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- (71) Applicant: **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA** [US/US]; 1111 Franklin Street, 12th Floor, Oakland, California 94607-5200 (US).
- (72) Inventors: **CHEN, Cheng-Han**; 66 Rossmore, Irvine, California 92620 (US). **WU, Benjamin M.**; 2740 Lorain Road, San Marino, California 91108 (US). **ARDEHALI, Reza**; 685 Thayer Avenue, Los Angeles, California 90024 (US). **NGUYEN, Ngoc Bao**; 11953 Rochester Avenue, Unit 8, Los Angeles, California 90025 (US).
- (74) Agent: **HUNTER, Tom** et al.; Weaver Austin Villeneuve & Sampson LLP, P.O. Box 70250, Oakland, California 94612-0250 (US).
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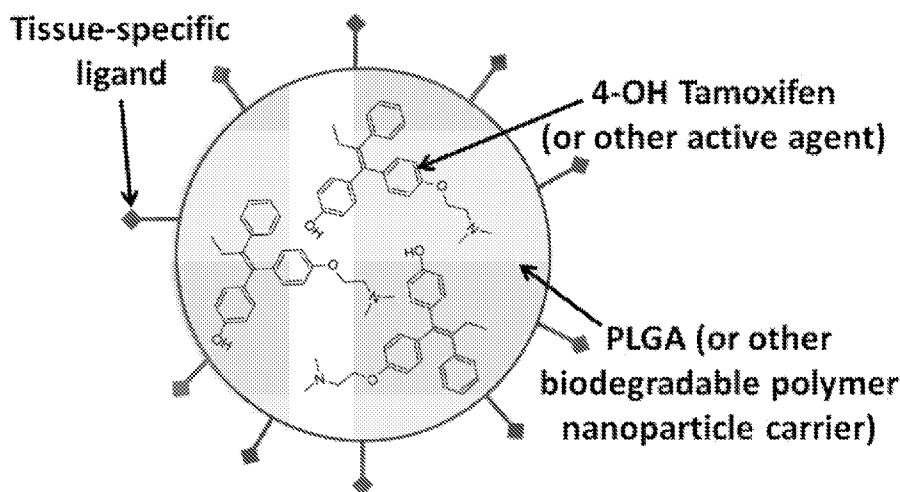


Fig. 1A

(57) Abstract: In certain embodiments METHODS for conditional and target-specific recombination are provided where the methods comprise: providing a mammal comprising cells that express an ER-ligand-inducible CRE recombinase under the control of a promoter, and that comprises a nucleic acid sequence flanked by a pair of loxP sequences; and administering to the mammal biodegradable polymer nanoparticles containing an estrogen receptor ligand where the nanoparticles provide specific delivery to a target tissue of the estrogen receptor ligand thereby activating said CRE recombinase which performs target specific recombination of the nucleic acid between the loxP sequences in said target tissue.

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METHOD FOR TEMPORAL AND TISSUE-SPECIFIC DRUG DELIVERY AND INDUCED NUCLEIC ACID RECOMBINATION

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims benefit of and priority to USSN 62/443,604, filed on
5 January 6, 2017, which is incorporated herein by reference in its entirety for all purposes.

STATEMENT OF GOVERNMENTAL SUPPORT

[0002] This invention was made with Government support under Grant Number
HL007895, awarded by the National Institutes of Health. The Government has certain rights
in the invention.

BACKGROUND

10 [0003] Site-specific nucleic acid recombination has been established as an important
research technique in modern molecular biology, especially to discover gene function in
normal and disease states. Current methods utilize recombinase technology, such as the Cre-
loxP system, to effect gene expression, deletion, inversion, or translocation, both *in vitro* and
15 *in vivo* (most commonly in mice). Other applications have included fate mapping and cell
ablation *in vivo*. Advances to the process have produced systems for conditional genetic
modification allowing for both temporal and cell-specific control of the gene modification.
For instance, the engineered CreER recombinase (consisting of fused Cre and mutated
ligand-binding domain of the estrogen receptor) has allowed for temporal control of Cre
20 activity; nucleic acid recombination is activated through systemic administration of
tamoxifen (or its active metabolite 4-hydroxy-tamoxifen) at a desired timepoint, which then
binds to the CreER recombinase (which is non-functional until 4-hydroxy-tamoxifen binds to
it). Cell-type specificity can be achieved by linking control of expression of the Cre or
CreER recombinase to a tissue-specific promoter. However, the generation of a mouse strain
25 linking a tissue-specific promoter to recombinase expression is an expensive and time-
consuming process. The ability to control cell- and tissue-specificity of recombinase
expression (and thus nucleic acid recombination) through an alternate means has to potential
to provide significant improvements to the cost and speed of biomedical research.

SUMMARY

[0004] Various embodiments contemplated herein may include, but need not be limited to, one or more of the following:

[0005] Embodiment 1: A method for conditional and target-specific recombination, said method comprising:

[0006] providing a mammal comprising cells that express an ER-ligand-inducible CRE recombinase under the control of a promoter, and that comprises a nucleic acid sequence flanked by a pair of *loxP* sequences; and

[0007] administering to said mammal biodegradable polymer nanoparticles containing an estrogen receptor ligand where said nanoparticles provide specific delivery to a target tissue of said estrogen receptor ligand thereby activating said CRE recombinase in said target tissue which performs target specific recombination of the nucleic acid between the *loxP* sequences in said target tissue.

[0008] Embodiment 2: The method of embodiment 1, wherein said mammal comprises cells that express an ER-ligand-inducible CRE recombinase under the control of a non-tissue specific promoter.

[0009] Embodiment 3: The method according to any one of embodiments 1-2, wherein said *loxP* sequences are inverted in orientation with respect to each other.

[0010] Embodiment 4: The method according to any one of embodiments 1-2, wherein said *loxP* sequences are in the same orientation with respect to each other.

[0011] Embodiment 5: The method according to any one of embodiments 1-4, wherein said nanoparticles comprise a polymer selected from the group consisting of Poly-D,L-lactide-co-glycolide (PLGA), Poly(L-lactic acid) (PLA), Poly-ε-caprolactone (PCL), chitosan, and gelatin.

[0012] Embodiment 6: The method of embodiment 5, wherein said nanoparticles comprise Poly-D,L-lactide-co-glycolide (PLGA).

[0013] Embodiment 7: The method of embodiment 6, wherein said nanoparticles comprise Poly-D,L-lactide-co-glycolide (PLGA) lactide:glycolide 50:50 (50:50 PLGA).

[0014] Embodiment 8: The method of embodiment 6, wherein said nanoparticles comprise Poly-D,L-lactide-co-glycolide (PLGA) lactide:glycolide 85:15 (85:15 PLGA).

[0015] Embodiment 9: The method of embodiment 5, wherein said nanoparticles comprise poly(L-lactic acid).

[0016] Embodiment 10: The method according to any one of embodiments 1-9, wherein said nanoparticles range in size from about 50 nm, or from about 100 nm, or from about 150 nm, up to about 500 nm, or up to about 400 nm, or up to about 300 nm, or up to about 200 nm.

[0017] Embodiment 11: The method of embodiment 10, wherein said nanoparticles range in size from about 150 nm up to about 200 nm.

[0018] Embodiment 12: The method according to any one of embodiments 1-11, wherein the zeta potential of said nanoparticles is at least about -10 mV, or at least about -15 mV, or at least about -20 mV, or at least about 24 mV.

[0019] Embodiment 13: The method according to any one of embodiments 1-12, wherein said estrogen receptor ligand is selected from the group consisting of tamoxifen, 4-hydroxy-tamoxifen, and a tamoxifen analogue.

[0020] Embodiment 14: The method of embodiment 13, wherein said estrogen receptor ligand is ICI 182,780.

[0021] Embodiment 15: The method according to any one of embodiments 1-14, wherein tissue specificity of said nanoparticles is by preferential passive accumulation in a target tissue.

[0022] Embodiment 16: The method of embodiment 15, wherein tissue specificity of said nanoparticles is by preferential passive accumulation in myocardium with damaged endothelium.

[0023] Embodiment 17: The method according to any one of embodiments 1-14, wherein tissue specificity of said nanoparticles is provided by tissue-specific binding moieties disposed on the surface of said nanoparticles.

[0024] Embodiment 18: The method of embodiment 17, wherein said tissue-specific binding moieties comprise targeting peptides.

[0025] Embodiment 19: The method of embodiment 18, wherein said targeting peptides comprise a peptide having an amino acid sequence selected from the group consisting of erythropoietin sequence, AGTFALRGDNPQG (SEQ ID NO:13), CDCRGDCFC (SEQ ID NO:14), NGRAHA (SEQ ID NO:15), SIGYPLP (SEQ ID NO:16),

MTPFPTSNEANLGGGS (SEQ ID NO:17), EYHHYNK (SEQ ID NO:18), CNHRYMQMC (SEQ ID NO:19), QPEHSST (SEQ ID NO:20), VNTANST (SEQ ID NO:21), ASSLNIA (SEQ ID NO:22), RSNVVP (SEQ ID NO:23), NRTWEQQ (SEQ ID NO:24), NQVGWS (SEQ ID NO:25), EARVRPP (SEQ ID NO:26), NSSRDLG (SEQ ID NO:27), NDVRAVS (SEQ ID NO:28), NDVRSAN (SEQ ID NO:29), VTAGRAP (SEQ ID NO:30), DLSNLTR (SEQ ID NO:31), RGDAVG (SEQ ID NO:32), RGDGLS (SEQ ID NO:33), PRSVTV (SEQ ID NO:34), DLGSARA (SEQ ID NO:35), ESGLSQS (SEQ ID NO:36), PRSTSDP (SEQ ID NO:37), NSSRSLG (SEQ ID NO:38), and MVNNFEW (SEQ ID NO:39).

[0026] Embodiment 20: The method of embodiment 17, wherein said tissue-specific binding moieties comprise antibodies.

[0027] Embodiment 21: The method of embodiment 20, wherein said tissue-specific binding moiety comprises an antibody that specifically binds a target selected from the group consisting of CD3, CD31, CD11b, CD45, CD3, erbB2, Her2, CD22, CD74, CD19, CD20, CD33, CD40, MUC1, IL-15R, HLA-DR, EGP-1, EGP-2, G250, prostate specific membrane antigen (PSMA), prostate specific antigen (PSA), prostatic acid phosphatase (PAP), and placental alkaline phosphatase.

[0028] Embodiment 22: The method of embodiment 21, wherein said tissue-specific binding moiety comprises an anti-CD3 antibody.

[0029] Embodiment 23: The method of embodiment 21, wherein said tissue-specific binding moiety comprises an anti-CD11b antibody.

[0030] Embodiment 24: The method of embodiment 21, wherein said tissue-specific binding moiety comprises an anti-CD31 antibody.

[0031] Embodiment 25: The method of embodiment 20, wherein said tissue-specific binding moiety comprises an antibody that specifically binds a target that is a cancer marker selected from the cancer markers shown in Table 3.

[0032] Embodiment 26: The method according to any one of embodiments 20-25, wherein said antibody is a full-length immunoglobulin.

[0033] Embodiment 27: The method according to any one of embodiments 20-25, wherein said antibody is selected from the group consisting of Fu, Fab, (Fab')₂, (Fab')₃, IgGACH2, a unibody, and a minibody.

[0034] Embodiment 28: The method according to any one of embodiments 20-25, wherein said antibody is a single chain antibody.

[0035] Embodiment 29: The method of embodiment 28 wherein said antibody is an scFv.

5 **[0036]** Embodiment 30: The method according to any one of embodiments 20-29, wherein said antibody is a human antibody.

[0037] Embodiment 31: The method according to any one of embodiments 17-30, wherein said tissue-specific binding moiety is adsorbed onto the surface of said nanoparticle.

10 **[0038]** Embodiment 32: The method according to any one of embodiments 17-30, wherein said tissue-specific binding moiety is attached to said nanoparticle by a non-covalent interaction.

[0039] Embodiment 33: The method of embodiment 32, wherein said non-covalent interaction comprises a biotin/avidin interaction.

15 **[0040]** Embodiment 34: The method of embodiment 32, wherein said tissue-specific binding moiety is an antibody and said non-covalent interaction comprises an interaction between an antibody-binding peptide and said antibody.

20 **[0041]** Embodiment 35: The method of embodiments 34, wherein said non-covalent interaction comprises an interaction between said antibody and a protein selected from the group consisting of Protein A, Protein G, Protein L, Protein Z, Protein LG, Protein LA, and Protein AG.

25 **[0042]** Embodiment 36: The method of embodiment 34, wherein said non-covalent interaction comprises an interaction between said an antibody and a moiety selected from the group consisting of PAM, D-PAM, D-PAM-θ, TWKTSRISIF (SEQ ID NO:40), FGRLVSSIRY (SEQ ID NO:41, Fc-III, EPIHRSTLTALL (SEQ ID NO:42), HWRGWV (SEQ ID NO:43), HYFKFD (SEQ ID NO:44), HFRRHL (SEQ ID NO:45), NKFRGKYK (SEQ ID NO:46), NARKFYKG (SEQ ID NO:47), KHRFNKD (SEQ ID NO:48).

[0043] Embodiment 37: The method of embodiment 34, wherein said non-covalent interaction comprises an interaction between said tissue-specific binding moiety and FcB6.1 peptide.

30 **[0044]** Embodiment 38: The method according to any one of embodiments 17-30, wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle.

[0045] Embodiment 39: The method of embodiment 38, wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle via a non-cleavable linker.

[0046] Embodiment 40: The method of embodiment 39 wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle via carbodiimide chemistry
5 utilizing EDC and sulfo-NHS.

[0047] Embodiment 41: The method of embodiment 38, wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle via a cleavable linker.

[0048] Embodiment 42: The method of embodiment 38, wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle via a cleavable linker
10 comprising a disulfide linker or an acid-labile linker.

[0049] Embodiment 43: The method of embodiment 38, wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle via an acid label linker comprising a moiety selected from the group consisting of a hydrazone, an acetal, a cis-
aconitate-like amide, a silyl ether.

[0050] Embodiment 44: The method of embodiment 38, wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle via a non-amino acid, non-peptide linker shown in Table 5.

[0051] Embodiment 45: The method according to any one of embodiments 1-44, wherein said mammal comprises cells that express an ER-ligand-inducible CRE recombinase
20 under the control of a constitutive promoter.

[0052] Embodiment 46: The method according to any one of embodiments 1-44, wherein said mammal comprises cells that express an ER-ligand-inducible CRE recombinase under the control of an inducible promoter.

[0053] Embodiment 47: The method according to any one of embodiments 1-44, wherein said mammal comprises cells that express an ER-ligand-inducible CRE recombinase
25 under the control of a CMV, Rosa26, or β -actin promoter.

[0054] Embodiment 48: The method according to any one of embodiments 1-47, wherein said inducible Cre recombinase comprises CreER^{T2}.

[0055] Embodiment 49: The method according to any one of embodiments 1-47, wherein said inducible Cre recombinase comprises an iCre.
30

- [0056] Embodiment 50: The method according to any one of embodiments 1-49, wherein said *loxP* sites comprise at least 2 pairs of *loxP* sites.
- [0057] Embodiment 51: The method according to any one of embodiments 1-49, wherein said *loxP* sites comprise at least 3 pairs of *loxP* sites.
- 5 [0058] Embodiment 52: The method according to any one of embodiments 1-51, wherein said *loxP* site(s) comprise a wild-type *loxP* site.
- [0059] Embodiment 53: The method according to any one of embodiments 1-52, wherein said *loxP* site(s) comprise a *loxP* variant selected from the group consisting of lox 511, lox 5171, lox 2272, M2, M3, M7, M11, lox 71, and lox 66.
- 10 [0060] Embodiment 54: The method according to any one of embodiments 1-53, wherein said nucleic acid flanked by said pair of *loxP* sequences comprises a gene or a portion of a gene and said recombination inhibits or blocks expression of a functional product from said gene.
- [0061] Embodiment 55: The method according to any one of embodiments 1-53, wherein said nucleic acid flanked by said pair of *loxP* sequences comprises a transcriptional stop element where said stop element is operably linked to a gene and said recombination removes said stop element allowing expression said gene.
- 15 [0062] Embodiment 56: The method of embodiment 55, wherein said gene is an oncogene.
- 20 [0063] Embodiment 57: The method of embodiment 56, wherein said gene is *K-ras*.
- [0064] Embodiment 58: The method according to any one of embodiments 1-57, wherein said mammal is a non-human mammal.
- [0065] Embodiment 59: The method of embodiment 58, wherein said mammal is a rat or mouse.
- 25 [0066] Embodiment 60: A transgenic murine, said murine comprising cells that express an ER-ligand-inducible CRE recombinase under the control of a non-tissue specific promoter.
- [0067] Embodiment 61: The transgenic murine of embodiment 60, wherein said promoter is a constitutive promoter.

[0068] Embodiment 62: The transgenic murine of embodiment 60, wherein said promoter is an inducible promoter.

[0069] Embodiment 63: The transgenic murine of embodiment 60, wherein said promoter is a CMV, Rosa26, or β -actin promoter.

5 [0070] Embodiment 64: The transgenic murine according to any one of embodiments 60-63, wherein said inducible Cre recombinase comprises CreER^{T2}.

[0071] Embodiment 65: The transgenic murine according to any one of embodiments 60-63, wherein said inducible Cre recombinase comprises an iCre.

10 [0072] Embodiment 66: The transgenic murine according to any one of embodiments 60-65, wherein said murine comprises a nucleic acid sequence flanked by a pair of *loxP* sequences.

[0073] Embodiment 67: The transgenic murine of embodiment 66, wherein said *loxP* sites comprise at least 2 pairs of *loxP* sites.

15 [0074] Embodiment 68: The transgenic murine of embodiment 66, wherein said *loxP* sites comprise at least 3 pairs of *loxP* sites.

[0075] Embodiment 69: The transgenic murine according to any one of embodiments 66-68, wherein said *loxP* site(s) comprise a wild-type *loxP* site.

20 [0076] Embodiment 70: The transgenic murine according to any one of embodiments 66-69, wherein said *loxP* site(s) comprise a *loxP* variant selected from the group consisting of *lox* 511, *lox* 5171, *lox* 2272, M2, M3, M7, M11, *lox* 71, and *lox* 66.

[0077] Embodiment 71: The transgenic murine according to any one of embodiments 66-70, wherein said nucleic acid flanked by said pair of *loxP* sequences comprises a gene or a portion of a gene and said recombination inhibits or blocks expression of a functional product from said gene.

25 [0078] Embodiment 72: The transgenic murine according to any one of embodiments 66-71, wherein said nucleic acid flanked by said pair of *loxP* sequences comprises a transcriptional stop element where said stop element is operably linked to a gene and said recombination removes said stop element allowing expression said gene.

30 [0079] Embodiment 73: The transgenic murine of embodiment 72, wherein said gene is an oncogene.

[0080] Embodiment 74: The transgenic murine of embodiment 73, wherein said gene is *K-ras*.

[0081] *Embodiment 75:* A nanoparticle for cell- or tissue-specific or preferential delivery of a pharmaceutical, said nanoparticle comprising a biodegradable polymer
5 containing said pharmaceutical.

[0082] Embodiment 76: The nanoparticle of embodiment 75, wherein said nanoparticle comprises a polymer selected from the group consisting of Poly-D,L-lactide-co-glycolide (PLGA), Poly(L-lactic acid) (PLA), Poly-ε-caprolactone (PCL), chitosan, and gelatin.

10 [0083] Embodiment 77: The nanoparticle of embodiment 76, wherein said nanoparticles comprise Poly-D,L-lactide-co-glycolide (PLGA).

[0084] Embodiment 78: The nanoparticle of embodiment 77, wherein said nanoparticles comprise Poly-D,L-lactide-co-glycolide (PLGA) lactide:glycolide 50:50 (50:50 PLGA).

15 [0085] Embodiment 79: The nanoparticle of embodiment 77, wherein said nanoparticles comprise Poly-D,L-lactide-co-glycolide (PLGA) lactide:glycolide 85:15 (85:15 PLGA).

[0086] Embodiment 80: The nanoparticle of embodiment 76, wherein said nanoparticles comprise poly(L-lactic acid).

