naturally occurring polymers), biologically active proteins (for example enzymes, other antibody or antibody fragments), a drug (small molecule (chemical entity), c, nucleic acids and fragments thereof (for example DNA, RNA and fragments thereof) a metal chelating agent, nanoparticles or a combination of two or more of the same.
31. A method according to any one of claims 1 to 22 and 24 to 30, which comprises administering the molecule comprising the GLA component and payload to a cancer patient.
32. A method according to claim 31, wherein the cancer is an epithelial cancer, for example colorectal cancer, testicular cancer, liver cancer, biliary tract cancer, prostate cancer, pancreatic cancer, breast cancer, ovarian cancer, cervical cancer, uterine cancer, gastric cancer, oesophageal cancer, thyroid cancer, renal cancer, bladder cancer, brain cancer, head and neck cancer or lung cancer or alternatively the cancer may be a haematological cancer, for example leukaemia, lymphoma, myeloma and chronic myeloproliferative diseases, such as AML, CML, ALL and CLL.
33. A vesicle or a population of vesicles obtainable from a method according to any one of claims 1 to 32.

Figure 1A


Thrombin
FACTOR Xa
NH2
GLA
KRINGE
CATALYTIC DOMAIN
COOH
S
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Factor VII
FACTOR Xa
NH2
GLA
EGF
CATALYTIC DOMAIN
COOH
S
S
Factor IX
FACTOR XIa
A
B
NH2
GLA
EGF
AP
CATALYTIC DOMAIN
COOH
S
S
Factor X
FACTOR Xase
NH2
GLA
EGF
AP
CATALYTIC DOMAIN
COOH
S
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Protein C
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NH2
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AP
CATALYTIC DOMAIN
COOH
S
S

Figure 1B

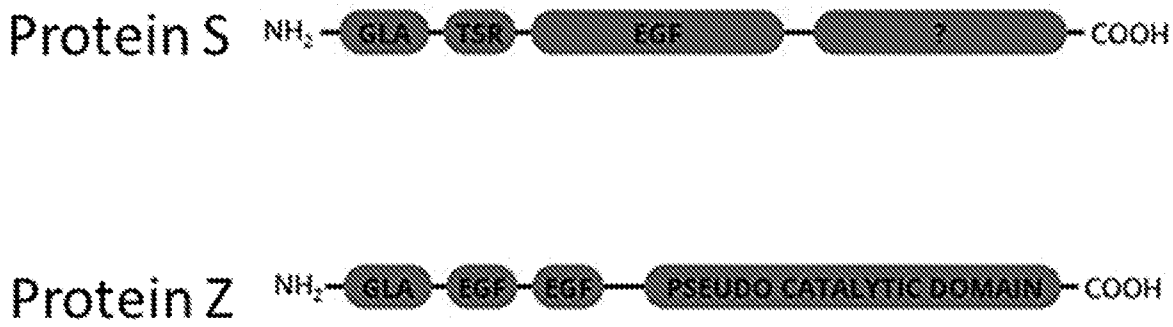
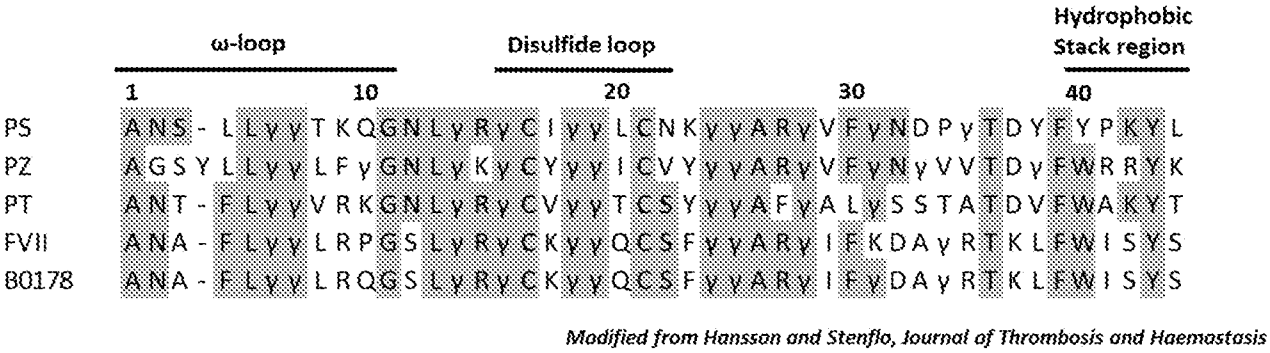


Figure 1C



Modified from Hansson and Stenflo, Journal of Thrombosis and Haemostasis

Figure 1D

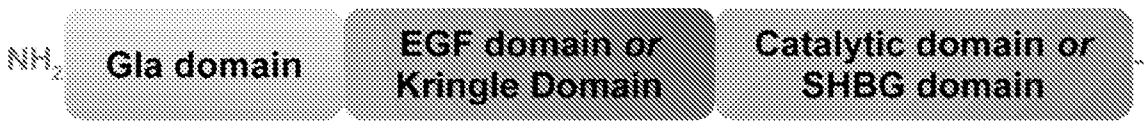


Figure 1E An embodiment of a GLA-component

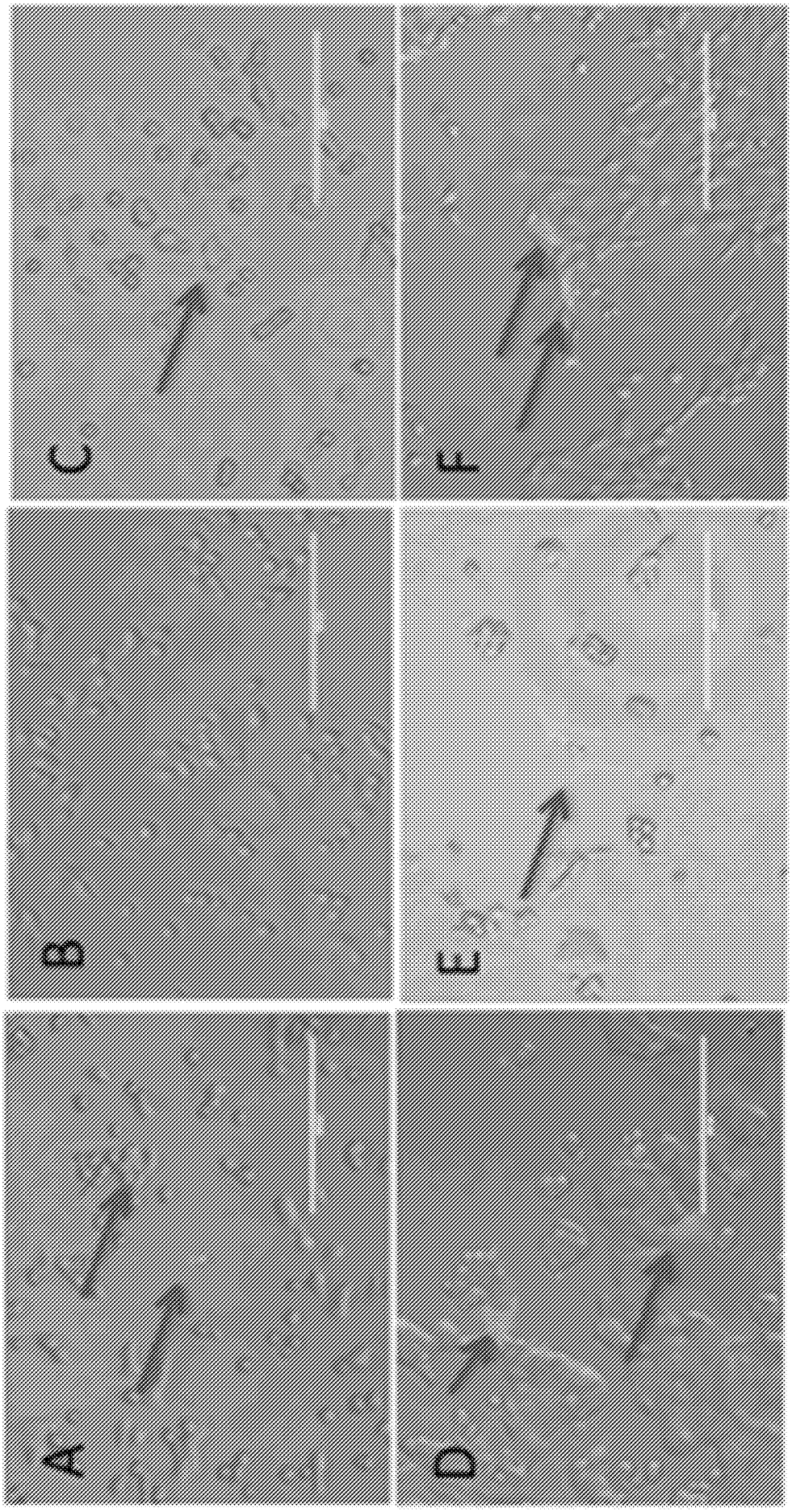
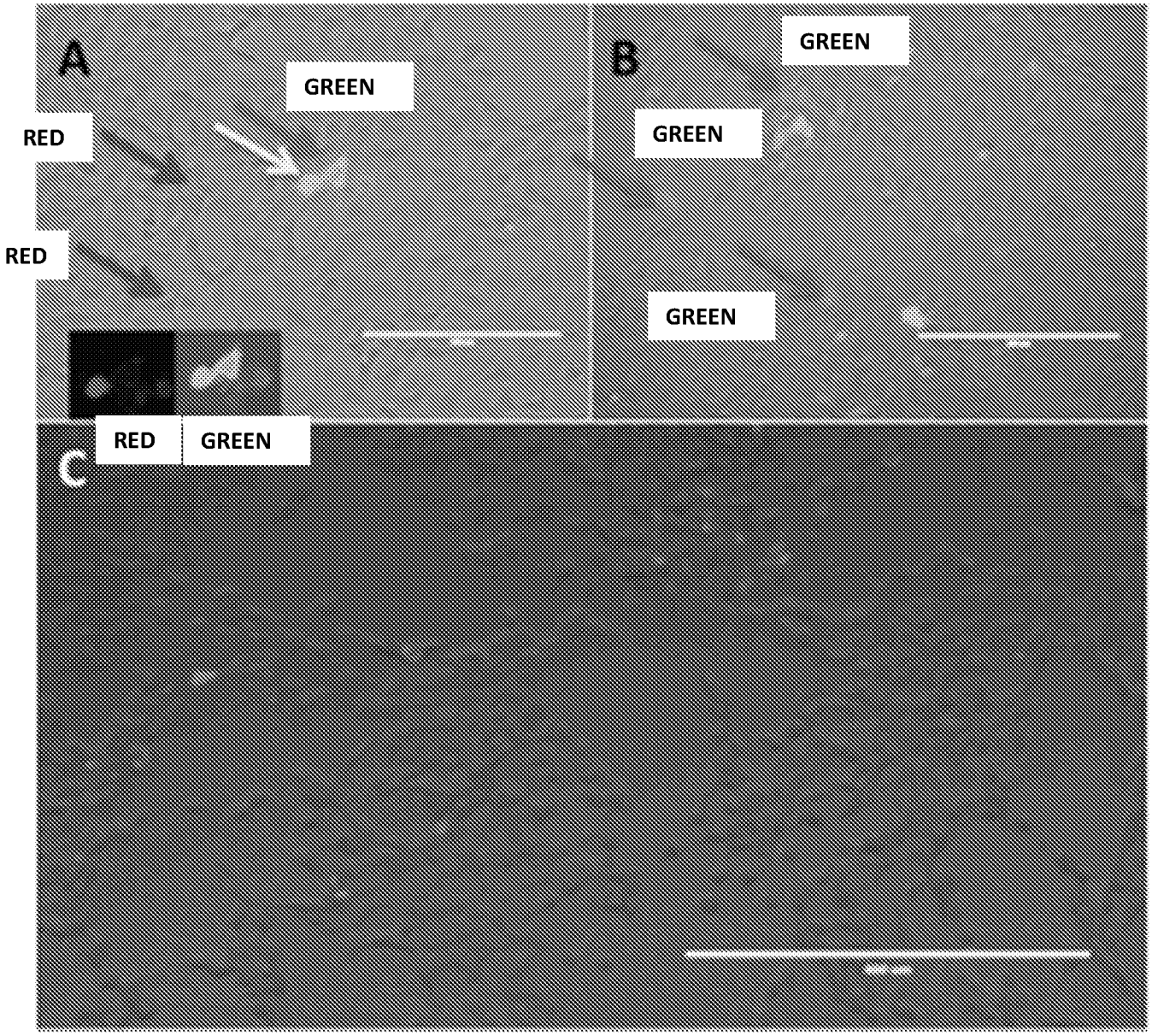
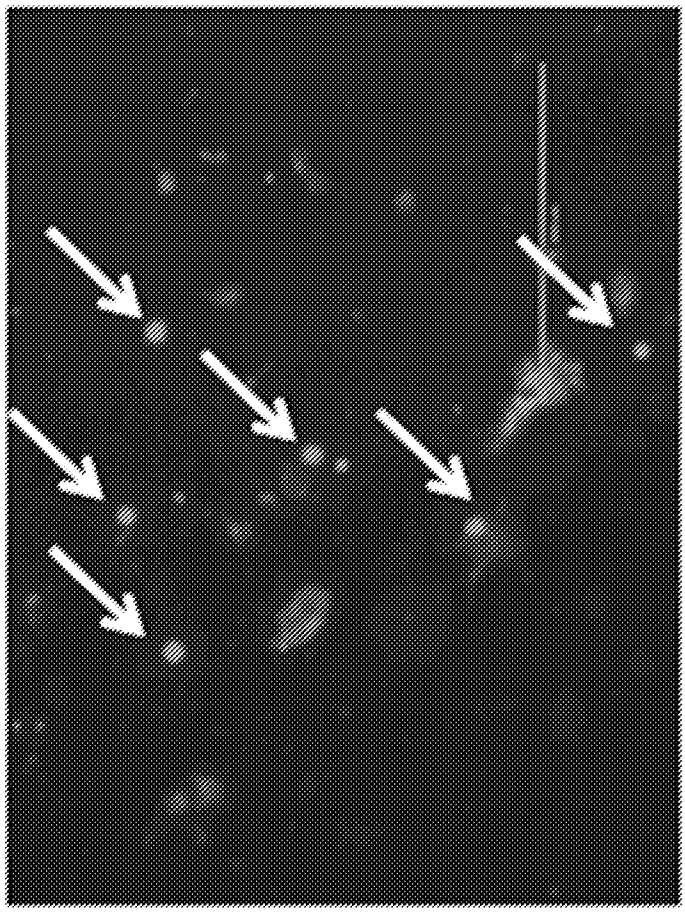