20 [0087] Embodiment 81: The nanoparticle according to any one of embodiments 75-80, wherein said nanoparticle ranges in size from about 50 nm, or from about 100 nm, or from about 150 nm, up to about 500 nm, or up to about 400 nm, or up to about 300 nm, or up to about 200 nm.

[0088] Embodiment 82: The nanoparticle of embodiment 81, wherein said
25 nanoparticle ranges in size from about 150 nm up to about 200 nm.

[0089] Embodiment 83: The nanoparticle according to any one of embodiments 75-82, wherein the zeta potential of said nanoparticle is at least about -10 mV, or at least about -15 mV, or at least about -20 mV, or at least about 24 mV.

[0090] Embodiment 84: The nanoparticle according to any one of embodiments 75-
30 83, wherein tissue specificity of said nanoparticles is by preferential passive accumulation in a target tissue.

[0091] Embodiment 85: The nanoparticle of embodiment 84, wherein tissue specificity of said nanoparticles is by preferential passive accumulation in myocardium with damaged endothelium.

[0092] Embodiment 86: The nanoparticle according to any one of embodiments 75-83, wherein tissue specificity of said nanoparticles is provided by tissue-specific binding moieties disposed on the surface of said nanoparticles.

[0093] Embodiment 87: The nanoparticle of embodiment 86, wherein said tissue-specific binding moieties comprise targeting peptides.

[0094] Embodiment 88: The nanoparticle of embodiment 87, wherein said targeting peptides comprise a peptide having an amino acid sequence selected from the group consisting of erythropoietin sequence, AGTFALRGDNPQG (SEQ ID NO:13), CDCRGDCFC (SEQ ID NO:14), NGRAHA (SEQ ID NO:15), SIGYPLP (SEQ ID NO:16), MTPFPTSNEANLGGGS (SEQ ID NO:17), EYHHYNK (SEQ ID NO:18), CNHRYMQMC (SEQ ID NO:19), QPEHSST (SEQ ID NO:20), VNTANST (SEQ ID NO:21), ASSLNIA (SEQ ID NO:22), RSNVVP (SEQ ID NO:23), NRTWEQQ (SEQ ID NO:24), NQVGWS (SEQ ID NO:25), EARVRPP (SEQ ID NO:26), NSSRDLG (SEQ ID NO:27), NDVRAVS (SEQ ID NO:28), NDVRSAN (SEQ ID NO:29), VTAGRAP (SEQ ID NO:30), DLSNLTR (SEQ ID NO:31), RGDAVG (SEQ ID NO:32), RGDGLS (SEQ ID NO:33), PRSVTV (SEQ ID NO:34), DLGSARA (SEQ ID NO:35), ESGLSQS (SEQ ID NO:36), PRSTSDP (SEQ ID NO:37), NSSRSLG (SEQ ID NO:38), and MVNNFEW (SEQ ID NO:39).

[0095] Embodiment 89: The nanoparticle of embodiment 86, wherein said tissue-specific binding moieties comprise antibodies.

[0096] Embodiment 90: The nanoparticle of embodiment 89, wherein said tissue-specific binding moiety comprises an antibody that specifically binds a target selected from the group consisting of CD3, CD11b, CD31, CD45, CD3, erbB2, Her2, CD22, CD74, CD19, CD20, CD33, CD40, MUC1, IL-15R, HLA-DR, EGP-1, EGP-2, G250, prostate specific membrane antigen (PSMA), prostate specific antigen (PSA), prostatic acid phosphatase (PAP), and placental alkaline phosphatase.

[0097] Embodiment 91: The nanoparticle of embodiment 90, wherein said tissue-specific binding moiety comprises an anti-CD3 antibody.

[0098] Embodiment 92: The nanoparticle of embodiment 90, wherein said tissue-specific binding moiety comprises an anti-CD11b antibody.

[0099] Embodiment 93: The nanoparticle of embodiment 90, wherein said tissue-specific binding moiety comprises an anti-CD31 antibody.

[0100] Embodiment 94: The nanoparticle of embodiment 89, wherein said tissue-specific binding moiety comprises an antibody that specifically binds a target that is a cancer marker selected from the cancer markers shown in Table 3.

[0101] Embodiment 95: The nanoparticle according to any one of embodiments 89-94, wherein said antibody is a full-length immunoglobulin.

[0102] Embodiment 96: The nanoparticle according to any one of embodiments 89-94, wherein said antibody is selected from the group consisting of Fv, Fab, (Fab')₂, (Fab')₃, IgGΔCH2, a unibody, and a minibody.

[0103] Embodiment 97: The nanoparticle according to any one of embodiments 89-94, wherein said antibody is a single chain antibody.

[0104] Embodiment 98: The nanoparticle of embodiment 97, wherein said antibody is an scFv.

[0105] Embodiment 99: The nanoparticle according to any one of embodiments 89-98, wherein said antibody is a human antibody.

[0106] Embodiment 100: The nanoparticle according to any one of embodiments 75-99, wherein pharmaceutical comprise an estrogen receptor ligand.

[0107] Embodiment 101: The nanoparticle of embodiment 100, wherein said pharmaceutical comprises an estrogen receptor ligand selected from the group consisting of tamoxifen, 4-hydroxy-tamoxifen, and a tamoxifen analogue.

[0108] Embodiment 102: The nanoparticle of embodiment 101, wherein said estrogen receptor ligand tamoxifen analog is ICI 182,780.

[0109] Embodiment 103: A method of delivering a pharmaceutical to a region of injury *in vivo*, said method comprising:

[0110] administering to a subject in need thereof a nanoparticle comprising a biodegradable polymer containing said pharmaceutical, wherein said nanoparticle selectively or preferentially accumulates in said region of injury.

[0111] Embodiment 104: The method of embodiment 103, wherein said nanoparticle comprises a polymer selected from the group consisting of Poly-D,L-lactide-co-glycolide (PLGA), Poly(L-lactic acid) (PLA), Poly-ε-caprolactone (PCL), chitosan, and gelatin.

[0112] Embodiment 105: The method of embodiment 104, wherein said nanoparticle comprises Poly-D,L-lactide-co-glycolide (PLGA).

[0113] Embodiment 106: The method of embodiment 105, wherein said nanoparticle comprises Poly-D,L-lactide-co-glycolide (PLGA) lactide:glycolide 50:50 (50:50 PLGA).

[0114] Embodiment 107: The method of embodiment 105, wherein said nanoparticle comprises Poly-D,L-lactide-co-glycolide (PLGA) lactide:glycolide 85:15 (85:15 PLGA).

[0115] Embodiment 108: The method of embodiment 104, wherein said nanoparticle comprise poly(L-lactic acid).

[0116] Embodiment 109: The method according to any one of embodiments 103-108, wherein said nanoparticle ranges in size from about 50 nm, or from about 100 nm, or from about 150 nm, up to about 500 nm, or up to about 400 nm, or up to about 300 nm, or up to about 200 nm.

[0117] Embodiment 110: The method of embodiment 109, wherein said nanoparticle ranges in size from about 150 nm up to about 200 nm.

[0118] Embodiment 111: The method according to any one of embodiments 103-110, wherein the zeta potential of said nanoparticle is at least about -10 mV, or at least about -15 mV, or at least about -20 mV, or at least about 24 mV.

[0119] Embodiment 112: The method according to any one of embodiments 103-111, wherein tissue specificity of said nanoparticle is by preferential passive accumulation in a target tissue.

[0120] Embodiment 113: The method of embodiment 112, wherein tissue specificity of said nanoparticle is by preferential passive accumulation in myocardium with damaged endothelium.

[0121] Embodiment 114: The method according to any one of embodiments 103-111, wherein tissue specificity of said nanoparticle is provided by tissue-specific binding moieties disposed on the surface of said nanoparticle.

[0122] Embodiment 115: The method of embodiment 114, wherein said binding moieties selected from the group consisting of an antibody, a DNA aptamer, an RNA aptamer, a peptide aptamer, a cell-binding peptide, an anticalin, a lectin, and a DARPIN.

[0123] Embodiment 116: The method according to any one of embodiments 114-115, wherein said tissue-specific binding moieties specifically or preferentially bind to markers on macrophages.

[0124] Embodiment 117: The method of embodiment 116, wherein said tissue specific binding moieties bind to CD11b.

[0125] Embodiment 118: The method according to any one of embodiments 114-117, wherein said tissue-specific binding moieties comprise antibodies.

[0126] Embodiment 119: The method of embodiment 118, wherein said antibody is a full-length immunoglobulin.

[0127] Embodiment 120: The method of embodiment 118 wherein said antibody is selected from the group consisting of Fv, Fab, (Fab')₂, (Fab')₃, IgGΔCH2, a unibody, and a minibody.

[0128] Embodiment 121: The method of embodiment 118, wherein said antibody is a single chain antibody.

[0129] Embodiment 122: The method according to any one of embodiments 103-121, wherein said pharmaceutical comprises an estrogen receptor ligand selected from the group consisting of tamoxifen, 4-hydroxy-tamoxifen, and a tamoxifen analogue.

[0130] Embodiment 123: The method of embodiment 122, wherein said estrogen receptor ligand tamoxifen analog is ICI 182,780.

DEFINITIONS.

[0131] The terms "tissue-specific" and "cell-specific" are used interchangeably and refer to the preferential or specific binding (targeting) of a nanoparticle described herein to a particular cell type and/or to a particular tissue type.

[0132] An "ER-ligand-inducible CRE recombinase" refers to a Cre recombinase that has been modified so that in a cell the Cre recombinase is inactive until contacted with a ligand that induced the Cre activity. Typically, an ER-ligand-inducible CRE recombinase refers to a Cre recombinase that is attached to ligand binding domain of an estrogen receptor

(ER). Binding of the estrogen receptor by a ligand (*e.g.*, and ER antagonist such as tamoxifen) results in the Cre being free to function as a recombinase in the cell.

[0133] As used herein, the term “selective targeting” or “specific binding” refers to use of targeting moieties attached to a nanoparticle described herein. Typically, the ligands interact specifically/selectively with receptors or other biomolecular components expressed on the target, *e.g.*, a cell or tissue of interest. The targeting ligands can include such molecules and/or materials as peptides, antibodies, aptamers, targeting peptides, polysaccharides, and the like.

[0134] The terms "polypeptide", "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residues is an artificial chemical analogue of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers

[0135] An "avidin/biotin" interaction refers to binding reaction between biotin and avidin or avidin variants including but not limited to streptavidin, neutravidin, and the like.

[0136] A "pharmaceutically acceptable carrier" as used herein includes, but need not be limited to, any of the standard pharmaceutically acceptable carriers. The pharmaceutical compositions contemplated herein can be formulated according to known methods for preparing pharmaceutically useful compositions. The pharmaceutically acceptable carrier can include diluents, adjuvants, and vehicles, as well as carriers, and inert, non-toxic solid or liquid fillers, diluents, or encapsulating material that does not react with the active ingredients of the invention. Examples include, but are not limited to: phosphate buffered saline, physiological saline, water, and emulsions, such as oil/water emulsions. The carrier can be a solvent or dispersing medium containing, for example, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils. Formulations are described in a number of sources that are well known and readily available to those skilled in the art. For example, Remington's Pharmaceutical Sciences (Martin E W [1995] Easton Pa., Mack Publishing Company, 19th ed.) describes formulations which can be used in connection with the drug delivery nanocarrier(s) (*e.g.*, LB-coated nanoparticle(s)) described herein.

[0137] As used herein, an "antibody" refers to a protein consisting of one or more polypeptides substantially encoded by immunoglobulin genes or fragments of

immunoglobulin genes or derived therefrom that is capable of binding (*e.g.*, specifically binding) to a target (*e.g.*, to a target polypeptide). The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon and mu constant region genes, as well as myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively.

[0138] A typical immunoglobulin (antibody) structural unit is known to comprise a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

[0139] Antibodies exist as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. Thus, for example, pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab)'_2$, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The $F(ab)'_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region thereby converting the $(Fab')_2$ dimer into a Fab' monomer. The Fab' monomer is essentially a Fab with part of the hinge region (*see, Fundamental Immunology*, W.E. Paul, ed., Raven Press, N.Y. (1993), for a more detailed description of other antibody fragments). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such Fab' fragments may be synthesized *de novo* either chemically or by utilizing recombinant DNA methodology. Thus, the term antibody, as used herein also includes antibody fragments either produced by the modification of whole antibodies or synthesized *de novo* using recombinant DNA methodologies. Certain preferred antibodies include single chain antibodies (antibodies that exist as a single polypeptide chain), more preferably single chain Fv antibodies (sFv or scFv) in which a variable heavy and a variable light chain are joined together (directly or through a peptide linker) to form a continuous polypeptide. The single chain Fv antibody is a covalently linked V_H-V_L heterodimer which may be expressed from a nucleic acid including V_H - and V_L - encoding sequences either joined directly or joined by a peptide-encoding linker. Huston, *et al.* (1988) *Proc. Nat. Acad. Sci. USA*, 85: 5879-5883. While the V_H and V_L are connected to each as a single polypeptide chain, the V_H and V_L domains associate non-covalently. The first functional

antibody molecules to be expressed on the surface of filamentous phage were single-chain Fv's (scFv), however, alternative expression strategies have also been successful. For example, Fab molecules can be displayed on a phage if one of the chains (heavy or light) is fused to g3 capsid protein and the complementary chain exported to the periplasm as a soluble molecule. The two chains can be encoded on the same or on different replicons; the important point is that the two antibody chains in each Fab molecule assemble post-translationally and the dimer is incorporated into the phage particle via linkage of one of the chains to, *e.g.*, g3p (*see, e.g.*, U.S. Patent No: 5733743). The scFv antibodies and a number of other structures converting the naturally aggregated, but chemically separated light and heavy polypeptide chains from an antibody V region into a molecule that folds into a three-dimensional structure substantially similar to the structure of an antigen-binding site are known to those of skill in the art (*see e.g.*, U.S. Patent Nos. 5,091,513, 5,132,405, and 4,956,778). In certain embodiments antibodies should include all that have been displayed on phage (*e.g.*, scFv, Fv, Fab and disulfide linked Fv (*see, e.g.*, Reiter *et al.* (1995) *Protein Eng.* 8: 1323-1331) as well as affibodies, nanobodies, unibodies, and the like.

[0140] The term "specifically binds", as used herein, when referring to a biomolecule (*e.g.*, protein, nucleic acid, antibody, *etc.*), refers to a binding reaction that is determinative of the presence of a biomolecule in heterogeneous population of molecules (*e.g.*, proteins and other biologics). Thus, under designated conditions (*e.g.* immunoassay conditions in the case of an antibody or stringent hybridization conditions in the case of a nucleic acid), the specified ligand or antibody binds to its particular "target" molecule and does not bind in a significant amount to other molecules present in the sample.

BRIEF DESCRIPTION OF THE DRAWINGS

[0141] Figure 1A schematically illustrates a nanoparticle system for tissue-specific delivery of an activating ligand for a Cre-Lox system. Figure 1B schematically illustrates targeting and Cre-mediated excision for expression of reporter protein and other Cre-Lox uses.

[0142] Figure 2 shows a schematic of process for producing nanoparticles.

[0143] Figure 3 shows scanning electron microscopy of final nanoparticles, showing uniform size distribution (approximately 200 microns in diameter).

[0144] Figure 4 shows results of an *in vivo* mouse study demonstrating absence of 4-hydroxy-tamoxifen in the bloodstream when encapsulated in nanoparticle form. Each condition consists of n=3 mice.

[0145] Figure 5 schematically illustrates a Rosa26-CreER x Td-Tomato model, in which Td-Tomato fluorescent reporter protein is expressed with successful CreER-induced recombination with delivery of 4-hydroxy-tamoxifen.

[0146] Figure 6 schematically illustrates a beta-actin-CreER x “Rainbow” model, in which one of three fluorescent reporter proteins (Cerulean, orange, or mCherry) is expressed with successful CreER-induced recombination with delivery of 4-hydroxy-tamoxifen.

[0147] Figure 7 illustrates one strategy to obtain conditional gene knockout using the methods described herein

[0148] Figure 8 schematically illustrates a Rosa26-CreER x Td-Tomato model. Nanoparticle-based 4-hydroxy-tamoxifen (bottom right frame) induces CreER-mediated recombination and expression of fluorescent reporter protein at a similar level as traditional free tamoxifen.

[0149] Figure 9 schematically illustrates a beta-actin-CreER x “Rainbow” model. Nanoparticle-based 4-hydroxy-tamoxifen (bottom right frame) induces CreER-mediated recombination and expression of fluorescent reporter proteins at a similar level as traditional free tamoxifen.

[0150] Figure 10 shows results of an *in vivo* study in the “beta-actin-CreER x Rainbow” transgenic mouse model. While the standard systemic delivery of tamoxifen (left column) results in recombination and expression of fluorescent reporter proteins in all tissues, delivery of 4-hydroxy-tamoxifen packaged in nanoparticle form (right column) results in DNA recombination specifically only liver and spleen tissue.

[0151] Figure 11 shows results of an *in vivo* study in the “Rosa26-CreER x Td-Tomato” transgenic mouse model. Shown is a cross-section of a mouse heart left ventricle, with the upper right corner (right panel) injured through ischemia-reperfusion. Close-up of the injured region demonstrates ischemia-specific DNA recombination and expression of fluorescent reporter protein (red) through nanoparticle delivery of 4-hydroxy-tamoxifen. In comparison, normal uninjured heart tissue (left panel) does not undergo DNA recombination.

[0152] Figure 12, panels a and b, shows a scanning electron microscopy of OH-Tam-loaded (panel a) PLGA(50:50) and (panel b) PLA nanoparticles created by the emulsion-solvent evaporation method. Particles are spherical in morphology and monodisperse on gross examination.

5 [0153] Figure 13 shows the average particle size, polydispersity, and zeta-potential of OH-Tam-loaded PLGA(50:50), PLGA(85:15), and PLA nanoparticles. All three formulations resulted in relatively monodisperse particle sizes in the range of 220 to 230 nanometers. Zeta-potentials are consistent between groups, and the negative potentials promote stable nanoparticle suspensions.

10 [0154] Figure 14 shows the elution of OH-Tam from PLGA(50:50), PLGA(85:15), and PLA nanoparticles. PLGA(50:50) nanoparticle formulations result in a rapid “burst” release of OH-Tam within the first hour, while the majority of the encapsulated drug released within 24 hours. Conversely, PLA nanoparticles control the release of encapsulated OH-Tam such that less than 10% is released within the first hour, and further release remains similarly
15 slow.

[0155] Figure 15, panels a and b, shows accumulation of nanoparticles within dermal fibroblasts *in vitro*. Fluorescence micrographs were taken after incubation for either (panel a) 1 hour or (panel b) 12 hours, at nanoparticle concentrations in culture media ranging from 1 µg/ml to 1000 µg/ml. There is clearly an increase in nanoparticle uptake with increased
20 nanoparticle concentration, as evidenced by increase in fluorescence detected from the cells. The fluorescence detected results from signal from Coumarin 6 encapsulated within the nanoparticles. There is no visible difference in nanoparticle uptake after incubation for either 1 hour or 12 hours.

[0156] Figure 16 shows confocal microscopy of dermal fibroblasts after incubation
25 with nanoparticles. Representative cells chosen from sample incubated at a concentration of 1mg/ml nanoparticles for 12 hours. The nanoparticles (as evidenced by fluorescent signal from the encapsulated Coumarin 6) are clearly dispersed throughout the cytoplasm of the cells. This is most clearly seen in the z-stack cross-section.

[0157] Figure 17, panels a-f, shows representative fluorescent micrographs of organ
30 sections after PLGA(50:50) nanoparticle tail vein injection and circulation. There is clear accumulation of nanoparticles within the liver and spleen sections, while there is no detectable nanoparticle uptake in either the heart, liver, kidney, or brain. This is consistent

with the known function of the mononuclear phagocyte system of the liver and spleen in clearing the bloodstream of foreign particles.

[0158] Figure 18, panels a and b, show that PLGA NP-delivered OH-Tam induces CreER-tdTomato recombination *in vitro*. Panel (a) shows the large number of recombined cells after incubation with PLGA(50:50) OH-Tam-loaded nanoparticles. Panel (b) shows fluorescence microscopy of the cells after expression of the tdTomato reporter protein.