Figure 2

Figure 3





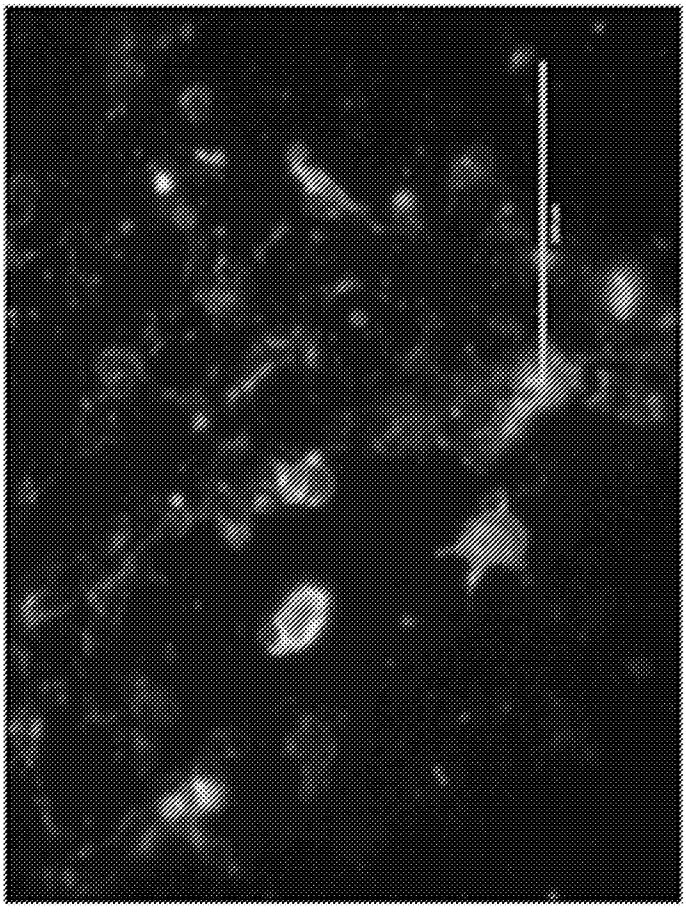
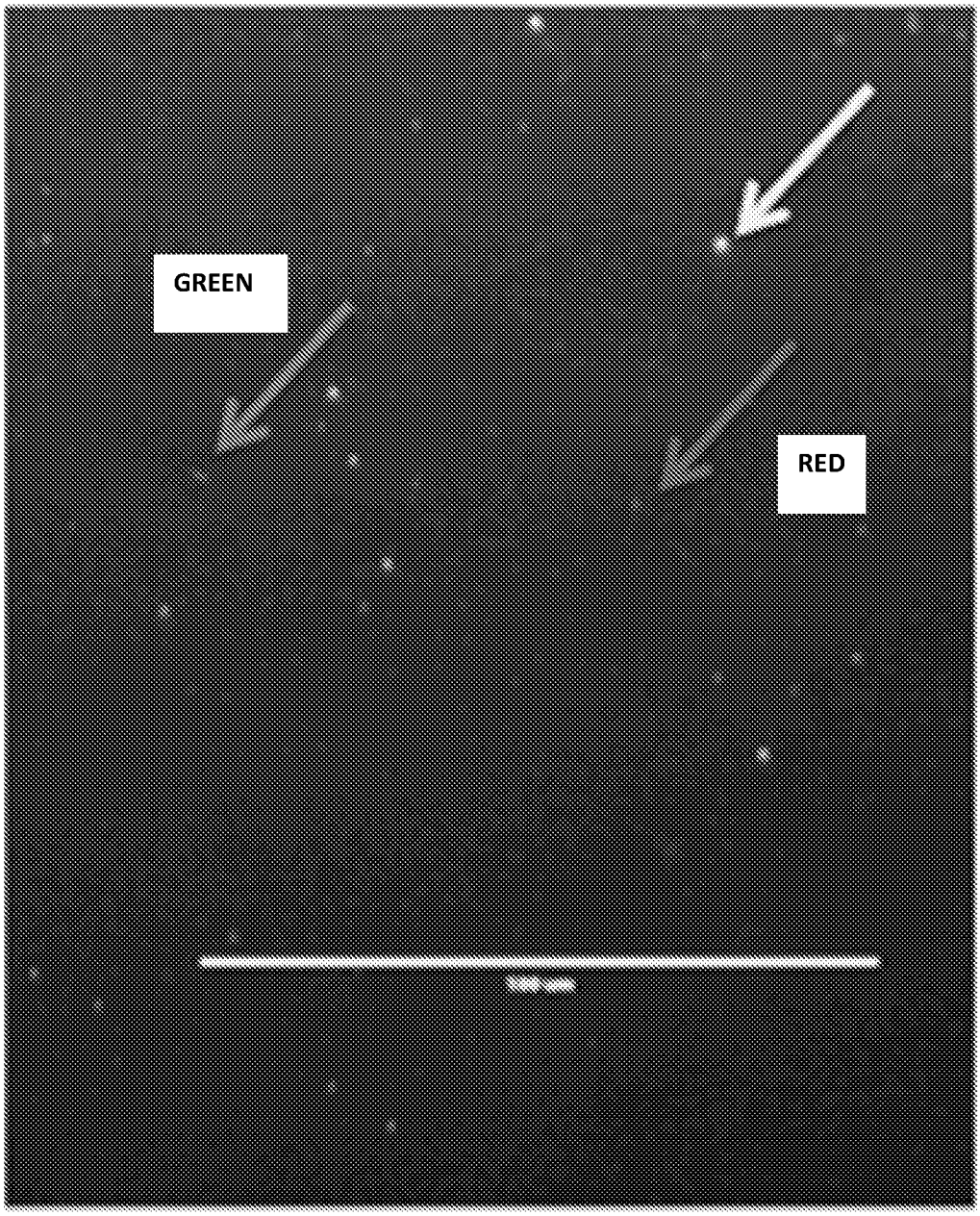
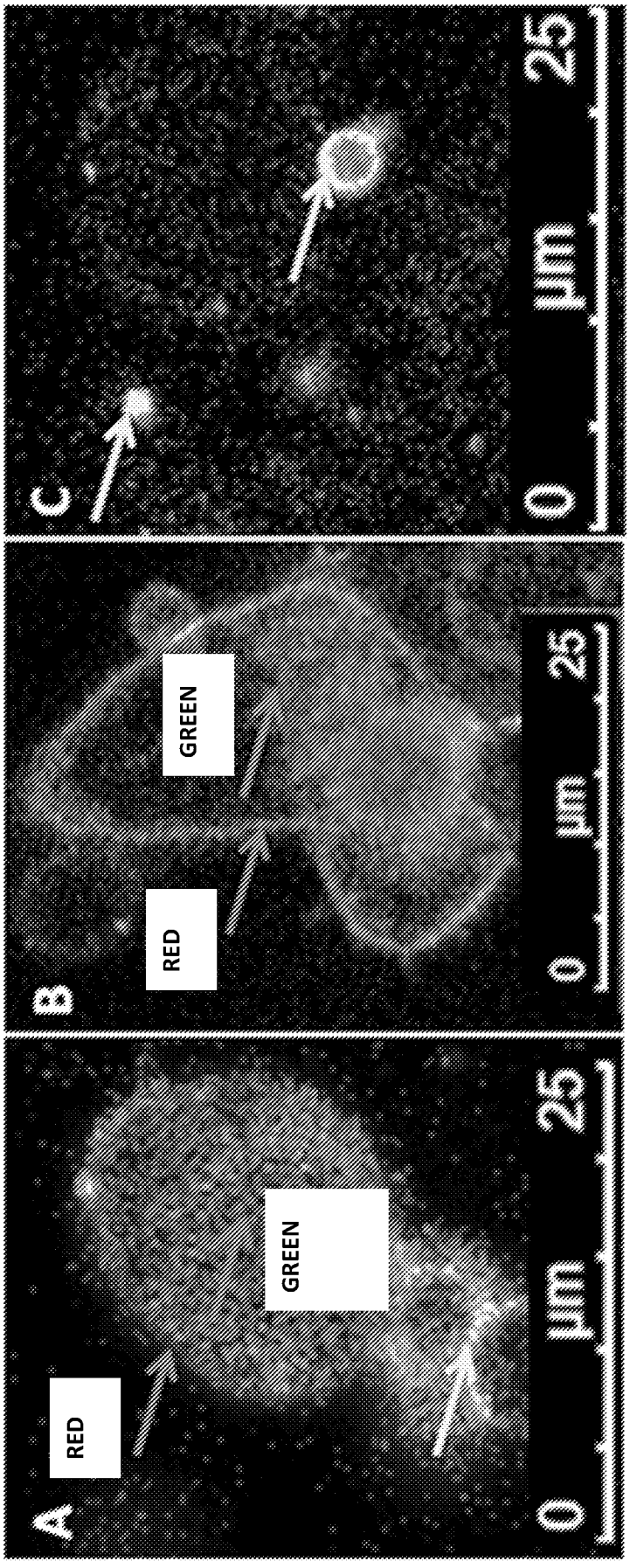