[0159] Figure 19, panels a and b, show that PLGA NP-delivered OH-Tam induces CreER-Rainbow recombination *in vitro*. Panel (a) shows the significant increase in recombined cells after incubation with PLGA(50:50) OH-Tam-loaded nanoparticles. Panel (b) shows fluorescence microscopy of the cells after expression of the cerulean, mOrange, and mhCherry reporter proteins.

[0160] Figure 20 shows HPLC-MS detection of 4-Hydroxytamoxifen. Tracing shows reliable detection of OH-Tam down to a serum level of 10 ng/mL. Transition m/z is 388.2, with peak at 2.37 minutes retention per the described test method.

[0161] Figure 21 shows a comparison of induced Cre recombination in the CreER-tdTomato system between IP injection of OH-Tam and IV injection of OH-Tam encapsulated PLGA(50:50) nanoparticles. With the standard intraperitoneal injection of OH-Tam, recombination (resulting in expression of the tdTomato fluorescent reporter protein) occurs in a majority of cells in every organ examined. However, with IV injection of encapsulated OH-Tam, the vast majority of the detected recombination events occurs in the liver and the spleen, with very few to no recombination events occurring in the other organs. This is consistent with known nanoparticle clearance by the liver and spleen, resulting in organ specific delivery of encapsulated OH-Tam.

[0162] Figure 22, panels a-c, shows co-localization of CreER-induced tdTomato reporter gene expression and nanoparticle accumulation in individual cells. These Confocal micrographs of representative tissue sections (labeled (panel a), (panel b), and (panel c)) from the liver reveal co-localization of nanoparticles (as evidenced by the encapsulated Coumarin 6 signal) with expression of tdTomato reporter gene (from CreEr-induced gene recombination after intracellular delivery of OH-Tam).

[0163] Figure 23 shows a comparison of induced Cre recombination in the CreER-Rainbow system between IP injection of OH-Tam and IV injection of OH-Tam encapsulated PLGA(50:50) nanoparticles. With the standard intraperitoneal injection of OH-Tam,

recombination (resulting in expression of the fluorescent reporter proteins cerulean, mOrange, and mCherry) occurs in many cells in every organ examined. However, with IV injection of encapsulated OH-Tam, the vast majority of the detected recombination events occurs in the liver and the spleen, with very few to no recombination events occurring in the other organs. This is consistent with known nanoparticle clearance by the liver and spleen, resulting in organ specific delivery of encapsulated OH-Tam.

[0164] Figure 24 shows changes in size and surface potential of PLGA(50:50) nanoparticles with sequential surface modification during carbodiimide-mediated conjugation to antibodies. At baseline, PLGA (50:50) OH-Tam-loaded nanoparticles are approximately 195nm in size, with a zeta-potential of -24 mV. With the addition of the sulfo-NHS ester crosslinker, particle size increases slightly, and the zeta potential becomes much less negative, possibly due to shielding of the previously available carboxylic acid end-groups. After conjugation of the antibody, average particle size has increased to approximately 242nm, consistent with the surface addition of an antibody approximately 15-20nm in size. This also results in a return to a more negative surface potential.

[0165] Figure 25 shows the effect of chemical conjugation on the degree of protein binding to the surface of PLGA(50:50) nanoparticles. This study examined and quantified the amount of antibody bound to the surface of nanoparticles for both the anti-CD3 and anti-CD31 antibody systems. The conjugation process was performed with and without the EDC crosslinker. It is apparent that even in the absence of crosslinker, antibodies non-covalently bind to the surface of the nanoparticles (approximately 357 CD3 antibodies and 302 CD31 antibodies per PLGA nanoparticle). However, the addition of crosslinker almost doubles the amount of antibody bound.

[0166] Figure 26, panels a-f, shows fluorescence microscopy of tissue sections after endothelial cell targeting of induced Cre recombination with anti-CD31 PLGA(50:50) OH-Tam-loaded nanoparticles. In the control samples (nanoparticles without targeting antibody), no tdTomato expression in cells resembling endothelial cells is apparent. In mice injected with anti-CD31 targeted nanoparticles, clear vascular structures are apparent in multiple organ systems.

[0167] Figure 27, panels a-h, shows tdTomato expression in endothelial cells following targeted OH-Tam delivery by anti-CD31 PLGA(50:50) nanoparticles. These Confocal micrographs of two different representative areas (panels a-d) and (panels e-h)

demonstrate clear co-localization of nanoparticles (Coumarin 6) and tdTomato gene expression in stained CD31+ endothelial cells.

[0168] Figure 28 illustrates a FACS analysis of lysed blood from Rosa26CreER-tdTomato mice after targeting with anti-CD11b PLGA(50:50) OH-Tam-loaded nanoparticles.

5 This representative analysis demonstrates a distinct population of tdTomato+ cells from the CD11b+ population in which recombination has occurred, due to uptake of the targeted nanoparticles.

[0169] Figure 29 shows a summary of FACS analysis data. These data show a significantly increased (by over a factor of 9) percentage of CD11b+ cells that undergo
10 recombination and express tdTomato after targeting with anti-CD11b PLGA(50:50) OH-Tam-loaded nanoparticles.

[0170] Figure 30, panels a and b, show cross-sectional microscopy of heart after ischemia-reperfusion injury. The area of injury (demarcated in red) is clearly seen in both the H&E stain (panel a) and the fluorescent micrograph (panel b) showing decrease in cardiac
15 myocardium auto-fluorescence (in the GFP channel) in the area of injury.

[0171] Figure 31, panels a-f, shows increase in nanoparticle uptake in regions of ischemia-reperfusion injury. Panels (a) and (b) are representative heart cross sections under fluorescent microscopy showing clear areas of injury, in which PLGA(50:50) OH-Tam loaded nanoparticles were injected in the lateral tail vein either 1 hour or 24 hours after injury
20 respectively. Panels (d) and (f) are representative fluorescent micrographs taken within the regions of injury, showing many cells that have undergone nanoparticle uptake with resulting recombination. Panels (c) and (e) are representative fluorescent micrographs taken in areas of normal myocardium, showing absence of cells that have undergone recombination.

[0172] Figure 32 panels a-g, shows increase in nanoparticle uptake in area of
25 ischemia-reperfusion injury after targeting with anti-CD11b PLGA(50:50) OH-Tam-loaded nanoparticles. Panel (a) is a representative heart cross section under fluorescent microscopy showing clear area of injury. Of note, many cells are visible in the ischemic region that have undergone recombination, a finding not present at this magnification in the experiment shown in Figure 31 using non-targeted nanoparticles. Confocal microscopy (panels b-g)
30 demonstrates that the tdTomato positive cells are CD68+ macrophages.

[0173] Figure 33, panels a-e, shows cardiomyocyte uptake of targeted anti-CD11b PLGA(50:50) OH-Tam-loaded nanoparticles *in vivo*. Panel (a) is a heart cross section (near

the heart apex) under fluorescent microscopy showing clear injury throughout the field of view as well as numerous tdTomato⁺ cells that have undergone recombination. There is a large cluster of cells in this view that appear to be cardiomyocytes in morphology. Confocal microscopy (panels b-e) confirms that the cluster of tdTomato⁺ cells are α -actinin⁺

cardiomyocytes.

DETAILED DESCRIPTION

[0174] The Cre/lox site-specific recombination system has emerged as an important tool for the generation of conditional somatic murine (*e.g.*, mouse) mutants. This method allows one to control gene activity in space and time in almost any tissue of the mouse, thus opening new avenues for studying gene function and for establishing sophisticated animal models of human diseases. A major technical advance in terms of *in vivo* inducibility was the development of ligand-dependent Cre recombinases that can be activated by administration of tamoxifen to the animal. Typically, cell- or tissue-specificity of the Cre/lox system has been achieved through the use of cell- or tissue-specific promoters driving the expression of the Cre component.

Nanoparticle directed Cre-Lox recombination.

[0175] In various embodiments methods of temporal and tissue-specific activation of Cre-mediated nucleic acid recombination are provided that do not require the use of cell- or tissue-specific promoters. This is accomplished by providing cell- or tissue specific delivery of a ligand (*e.g.*, 4-hydroxy-tamoxifen) that activates the inducible Cre (CreER) (*see, e.g.*, Figures 1A and 1B). In particular, in certain embodiments, the cell- or tissue-specific delivery of the Cre activating ligand (*e.g.*, 4-hydroxy-tamoxifen) is accomplished by packaging the ligand in a biodegradable nanoparticle (*see, e.g.*, Figure 1A). The target specificity of the nanoparticle provides the cell- and tissue-specificity of the Cre recombinase, while timing of the administration of the nanoparticle determines the timing and conditional expression of the Cre in the target cells and/or tissues.

[0176] Accordingly, in certain illustrative embodiments this invention comprises a nanoparticle system that packages and delivers the Cre-inducing ligand (*e.g.*, 4-hydroxy-tamoxifen) to specific cells and tissues in animal models containing constitutively expressed CreER recombinase and a *loxP*-flanked region. The cell and tissue specificity of the nanoparticles can be induced through either passive preferential accumulation of the nanoparticles in certain tissues of interest (such as sites of ischemic damage, or in certain

cancer tumors), or through active targeting of the cell or tissue of interest through specific targeting ligands on the surface of the nanoparticle. Figure 1 shows an illustrative schematic of the nanoparticle system. One particular illustrative, but non-limiting application would involve delivery to ischemic tissue (such as in myocardial infarction resulting from
5 obstruction in the flow of coronary arterial blood) through passive accumulation in myocardium with damaged endothelium as well as active targeting of cardiac-specific ligands.

[0177] Accordingly in various embodiments, a transgenic CreER-*loxP* murine system in which the CreER recombinase is under the control of a non-tissue specific promoter is
10 provided. This can be generated through crossing of an inducible Cre strain (such as CreER recombinase inducible by 4-hydroxy-tamoxifen, in which the expression of CreER is under the control of a general promoter) with a *loxP*-flanked strain (*e.g.*, a strain in which a segment of the nucleic acid is flanked by *loxP* sites).

[0178] Figure 7 illustrates one strategy to obtain a tissue-specific conditional gene
15 knockout using the methods described herein. Mice harboring the conditional allele (*e.g.*, a gene or exon flanked by *loxP* (floxed gene) are crossed with mice carrying a CreER fusion protein that is under the control of a constitutive (tissue independent) promoter (CP). Breeding of these two mice will produce heterozygous offspring that contain the floxed gene of interest and the CreER fusion transgene. These animals are crossed to homozygous
20 conditional mutant mice (G1). Offspring that are homozygous for the conditional allele and heterozygous for the CreER transgene (G2) can be generated at a frequency of 25% and represent the experimental animals. In the presence of ligand (*e.g.*, tamoxifen [4-OH]) nanoparticles) the floxed gene, or gene fragment, of interest will be excised only in the CreER-expressing cells/tissue (gray) targeted by the nanoparticles, while the gene of interest
25 remains functional in other cell/tissue types (white) that are not targeted by the nanoparticles. Control animals are treated with vehicle and the floxed gene of interest remains functional in all cell/tissue types since the CreER transgene is not activated by the ligand.

[0179] In certain embodiments the *loxP*-flanked strain is a reporter strain, in which the *loxP* sites are combined with visible marker proteins used to trace CreER recombination
30 success. However, in certain embodiments, the *loxP*-flanked strain is a strain in which a gene or portion of a gene (*e.g.*, an exon) is floxed to provide an inducible Cre-mediated knockout in which the expression produce of the gene is reduced or eliminated.

[0180] This Cre-lox system can also be used to obtain gain-of-function mutations. In this embodiment, the gene of interest is operably linked to a removable (floxed) transcriptional stop element (Lox-STOP-Lox). Thus, for example, mice harboring the conditional stop element (Lox-STOP-Lox) operably linked to the gene of interest, are crossed with mice carrying a CreER fusion protein that is under the control of a constitutive (tissue independent) promoter (CP). Breeding of these two mice will produce heterozygous offspring that contain the floxed stop element and the CreER fusion transgene. These animals are crossed to homozygous conditional mutant mice (G1). Offspring that are homozygous for the conditional allele and heterozygous for the CreER transgene (G2) can be generated at a frequency of 25% and represent the experimental animals. In the presence of ligand (*e.g.*, tamoxifen [4-OH]) nanoparticles) the floxed stop element will be excised only in the CreER-expressing cells/tissue (gray) targeted by the nanoparticles leading to transcription of the operably linked gene of interest. In contrast, the gene of interest remains stopped in other cell/tissue types (white) that are not targeted by the nanoparticles. Control animals are treated with vehicle and the floxed stop element remains functional blocking transcription of the operably linked gene. In certain embodiments the stop element can comprise a combination of both *neo* and stop element (*neostop*), *e.g.*, as described by Dragatsis & Zeitlin (2001) *Nucleic Acids Res.*, 29(3): e10.

[0181] This gain of function approach has been used, *inter alia*, to generate models of human cancer. Mice harboring a conditionally activatable allele of oncogenic K-ras have been used to generate various mouse models of human cancer, including lung adenocarcinoma (*see, e.g.*, Jackson *et al.* (2001) *Genes Dev.* 15: 3243-3248). Expression of oncogenic K-ras is controlled by a removable transcriptional stop element (Lox-STOP-LOX). In the presence of Cre, the STOP element was removed and oncogenic K-ras is expressed only in cells where the recombinase is expressed (*see, e.g.*, Tuveson *et al.* (2004) *Cancer Cell*, 5: 375-387). Combining gain-of-function and loss-of-function mutations has led to the development of more advanced cancer models (*see, e.g.*, Babaei-Jadidi *et al.* (2011) *J. Exp. Med.* 208: 295-312; Young *et al.* (2011) *Cancer Res.* 71: 4040-4047).

[0182] As proof of principle, the use of nanoparticle-based deliver of 4-hydroxy-tamoxifen to achieve tissue-specific Cre recombination has been demonstrated in two models:

[0183] a) A “Rosa26-CreER x Td-Tomato” model (see Figure 5), in which control of CreER expression is under the control of the constitutive Rosa26 locus, and

successful CreER recombination is reported through expression of the fluorescent Td-Tomato protein; and.

[0184] b) A “beta-actin-CreER x Rainbow” model (see Figure 6), in which control of CreER expression is under the control of the constitutive beta-actin protein, and

5 successful CreER recombination is reported through expression of either of the fluorescent proteins Cerulean, mOrange, or mCherry.

[0185] Successful 4-hydroxy-tamoxifen-nanoparticle-based inducible-CreER DNA recombination was first demonstrated *in vitro* in each of the above systems through the following experiment (*see, also* Example 1):

10 [0186] a) Nanoparticles consisting of 4-hydroxy-tamoxifen encapsulated in polymer (either 50:50 PLGA, 85:15 PLGA, or PLLA) were created as described below and in Example 1.

[0187] b) Skin fibroblasts were cultured *in vitro* from the above two transgenic mouse models through traditional cell culture techniques.

15 [0188] c) When the cell cultures reached confluency, the nanoparticles were introduced into the cell culture media and incubated along with the cells. As controls, cell cultures were also performed with 1) incubation of polymer-only (drug-free) nanoparticles, 2) incubation with free 4-hydroxytamoxifen drug (not in nanoparticle form), and 3) baseline control without any modification.

20 [0189] d) After 24 hours, the cells are fixed in 4% paraformaldehyde as per standard technique.

[0190] e) Imaging of the cells was performed using standard fluorescence microscopy. The degree of recombination was quantified as the number of cells expressing the reporter fluorescent protein, as analyzed by “Image-J” image analysis software.

25 [0191] Figures 8 and 9 show representative images for the Rosa26-CreER x Td-Tomato model and the beta-actin-CreER x “Rainbow” model respectively. Nanoparticle-delivered 4-hydroxy-tamoxifen is clearly able to induce expression of fluorescent reporter protein (through CreER-*loxP* recombination) in the same degree as the standard technique using free tamoxifen.

30 [0192] Successful *in vivo* differential tissue-specific induced nucleic acid recombination using 4-hydroxy-tamoxifen delivered in nanoparticle form was first demonstrated/reduced to practice in transgenic mice of the “beta-actin-CreER x Rainbow”

model. First, nanoparticles of 4-hydroxy-tamoxifen packaged in 50:50 PLGA were produced as described below and in Example 1. These nanoparticles were then injected intravenously through the tail vein of beta-actin-CreER x “Rainbow” transgenic mice, and allowed to circulate for 72 hours. The mice were then sacrificed, and the following organs were collected: heart, lungs, liver, spleen, kidney, and brain. Each of the organs was fixed, mounted in OCT, and frozen at -80C. Sections were cut using a cryostat, and imaging was performed under fluorescence microscopy. As a positive control, transgenic mice of the same model were injected with intra-peritoneal free tamoxifen, and the organs similarly harvested. As a negative control, drug-free 50:50 PLGA nanoparticles were produced and injected intravenously through the tail vein of the same model of transgenic mice, and the organs similarly harvested. Figure 10 shows representative images of each organ for each of the conditions. Whereas intra-peritoneal systemic injection of free tamoxifen results in expression of the reporter fluorescent proteins in all tissues, the 4-hydroxy-tamoxifen – 50:50 PLGA nanoparticles caused liver- and spleen-specific DNA recombination and expression of the reporter fluorescent proteins. Nanoparticles without drug were not able to induce any recombination.

[0193] The second *in vivo* demonstration/reduction to practice of differential tissue-specific induced nucleic acid recombination using 4-hydroxy-tamoxifen nanoparticles was in the Rosa26-CreER x Td-Tomato transgenic mouse model (see Figure 11 for representative results). This study demonstrated that nanoparticles can be targeted specifically to tissues that are specifically under ischemic conditions. First, nanoparticles of 4-hydroxy-tamoxifen packaged in 50:50 PLGA were produced as described above. Mice of the “Rosa26-CreER x Td-Tomato” transgenic strain underwent surgical knot ligation of their left anterior descending artery for 45 minutes, followed by release of the knot, resulting in ischemia-reperfusion injury. The nanoparticles were then injected intravenously through the tail vein of the mice after 1, 4, and 24 hours, and allowed to circulate for 7 days. The mice were then sacrificed, and the heart from each mouse was harvested, fixed, mounted in OCT, and frozen at -80C. Sections were cut using a cryostat, and imaging was performed under fluorescence microscopy. The ischemic regions of the heart were compared with the normal non-injured regions. It was demonstrated that the ischemic cells undergo DNA recombination and expression of reporter protein due to targeting by the 4-hydroxy-tamoxifen nanoparticles, whereas no DNA recombination occurs in normal uninjured tissue.

[0194] Accordingly, in view of the foregoing, in certain embodiments a method for conditional and target-specific recombination is provided where the method involves providing a mammal comprising cells that express an ER-ligand-inducible CRE recombinase under the control of a non-tissue specific promoter and that comprises a nucleic acid sequence flanked by a pair of *loxP* sequences, inverted in orientation with respect to each other; and administering to the mammal biodegradable polymer nanoparticles containing an estrogen receptor ligand where the nanoparticles provide specific delivery to a target tissue of the estrogen receptor ligand activating the CRE recombinase which performs target specific recombination of the nucleic acid between the *loxP* sequences in the target tissue. As noted above, in certain embodiments, the *loxP* sequences flank a gene or portion of a gene and the recombination provides inactivation or replacement (depending on the orientation of the *loxP* sequences) of the gene. In certain embodiments, the *loxP* sequence flank a stop element that is operably linked to a gene and the recombination provides a gain of function. Typically, the Cre recombinase is constitutively expressed (*e.g.*, under the control of a constitutive promoter), however, in certain embodiments, the Cre recombinase can be expressed under the control of an inducible promoter.