Figure 4

Figure 5





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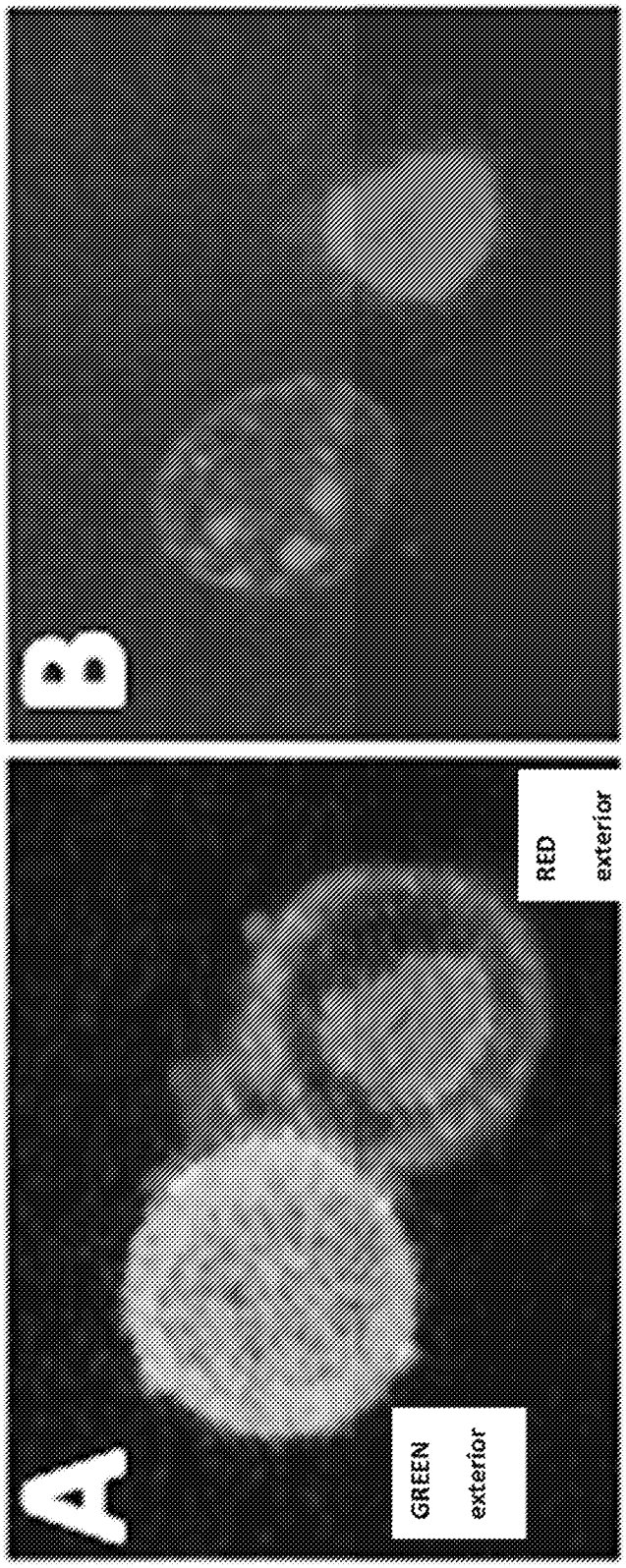


Figure 7

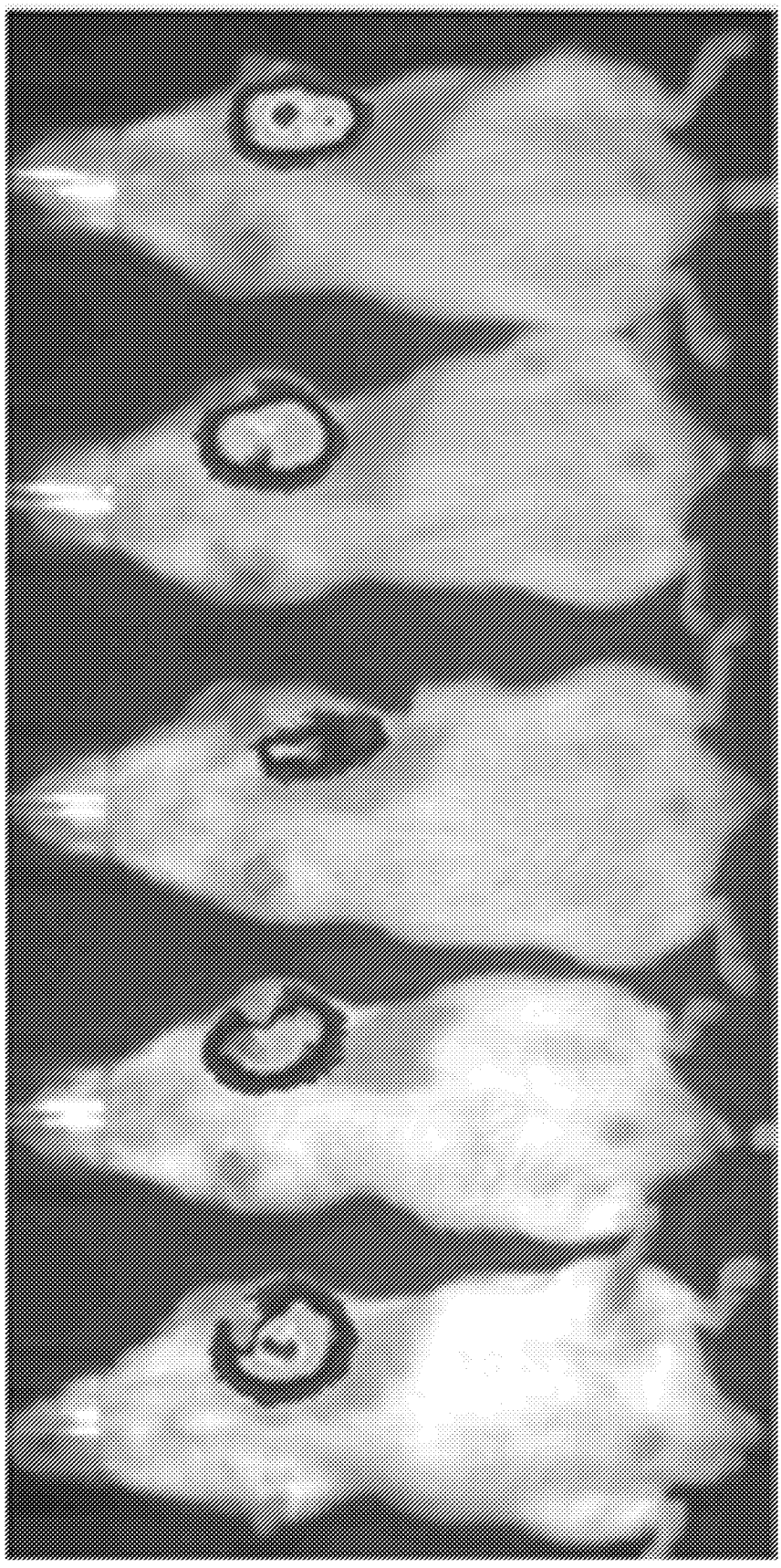
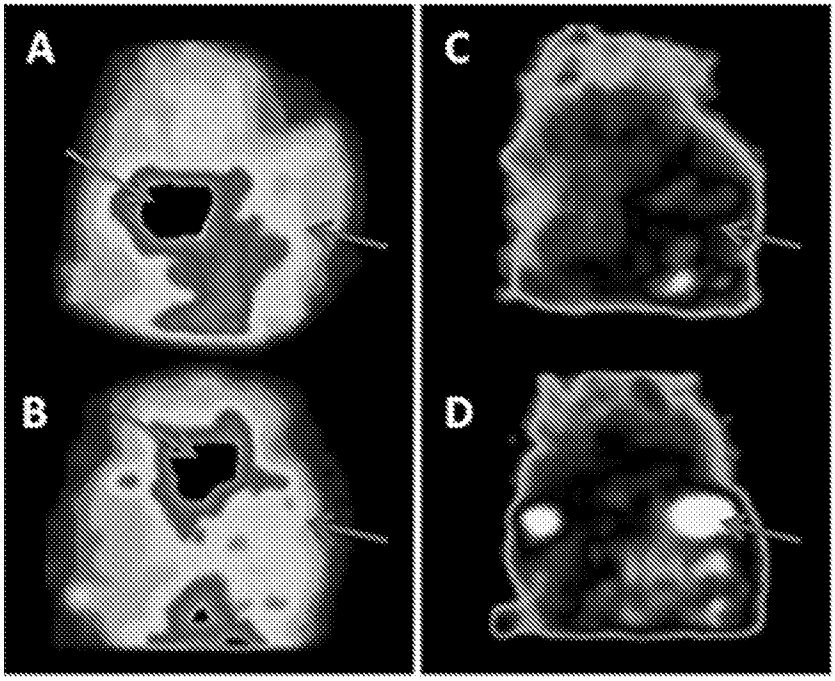


Figure 8

Figure 9



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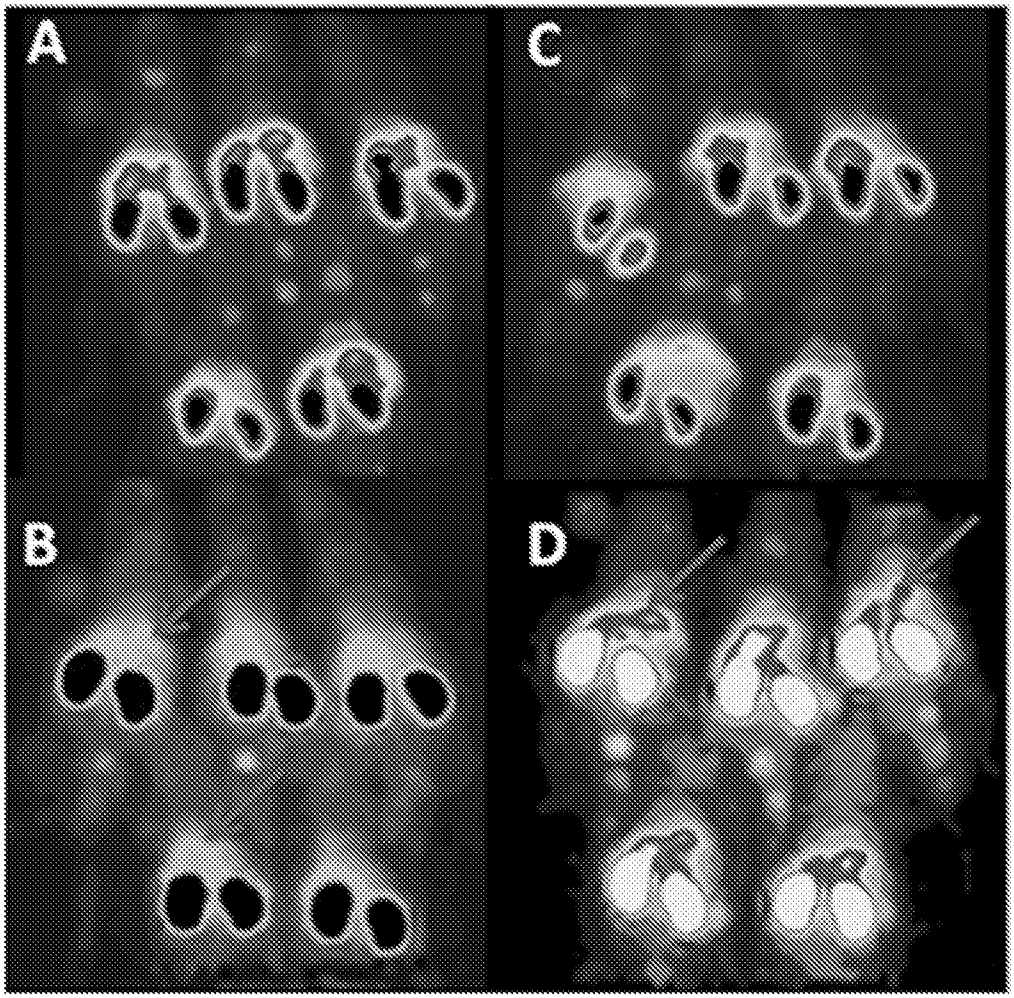
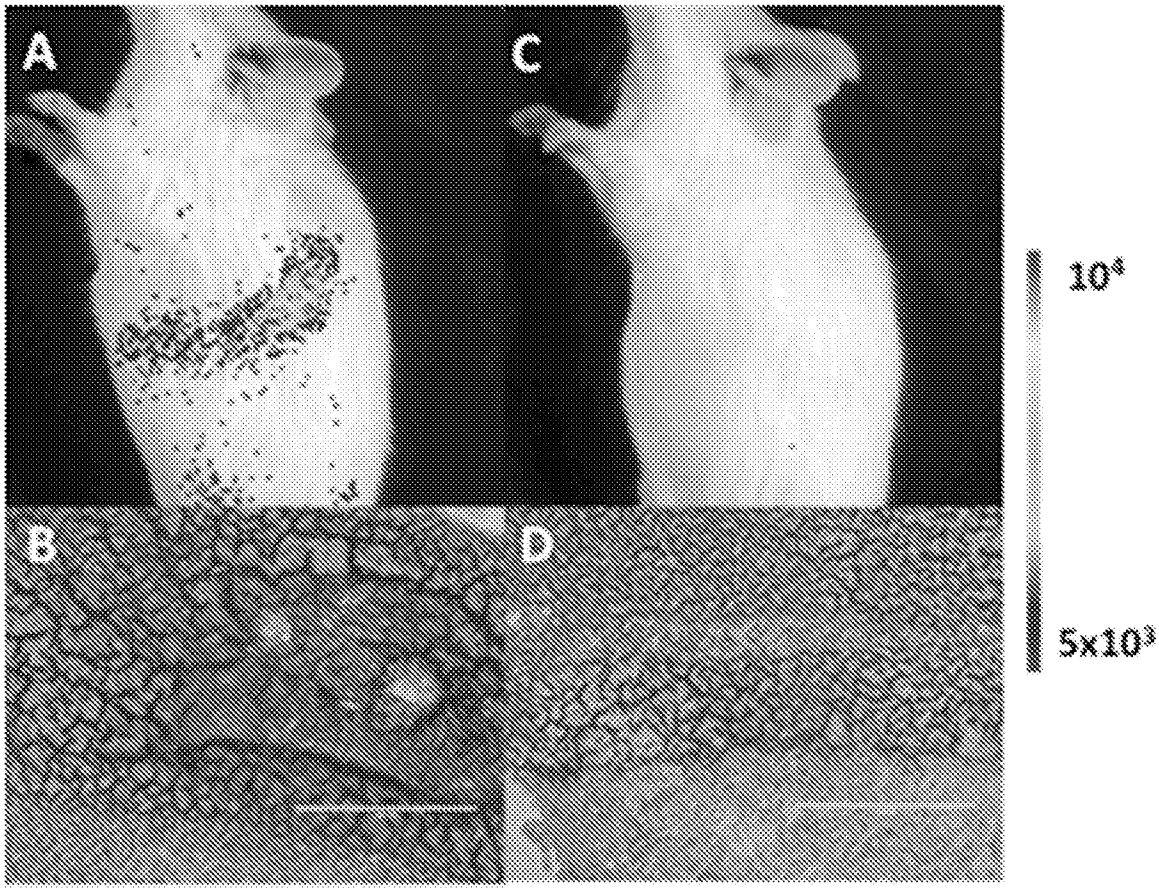