[0195] Cre (cyclization recombination) is a 38 kDa, site-specific DNA tyrosine recombinase derived from P1 bacteriophage. It catalyzes DNA recombination between two 34-bp recognition sequences referred to as *loxP* sites (locus of crossing over {X} of P1). Since Cre does not require high-energy cofactors or accessory proteins and exhibits optimal recombinase activity at 37°C (Buchholz *et al.* (1998) *Nat. Biotechnol.* 16: 657-662), the system is optimal for genetic engineering in cultured mammalian cells and in mice. A major technical advance in terms of *in vivo* inducibility was the development of ligand-dependent Cre recombinases that can be activated by administration of tamoxifen (or other ER binding ligands) to the animal. A number of CreER recombinase variants are known to those of skill in the art (see, *e.g.* U.S. Patent Pub. No: 2016/0017298 describing Cre recombinase comprising mutations represented by R32V, and/or R32M, and/or 303GVSDup, and the like). In certain embodiments the CreER is a CreER(T2) (a.k.a. CreERT2) which comprises a Cre fused to a mutated human estrogen receptor (ER) ligand-binding domain (LBD) where the binding domain contains either the G400V/M543A/L544A or the G400V/L539A/L540A triple mutation of the human ER LBD (see, *e.g.*, Feil *et al.* (1997) *Biochem. Biophys. Res. Commun.* 237(3): 752-757). In certain embodiments a cassette, including a codon-optimized Cre (iCre), flanked by two Cre-ERT2 moieties, has been reported to have good inducibility

and negligible background (see, *e.g.*, Casanova *et al.* (2002) *Genesis*, 34: 208-214; Jullien *et al.* (2008) *Genesis*, 46(4): 193-199).

[0196] The Cre recombinase or inducible Cre recombinase (*e.g.*, CreER) induces recombination at *loxP* sites. *loxP* (locus of X-over P1) is a site on the bacteriophage P1 typically consisting of 34 bp. The site typically includes an asymmetric 8 bp sequence, variable except for the middle two bases, in between two sets of symmetric, 13 bp sequences. The is given below:

13 bp 8bp 13bp
ATAACTTCGTATA - NNNTANNN - TATACGAAGTTAT (SEQ ID NO:1)

where 'N' indicates bases that may vary, and lowercase letters indicate bases that have been mutated from the wild-type. The 13 bp sequences are palindromic but the 8 bp spacer is not, thus giving the *loxP* sequence a certain direction. Usually *loxP* sites come in pairs for genetic manipulation. If the two *loxP* sites are in the same orientation, the floxed sequence (sequence flanked by two *loxP* sites) is excised. However, if the two *loxP* sites are in the opposite orientation, the floxed sequence is inverted. If there exists a floxed donor sequence, the donor sequence can be swapped with the original sequence. This technique is called recombination-mediated cassette exchange and is a very convenient and time-saving way for genetic manipulation. The caveat, however, is that the recombination reaction can happen backwards, rendering cassette exchange inefficient. In addition, sequence excision can happen *in trans* instead of an *in cis* cassette exchange event. Various *loxP* mutants are created to avoid these problems (see, *e.g.*, Araki (1997) *Nucl. Acids Res.*, 25(4): 868-872). Wild-type and illustrative, but non-limiting *loxP* variants are shown in Table 1.

Table 1. Illustrative, but non-limiting examples of *loxP* sites (see, *e.g.*, Missirlis *et al.* (2006) *BMC Genomics*. 7: 73).

Name	13bp Recognition Region	8bp Spacer Region	13bp Recognition Region	SEQ ID NO
Wild-Type	ATAACTTCGTATA	ATGTATGC	TATACGAAGTTAT	2
lox 511	ATAACTTCGTATA	ATGTATaC	TATACGAAGTTAT	3
lox 5171	ATAACTTCGTATA	ATGTgTaC	TATACGAAGTTAT	4
lox 2272	ATAACTTCGTATA	AaGTATcC	TATACGAAGTTAT	5
M2	ATAACTTCGTATA	AgaaAcca	TATACGAAGTTAT	6
M3	ATAACTTCGTATA	taaTACCA	TATACGAAGTTAT	7
M7	ATAACTTCGTATA	AgatAGAA	TATACGAAGTTAT	8

M11	ATAACTTCGTATA	aGATAgaa	TATACGAAGTTAT	9
lox 71	taccgTTCGTATA	NNNTANNN	TATACGAAGTTAT	10
lox 66	ATAACTTCGTATA	NNNTANNN	TATACGAAcggtta	11

[0197] Multiple variants of *loxP*, in particular lox2272 and loxN, can be used by with the combination of different Cre actions (transient or constitutive) to create a "Brainbow" system that allows, for example, multi-coloring of mice's brain with four fluorescent proteins. Another report using two lox variants pair but through regulating the length of DNA in one pair results in stochastic gene activation with regulated level of sparseness (*see, e.g., Wang et al. (2009) PLoS ONE, 4(1): e4200*).

[0198] While activation of CreER is illustrated using 4-hydroxy-tamoxifen, it will be recognized that other ligands that bind the estrogen receptor (ER) binding domain can similarly be used. Accordingly, in certain embodiments, the ligand comprises tamoxifen or other ER antagonists, *e.g., a tamoxifen analogue, 7 α ,17 β -[9-[(4,4,5,5,5-Pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol (ICI 182,780), and the like.*

[0199] As noted above, in certain embodiments, the nanoparticles are used without targeting moieties and are selectively directed to cells and/or tissues that accumulate nanoparticles. In certain embodiments the nanoparticles are attached to one or more targeting moieties that direct the nanoparticles to particular cells and/or tissues. In certain embodiments cell- or tissue-specificity of the nanoparticles is altered by modification of the polymer composition, size, shape, or surface chemistry.

[0200] In certain embodiments the transgenic murine model is modified which results in differential targeting of the nanoparticle resulting in differential DNA recombination. For example, mouse models of cancerous tumors can be studied in this fashion as DNA recombination would likely preferentially occur in tumors with increased uptake of nanoparticles.

Nanoparticle systems.

[0201] In various embodiments nanoparticles are used to achieve cell- and/or tissue-specific delivery of the ligand that activate the Cre recombinase (*e.g., CreER*). Typically, the nanoparticles comprise one or more biodegradable polymers that package a payload of the CreER activating ligand (*e.g., tamoxifen, 4-hydroxytamoxifen, etc.*). Illustrative biodegradable polymers include, but are not limited to Poly-D,L-lactide-co-glycolide (PLGA), Poly(L-lactic acid) (PLA), Poly- ϵ -caprolactone (PCL), chitosan, and gelatin.

[0202] Nanoparticles of different compositions have been created by us, using polymers such as Poly(D,L-lactide-co-glycolide) lactide:glycolide 50:50 (a.k.a. “50:50 PLGA”), Poly(D,L-lactide-co-glycolide) lactide:glycolide 85:15 (a.k.a. “85:15 PLGA”), and Poly(L-lactic acid) (a.k.a. “PLLA”). The production process we used is based on a technique known as “emulsification-solvent evaporation” (*see, e.g., Rocca et al. (2004) J. Control Release*, 99, 271–280), and is schematically illustrated in Figure 2. In one illustrative, but non-limiting embodiment, steps for producing a batch of nanoparticles are summarized as follows:

[0203] a) Under low light conditions, dissolve polymer and 4-hydroxy-tamoxifen in dichloromethane.

[0204] b) Dissolution may be facilitated by placing solution on a shaker for up to 4 hours.

[0205] c) Prepare an aqueous solution of 1% polyvinyl alcohol.

[0206] d) Combine organic solution with aqueous solution in a 1:4 ratio.

[0207] e) Emulsify the combined solution by ultrasonic probe cavitation for 120 seconds.

[0208] f) Evaporate off dichloromethane by stirring the emulsification for 12 hours.

[0209] g) Wash the resultant nanoparticles by centrifuging the suspension at 14,500 rpm for 10 minutes and re-suspending the pellet in in water. Repeat 3 times.

[0210] h) Freeze the suspension at -80C.

[0211] i) Place the frozen suspension in a lyophilizer for 48 hours.

[0212] j) Store the final product in dark conditions.

[0213] This protocol is illustrative and non-limiting. Using the teachings provided herein, numerous methods of nanoparticle fabrication will be available to one of skill in the art.

[0214] Figure 3 below shows scanning electron microscopy (SEM) of the final nanoparticle product, demonstrating a uniform product with even size distribution.

[0215] For there to be cell- and/or tissue-specific delivery of the 4-hydroxy-tamoxifen, there should be no/negligible release of the drug in the bloodstream prior to nanoparticle uptake by a cell. Figure 4 shows the results of an *in vivo* mouse experiment demonstrating the absence of bloodstream release of 4-hydroxy-tamoxifen when encapsulated in nanoparticle form. As a control, free tamoxifen was injected in the

peritoneum of wild-type mice, with the result that large quantities of the drug are detected in the mouse serum by high-performance liquid chromatography – mass spectroscopy (HPLC-MS). When the drug was encapsulated in nanoparticle form as described above and directly injected intravenously in mice, there was no detectable drug in the mouse serum.

5 **Cell- and/or tissue-specific binding moieties (targeting moieties)**

[0216] In certain embodiments the nanoparticles described herein are attached to one or more targeting moieties (cell- and/or tissue-specific binding moieties) that bind to a target cell and/or tissue and thereby determine and/or increase the cell- and/or tissue-specificity of the nanoparticle. In various embodiments, the targeting moiety can include any moiety
10 capable of binding to a cell surface marker.

[0217] Illustrative binding moieties include, but are not limited to a DNA aptamer, an RNA aptamer, a peptide aptamer, a cell-binding peptide, an anticalin, a lectin, a DARPIN, an antibody, and the like.

[0218] Nucleic acid aptamers are nucleic acid species that have been engineered
15 through repeated rounds of *in vitro* selection or equivalently, SELEX (systematic evolution of ligands by exponential enrichment) to bind to various molecular targets such as small molecules, proteins, nucleic acids, and even cells, tissues and organisms. Aptamers offer molecular recognition properties that rival that of antibodies. In addition to their discriminate recognition, aptamers offer advantages over antibodies as they can be engineered completely
20 in a test tube, are readily produced by chemical synthesis, possess desirable storage properties, and elicit little or no immunogenicity in therapeutic applications.

[0219] Methods of aptamer selection/preparation are well known to those of skill in the art. Moreover, the process *in vitro* selection has been automated (see, *e.g.*, Cox and Ellington (2001) *Bioorganic & Med. Chem.* 9(10): 2525–2531; Cox *et al.* (2002) *Comb.*
25 *Chem. High Throughput Screen.* 5(4): 289-299; Cox *et al.* (2002) *Nucl. Acids Res.* 30(20): e108; and the like) reducing the duration of a selection experiment from six weeks to three days.

[0220] Both DNA and RNA aptamers show robust binding affinities for various targets (see, *e.g.*, Neves *et al.* (2010) *Biophys. Chem.* 153(1): 9-16; Baugh *et al.* (2000) *J.*
30 *Mol. Biol.* 301(1): 117-128; Dickman *et al.* (1995) *J. Cell. Biol.* 59:56-56). DNA and RNA aptamers have been selected for the same target. Lately, a concept of smart aptamers, and smart ligands in general, has been introduced in which aptamers are selected with pre-defined

equilibrium (K_d) rate (k_{off}/k_{on}), and thermodynamic (ΔH , ΔS) parameters of aptamer-target interaction. Kinetic capillary electrophoresis is the technology used for the selection of smart aptamers. It obtains aptamers in a few rounds of selection.

[0221] Peptide aptamers (Colas *et al.* (1996) *Nature*, 380: 548-550) are artificial proteins selected or engineered to bind specific target molecules. These proteins typically consist of one or more peptide loops of variable sequence displayed by a protein scaffold. They are typically isolated from combinatorial libraries and often subsequently improved by directed mutation or rounds of variable region mutagenesis and selection. *In vivo*, peptide aptamers can bind cellular protein targets. In certain embodiments the peptides that form the aptamer variable regions are synthesized as part of the same polypeptide chain as the scaffold and are constrained at their N and C termini by linkage to it. This double structural constraint decreases the diversity of the conformations that the variable regions can adopt (Spolar *et al.* (1994) *Science*, 263: 777-784), and this reduction in conformational diversity lowers the entropic cost of molecular binding when interaction with the target causes the variable regions to adopt a single conformation. As a consequence, peptide aptamers can bind their targets tightly, with binding affinities comparable to those shown by antibodies (nanomolar range).

[0222] Peptide aptamer scaffolds are typically small, ordered, soluble proteins. The first scaffold, which is still widely used, is *Escherichia coli* thioredoxin, the *trxA* gene product (TrxA) (see, *e.g.*, Colas *et al.* (1996) *Nature*, 380: 548-550; Reverdatto *et al.* (2015) *Curr. Top. Med. Chem.* 15: 1082-1101). In these molecules, a single peptide of variable sequence is displayed instead of the Gly-Pro motif in the TrxA -Cys-Gly-Pro-Cys- (SEQ ID NO:12) active site loop. Improvements to TrxA include substitution of serines for the flanking cysteines, that prevents possible formation of a disulfide bond at the base of the loop, introduction of a D26A substitution to reduce oligomerization, and optimization of codons for expression in particular cells.

[0223] Peptide aptamer selection can be made using different systems, but the most used is currently the yeast two-hybrid system. Peptide aptamers can also be selected from combinatorial peptide libraries constructed by phage display and other surface display technologies such as mRNA display, ribosome display, bacterial display and yeast display. These experimental procedures are also known as biopannings. Among peptides obtained from biopannings, mimotopes can be considered as a kind of peptide aptamers. All the

peptides panned from combinatorial peptide libraries have been stored in a special database with the name MimoDB (see, *e.g.*, Huang *et al.* (2011) *Nucl. Acids Res.* 40(1): D271-277).

[0224] The affimer proteins, an evolution of peptide aptamers, are small, highly stable proteins engineered to display peptide loops that provides a high affinity binding surface for a specific target protein. Affimer proteins are typically of low molecular weight, *e.g.*, 12-14 kDa, derived from the cysteine protease inhibitor family of cystatins (see, *e.g.*, Woodman *et al.* (2005) *J. Mol Biol.* 352: 1118-1133; Hoffmann *et al.* (2010) *PEDS*, 23(5): 403-413; Stadler *et al.* (2011) *PEDS*, 24(9): 751-763; Tiede *et al.* (2014) *PEDS*, 27(5): 145-155; and the like).

[0225] The affimer scaffold is a stable protein based on the cystatin protein fold. It displays two peptide loops and an N-terminal sequence that can be randomized to bind different target proteins with high affinity and specificity similar to antibodies. Stabilization of the peptide upon the protein scaffold constrains the possible conformations which the peptide may take, thus increasing the binding affinity and specificity compared to libraries of free peptides.

[0226] Other protein scaffolds include, but are not limited to designed ankyrin repeat proteins (DARPs) are a class of non-immunoglobulin proteins that can offer advantages over antibodies for target binding (see, *e.g.*, Stumpp and Amstutz (2007) *Curr. Opin. Drug Discov. Devel.* 10(2): 153-159). DARPs have been successfully used, for example, for the inhibition of kinases, proteases and drug-exporting membrane proteins. DARPs specifically targeting the cell surface markers (*e.g.*, HER2) also been generated and were shown to function in both *in vitro* diagnostics and *in vivo* tumor targeting. DARPs are useful because of their favorable molecular properties, including small size and high stability. The low-cost production in bacteria and the rapid generation of many target-specific DARPs make the DARP well suited for targeting moieties for essentially any desired target. Additionally, DARPs can be easily generated in multispecific formats, offering the potential to target an effector DARP to a specific organ or to target multiple receptors with one molecule composed of several DARPs.

[0227] Anticalins are another class of engineered ligand-binding proteins that are based on the lipocalin scaffold (see, *e.g.*, Schlehuber & Skerra (2005) *Expert. Opin. Biol. Ther.* 5(11): 1453-1462). The lipocalin protein architecture is characterized by a compact, rigid β -barrel that supports four structurally hypervariable loops. These loops form a pocket

for the specific complexation of differing target molecules. Natural lipocalins occur in human plasma and body fluids, where they usually function in the transport of vitamins, steroids or metabolic compounds. Using targeted mutagenesis of the loop region and biochemical selection techniques, variants with novel ligand specificities, both for low-molecular weight substances and for macromolecular protein targets, can be generated. Due to their small size, typically between 160 and 180 residues, robust tertiary structure and composition of a single polypeptide chain, such "anticalins" can provide several advantages over antibodies concerning economy of production, stability during storage, faster pharmacokinetics and better tissue penetration.

[0228] In certain embodiments the targeting moieties attached to the nanoparticles described herein comprise antibodies. In certain embodiments the antibodies are monoclonal antibodies. Such antibodies include full length immunoglobulins (*e.g.*, IgG, IgA, IgM, *etc.*) as well as antibody fragments including, but are not limited to, Fab, Fab', Fab'-SH, F(ab')₂, Fv, Fv', Fd, Fd', scFv, hsFv fragments, single-chain antibodies, cameloid antibodies, diabodies, and the like. Methods of producing such antibodies are well known to those of skill in the art. Such antibodies are commercially available (see, *e.g.*, Pacific Immunology, Ramona CA, ABClonal, Woburn, MA, *etc.*).

[0229] In certain embodiments the antibody targeting moieties can be constructed as unibodies. UniBody technology is an antibody technology that produces a stable, smaller antibody format with an anticipated longer therapeutic window than certain small antibody formats. In certain embodiments unibodies are produced from IgG4 antibodies by eliminating the hinge region of the antibody. Unlike the full size IgG4 antibody, the half molecule fragment is very stable and is termed a uniBody. Halving the IgG4 molecule leaves only one area on the UniBody that can bind to a target. Methods of producing unibodies are described in detail in PCT Publication WO2007/059782, which is incorporated herein by reference in its entirety (see, also, Kolfschoten *et al.* (2007) *Science*, 317: 1554-1557).

[0230] In certain embodiments the antibody targeting moieties can be constructed affibody molecules. Affibody molecules are class of affinity proteins based on a 58-amino acid residue protein domain, derived from one of the IgG-binding domains of staphylococcal protein A. This three helix bundle domain has been used as a scaffold for the construction of combinatorial phagemid libraries, from which affibody variants that target the desired molecules can be selected using phage display technology (see, *e.g.*, Nord *et al.* (1997) *Nat. Biotechnol.* 15: 772-777; Ronmark *et al.* (2002) *Eur. J. Biochem.*, 269: 2647-2655; and the

like). Details of Affibodies and methods of production are known to those of skill (see, *e.g.*, US Patent No 5,831,012 which is incorporated herein by reference in its entirety).

[0231] In certain embodiments the antibodies used for targeting moieties are internalizing antibodies. Methods of producing internalizing antibodies, *e.g.*, from phage display libraries, are well known to those of skill in the art (see, *e.g.*, Nielsen *et al.* (2000) *Pharmaceut. Sci. Technol. Today*, 3(8): 282-291) Zhou and Marks (2012) *Meth. Enzym.* 502: 43-66; and the like).

[0232] In certain embodiments, the cell- and/or tissue-specific binding moiety (targeting moiety) comprise a peptide that binds to cell surface markers. Peptides that bind to particular cell surface markers are well known to those of skill in the art. Illustrative, but non-limiting list of targeting peptides and their corresponding targets are shown in Table 2.

Table 2. Illustrative cell-marker specific binding peptides (see, *e.g.*, Aquilino & Arap, eds. (2009) *Tissue-Specific Vascular Endothelia signals and Vector Targeting Part A*, Elsevier San Diego, CA).

Target cell/tissue	Amino acid sequence	SEQ ID NO
B2, β 3 integrin	AGTFALRGDNPQG	13
AvB3 and α v β 5 integrin	CDCRGDCFC	14
CD13	NGRAHA	15
HUVEC cells	SIGYPLP	16
HUVEC cells	MTPFPPTSNEANLGGGS	17
Human saphenous vein and arterial SMC	EYHHYNK	18
Atherosclerotic lesions via membrane type I matrix metalloproteinase (MT1-MMP)	CNHRYMQMC	19
Brain endothelium <i>i.v.</i>	QPEHSST	20
Lung endothelium <i>i.v.</i>	VNTANST	21
Muscle <i>i.v.</i>	ASSLNIA	22
Mec1	RSNAVVP	23
PBPC	NRTWEQQ	24
Kasumi-1	NQVGWS	25
K562	EARVRPP	26
Coronary artery endothelial cells	NSSRDLG	27
HSaVEC	NDVRAVS	28
HSaVEC	NDVRSAN	29
Calu6	VTAGRAP	30
PC3	DLSNLTR	31

M07e	RGDAVGV	32
Primary PymT tumor cells	RGDLGLS	33
Coronary artery endothelial cells	PRSVTVP	34
PymT tumor tissue <i>i.v.</i>	DLGSARA	35
PymT tumor tissue <i>i.v.</i>	ESGLSQS	36
Lung tissue <i>i.v.</i>	PRSTSDP	37
Lung tissue after topic application	NSSRSLG	38
Lung tissue after topic application	MVNNFEW	39

[0233] The "targets" shown in in Table 2 are illustrative and non-limiting. Other cell-surface markers (targets) for the binding moieties attached to the nanoparticle described herein include, but are not limited to CD3, CD31, CD45, CD3, erbB2, Her2, CD22, CD74, CD19, CD20, CD33, CD40, MUC1, IL-15R, HLA-DR, EGP-1, EGP-2, G250, prostate specific membrane antigen (PSMA), prostate specific antigen (PSA), prostatic acid phosphatase (PAP), placental alkaline phosphatase, and the like. In certain embodiments, the marker comprises CD3, CD31, or CD45. Antibodies that specifically bind to these markers are readily created using standard methods known to those of skill in the art. Additionally, such antibodies are also commercially available from numerous suppliers.