Figure 11



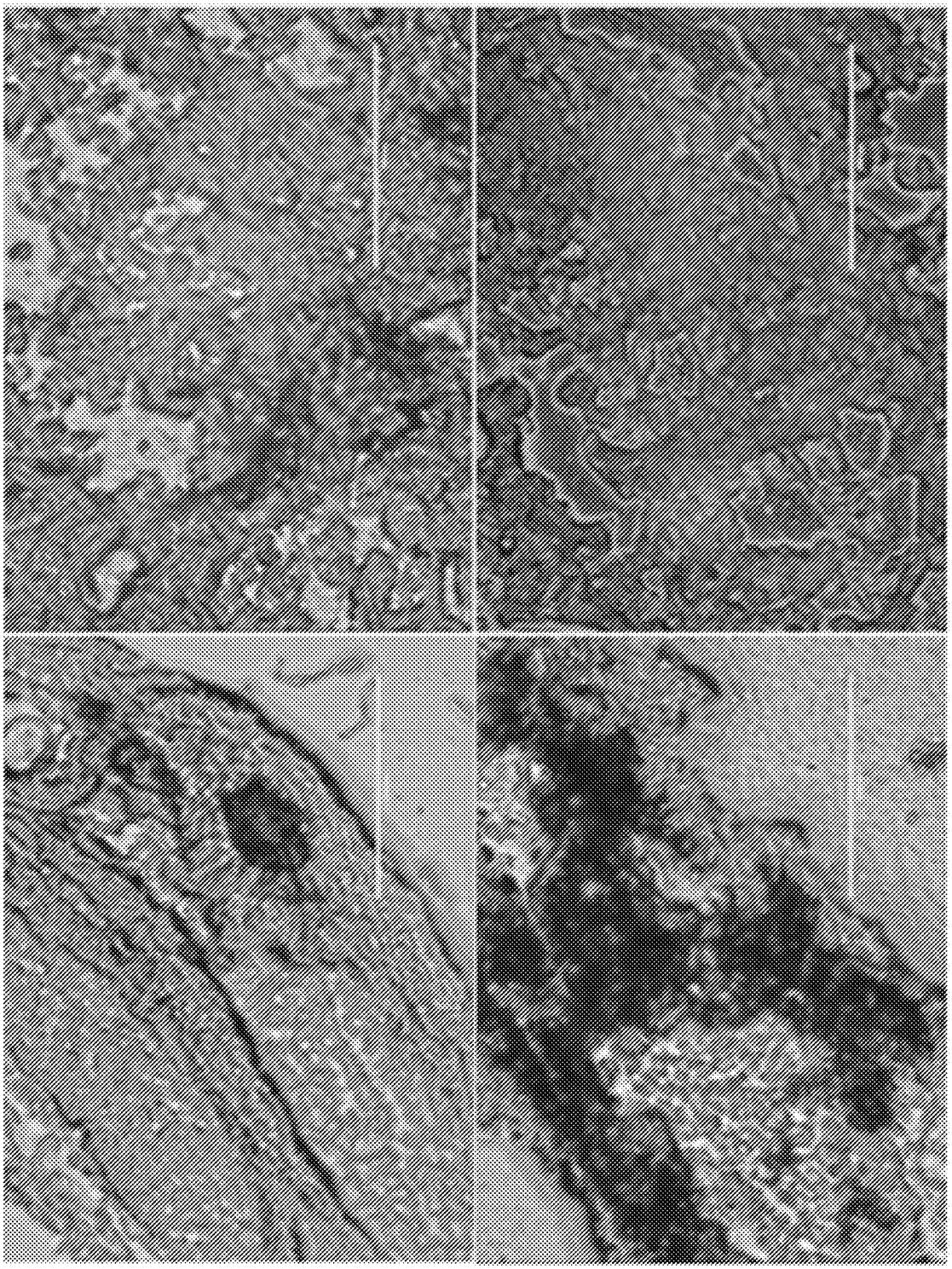


Figure 12

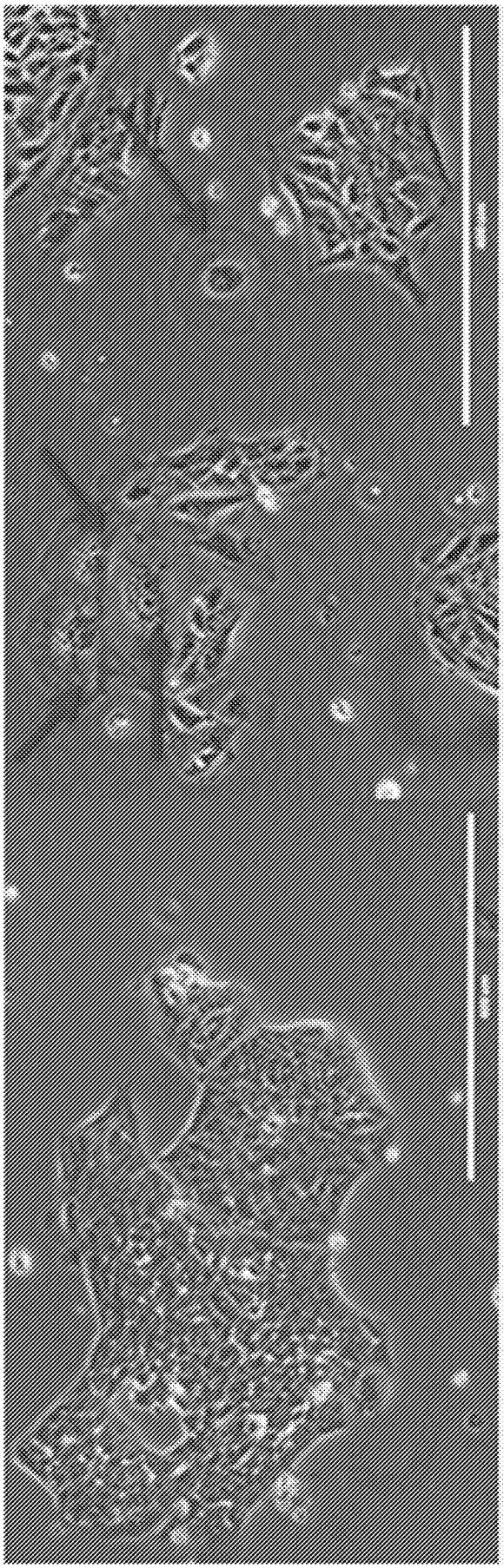


Figure 13

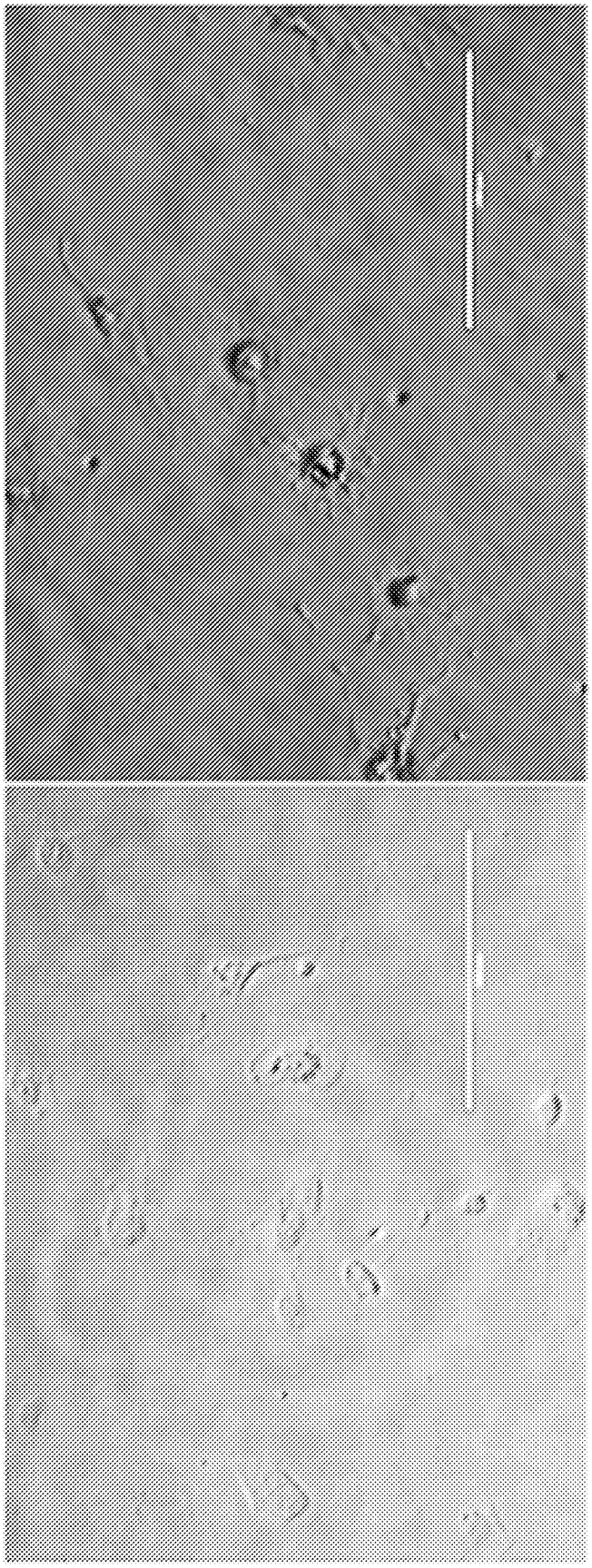


Figure 14

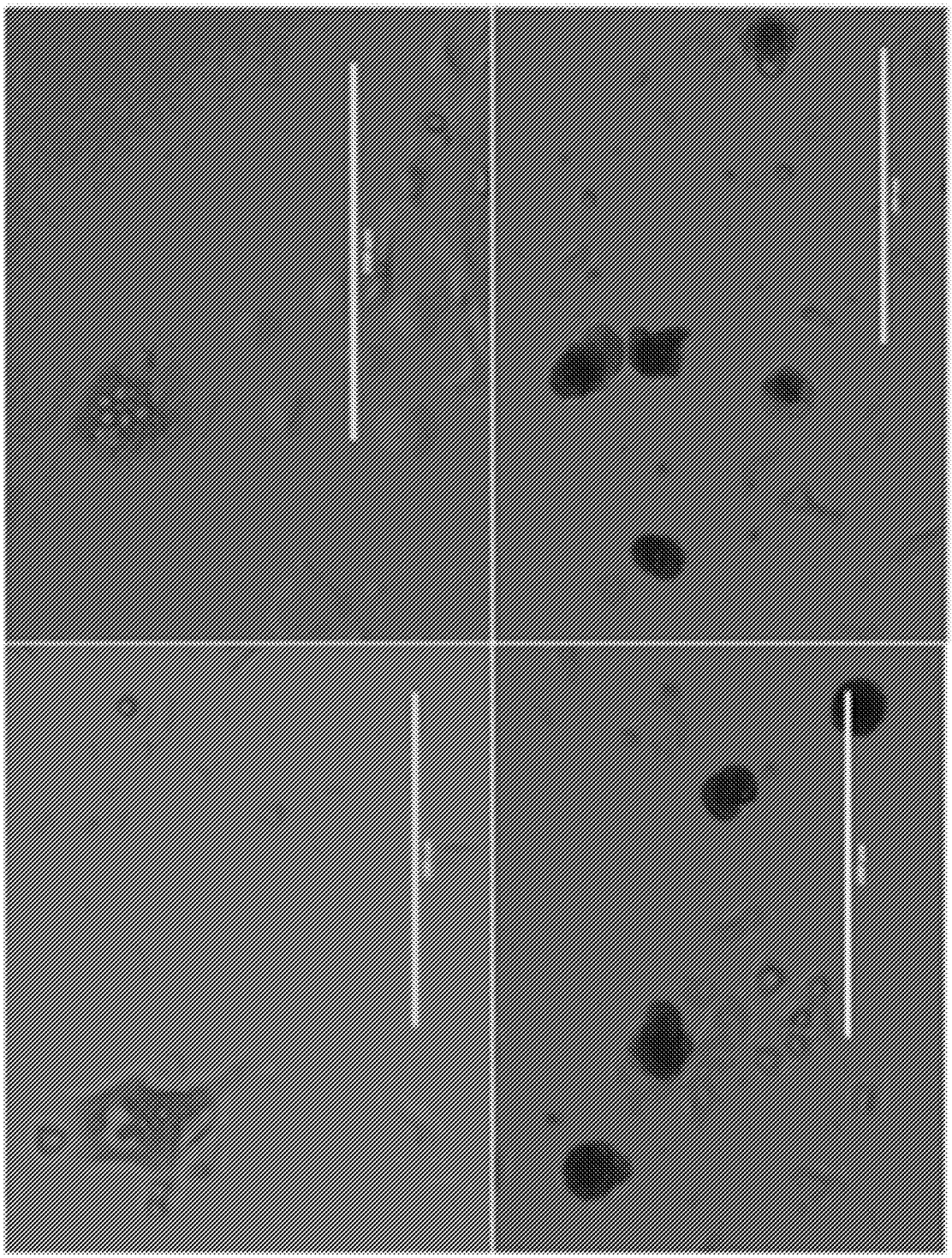