10 **[0234]** In certain embodiments, the targeting moieties are utilized that specifically bind to markers present on cancer cells. Such markers are well-known to those of skill in the art and an illustrative list of such markers is shown in Table 3.

[0235] Table 3. Illustrative cancer markers and associated references, all of which are incorporated herein by reference for the purpose of identifying the referenced tumor markers.

Marker	Reference
5 alpha reductase	Délos <i>et al.</i> (1998) <i>Int J Cancer</i> , 75:6 840-846
α -fetoprotein	Esteban <i>et al.</i> (1996) <i>Tumour Biol.</i> , 17(5): 299-305
AM-1	Harada <i>et al.</i> (1996) <i>Tohoku J Exp Med.</i> , 180(3): 273-288
APC	Dihlmann <i>et al.</i> (1997) <i>Oncol Res.</i> , 9(3) 119-127
APRIL	Sordat <i>et al.</i> (1998) <i>J Exp Med.</i> , 188(6): 1185-1190
BAGE	Böel <i>et al.</i> (1995) <i>Immunity</i> , 2: 167-175.
β -catenin	Hugh <i>et al.</i> (1999) <i>Int J Cancer</i> , 82(4): 504-11
Bc12	Koty <i>et al.</i> (1999) <i>Lung Cancer</i> , 23(2): 115-127
bcr-abl (b3a2)	Verfaillie <i>et al.</i> (1996) <i>Blood</i> , 87(11): 4770-4779
CA-125	Bast <i>et al.</i> (1998) <i>Int J Biol Markers</i> , 13(4): 179-187

CASP-8/FLICE	Mandruzzato <i>et al.</i> (1997) <i>J Exp Med.</i> , 186(5): 785-793.
Cathepsins	Thomssen <i>et al.</i> (1995) <i>Clin Cancer Res.</i> , 1(7): 741-746
CD19	Scheuermann <i>et al.</i> (1995) <i>Leuk Lymphoma</i> , 18(5-6): 385-397
CD20	Knox <i>et al.</i> (1996) <i>Clin Cancer Res.</i> , 2(3): 457-470
CD21, CD23	Shubinsky <i>et al.</i> (1997) <i>Leuk Lymphoma</i> , 25(5-6): 521-530
CD22, CD38	French <i>et al.</i> (1995) <i>Br J Cancer</i> , 71(5): 986-994
CD33	Nakase <i>et al.</i> (1996) <i>Am J Clin Pathol.</i> , 105(6): 761-768
CD35	Yamakawa <i>et al.</i> <i>Cancer</i> , 73(11): 2808-2817
CD44	Naot <i>et al.</i> (1997) <i>Adv Cancer Res.</i> , 71: 241-319
CD45	Buzzi <i>et al.</i> (1992) <i>Cancer Res.</i> , 52(14): 4027-4035
CD46	Yamakawa <i>et al.</i> (1994) <i>Cancer</i> , 73(11): 2808-2817
CD5	Stein <i>et al.</i> (1991) <i>Clin Exp Immunol.</i> , 85(3): 418-423
CD52	Ginaldi <i>et al.</i> (1998) <i>Leuk Res.</i> , 22(2): 185-191
CD55	Spendlove <i>et al.</i> (1999) <i>Cancer Res.</i> , 59: 2282-2286.
CD59 (791Tgp72)	Jarvis <i>et al.</i> (1997) <i>Int J Cancer</i> , 71(6): 1049-1055
CDC27	Wang <i>et al.</i> (1999) <i>Science</i> , 284(5418): 1351-1354
CDK4	Wölfel <i>et al.</i> (1995) <i>Science</i> , 269(5228): 1281-1284
CEA	Kass <i>et al.</i> (1999) <i>Cancer Res.</i> , 59(3): 676-683
c-myc	Watson <i>et al.</i> (1991) <i>Cancer Res.</i> , 51(15): 3996-4000
Cox-2	Tsujii <i>et al.</i> (1998) <i>Cell</i> , 93: 705-716
DCC	Gotley <i>et al.</i> (1996) <i>Oncogene</i> , 13(4): 787-795
DeR3	Pitti <i>et al.</i> (1998) <i>Nature</i> , 396: 699-703
E6/E7	Steller <i>et al.</i> (1996) <i>Cancer Res.</i> , 56(21): 5087-5091
EGFR	Yang <i>et al.</i> (1999) <i>Cancer Res.</i> , 59(6): 1236-1243.
EMBP	Shiina <i>et al.</i> (1996) <i>Prostate</i> , 29(3): 169-176.
Ena78	Arenberg <i>et al.</i> (1998) <i>J. Clin. Invest.</i> , 102: 465-472.
FGF8b and FGF8a	Dorkin <i>et al.</i> (1999) <i>Oncogene</i> , 18(17): 2755-2761
FLK-1/KDR	Annie and Fong (1999) <i>Cancer Res.</i> , 59: 99-106
Folic Acid Receptor	Dixon <i>et al.</i> (1992) <i>J Biol Chem.</i> , 267(33): 24140-72414
G250	Divgi <i>et al.</i> (1998) <i>Clin Cancer Res.</i> , 4(11): 2729-2739
GAGE-Family	De Backer <i>et al.</i> (1999) <i>Cancer Res.</i> , 59(13): 3157-3165
gastrin 17	Watson <i>et al.</i> (1995) <i>Int J Cancer</i> , 61(2): 233-240

Gastrin-releasing hormone (bombesin)	Wang <i>et al.</i> (1996) <i>Int J Cancer</i> , 68(4): 528-534
GD2/GD3/GM2	Wiesner and Sweeley (1995) <i>Int J Cancer</i> , 60(3): 294-299
GnRH	Bahk <i>et al.</i> (1998) <i>Urol Res.</i> , 26(4): 259-264
GnTV	Hengstler <i>et al.</i> (1998) <i>Recent Results Cancer Res.</i> , 154: 47-85
gp100/Pmel17	Wagner <i>et al.</i> (1997) <i>Cancer Immunol Immunother.</i> , 44(4): 239-247
gp-100-in4	Kirkin <i>et al.</i> (1998) <i>APMIS</i> , 106(7): 665-679
gp15	Maeurer <i>et al.</i> (1996) <i>Melanoma Res.</i> , 6(1): 11-24
gp75/TRP-1	Lewis <i>et al.</i> (1995) <i>Semin Cancer Biol.</i> , 6(6): 321-327
hCG	Hoermann <i>et al.</i> (1992) <i>Cancer Res.</i> , 52(6): 1520-1524
Heparanase	Vlodavsky <i>et al.</i> (1999) <i>Nat Med.</i> , 5(7): 793-802
Her2/neu	Lewis <i>et al.</i> (1995) <i>Semin Cancer Biol.</i> , 6(6): 321-327
Her3	
HMTV	Kahl <i>et al.</i> (1991) <i>Br J Cancer</i> , 63(4): 534-540
Hsp70	Jaattela <i>et al.</i> (1998) <i>EMBO J.</i> , 17(21): 6124-6134
hTERT (telomerase)	Vonderheide <i>et al.</i> (1999) <i>Immunity</i> , 10: 673-679. 1999.
IGFR1	Ellis <i>et al.</i> (1998) <i>Breast Cancer Res. Treat.</i> , 52: 175-184
IL-13R	Murata <i>et al.</i> (1997) <i>Biochem Biophys Res Commun.</i> , 238(1): 90-94
iNOS	Klotz <i>et al.</i> (1998) <i>Cancer</i> , 82(10): 1897-1903
Ki 67	Gerdes <i>et al.</i> (1983) <i>Int J Cancer</i> , 31: 13-20
KIAA0205	Guéguen <i>et al.</i> (1998) <i>J Immunol.</i> , 160(12): 6188-6194
K-ras, H-ras, N-ras	Abrams <i>et al.</i> (1996) <i>Semin Oncol.</i> , 23(1): 118-134
KSA (CO17-1A)	Zhang <i>et al.</i> (1998) <i>Clin Cancer Res.</i> , 4(2): 295-302
LDLR-FUT	Caruso <i>et al.</i> (1998) <i>Oncol Rep.</i> , 5(4): 927-930
MAGE Family (MAGE1, MAGE3, etc.)	Marchand <i>et al.</i> (1999) <i>Int J Cancer</i> , 80(2): 219-230
Mammaglobin	Watson <i>et al.</i> (1999) <i>Cancer Res.</i> , 59: 13 3028-3031
MAP17	Kocher <i>et al.</i> (1996) <i>Am J Pathol.</i> , 149(2): 493-500

Melan-A/ MART-1	Lewis and Houghton (1995) <i>Semin Cancer Biol.</i> , 6(6): 321-327
mesothelin	Chang <i>et al.</i> (1996) <i>Proc. Natl. Acad. Sci., USA</i> , 93(1): 136-140
MIC A/B	Groh <i>et al.</i> (1998) <i>Science</i> , 279: 1737-1740
MT-MMP's, such as MMP2, MMP3, MMP7, MMP9	Sato and Seiki (1996) <i>J Biochem (Tokyo)</i> , 119(2): 209-215
Mox1	Candia <i>et al.</i> (1992) <i>Development</i> , 116(4): 1123-1136
Mucin, such as MUC- 1, MUC-2, MUC-3, and MUC-4	Lewis and Houghton (1995) <i>Semin Cancer Biol.</i> , 6(6): 321-327
MUM-1	Kirkin <i>et al.</i> (1998) <i>APMIS</i> , 106(7): 665-679
NY-ESO-1	Jager <i>et al.</i> (1998) <i>J. Exp. Med.</i> , 187: 265-270
Osteonectin	Graham <i>et al.</i> (1997) <i>Eur J Cancer</i> , 33(10): 1654-1660
p15	Yoshida <i>et al.</i> (1995) <i>Cancer Res.</i> , 55(13): 2756-2760
P170/MDR1	Trock <i>et al.</i> (1997) <i>J Natl Cancer Inst.</i> , 89(13): 917-931
p53	Roth <i>et al.</i> (1996) <i>Proc. Natl. Acad. Sci., USA</i> , 93(10): 4781-4786.
p97/melanotransferrin	Furukawa <i>et al.</i> (1989) <i>J Exp Med.</i> , 169(2): 585-590
PAI-1	Grøndahl-Hansen <i>et al.</i> (1993) <i>Cancer Res.</i> , 53(11): 2513-2521
PDGF	Vassbotn <i>et al.</i> (1993) <i>Mol Cell Biol.</i> , 13(7): 4066-4076
Plasminogen (uPA)	Naitoh <i>et al.</i> (1995) <i>Jpn J Cancer Res.</i> , 86(1): 48-56
PRAME	Kirkin <i>et al.</i> (1998) <i>APMIS</i> , 106(7): 665-679
Probasin	Matuo <i>et al.</i> (1985) <i>Biochem Biophys Res Commun.</i> , 130(1): 293-300
Progenipoiectin	-----
PSA	Sanda <i>et al.</i> (1999) <i>Urology</i> , 53(2): 260-266.
PSM	Kawakami <i>et al.</i> (1997) <i>Cancer Res.</i> , 57(12): 2321-2324
RAGE-1	Gaugler <i>et al.</i> (1996) <i>Immunogenetics</i> , 44(5): 323-330
Rb	Dosaka-Akita <i>et al.</i> (1997) <i>Cancer</i> , 79(7): 1329-1337
RCAS1	Sonoda <i>et al.</i> (1996) <i>Cancer</i> , 77(8): 1501-1509.
SART-1	Kikuchi <i>et al.</i> (1999) <i>Int J Cancer</i> , 81(3): 459-466
SSX gene family	Gure <i>et al.</i> (1997) <i>Int J Cancer</i> , 72(6): 965-971

STAT3	Bromberg <i>et al.</i> (1999) <i>Cell</i> , 98(3): 295-303
STn (mucin assoc.)	Sandmaier <i>et al.</i> (1999) <i>J Immunother.</i> , 22(1): 54-66
TAG-72	Kuroki <i>et al.</i> (1990) <i>Cancer Res.</i> , 50(16): 4872-4879
TGF- α	Imanishi <i>et al.</i> (1989) <i>Br J Cancer</i> , 59(5): 761-765
TGF- β	Picon <i>et al.</i> (1998) <i>Cancer Epidemiol Biomarkers Prev</i> , 7(6): 497-504
Thymosin β 15	Bao <i>et al.</i> (1996) <i>Nature Medicine</i> . 2(12), 1322-1328
IFN- α	Moradi <i>et al.</i> (1993) <i>Cancer</i> , 72(8): 2433-2440
TPA	Maulard <i>et al.</i> (1994) <i>Cancer</i> , 73(2): 394-398
TPI	Nishida <i>et al.</i> (1984) <i>Cancer Res</i> 44(8): 3324-9
TRP-2	Parkhurst <i>et al.</i> (1998) <i>Cancer Res.</i> , 58(21) 4895-4901
Tyrosinase	Kirkin <i>et al.</i> (1998) <i>APMIS</i> , 106(7): 665-679
VEGF	Hyodo <i>et al.</i> (1998) <i>Eur J Cancer</i> , 34(13): 2041-2045
ZAG	Sanchez <i>et al.</i> (1999) <i>Science</i> , 283(5409): 1914-1919
p16INK4	Quelle <i>et al.</i> (1995) <i>Oncogene</i> Aug. 17, 1995; 11(4): 635-645
Glutathione S-transferase	Hengstler (1998) <i>et al. Recent Results Cancer Res.</i> , 154: 47-85

[0236] Any of the foregoing markers can be used as targets for the targeting moieties on the nanoparticles described herein. In certain embodiments the target markers include, but are not limited to members of the epidermal growth factor family (*e.g.*, HER2, HER3, EGF, HER4), CD1, CD2, CD3, CD5, CD7, CD13, CD14, CD15, CD19, CD20, CD21, CD23, CD25, CD33, CD34, CD38, 5E10, CEA, HLA-DR, HM 1.24, HMB 45, 1a, Leu-M1, MUC1, PMSA, TAG-72, phosphatidyl serine antigen, and the like.

[0237] The foregoing targets and targeting moieties are illustrative and non-limiting. Using the teachings provided herein, nanoparticles bearing numerous other targeting moieties will be available to one of skill in the art.

Attachment of the tissue- or cell-specific binding (targeting) moieties to the nanoparticles.

[0238] Methods of coupling the nanoparticle to the tissue- or cell-specific binding moiety (targeting moiety) include covalent and non-covalent methods. Noncovalent coupling

includes, *inter alia*, simple adsorption of the tissue- or cell-specific binding moiety (targeting moiety) to the surface of the nanoparticle. Other methods of non-covalent binding include, *inter alia*, the use of biotin and avidin or streptavidin (see, *e.g.*, U.S. Patent No: US 4,885,172 A), as well as typical biotin/avidin alternatives (*e.g.*, FITC/anti-FITC (see, *e.g.*, Harmer and Samuel (1989) *J. Immunol. Meth.* 122(1): 115-221), dioxigenin/anti-dioxigenin, and the like),

[0239] In certain embodiment the tissue- or cell-specific binding moiety is covalently coupled to the nanoparticle, *e.g.*, by traditional chemical conjugation using, for example, bifunctional coupling agents such as glutaraldehyde, diimide esters, aromatic and aliphatic diisocyanates, bis-p-nitrophenyl esters of dicarboxylic acids, aromatic disulfonyl chlorides and bifunctional arylhalides such as 1,5-difluoro-2,4-dinitrobenzene; p,p'-difluoro m,m'-dinitrodiphenyl sulfone, sulfhydryl-reactive maleimides, and the like.

[0240] As noted above, in certain embodiments the tissue- or cell-specific binding moiety (targeting moiety) (*e.g.*, antibody, lectin, aptamer, anticaline, lectin, DarPIN, binding peptide) is attached to the nanoparticle directly (*e.g.*, through a functional group on the nanoparticle such as COOH, OH, *etc.*), or via a linker (linking agent). A "linker" or "linking agent" as used herein, is a molecule that is used to join two or more molecules. In certain embodiments, the linker is typically capable of forming covalent bonds to both molecule(s) (*e.g.*, the tissue- or cell-specific binding moiety (targeting moiety) and the nanoparticle). Suitable linkers are well known to those of skill in the art and include, but are not limited to, straight or branched-chain carbon linkers, heterocyclic carbon linkers, or peptide linkers

[0241] Typically the linker comprises a functional group that is reactive with a corresponding functional group on the tissue- or cell-specific binding moiety (targeting moiety) and/or nanoparticle. A bifunctional linker has one functional group reactive with a group on the tissue- or cell-specific binding moiety (targeting moiety) (*e.g.*, antibody) and another functional group reactive on the nanoparticle and can be used to form the desired conjugate. A heterobifunctional linker typically comprises two or more different reactive groups that react with sites on the tissue- or cell-specific binding moiety (targeting moiety) and on the nanoparticle, respectively. For example, a heterobifunctional crosslinker such as cysteine may comprise an amine reactive group and a thiol-reactive group can interact with an aldehyde on a derivatized peptide. Additional combinations of reactive groups suitable for heterobifunctional crosslinkers include, for example, amine- and sulfhydryl reactive groups; carbonyl and sulfhydryl reactive groups; amine and photoreactive groups; sulfhydryl and

photoreactive groups; carbonyl and photoreactive groups; carboxylate and photoreactive groups; and arginine and photoreactive groups.

[0242] Such reactions and functional groups are illustrative and non-limiting. Other illustrative suitable reactive groups include, but are not limited to thiol (-SH), carboxylate (COOH), carboxyl (-COOH), carbonyl, amine (NH₂), hydroxyl (-OH), aldehyde (-CHO), alcohol (ROH), ketone (R₂CO), active hydrogen, ester, sulfhydryl (SH), phosphate (-PO₃), or photoreactive moieties. Amine reactive groups include, but are not limited to *e.g.*, isothiocyanates, isocyanates, acyl azides, NHS esters, sulfonyl chlorides, aldehydes and glyoxals, epoxides and oxiranes, carbonates, arylating agents, imidoesters, carbodiimides, and anhydrides. Thiol-reactive groups include, but are not limited to *e.g.*, haloacetyl and alkyl halide derivatives, maleimides, aziridines, acryloyl derivatives, arylating agents, and thiol-disulfides exchange reagents. Carboxylate reactive groups include, but are not limited to *e.g.*, diazoalkanes and diazoacetyl compounds, such as carbonyldiimidazoles and carbodiimides. Hydroxyl reactive groups include, but are not limited to *e.g.*, epoxides and oxiranes, carbonyldiimidazole, oxidation with periodate, N,N'-disuccinimidyl carbonate or N-hydroxysuccinimidyl chloroformate, enzymatic oxidation, alkyl halogens, and isocyanates. Aldehyde and ketone reactive groups include, but are not limited to *e.g.*, hydrazine derivatives for schiff base formation or reduction amination. Active hydrogen reactive groups include, but are not limited to *e.g.*, diazonium derivatives for mannich condensation and iodination reactions. Photoreactive groups include, but are not limited to *e.g.*, aryl azides and halogenated aryl azides, benzophenones, diazo compounds, and diazirine derivatives.

[0243] In certain embodiments, the tissue- or cell-specific binding moiety (targeting moiety) (*e.g.*, an anti-CD3, or anti-CD31 antibody) is chemically conjugated to the nanoparticle. Means of chemically conjugating molecules are well known to those of skill. In one illustrative, but non-limiting approach, the tissue- or cell-specific binding moiety (targeting moiety) is attached to the nanoparticle via carbodiimide chemistry utilizing EDC and sulfo-NHS (*see, e.g.*, Example 1).

[0244] This procedure is illustrative, but not limiting. Numerous other methods of coupling a tissue- or cell-specific binding moiety to the nanoparticle will be available to one of skill in the art. Particular procedures for conjugating two molecules varies according to the chemical structure of the moieties to be joined. Polypeptides (*e.g.*, antibodies or peptide targeting moieties) typically contain a variety of functional groups; *e.g.*, carboxylic acid (COOH) or free amine (-NH₂) groups that are available for reaction with a suitable functional

group on the nanoparticle, functionalized nanoparticle, , or on a linker to join the molecules thereto.

[0245] For example, a common approach to the conjugation of antibodies (or other polypeptide targeting moieties), can involve the use of available lysines or reduced cysteine disulfides to form the conjugates. Lysine and cysteine as natural amino acids frequently exist in the antibodies and are readily available for reaction. For example, the thiol groups produced from reduction of cystines and primary amino group of lysines can be directly exploited. In certain illustrative, but non-limiting embodiments the primary amine in the lysines is easily reacted with *N*-hydroxysuccinimide (NHS) esters linker to form stable amide bonds and a great number of commercial linkers depend on this method. In certain embodiments, the amine of lysine can also be used to make an amidine with a pendant thiol for connection to a linker or payload via 2-imiothiolane (Traut's reagent).

[0246] In another illustrative, but non-limiting example, cysteins, as natural amino acids in the targeting moieties (*e.g.*, antibodies, targeting peptides, *etc.*) can be tethered through disulfide bridges. Under appropriate conditions, the disulfide bonds can be selectively reduced by the DL-Dithiothreitol (DTT) or Tris(2-carboxyethyl)phosphine (TCEP) and provide reactive thiol groups. The free thiol groups as attachment sites on the antibodies can be conjugated with a small linker molecule through different chemical reactions, such as Michael additions, α -halo carbonyl alkylations and disulfide formation. The hydrolyzed succinimide-thioether linker is a common useful linkage.

[0247] In certain embodiments the antibodies and/or targeting peptides can include genetically encoded unnatural amino acids to provide linkage sites. Commonly utilized unnatural amino acids in the targeting moieties can include, *inter alia*, *para*-acetyl Phe, *para*-azido Phe, propynyl-Tyr, and the like.

[0248] In certain embodiments, the linker comprises a cleavable linker. Cleavable linkers include both chemically cleavable linkers and enzymatically cleavable linkers.

[0249] A number of different chemically cleavable linkers are known to those of skill in the art (see, *e.g.*, U.S. Pat. Nos. 4,618,492; 4,542,225, and 4,625,014). Illustrative chemically cleavable linkers include, but are not limited to, acid-labile linkers, disulfide linkers, and the like. Acid-labile linkers are designed to be stable at pH levels encountered in the blood, but become unstable and degrade when the low pH environment in lysosomes is encountered. Acid-sensitive linkers include, but are not limited to hydrazones, acetals, cis-

aconitate-like amides, and silyl ethers (*see, e.g.,* Perez *et al.* (2013) *Drug Discov. Today*, 1–13). Hydrazones are easily synthesized and have a plasma half-life of 183 hours at pH 7 and 4.4 hours at pH 5, indicating that they are selectively cleavable under acidic conditions such as those found in the lysosome (*see, e.g.,* Doronina *et al.* 2013) *Nat. Biotechnol.* 21(7): 778–784).

[0250] Disulfide bridges are cleavable linkers that take advantage of the cellular reducing environment (*see, e.g.,* Saito *et al.* (2013) *Adv. Drug Deliv. Rev.* 55(2): 199-215). After internalization and degradation, disulfide bridges can release drugs in the lysosome.

[0251] Enzymatically cleavable linkers are selected to be cleaved by an enzyme (*e.g.,* a protease). Protease-cleavable linkers are typically designed to be stable in blood/plasma, but rapidly release free drug inside lysosomes in target cells upon cleavage by lysosomal enzymes. In various embodiments, they can take advantage of the high levels of protease activity inside lysosomes. The most popular enzymatic cleavage sequence is the dipeptide valine-citrulline, combined with a self-immolative linker *p*-aminobenzyl alcohol (PAB).

Cleavage of an amide-linked PAB triggers a 1,6-elimination of carbon dioxide and concomitant release of the free drug in parent amine form (*see, e.g.,* Burke *et al.* (2009) *Bioconjug. Chem.* 20(6): 1242-1250).

[0252] A library of dipeptide linkers was screened by Debowchik and co-workers to measure the rate of doxorubicin release by enzymatic hydrolysis (*see, e.g.,* Dubowchik *et al.* (2002) *Bioconjug. Chem.* 13(4): 855-869; Dubowchik *et al.* (2002) *Bioorg. Med. Chem. Lett.* 12(11): 1529-1532). They found that Phe-Lys was cleaved most rapidly with a half-life of 8 min, followed closely by Val-Lys with a half-life of 9 min. In stark contrast, Val-Cit showed a half-life of 240 min. They also found that removal of the PAB group reduced the cleavage rate, presumably through steric interference with enzyme binding.

[0253] Another study compared the potency of auristatin derivative MMAE linked by dipeptide linkers Phe-Lys and Val-Cit and an analogous hydrazone linker. The Val-Cit linker proved to be over 100 times as stable as the hydrazone linker in human plasma. Most significantly, the Phe-Lys linker was substantially less stable than Val-Cit in human plasma, which accounts for its current popularity (*see, e.g.,* Doronina *et al.* (2003) *Nat. Biotechnol.* 21(7): 778-784).

[0254] Non-peptide enzymatically cleavable linkers are also known to those of skill in the art. A glucuronide linker incorporates a hydrophilic sugar group that is cleaved by the

lysosomal enzyme beta glucuronidase. Once the sugar is cleaved from the phenolic backbone, self-immolation of the PAB group releases the conjugated moiety (*see, e.g., Jeffrey et al. (3006) Bioconjug. Chem. 17(3): 831-840*).

[0255] In certain embodiments, the linker used to join an antibody to a nanoparticle described herein comprises a protein that binds (*e.g., non-covalently binds to the antibody (e.g., to the Fc region of the antibody)*). A number of bacterial proteins are known to bind mammalian immunoglobins and include, but are not limited to, protein A, G, L, Z, and recombinant (fusion proteins) derivatives thereof (*see, e.g., Table 4; Rodrigo et al. (2015) Antibodies, 4: 259-277; Konrad et al. (2011) Bioconjug. Chem. 22: 2395-2403; Kihlberg et al. (1996) Eur. J. Biochem. 240: 556-563; Nilsson et al. (1987) Protein Eng. Des. Sel. 1: 107-113; Ghitescu et al. (1991) J. Histochem. Cytochem. 39: 1057-1065; Akerstrom & Bjorck (1986) J. Biol. Chem. 261: 10240-10247; Svensson et al. (1998) Eur. J. Biochem. 258: 890-896*).

Table 4. Illustrative proteins that can be incorporated into linkers to bind cell targeting moieties (*e.g., cell-targeting antibodies*).

Protein Used in Linkage	Reference
Protein A	Ey <i>et al.</i> (1978) <i>Immunochimistry</i> , 15: 429-436; Svensson <i>et al.</i> (1998) <i>Eur. J. Biochem.</i> 258: 890-896
Protein G	Akerstrom <i>et al.</i> (1986) <i>J. Biol. Chem.</i> 261: 10240-10247
Protein L	Rodrigo <i>et al.</i> (2015) <i>Antibodies</i> , 4: 259-277
Protein Z	Konrad <i>et al.</i> (2011) <i>Bioconjug. Chem.</i> 22: 2395-2403
Protein LG	Kihlberg <i>et al.</i> (1996) <i>Eur. J. Biochem.</i> 240: 556-563
Protein LA	Nilsson <i>et al.</i> (1987) <i>Protein Eng. Des. Sel.</i> 1: 107-113
Protein AG	Ghitescu <i>et al.</i> (1991) <i>J. Histochem. Cytochem.</i> 39: 1057-1065

[0256] A number of cyclic peptides are known that bind to antibody constant regions and can be used to link antibodies to the nanoparticle. Examples of such peptides include, but are not limited to PAM (Fassina *et al.* (2006) *J. Mol. Recognit.* 9: 564-569), D-PAM (Verdoliva *et al.* (2002) *J. Immunol. Meth.* 271: 77-88), D-PAM- θ (Dinon *et al.* (2011) *J.*

Mol. Recognit. 24: 1087-1094), TWKTSRISIF (SEQ ID NO:40) and FGRLVSSIRY (SEQ ID NO:41, Krook *et al.* (1998) *J. Immunol. Meth.* 221: 151-157), Fc-III (DeLano *et al.* (2000) *Science*, 287: 1279-1283), EPIHRSTLTALL (SEQ ID NO:42, Ehrlich *et al.* (2001) *J. Biochem. Biophys. Meth.* 49: 443-454), HWRGWV (SEQ ID NO:43, Yang *et al.* (2006) *J. Peptide Res.* 66: 110-137), HYFKFD (SEQ ID NO:44, Yang *et al.* (2009) *J. Chromatogr. A*, 1216: 910-918), HFRRHL (SEQ ID NO:45, Menegatti *et al.* (2016) *J. Chromatogr. A*, 1445: 93-104), NKFRGKYK (SEQ ID NO:46) and NARKFYKG (SEQ ID NO:47, Sugita *et al.* (2013) *Biochem. Eng. J.* 79: 33-40), KHRFNKD (SEQ ID NO:48, Yoo & Choi (2015) *BioChip J.* 10: 88-94), and the like (*see, e.g.,* Choe *et al.* (2016) *Materials*, 9: 994).

10 **[0257]** In certain embodiments the nanoparticle is linked to a tissue- or cell-specific binding moiety (antibody targeting moiety) by a linker comprising a peptide that binds to an antibody (*e.g.,* to Fc region of an antibody) at high pH, but releases the antibody at lower pH. In certain embodiments, the peptide comprises the FcB6.1 peptide (*see, e.g.,* Strauch *et al.* (2014) *Proc. Natl. Acad. Sci. USA*, 111(2): 675-680).

15 **0258]** Many procedures and linker molecules for attachment of various molecules to peptides or proteins are known (*see, e.g.,* European Patent Application No. 188,256; U.S. Patent Nos. 4,671,958, 4,659,839, 4,414,148, 4,699,784; 4,680,338; 4,569,789; and 4,589,071; and Borlinghaus *et al.* (1987) *Cancer Res.* 47: 4071-4075). Illustrative non-peptide linkers suitable for chemical conjugation are shown in Table 5.

20 Table 5. Illustrative linkers.

Linker	Linker
2-nitrobenzene or <i>O</i> -nitrobenzyl	2-[2-(tert-Butyldimethylsilyloxy)ethoxy]ethanamine
Nitropyridyl disulfide	tert-Butyl 4-hydroxybutyrate
Dioleoylphosphatidylethanolamine (DOPE)	4-(2-Chloropropionyl)phenylacetic acid
S-acetylmercaptosuccinic acid	1,11-Diamino-3,6,9-trioxaundecane
1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetracetic acid (DOTA)	di-Boc-cystamine
β -glucuronide and β -glucuronide variants	Diethylene glycol monoallyl ether
Poly(alkylacrylic acid)	3,4-Dihydro-2H-pyran-2-methanol
Benzene-based linkers (for example: 2,5-Bis(hexyloxy)-1,4-bis[2,5-bis(hexyloxy)-4-formyl-phenylenevinylene]benzene) and like molecules	4-[(2,4-Dimethoxyphenyl)(Fmoc-amino)methyl]phenoxyacetic acid

Disulfide linkages	4-(Diphenylhydroxymethyl)benzoic acid
Poly(amidoamine) or like dendrimers linking multiple target and killing peptides in one molecule	4-(Fmoc-amino)-1-butanol
Hydrazone and hydrazone variant linkers	2-(Fmoc-amino)ethanol
PEG of any chain length	2-[2-(Fmoc-amino)ethoxy]ethylamine hydrochloride
Succinate, formate, acetate butyrate, other like organic acids	2-(Fmoc-amino)ethyl bromide
Aldols, alcohols, or enols	6-(Fmoc-amino)-1-hexanol
Peroxides	5-(Fmoc-amino)-1-pentanol
alkane or alkene groups of any chain length	3-(Fmoc-amino)-1-propanol
Variants of any of the above linkers containing halogen or thiol groups	3-(Fmoc-amino)propyl bromide
Quaternary-ammonium-salt linkers	N-Fmoc-2-bromoethylamine
Allyl(4-methoxyphenyl)dimethylsilane	N-Fmoc-1,4-butanediamine hydrobromide
6-(Allyloxycarbonylamino)-1-hexanol	N-Fmoc-cadaverine hydrobromide
3-(Allyloxycarbonylamino)-1-propanol	N-Fmoc-ethylenediamine hydrobromide
4-Aminobutyraldehyde diethyl acetal	N-Fmoc-1,6-hexanediamine hydrobromide
(E)-N-(2-Aminoethyl)-4-{2-[4-(3-azidopropoxy)phenyl]diazenyl}benzamide hydrochloride	N-Fmoc-1,3-propanediamine hydrobromide
N-(2-Aminoethyl)maleimide trifluoroacetate	N-Fmoc-N''-succinyl-4,7,10-trioxa-1,13-tridecanediamine
Amino-PEG4-alkyne	(3-Formyl-1-indolyl)acetic acid
Benzyl N-(3-hydroxypropyl)carbamate	6-Guanidinoheptanoic acid
4-(Boc-amino)-1-butanol	4-Hydroxybenzyl alcohol
4-(Boc-amino)butyl bromide	N-(4-Hydroxybutyl)trifluoroacetamide
2-(Boc-amino)ethanethiol	4'-Hydroxy-2,4-dimethoxybenzophenone
2-[2-(Boc-amino)ethoxy]ethoxyacetic acid (dicyclohexylammonium) salt	N-(2-Hydroxyethyl)maleimide
2-(Boc-amino)ethyl bromide	4-[4-(1-Hydroxyethyl)-2-methoxy-5-nitrophenoxy]butyric acid
6-(Boc-amino)-1-hexanol 21-(Boc-amino)-4,7,10,13,16,19-hexaoxaheneicosanoic acid	N-(2-Hydroxyethyl)trifluoroacetamide
6-(Boc-amino)hexyl bromide	N-(6-Hydroxyhexyl)trifluoroacetamide
5-(Boc-amino)-1-pentanol	4-Hydroxy-2-methoxybenzaldehyde
3-(Boc-amino)-1-propanol	4-Hydroxy-3-methoxybenzyl alcohol
3-(Boc-amino)propyl bromide	4-(Hydroxymethyl)benzoic acid

15-(Boc-amino)-4,7,10,13-tetraoxapentadecanoic acid	4-(4-Hydroxymethyl-3-methoxyphenoxy)butyric acid
N-Boc-1,4-butanediamine	4-(Hydroxymethyl)phenoxyacetic acid
N-Boc-cadaverine	3-(4-Hydroxymethylphenoxy)propionic acid
N-Boc-ethanolamine	N-(5-Hydroxypentyl)trifluoroacetamide
N-Boc-ethylenediamine	4-(4'-Hydroxyphenylazo)benzoic acid
N-Boc-2,2'-(ethylenedioxy)diethylamine	N-(3-Hydroxypropyl)trifluoroacetamide
N-Boc-1,6-hexanediamine	2-Maleimidoethyl mesylate technical
N-Boc-1,6-hexanediamine hydrochloride	4-Mercapto-1-butanol
N-Boc-4-isothiocyanatoaniline	6-Mercapto-1-hexanol
N-Boc-4-isothiocyanatobutylamine	Phenacyl 4-(bromomethyl)phenylacetate
N-Boc-2-isothiocyanatoethylamine	4-Sulfamoylbenzoic acid
N-Boc-3-isothiocyanatopropylamine	N-Trityl-1,2-ethanediamine hydrobromide
N-Boc-N-methylethylenediamine	4-(Z-Amino)-1-butanol
N-Boc-m-phenylenediamine	6-(Z-Amino)-1-hexanol
N-Boc-p-phenylenediamine	5-(Z-Amino)-1-pentanol
2-(4-Boc-1-piperazinyl)acetic acid	N-Z-1,4-Butanediamine hydrochloride
N-Boc-1,3-propanediamine	N-Z-Ethanolamine
N-Boc-1,3-propanediamine	N-Z-Ethylenediamine hydrochloride
N-Boc-N'-succinyl-4,7,10-trioxa-1,13-tridecanediamine	N-Z-1,6-hexanediamine hydrochloride
N-Boc-4,7,10-trioxa-1,13-tridecanediamine	N-Z-1,5-pentanediamine hydrochloride
N-(4-Bromobutyl)phthalimide	N-Z-1,3-Propanediamine hydrochloride
4-Bromobutyric acid	1,4-Bis[3-(2-pyridyldithio)propionamido]butane
4-Bromobutyryl chloride purum	BMOE (bis-maleimidoethane)
4-Bromobutyryl chloride	BM(PEG)2 (1,8-bismaleimido-diethyleneglycol)
N-(2-Bromoethyl)phthalimide	BM(PEG)3 (1,11-bismaleimido-triethyleneglycol)
6-Bromo-1-hexanol	DTME (dithio-bis-maleimidoethane)
3-(Bromomethyl)benzoic acid N-succinimidylester	BMOE (bis-maleimidoethane)
4-(Bromomethyl)phenyl isothiocyanate	DTME (dithio-bis-maleimidoethane)
8-Bromo-octanoic acid	Maleimidoacetic acid N-hydroxysuccinimide ester
8-Bromo-1-octanol	4-(N-Maleimidomethyl)cyclohexanecarboxylic

	acid N-hydroxysuccinimide ester
4-(2-Bromopropionyl)phenoxyacetic acid	4-(N-Maleimidomethyl)cyclohexane-1-carboxylic acid 3-sulfo-N-hydroxysuccinimide ester
N-(3-Bromopropyl)phthalimide	4-(4-Maleimidophenyl)butyric acid N-hydroxysuccinimide ester
4-(tert-Butoxymethyl)benzoic acid	3-(Maleimido)propionic acid N-hydroxysuccinimide ester
tert-Butyl 2-(4-{[4-(3-azidopropoxy)phenyl]azo}benzamido)ethylcarbamate	

[0259] The foregoing methods of attaching a cell- or tissue-specific binding moiety to a nanoparticle described herein are illustrative and non-limiting. Using the aching provided herein numerous other attachment strategies will be available to one of skill in the art.

Pharmaceutical delivery agent

5 **[0260]** In addition to their use in Cre/lox systems, the nanoparticles described herein find use in the active targeting and nanoparticle-based delivery of therapeutic agents for many disease states. In particular, in certain embodiments, the nanoparticles described herein can greatly improve treatment of myocardial ischemia and infarction (caused by obstruction of blood flow in the coronary arteries), the disease that is the leading cause of morbidity and

10 mortality worldwide. While much advancement has been made in the treatment of myocardial ischemia and infarction of the past decades, approximately 1 million myocardial infarctions still occur in the United States annually. Many of these patients go on to develop heart failure, which now affects over 5 million patients. Potential treatments for myocardial ischemia include therapeutic agents such as vasodilators, anti-oxidants, calcium-channel

15 blockers, and anti-inflammatory agents. However, effective treatments using these drugs have been limited by the pharmacokinetics of traditional systemic drug administration. With usual oral ingestion or intravascular injection of a therapeutic agent, only a small amount of the agent reaches the desired tissue. Increasing the administered systemic dose of a drug raises the risk of undesired side effects to other organs. The nanoparticles described herein allow for

20 active targeting and delivery of therapeutic agents to only the specific tissue (such as ischemic myocardium) of interest, thereby increasing drug delivery to an effective level as well as minimizing adverse effects on organs other than the tissue of interest. Besides myocardial ischemia and infarction, the nanoparticles described herein have important applications in many other diseases where higher therapeutic index is desirable.