Figure 15

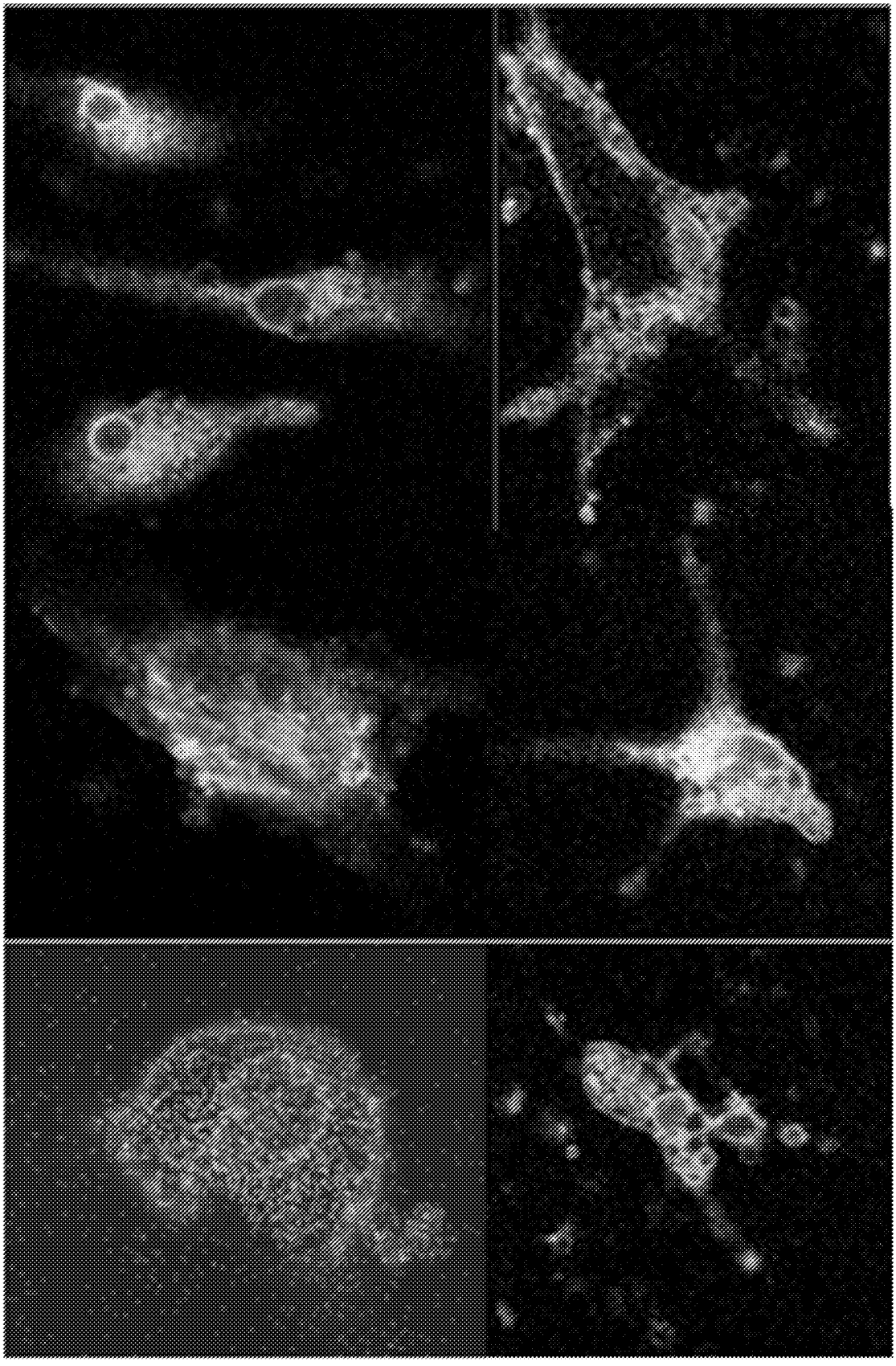


Figure 16

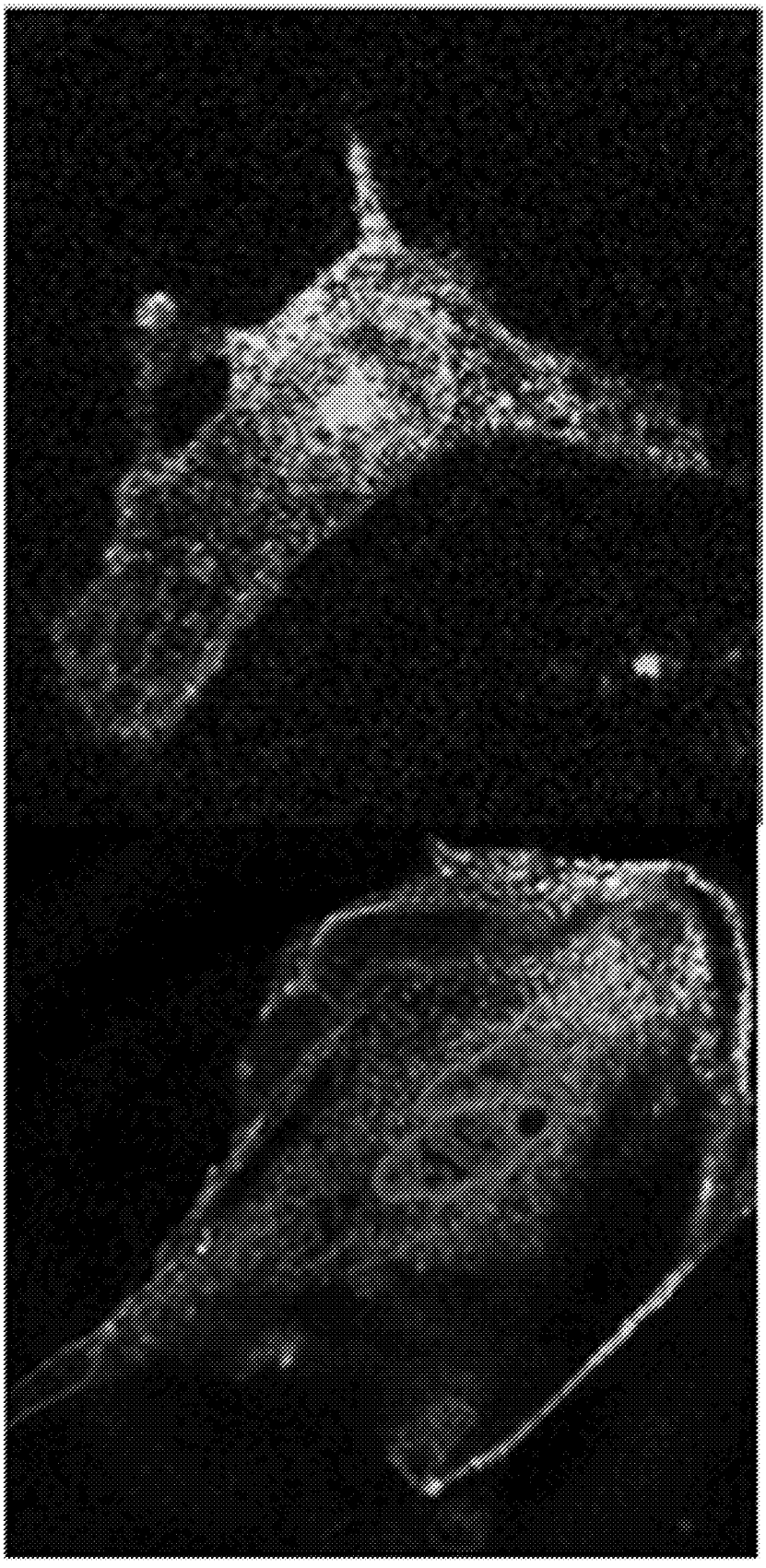


Figure 17

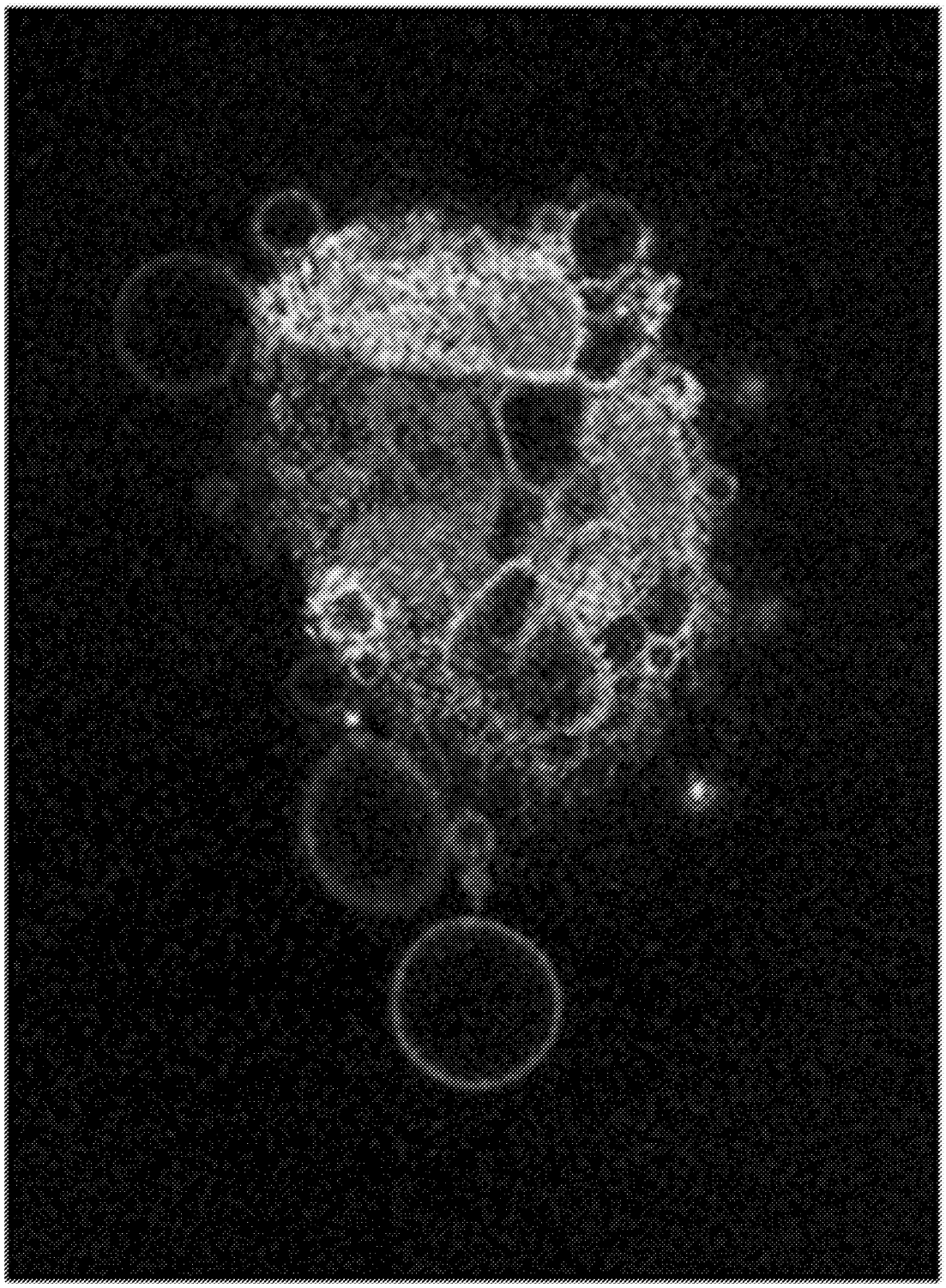


Figure 18

Figure 19