[0261] Accordingly, an important application of the nanoparticles described herein is in their use in methods for targeted delivery of pharmaceutical agents to desired organs or tissues of interest. One embodiment is a nanoparticle system specifically targeted to ischemic or infarcted cardiac tissue, such as during or after myocardial infarction and subsequent
5 revascularization. There is currently a large unmet need in ameliorating the ischemic reperfusion injury that results from coronary occlusion and revascularization. This injury can account for up to up to 50% of the final size of a myocardial infarct, resulting in significant morbidity and mortality to millions of patients in the United States and worldwide. Current medical therapy for treating myocardial infarction and resultant reperfusion injury has
10 reached a plateau, with numerous clinical trials over the past decade involving thousands of patients unable to demonstrate significant benefits to reperfusion injury. By delivery of therapeutic agents preferentially to the site of myocardial injury, this invention would raise the local levels of the agent, and thus likely improve its efficacy.

[0262] In certain embodiments the nanoparticles deliver a therapeutic agent to an
15 ischemic tissue (*e.g.*, damaged myocardial tissue) by passive accumulation at the target site. In certain embodiments the nanoparticle(s) bearer targeting moieties that bind macrophages, *e.g.* targeting moieties that bind CD11b. It was a surprising discovery that targeting the nanoparticles to macrophages can actually enable nanoparticle uptake and drug delivery into cardiomyocytes at a region of injury. This was an unexpected result, and, without being
20 bound to a particular theory, may be due to increase in the local concentration of nanoparticles from macrophage accumulation/processing and exocytosis into the surrounding environment. Taken together, these findings offer a roadmap for rational development of improved nanoparticle systems for delivery of therapeutic agents to areas of ischemia-reperfusion injury.

[0263] Commercial application would best involve production of the nanoparticle system by a Pharmaceutical company. In the embodiment of a nanoparticle system targeted towards ischemic myocardium, this invention would be of interest to all of the major
25 Pharmaceutical companies in the Cardiovascular space such as Pfizer, Novartis, Merck, Sanofi, Roche, and GlaxoSmithKline. In addition, this invention would be of interest to
30 Cardiovascular medical device manufacturers such as Abbott, Medtronic, and Boston Scientific who produce products for percutaneous coronary intervention, as this nanoparticulate system could be directly infused into the revascularized coronary artery after intervention.

Enabling biomedical research tool

[0264] Current techniques for temporal and cell-specific induction of DNA recombination rely on expensive and time-consuming generation of transgenic mice strains that express CreER under the control of a tissue- or cell-specific promoter. This

5 compositions and methods described herein provide a means for both temporal and tissue-specific activation of CreER-mediated nucleic acid recombination through cell- and tissue-specific delivery of the Cre activating ligand (*e.g.*, 4-hydroxy-tamoxifen) itself, without the need for tissue-specific promoter expression of CreER. By modifying various characteristics of the nanoparticles such as surface chemistry and addition of specific targeting ligands, one
10 can design a library of particles that target specific cells and tissues of interest. Each specific design of particle can then be produced in bulk and kept in storage. One could then use these particles as necessary to induce cell- and tissue-specific DNA recombination in standard readily-available transgenic mice expressing CreER under the control of a general promoter. It is anticipated that this technique could replace the current system of generating new mouse
15 lines each time cell- or tissue-specificity needed to be achieved. Instead, scientists could immediately purchase the specific nanoparticle with the desired cell specificity, saving up to a year or more in time and associated costs.

Kits.

[0265] In certain embodiments kits are provided for the practice of the methods
20 described herein. In certain embodiments the kits comprise a container contain a tissue- or cell-specific nanoparticle comprising a CreER-inducing ligand as described herein. In certain embodiments the CreER-inducing ligand comprise tamoxifen, 4-hydroxy-tamoxifen, and/or a tamoxifen analogue.

[0266] In certain embodiments the kit further comprises instructional materials
25 teaching the use of the nanoparticles to induce tissue- and/or cell-specific Cre recombination of one or more floxed sequence to produce a loss-of-function (knockout) or a gain-of-function.

[0267] While the instructional materials in the various kits typically comprise written or printed materials they are not limited to such. Any medium capable of storing such
30 instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to electronic storage media (*e.g.*, magnetic discs, tapes,

cartridges, chips), optical media (*e.g.*, CD ROM), and the like. Such media may include addresses to internet sites that provide such instructional materials.

EXAMPLES

[0268] The following examples are offered to illustrate, but not to limit the claimed invention.

Example 1

Nanoparticles for Targeted Induced Cre Recombination

[0269] Nanoparticles have long been studied as a method for targeted as well as controlled drug delivery. In order to develop a drug delivery platform for the targeted delivery of therapeutics to ischemic myocardium, we first created a reliable model to evaluate and refine nanoparticle design and performance.

[0270] Methods to directly track nanoparticle targeting and distribution rely on imaging techniques such as fluorescence microscopy, optical imaging, or magnetic resonance imaging. However, they rarely address the success of drug delivery itself. The induced Cre/*loxP* system has been a powerful tool in developmental and molecular biology through its ability to temporally control gene recombination through the administration of the drug tamoxifen, and spatially control gene recombination through selective design of mouse strains in which the CreER gene is under the control of ubiquitous vs. tissue-specific promoters.

[0271] We hypothesized that this system can be used as a reporter system for drug delivery by nanoparticles if the encapsulated drug was 4-Hydroxytamoxifen (the active form of tamoxifen), and the CreER gene left under the control of a ubiquitous promotor. In this scenario, both temporal and spatial control of gene recombination would be governed by the intracellular uptake of nanoparticles delivering the OH-Tam, resulting in expression of a reporter gene specifically if drug delivery were successful. Modifications in nanoparticle design could then be analyzed for their effects on reporter gene expression.

Materials and Methods

Materials

[0272] Poly(lactic-co-glycolic) acid (50:50) (PLGA 50:50) (M_w 7,000-17,000, Cat#719897), Poly(lactic-co-glycolic) acid (85:15) (PLGA 85:15) (M_w 50,000-75,000,

Cat#430471), Poly(vinyl alcohol) (PVA) (M_w 31,000-50,000, Cat# 363073), 4-Hydroxytamoxifen (OH-Tam) (Cat# H7904), Coumarin 6 (Cat# 442631), Sodium dodecyl sulfate (98%, Cat# 862010), Nunc Lab-Tek 2-well chamber slide system (Cat# C6682), Methanol ($\geq 99.9\%$, for HPLC, Cat# 34860), EDC (Cat# 39391), and Sulfo-NHS (Cat# 56485), were obtained from Sigma-Aldrich (St. Louis, MO).

[0273] Dichloromethane (DCM) (Cat# D1511), Dulbecco's Modified Eagle Medium w/ 4.5g/L glucose, L-glutamine & sodium pyruvate (DMEM 1X, Cellgro, Cat # 10-013-CV), fetal bovine serum (FBS, Cat# MT35015CV), 70 μ m nylon meshes (Cat# 22363548), BCA Protein Assay Kit (Cat# PI-23227), anti-CD31 Ab (clone 2H8, Cat# EN-MA3105), anti-CD3e Ab (clone 145-2C11, Cat# PIMA517655), and Gibco ACK Lysing Buffer (Cat# A10492), were obtained from Fisher Scientific (Waltham, MA).

[0274] Acetonitrile (Cat# 9017-33) was obtained from Thomas Scientific (Swedesboro, NJ).

[0275] Medium 199 (Cat# 11150-059), and AlexaFluor 647-conjugated anti-rabbit Ab (Cat# A-31573) were obtained from Life Technologies (Grand Island, NY).

[0276] Liberase TM (Cat# 05401127001) was obtained from Roche (Indianapolis, IN).

[0277] Poly(L-lactide) acid (PLA) (IV 0.90-1.20 Cat#B6002-2) was obtained from Lactel (Birmingham, AL).

[0278] Anti-CD11b Ab (Cat# 553308), and AlexaFluor 647-conjugated anti-CD11b Ab (Cat# 557686) was obtained from BD Biosciences (Franklin Lakes, NJ)

[0279] Anti-CD31 Ab (Cat# ab2364) was obtained from Abcam (Cambridge, United Kingdom).

Fabrication and characterization of 4-Hydroxytamoxifen-loaded nanoparticles

[0280] Nanoparticles encapsulating 4-Hydroxytamoxifen were created using an emulsion-solvent evaporation technique. Three different carrier polymers were used: Poly(lactic-co-glycolic) acid (PLGA) (50:50), PLGA (85:15), and Poly(L-lactide) acid (PLA). Briefly, the "oil" phase of the emulsion was prepared by mixing 50mg of the polymer of interest, 4mg of 4-Hydroxytamoxifen, and 0.5mg of Coumarin 6 in 3ml of dichloromethane for 2 hours. 2ml of this solution was then slowly poured into 8ml of cold 1% PVA (in ddH₂O) solution. This solution was then sonicated on ice using a Fisher

Scientific Model 500 ultrasonic dismembrator, with a microtip probe at 30% output for 120 seconds. The emulsion was then stirred overnight (minimum of 12 hours) with a magnetic stir-bar at 700 rpm to allow the organic solvent to evaporate. The resulting nanoparticles were washed of the PVA surfactant by 2 successive rinses in ddH₂O (solution centrifugation at 14.5k rpm for 8 minutes followed by sonication of the pellet at 10% output for 10 seconds). With the final rinse, the nanoparticles were resuspended in ddH₂O at the desired concentration, frozen at -80°C for 24 hours, and lyophilized for 48 hours with the Labconco FreeZone 4.5. As controls for some of the experiments, nanoparticles without 4-Hydroxytamoxifen were also fabricated as per the above procedure.

[0281] Lyophilized nanoparticles were imaged under scanning electron microscopy using a Nova Nano SEM 230, with low vacuum detector, 5.0 to 10.0 keV accelerating voltage, and 2.0 to 3.0 spot size. Of note, gold sputter coating was not performed as this was found in preliminary experiments to distort the structure of the nanoparticles.

[0282] To determine nanoparticle size and surface zeta-potential, lyophilized samples were suspended in solution which was placed in a cuvette (Malvern DTS0012) and inserted into a Zetasizer Nano ZS (Malvern, Worcestershire, UK) where the zeta-size, polydispersity index, and zeta-potential of triplicate samples was determined. The Zetasizer measures nanoparticle zeta-size by dynamic light scattering and zeta-potential by the electrophoretic mobility of the particles, which is then related to the potential using the Smoluchowski approximation. Numerical results are presented as a zeta-average size or a zeta-potential $\pm$ standard deviation.

[0283] 4-Hydroxytamoxifen elution from the prepared nanoparticles was measured by preparing 1 mg/ml suspensions of lyophilized nanoparticles in 0.5% SDS/PBS. At the specified time points of 1 hour, 24 hours, and 72, hours, the suspensions were centrifuged at 14.5k rpm for 8 minutes, and supernatant collected. The UV absorbance of the supernatant at 280nm was then analyzed and quantified using a Tecan Infinite 200 plate reader (Tecan, Männedorf, Switzerland). Concentration of 4-Hydroxytamoxifen in the supernatant was determined by relating the absorbance with a generated standard curve. All experimental samples were generated in triplicate.

In vitro uptake of OH-Tam-loaded nanoparticles

[0284] The *in vitro* uptake of OH-Tam-loaded nanoparticles into cells was studied by incubating nanoparticles with cultured murine dermal fibroblasts. Briefly, ear biopsies of

wild-type C57BL/6 mice were performed, cut into small pieces, and digested with Liberase Blendzyme TH and TM in Medium 199 plus DNase I and polaxamer at 37°C for 1 hour. Cells were passed through a 70 µm cell strainer and centrifuged. The cells were resuspended in culture media (DMEM with 10% FBS and 1% Pen/Strep), and plated on 2-well chamber slides. After the cells were cultured to approximately 60-70% confluence, PLGA (50:50) nanoparticles were suspended in culture media at the experimental concentrations (1 µg/ml, 10 µg/ml, 100 µg/ml, and 1000 µg/ml). For each condition, the nanoparticle/culture media suspension was incubated with the cells for either 1 hour or 12 hours. At the end of the incubation, the media was aspirated from the chamber, and the cells were fixed by first washing with cold PBS x 3, followed by cold 4% paraformaldehyde for 15 minutes, followed by repeat cold PBS rinse x 3. VectaShield DAPI mounting medium was then placed on each slide, and the slide was sealed with a coverslip. Imaging was performed under fluorescence microscopy (AF6000LX, Leica Microsystems, Wetzlar, Germany) as well as confocal microscopy (TCS SP5-STED, Leica Microsystems, Wetzlar, Germany).

In vivo biodistribution of OH-Tam-loaded nanoparticles

[0285] The *in vivo* biodistribution of OH-Tam-loaded nanoparticles was studied by intravenous injection of generated nanoparticles into C57BL/6 wild-type mice. Briefly, PLGA (50:50) nanoparticles were created as described above. The lyophilized nanoparticles were then suspended in PBS at a concentration of 1 mg/ml. The suspension was filtered through a 0.22 µm sterile filter. For each mouse, 200 µl of the nanoparticle suspension was injected in a lateral tail vein with a 29G hubless needle, and allowed to circulate for 2 hours. The mice were then euthanized by cervical dislocation after anesthesia with 5% isoflurane. For each mouse, the heart, one lung, one kidney, the liver, the spleen, and the brain were harvested. The organs were then fixed in 4% paraformaldehyde overnight at 4°C, followed by dehydration with sucrose solution for 24 hours. The organs were then mounted in OCT embedding compound, and frozen at -80°C for 48 hours. Sections of 6-8µm thick tissue were then cut with a cryostat, and mounted on standard histological slides. Imaging was then performed under fluorescence microscopy (AF6000LX, Leica Microsystems, Wetzlar, Germany) as well as confocal microscopy (TCS SP5-STED, Leica Microsystems, Wetzlar, Germany).

Generation of transgenic mice

[0286] Rosa26^{CreER};tdT^{F/F} mouse lines were obtained by crossing Rosa26-CreER mice with lineage reporter R26R-tdTomato mice. Rosa26^{CreER};R26^{VT2/GK3} mouse lines were obtained by crossing Rosa26-CreER mice with the multicolor reporter R26-“Rainbow” mice.

5 All mice were on a C57BL/6 background.

[0287] All animal experiments were performed in accordance with policies established by the UCLA Chancellor’s animal research committee as well as U.S. National Institute of Health guidelines.

OH-Tam induced CreER recombination *in vitro*

10 [0288] Induction of CreER-mediated recombination was studied *in vitro* in dermal fibroblasts from both the Rosa26^{CreER};tdT^{F/F} and the Rosa26^{CreER};R26^{VT2/GK3} transgenic mice models. Briefly, the ear biopsies from mice from each transgenic model were performed, cut into small pieces, and digested with Liberase Blendzyme TH and TM in Medium 199 plus DNase I and polaxamer at 37°C for 1 hour. Cells were passed through a 70 µm cell strainer and centrifuged. The cells were resuspended in culture media (DMEM with 10% FBS and 1% Pen/Strep), and plated on 2- well chamber slides. After the cells were cultured to approximately 60-70% confluence, PLGA (50:50) nanoparticles (containing OH-Tam and Coumarin 6 as previously described) were suspended in culture media at 100 µg/ml, and the nanoparticle/culture media suspension was incubated with the cells for 24 hours. As controls, 20 cells were also incubated with: a) no a concentration of 100nM. All experimental conditions were run in triplicate. At the end of the 24 hour incubation, the media was aspirated from the chamber, and the cells were fixed by first washing with cold PBS x 3, followed by cold 4% paraformaldehyde for 15 minutes, followed by repeat cold PBS rinse x 3. VectaShield DAPI mounting medium was then placed on each slide, and the slide was sealed with a coverslip. 25 Imaging was performed under fluorescence microscopy (AF6000LX, Leica Microsystems, Wetzlar, Germany), with 5 20x images recorded for each experimental condition. Quantification of cells in each image was performed using ImageJ image processing and analysis software (NIH). Numerical results are presented as a percentage of positive to total cells in field of view ± standard deviation. Statistical testing was performed using the 30 Student’s t-test, and statistical significance was achieved with a two-sided *P* value ≤0.05.

Serum detection of OH-Tam

[0289] To study the bloodstream release of 4-Hydroxytamoxifen from its carrier nanoparticles, serum quantification was performed using a capillary high-performance liquid chromatography/tandem mass spectrometry (HPLC-MS) method. Briefly, OH-Tam nanoparticles were generated as described above, injected (400 µg) into the lateral tail vein of wild-type C57BL/6 mice, and allowed to circulate for either 1 hour, 12 hours, and 5 days. As a control, other mice were injected with 1mg 4-Hydroxytamoxifen (dissolved in corn oil) with standard intraperitoneal technique, which was then allowed to circulate for 12 hours. At the end of the specified time points, blood from each mouse was collected through retro-orbital capillary collection. The blood was allowed to clot, and then centrifuged at 4°C for 8 minutes at 15K rpm. The serum supernatant was removed, and then further processed as follows for HPLC. 40µl of serum from each mouse was mixed with 80 µl of acetonitrile, vortexed, and then centrifuged at 14.5k for 8 minutes. The supernatant was removed, and then dried under a gentle argon stream. The resulting residue was then resuspended in 40µl of 100% methanol, vortexed for 1 minute, and centrifuged at 14.5k for 8 minutes. The supernatant was then removed, and 4-hydroxytamoxifen measured as described by Plumb *et al.* (2001) Rapid Comm. Mass Spect. 15: 297-303, with the following modifications: a 3- µl aliquot of sample was injected into a C18 analytical column, maintained at 40°C, eluted with a 5-95% B gradient over 5 minutes at 400 µl/min, where solution A was aqueous formic acid (0.3%) and solution B was acetonitrile. The column effluent was monitored by tandem mass spectrometry (MS/MS) detection of the transition m/z 388.2→71.9 using a Waters LCT Premier UPLC/MS system. The area under the curve of the peak at a retention time of 2.37 minutes was quantified, and the concentration of 4-Hydroxytamoxifen was calculated using a generated standard curve. Numerical results are presented as nanograms of OH-Tam per mL of blood ± standard deviation.

OH-Tam induced CreER recombination *in vivo*

[0290] The tissue distribution of induced CreER-mediated recombination was studied *in vivo* in both the Rosa26^{CreER};tdT^{F/F} and the Rosa26^{CreER};R26^{VT2/GK3} transgenic mice models. Briefly, OH-Tam nanoparticles were generated as described above. Nanoparticles without OH-Tam were also generated for use as a control. For each condition, 1mg (200 µg for 5 consecutive days) of nanoparticles were injected into the lateral tail vein of each mouse, for both the Rosa26^{CreER};tdT^{F/F} and the Rosa26^{CreER};R26^{VT2/GK3} transgenic mouse models, and allowed to circulate for a total of 6 days (counting from the first injection). As a control,

other mice were injected with 1mg 4-Hydroxytamoxifen (dissolved in corn oil) on day 1 with standard intraperitoneal technique, and then no more OH-Tam was given for the remainder of the 6 days. The mice were then euthanized by cervical dislocation after anesthesia with 5% isoflurane.

5 **[0291]** Heparin was injected prior to anesthesia to prevent coagulation of blood in the coronary arteries. For each mouse, the heart, one lung, one kidney, the liver, and the spleen, were harvested. The organs were then fixed in 4% paraformaldehyde overnight at 4°C, followed by dehydration with sucrose solution for 24 hours. The organs were then mounted in OCT embedding compound, and frozen at -80°C for 48 hours. Sections of 7-8 µm thick
10 tissue were then cut with a cryostat, and mounted on standard histological slides. Imaging was then performed under fluorescence microscopy (AF6000LX, Leica Microsystems, Wetzlar, Germany) as well as confocal microscopy (TCS SP5-STED, Leica Microsystems, Wetzlar, Germany).