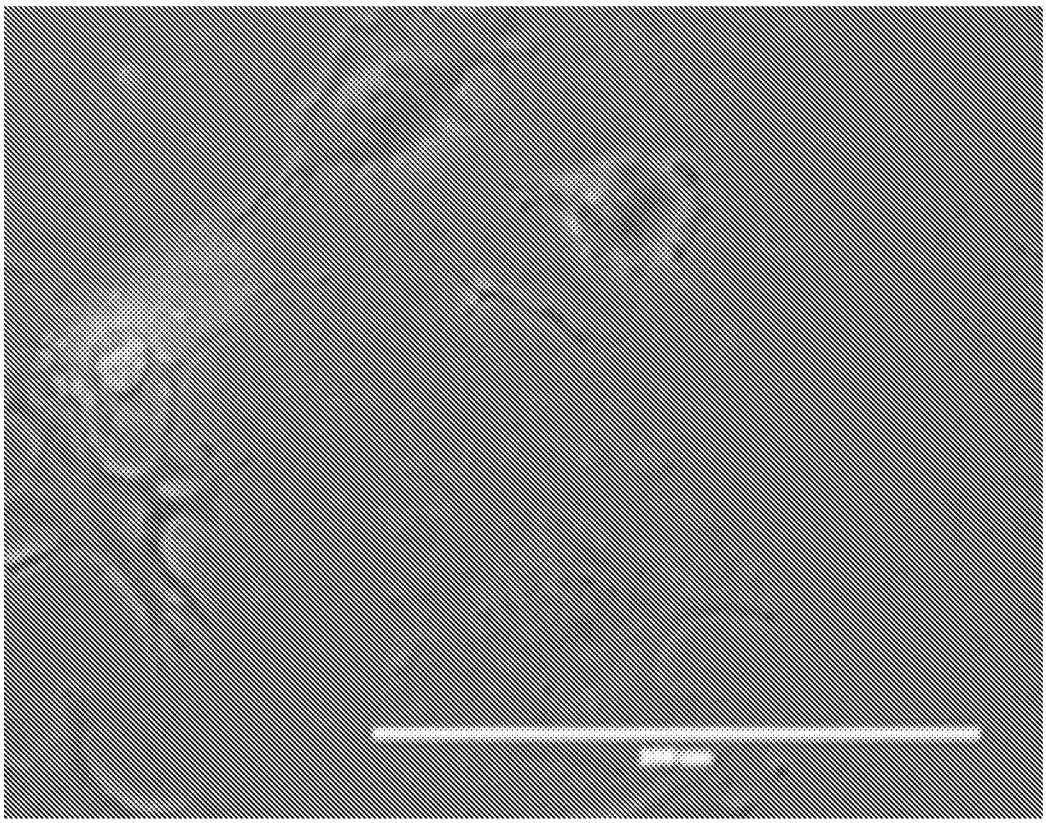
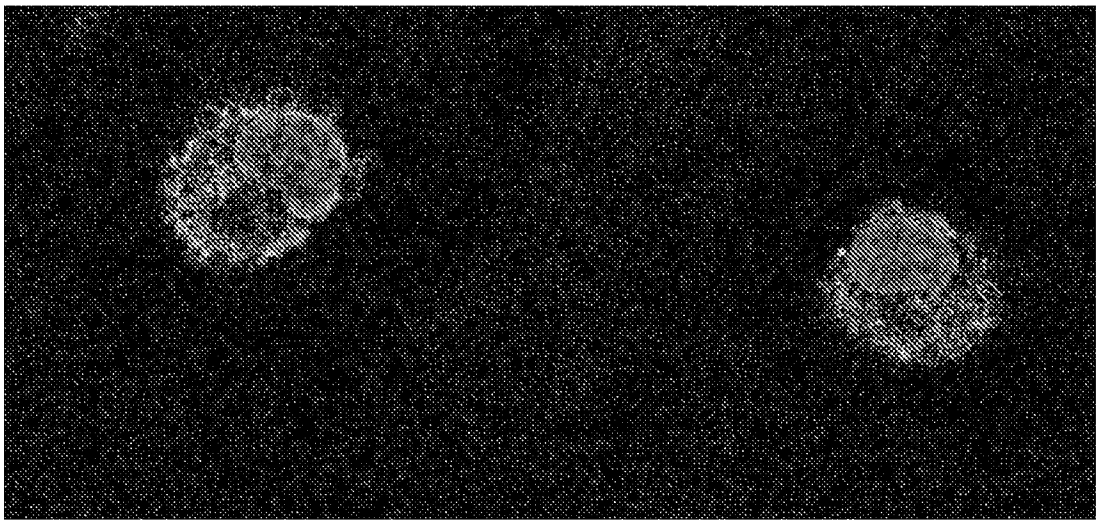
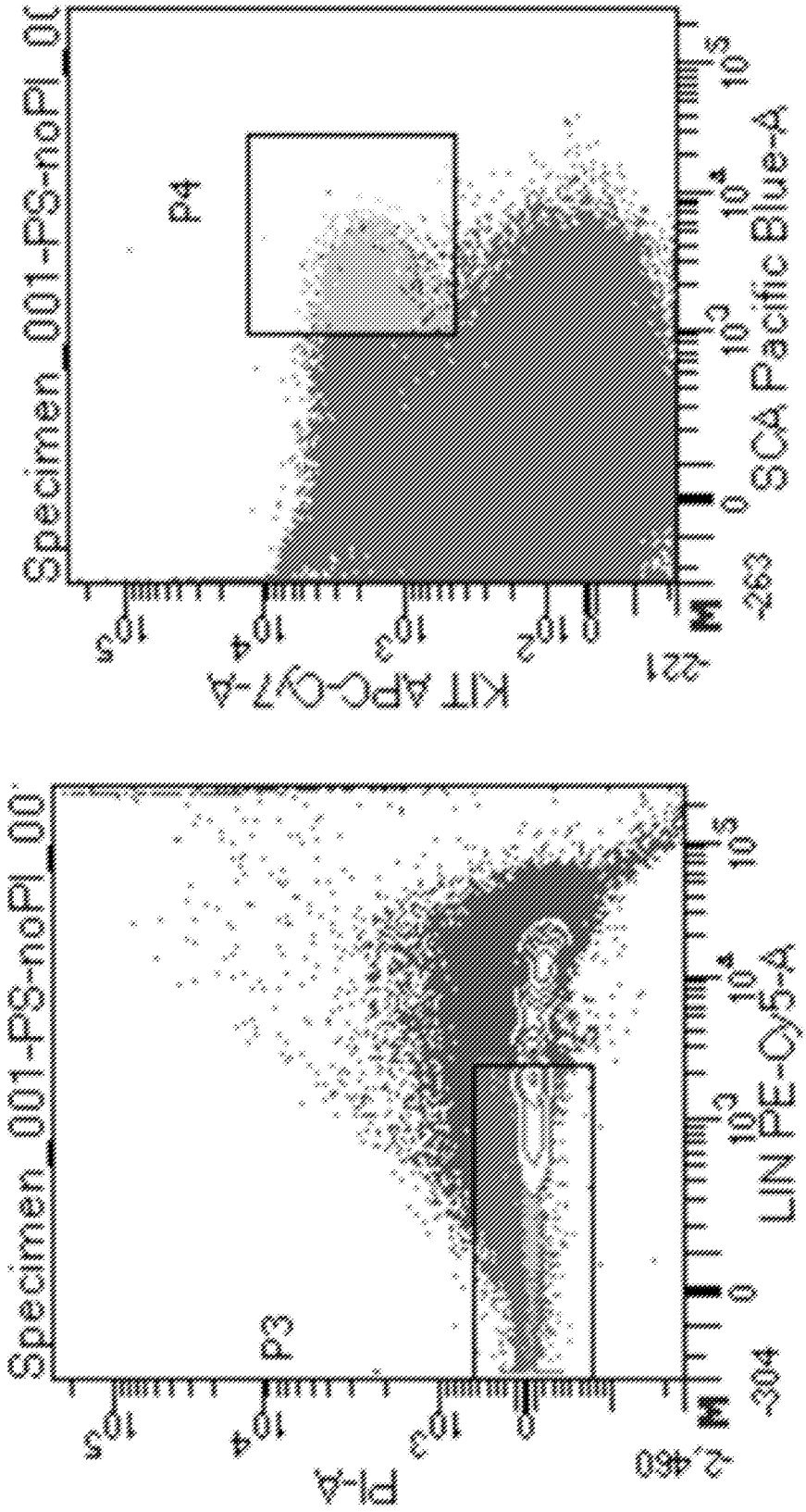


Figure 20

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P4
KIT APC-Cy7-A
SCA Pacific Blue-A

Figure 21

Figure 22

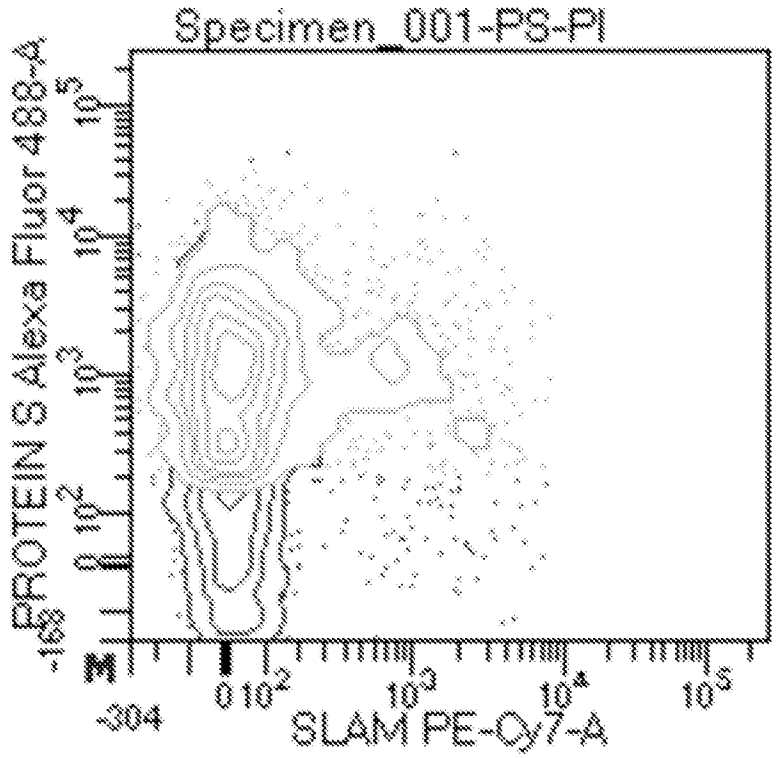


Figure 23

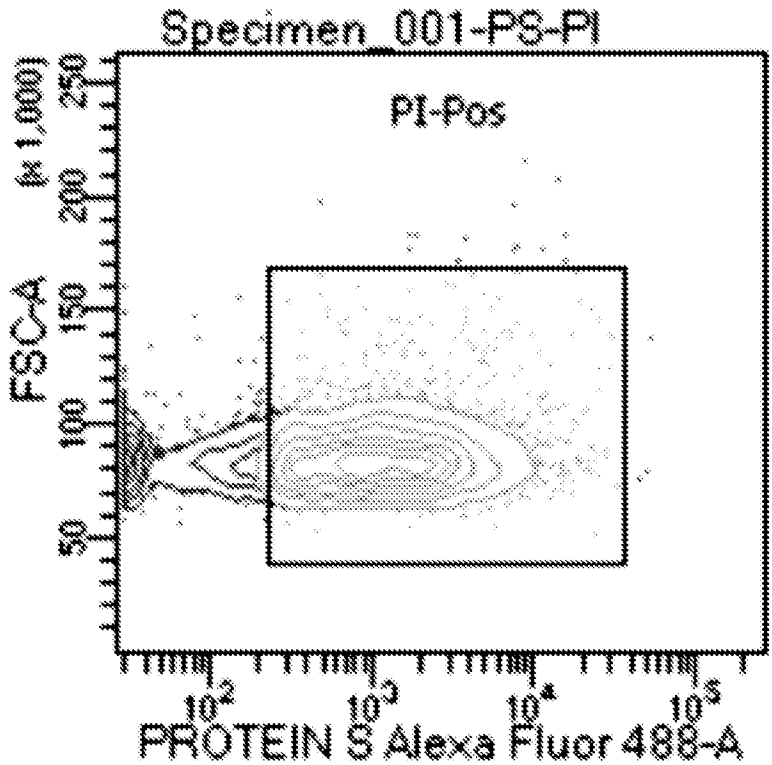


Figure 24

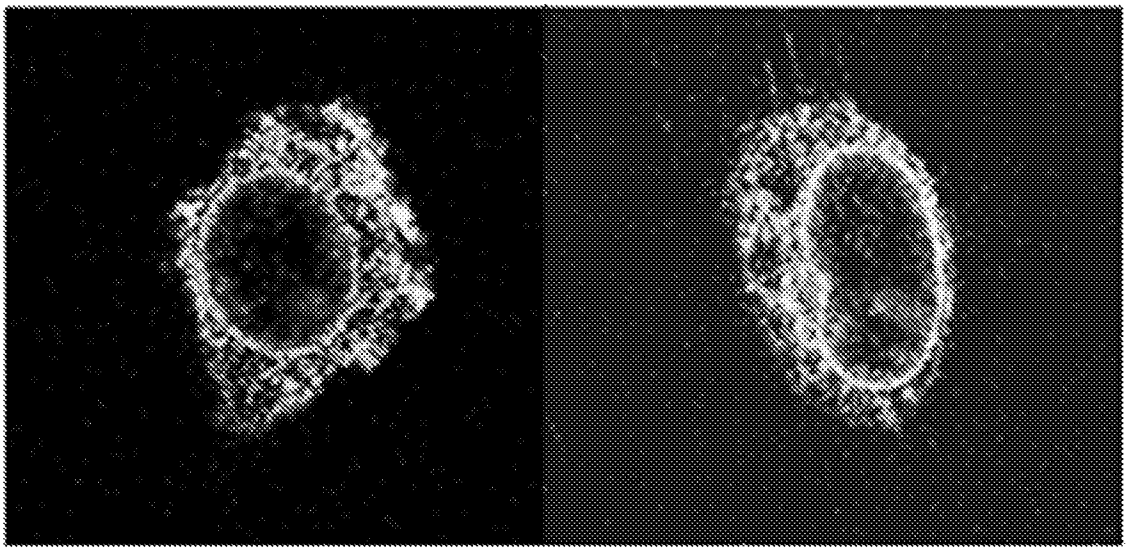
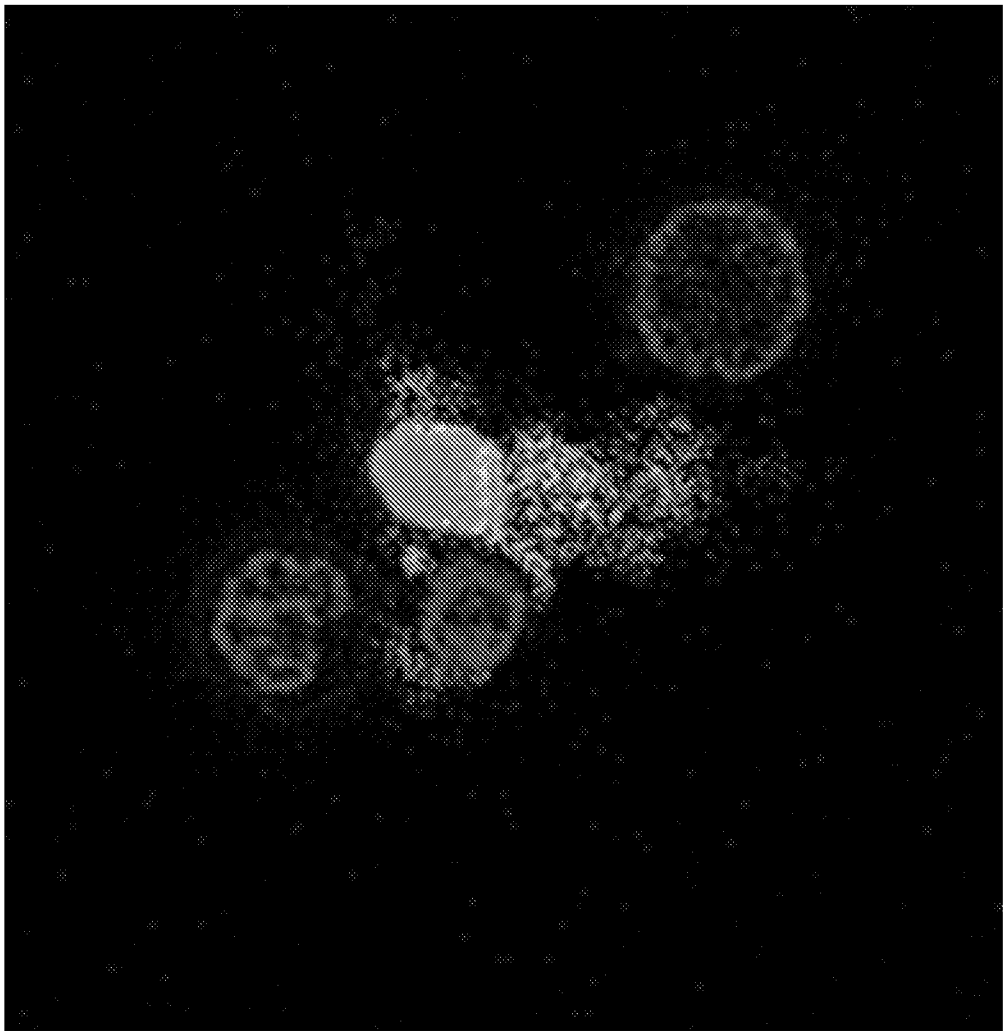


Figure 25



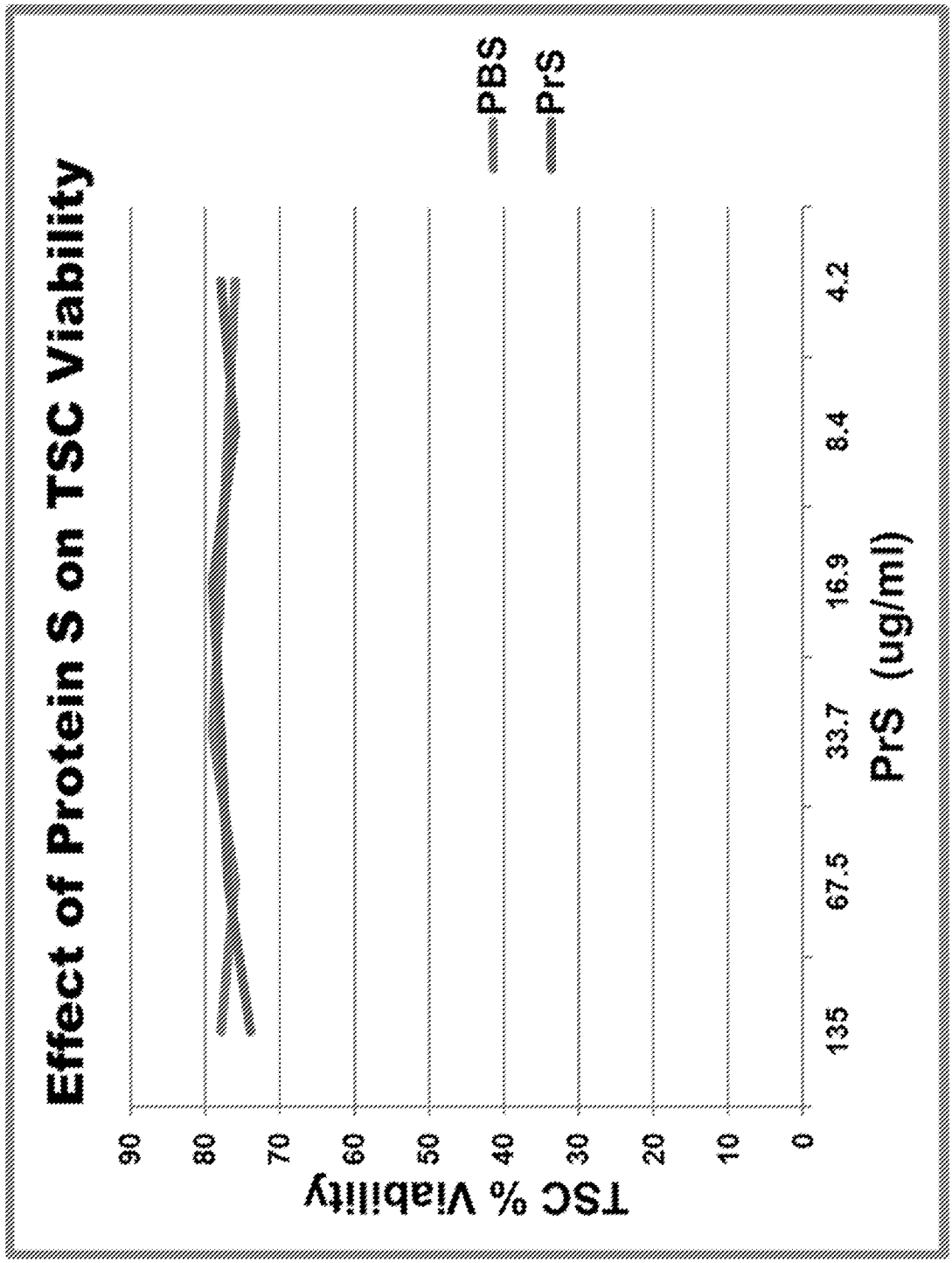

Effect of Protein S on TSC Viability
PBS
PrS
TSC % Viability
90
80
70
60
50
40
30
20
10
0
135
67.5
33.7
16.9
8.4
4.2
PrS (ug/ml)

Figure 26

INTERNATIONAL SEARCH REPORT

International application No
PCT/US2018/049619

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N5/09 C12N9/74 C12N9/64 C07K14/755 C07K19/00
C12N5/071 C12N5/0775 C12N5/0789 C12N5/073 C07K14/745
A61K38/36

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)
EPO-Internal, WPI Data, BIOSIS, Sequence Search

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2014/151535 A1 (BAYER HEALTHCARE LLC [US]) 25 September 2014 (2014-09-25) cited in the application page 3; claims 1-9; example 1; sequence 6	1-33
X	WO 2012/087241 A1 (AGENCY SCIENCE TECH & RES [SG]; LIM SAI KIANG [SG]) 28 June 2012 (2012-06-28) page 2	33

-/--

[X] Further documents are listed in the continuation of Box C.

[X] 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	Date of mailing of the international search report
21 November 2018	06/12/2018

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Paresce, Donata

2

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WATARU NAKAI ET AL: "A novel affinity-based method for the isolation of highly purified extracellular vesicles", SCIENTIFIC REPORTS, vol. 6, no. 1, 23 September 2016 (2016-09-23), XP055519274, DOI: 10.1038/srep33935	33
A	page 1 - page 2; figure 1	1-32
X	ANIRBAN GHOSH ET AL: "Rapid Isolation of Extracellular Vesicles from Cell Culture and Biological Fluids Using a Synthetic Peptide with Specific Affinity for Heat Shock Proteins", PLOS ONE, vol. 9, no. 10, 17 October 2014 (2014-10-17), page e110443, XP055286213, DOI: 10.1371/journal.pone.0110443	33
A	page 1 - page 4; figure 2	1-32
A	KRISTOF SCHUTTERS ET AL: "Phosphatidylserine targeting for diagnosis and treatment of human diseases", APOPTOSIS ; AN INTERNATIONAL JOURNAL ON PROGRAMMED CELL DEATH, KLUWER ACADEMIC PUBLISHERS, BO, vol. 15, no. 9, 4 May 2010 (2010-05-04), pages 1072-1082, XP019812780, ISSN: 1573-675X page 1072 page 1076; table 1	1-33
A	US 2011/159571 A1 (BARRY MICHAEL A [US] ET AL) 30 June 2011 (2011-06-30) page 1 - page 2; example 1	1-33
A	WO 2017/118764 A1 (BROCKER THOMAS [DE]; KRANICH JAN [DE]) 13 July 2017 (2017-07-13) claims 1-4	1-33
A	LEMKE GREG: "Phosphatidylserine Is the Signal for TAM Receptors and Their Ligands", TRENDS IN BIOCHEMICAL SCIENCES, ELSEVIER, AMSTERDAM, NL, vol. 42, no. 9, 19 July 2017 (2017-07-19), pages 738-748, XP085195731, ISSN: 0968-0004, DOI: 10.1016/J.TIBS.2017.06.004 page 738 - page 740	1-33

-/--

2

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MINGDONG HUANG ET AL: "Structural basis of membrane binding by Gla domains of vitamin K-dependent proteins", NATURE STRUCTURAL BIOLOGY, vol. 10, no. 9, 1 September 2003 (2003-09-01), pages 751-756, XP055092113, ISSN: 1072-8368, DOI: 10.1038/nsb971 the whole document	1-33
A	G. T. TIETJEN ET AL: "Molecular mechanism for differential recognition of membrane phosphatidylserine by the immune regulatory receptor Tim4", PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, vol. 111, no. 15, 31 March 2014 (2014-03-31), pages E1463-E1472, XP055524812, US ISSN: 0027-8424, DOI: 10.1073/pnas.1320174111 the whole document	1-33

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/US2018/049619

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2014151535 A1	25-09-2014	AU 2014233885 A1	17-09-2015
		AU 2014236990 A1	10-09-2015
		CA 2905972 A1	25-09-2014
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		EP 2970442 A1	20-01-2016
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		HK 1216649 A1	25-11-2016
		JP 2016513463 A	16-05-2016
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		SG 11201506307X A	29-09-2015
		SG 11201506880W A	29-09-2015
		US 2016008482 A1	14-01-2016
		US 2016106806 A1	21-04-2016
		US 2017296624 A1	19-10-2017
		WO 2014151535 A1	25-09-2014
		WO 2014151683 A1	25-09-2014
		ZA 201507670 B	29-11-2017
		ZA 201507674 B	30-08-2017
WO 2012087241 A1	28-06-2012	SG 190450 A1	28-06-2013
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		WO 2010006136 A2	14-01-2010
WO 2017118764 A1	13-07-2017	NONE	