Antibody conjugation to OH-Tam-loaded nanoparticles

15 **[0292]** To covalently conjugate antibodies onto the OH-Tam nanoparticle surface, carbodiimide chemistry utilizing EDC and sulfo-NHS. EDC was selected as it is well known to react with a carboxylic acid group (in our case the terminal end of the PLGA molecule) to form a highly reactive O-acylisourea intermediate. The addition of sulfo-NHS then leads to a
20 stable amine-reactive sulfo-NHS ester, which then reacts with primary amines on the antibody to form a stable amide bond. For this study, conjugation of anti-CD3 Ab, anti-CD11b Ab, and anti-CD31 Ab to OH-Tam nanoparticles were all performed. Briefly, OH-Tam-loaded nanoparticles were prepared as described previously. The nanoparticles were activated by suspending in MES buffer (pH 6.0) at a concentration of 2mg/ml, to which 500 µg of EDC and 500 µg of sulfo-NHS was added. This mixture was kept at room temperature
25 for 30 minutes. To remove unused reagents, 2 successive rinses in PBS were performed (solution centrifugation at 14.5k rpm for 8 minutes followed by sonication of the pellet at 10% output for 10 seconds). The final resuspension of the nanoparticles was into PBS. A control experiment was also performed in which EDC was withheld from the above steps, in order to examine antibody adsorption onto the nanoparticle surface. Antibody conjugation to
30 the activated nanoparticles was then performed by incubating 1mg of activated nanoparticles with 250 µg of the desired antibody for 6 hours. Excess antibody was then removed through 2 successive rinses in PBS were performed (solution centrifugation at 14.5k rpm for 8 minutes followed by sonication of the pellet at 10% output for 10 seconds).

Characterization of antibody-conjugated OH-Tam-loaded nanoparticles

[0293] The zeta-average size and zeta-potential of the nanoparticles were determined at multiple steps of the conjugation process of Anti-CD3 Ab to OH-Tam loaded nanoparticles: at baseline, after activation of the nanoparticles with EDC/sulfo-NHS, and after antibody conjugation. This was performed in triplicate at each condition with the Zetasizer Nano ZS as previously described.

[0294] In addition, the degree of antibody binding to nanoparticles was also studied using a protein quantitation assay. First, the antibody conjugation process was performed as described above to produce nanoparticles conjugated to Anti-cd3 Ab as well as nanoparticles conjugated to Anti-CD31. In order to determine the effect of the conjugation process itself on the degree of antibody bound to the nanoparticles, a control set of nanoparticles was created in which the entire conjugation process was followed except for the addition of EDC crosslinker.

Nanoparticles created by these processes were then tested with the BCA Protein Assay Kit (Pierce), which is a colorimetric assay based on the known reduction of Cu^{2+} to Cu^{+} by protein in a sample. Each sample was incubated with the assay working reagent for 30 minutes at 37°C in the dark. The absorbance of each sample at 560nm was measured on a Tecan Infinite 200 plate reader (Tecan, Männedorf, Switzerland). Protein amount was then determined by comparing the result with that from a generated standard curve. To calculate the degree of antibody binding to nanoparticles (ratio of number of antibodies detected to the number of nanoparticles), a theoretical antibody molecular weight of 150 kDa and a nanoparticle size of 200nm diameter (resulting in a calculation of 1.32275 E-13 moles of NP per mg) was used. All experimental samples were tested in triplicate. Numerical results are presented as number of antibody molecules per nanoparticle $\pm$ standard deviation.

In vivo targeting of anti-CD31 OH-Tam-loaded nanoparticles

Targeting of endothelial cells by anti-CD31 OH-Tam-loaded nanoparticles was studied in the Rosa26^{CreER};tdT^{F/F} transgenic mouse model. Briefly, anti-CD31-conjugated nanoparticles were prepared as described above. As a control, OH-Tam-loaded nanoparticles that did not undergo any antibody conjugation were also prepared. For each of these conditions, 500 μg of conjugated nanoparticles (250 μg for 2 consecutive days) were injected into the lateral tail vein of Rosa26^{CreER};tdT^{F/F} transgenic mice. Triplicates were performed of each condition. After 5 days, the mice were euthanized by cervical dislocation after anesthesia with 5%

isoflurane. Heparin was injected prior to anesthesia to prevent coagulation of blood in the coronary arteries. For each mouse, the heart, one lung, one kidney, the liver, and the spleen, were harvested. The organs were then fixed in 4% paraformaldehyde overnight at 4°C, followed by dehydration with sucrose solution for 24 hours. The organs were then mounted in OCT embedding compound, and frozen at -80°C for 48 hours. Sections of 7-8µm thick tissue were then cut with a cryostat, and mounted on standard histological slides.

Immunofluorescent staining on the frozen sections was performed using a primary antibody to CD31 (Abcam) and the appropriate AlexaFluor 647-conjugated secondary antibody (Life).

Imaging was then performed under fluorescence microscopy (AF6000LX, Leica

Microsystems, Wetzlar, Germany) as well as confocal microscopy (TCS SP5-STED, Leica Microsystems, Wetzlar, Germany).

In vivo targeting of anti-CD11b OH-Tam-loaded nanoparticles

[0295] Targeting of bloodstream monocytes and neutrophils by anti-CD11b OH-Tam-loaded nanoparticles was studied in the Rosa26^{CreER};tdT^{F/F} transgenic mouse model. Briefly, anti-CD11b-conjugated nanoparticles were prepared as described above. As a control, OH-Tam-loaded nanoparticles that did not undergo any antibody conjugation were also prepared. For each of these conditions, 900 µg of conjugated nanoparticles (300 µg for 3 consecutive days) were injected into the lateral tail vein of Rosa26^{CreER};tdT^{F/F} transgenic mice. Triplicates were performed of each condition. After the fourth day, the mice were injected with heparin, and blood from each mouse was collected through retro-orbital capillary collection. For each mouse, 500 µL of blood was mixed with 10 mL RBC lysing buffer at room temperature for 5 minutes. The mixture was then centrifuged at 300g for 5 minutes, the aspirate removed, and the cell pellet gently resuspended and washed with cold PBS. This was again centrifuged at 300g for 5 minutes. The pellet was resuspended in 200 µL FACS buffer containing 1:50 AlexaFluor 647-conjugated anti-CD11b Ab. The antibody was allowed to incubate for 30 minutes at room temperature in the dark, after which excess antibody was washed off by adding 3ml of PBS and centrifuging at 300g for 3 minutes. The cells were resuspended in FACS buffer for analysis. Samples were acquired on a FACS Aria Cell Sorter (BD Biosciences), and data were analyzed with FlowJo software. Numerical results are presented as a percentage of tdTomato positive cells to CD11b positive cells in the analyzed samples ± standard deviation. Statistical testing was performed using the Student's t-test, and statistical significance was achieved with a two-sided *P* value ≤0.05.

Results

Nanoparticle characterization

[0296] Nanoparticles composed of PLGA (50:50), PLGA(85:15), or PLA and encapsulating 4-Hydroxytamoxifen and Coumarin 6 were fabricated by the emulsion-solvent evaporation method. Characterization of the nanoparticles included evaluation of NP morphology, size, polydispersity, and drug elution properties. SEM (Figure 12) revealed that the NP were spherical in shape, and relatively monodisperse.

[0297] Zetasizer analysis (Figure13) showed that all three formulations resulted in particle sizes in the range of 220 to 230 nm with polydispersity index ranging from 0.05 to 0.20. Zeta potential analysis of surface charge demonstrated that all three formulations resulted in a zeta potential of approximately -30 mV, in the range that would promote stable suspensions with lower risk of aggregation.

[0298] *In vitro* drug elution testing of all three formulations (Figure 14) demonstrated 3 distinct drug release patterns. The PLGA (50:50) nanoparticle formulations resulted in a rapid “burst” release of OH-Tam within the first hour, while the majority of the encapsulated drug released within 24 hours. Conversely, PLA nanoparticles control the release of encapsulated OH-Tam such that less than 10% is released within the first hour, and further release remains similarly slow. The drug elution behavior of PLGA (85:15) nanoparticles falls between these two extremes. These results are consistent with the known behavior of these materials as agents for controlled delivery of therapeutics (*see, e.g., Makadia et al. (2011) Polymers, 3: 1377-1397*).

[0299] Based on these results, and on the desired performance criteria for our drug delivery system, it was decided to focus choose the PLGA (50:50) formulation as the material of choice for the nanoparticles. Chiefly, the requirement for the nanoparticle to quickly release its payload of OH- Tam into the cytoplasm of cells after cell uptake necessitates a drug elution curve with a quick “burst” release, such as that demonstrated by PLGA (50:50).

In vitro uptake of OH-Tam-loaded nanoparticles

[0300] In this experiment (Figure 15) examining the ability of OH-Tam-loaded PLGA(50:50) nanoparticles to be adequately endocytosed by cells, multiple NP concentrations (ranging from 1 µg/ml to 1000 µg/ml) as well as 2 different incubation time points (1 hour and 2 hours) were studied. Nanoparticle uptake was evaluated by determining

the relative level of fluorescence detected in the cell, as the encapsulated Coumarin 6 would theoretically be detectable in the GFP range. The fluorescence seen increased with nanoparticle concentration. However, it did not appear that incubation for 12 hours resulted in further nanoparticle uptake after 1 hour.

- 5 **[0301]** Cells from this study were then examined under Confocal microscopy (Figure 16), which again evaluated nanoparticle uptake into the cell by the basis of GFP signal detected. This demonstrated (most clearly seen in the z-stack cross-section) that the nanoparticles were clearly well distributed within the cells.

In vivo biodistribution of OH-Tam-loaded nanoparticles

- 10 **[0302]** This experiment focused on the examining the biodistribution of OH-Tam PLGA (50:50) nanoparticles after lateral tail vein injection into mice. There is clear accumulation of nanoparticles within the liver and spleen sections, while there is no detectable nanoparticle uptake in either the heart, liver, kidney, or brain. This is consistent with the known function of the mononuclear phagocyte system of the liver and spleen in
15 clearing the bloodstream of foreign particles.

OH-Tam induced CreER recombination *in vitro*

- [0303]** The first study (Figure 18) evaluated the ability of OH-Tam-loaded nanoparticles to induce recombination in Rosa26-CreERxtdTomato dermal fibroblasts. These nanoparticles induced recombination in the vast variety of cells in this model. The
20 second study (Figure 19) evaluated the ability of OH-Tam-loaded nanoparticles to induce recombination in a more complicated induced Cre system (Rosa26-CreERxRainbow). Again, the majority of cells in this model did undergo recombination. However, it was noted that this model perhaps might be “leaky”, as there was baseline expression of the reporter proteins (cerulean, mOrange, and mCherry) in the control samples which did not deliver any OH-
25 Tam.

Serum detection of OH-Tam

- [0304]** For the purposes of this nanoparticle application, it is imperative that there be minimal to no bloodstream release of OH-Tam prior to the nanoparticle reaching its cell destination. This would thus ensure that there would be targeted delivery to the cell of
30 interest. In addition, released free OH-Tam may induce recombination in cells which had not taken up nanoparticles, resulting in false positive results in our experiments.

[0305] To determine the degree of bloodstream release of OH-Tam from PLGA(50:50) nanoparticles, blood from mice injected with IV nanoparticles as well as IP OH-Tam was evaluated by a HPLC-MS test method as previously described. Figure 20 shows a representative tracing of a standard sample of OH-Tam at a concentration of 10 ng/mL, demonstrating that a clear signal can be obtained even down to this low level.

[0306] While a bloodstream level of 300 ng/mL (Figure 4) could be detected in mice injected with IP free 4-Hydroxytamoxifen, those mice injected with PLGA(50:50) NP encapsulating OH-Tam which was allowed to circulate for either 1 hour, 12 hours, or 5 days all had non-detectable levels of OH-Tam in their blood. This indicates that no significant bloodstream release of OH-Tam occurs when the OH-Tam is packaged in nanoparticle form.

OH-Tam induced CreER recombination *in vivo*

[0307] Once it was established that OH-Tam-loaded nanoparticles could successfully induce recombination *in vitro*, and that no bloodstream release of the encapsulated drug occurs, it was decided to proceed to evaluation of the nanoparticle formulation ability to induce CreER recombination *in vivo*. The first study (Figure 21) compared the degree and pattern of induced CreER recombination (in the CreER-tdTomato transgenic system) after standard IP injection of 4-Hydroxytamoxifen with that from IV injection of OH-Tam-loaded PLGA(50:50) nanoparticles. The second study (Figure 23) examined the same premise but using the more complicated CreER-Rainbow transgenic system. In both systems, standard intraperitoneal injection of OH-Tam resulted in recombination and expression of fluorescent reporter protein(s) in a majority of cells in every organ examined. However, with IV injection of encapsulated OH-Tam, the vast majority of the detected recombination events occurs in the liver and the spleen, with very few to no recombination events occurring in the other organs. This is consistent with known nanoparticle clearance by mononuclear phagocyte system in the liver and spleen, resulting in organ specific delivery of encapsulated OH-Tam.

[0308] To further examine the question of whether recombination occurs from direct nanoparticle delivery of OH-Tam or possibly from release of OH-Tam into the bloodstream by nanoparticles prior to uptake into a cell, confocal microscopy was performed of multiple areas of positive recombination (Figure 22). These results show that nanoparticles co-localize to cells with tdTomato reporter gene expression, further supporting the claim that

recombination is being driven by OH-Tam that has been directly carried to the cytoplasm of a cell by the nanoparticle.

Characterization of antibody-conjugated OH-Tam-loaded nanoparticles

[0309] While the liver- and spleen- specific induced recombination seen after delivery of OH-Tam-loaded nanoparticles is a direct byproduct of natural particle clearing mechanisms in the organs of the mononuclear phagocyte system, true tissue specific control of recombination would necessitate modification of the nanoparticle design to include active targeting. Three different model antibodies were chosen for straightforward bloodstream path to their targets: CD31 (endothelial cells), CD11b (monocytes/macrophages/neutrophils), and CD3 (T-cells). Antibody conjugation through carbodiimide linker chemistry to the surface of the nanoparticles was felt to be a straightforward method to endow the NP with tissue targeting capabilities. Figure 24 shows characterization of the nanoparticles during the conjugation process. It can be seen that the size of the nanoparticle steadily increases from a baseline of approximately 195nm, to approximately 242nm after antibodies have been bound to the surface. This size increase would be consistent with the averages size of an antibody. Regarding zeta potential, the initial zeta-potential measured was -24 mV. With the addition of the sulfo-NHS ester crosslinker, the zeta potential becomes much less negative, possibly due to shielding of the previously available carboxylic acid end-groups. After conjugation of the antibody, there is a return to a more negative surface potential as the surface charge becomes dependent on the specific amino acid composition of the antibody used.

[0310] Quantitation of the degree of antibody binding to the surface of a nanoparticle was also examined. The conjugation process was performed with and without the EDC crosslinker. It is apparent (Figure 25) that even in the absence of crosslinker, antibodies non-covalently bind to the surface of the nanoparticles (approximately 357 CD3 antibodies and 302 CD31 antibodies per PLGA nanoparticle). However, the addition of crosslinker almost doubles the amount of antibody bound (to 666 CD3 antibodies and 726 CD31 antibodies per PLGA nanoparticle). It should be noted that the conjugation process results in an amide bond being produced with any free amine on the antibody surface, which may potentially be in the active site of the antibody. This would then result in shielding of the active site, rendering that antibody molecule inactive. However, given the large number of antibodies calculated to be bound to each nanoparticle, it is more than likely that each nanoparticle has more than 1 active antibody able to bind to its target.

In vivo targeting of anti-CD31 OH-Tam-loaded nanoparticles

[0311] The ability for anti-CD31 OH-Tam-loaded nanoparticles to effect tissue-specific (endothelial cell) recombination was tested (Figure 26) in the CreER-tdTomato transgenic mouse model. IV injection of control nanoparticles (OH-Tam loaded nanoparticles without targeting antibody) resulted in the known pattern of recombination primarily in the liver and spleen parenchymal cells. However, with the addition of CD31 antibody to the surface of the nanoparticle, IV injection resulted in recombination in cells each different organ that appeared to resemble endothelial cells by morphology (Figure 2, panels b, d, f). This was further examined by staining the slides with CD31 antibody (abcam), and examining under Confocal microscopy (Figure 27). This revealed that the tdTomato positive cells co-localized with Coumarin 6, and indeed did stain positive for CD31, indicating successful targeting of endothelial cells by the nanoparticles.

In vivo targeting of anti-CD11b OH-Tam-loaded nanoparticles

[0312] The ability for anti-CD11b OH-Tam-loaded nanoparticles to enhance targeting to bloodstream monocytes and neutrophils was tested in the CreEr-tdTomato mouse model (Figures 19 and 20). FACS analysis for CD11b positive cells revealed that a distinct population of tdTomato positive cells did exist from the CD11b+ population. This would indicate that recombination had occurred. Blood from each mouse injected with nanoparticles was analyzed with FACS. All of the data was combined, and this revealed that the addition of CD11b antibody to the surface of the nanoparticle resulted in an almost 9-fold increase in the uptake of nanoparticles in bloodstream CD11b+ (monocytes and neutrophils) cells.

Discussion

[0313] These results demonstrate that the emulsification-solvent evaporation method can reliably and reproducibly create monodisperse formulations of PLGA nanoparticles encapsulating 4-Hydroxytamoxifen. These nanoparticles have been shown for the first time to induce gene recombination and reporter protein expression both *in vitro* and *in vivo*, in two different CreER/*loxP* systems. In addition, it was shown that the successful delivery of OH-Tam was based on direct nanoparticle uptake into the cell and release of the drug into the cytoplasm. This was confirmed with co-localization of particles with cells expressing the reporter protein, and verification that the nanoparticles do not release the OH-Tam into the bloodstream at detectable levels. Furthermore, it was demonstrated that targeting ligands

could be added to the nanoparticles to preferentially increase nanoparticle uptake into the cell and tissue types of interest.

Example 2

Nanoparticle Targeting of Ischemic Myocardium

5 **[0314]** Myocardial ischemia-reperfusion injury remains a major unmet medical need in the field of cardiology and a subject of intense therapeutic interest. Countless drugs have been studied for the alleviation of ischemia-reperfusion injury, but have always fallen short of clinical efficacy despite promising results at the pre-clinical level. While many explanations have been offered for these failures, a few of them involve technical limitations of drug
10 delivery itself that may lend themselves to alternative drug delivery technologies such as nanoparticles.

[0315] For instance, many drugs have short blood circulation or solubility, or may require such high doses to be efficacious that they result in significant systemic or off-target side effects. By packaging drugs in nanoparticle form, and engineering the characteristics of
15 the nanoparticles themselves, it is possible to increase drug circulation times, use less dose, target certain tissue, and control the release rate of the delivered drug.

[0316] In this example, we describe experimental methods and results that demonstrate the feasibility of targeted nanoparticles for enhanced accumulation and drug delivery in myocardium subjected to ischemia reperfusion injury.

20 **Materials and Methods**

Materials

[0317] AlexaFluor 647-conjugated anti-mouse Ab (Cat# A-21239), AlexaFluor 647-conjugated anti-rat Ab (Cat# A-21472), AlexaFluor 647-conjugated anti-rabbit Ab (Cat# A-31573) were obtained from Life Technologies (Grand Island, NY).

25 **[0318]** α -actinin Ab (Cat# A7811) was obtained from Sigma-Aldrich (St. Louis, MO).

[0319] CD68 Ab (Cat# MCA1957T) was obtained from AbD Serotec (Kidlington, United Kingdom).

[0320] CD11b Ab (Cat# 553308) was obtained from BD Biosciences (Franklin Lakes, NJ).

[0321] CD31 Ab (Cat# ab2364) was obtained from Abcam (Cambridge, United Kingdom).

[0322] PDGFR- α Ab (Cat# sc-338) was obtained from Santa Cruz Biotechnology (Dallas, TX).

5 [0323] All other materials used were previously delineated above.

Mouse Ischemia-reperfusion injury model

[0324] Adult mice (both male and female) approximately 12-15 weeks old were subjected to myocardial ischemia-reperfusion injury through LAD ligation and release in the following manner. Briefly, the animals were anesthetized with 1-3% isoflurane and intubated
10 through the trachea. The heart was exposed through lateral thoracotomy. Ligation of the left anterior descending artery was then performed. After 45 minutes of occlusion, the suture was released. The chest was then closed and sutured, and the animals allowed to recover on a heating pad. Per each specific study protocol, nanoparticles were injected either directly into the injury zone, or through a lateral tail vein at the specified time point. At the indicated time
15 points, the mice were then euthanized by cervical dislocation after anesthesia with 5% isoflurane. Heparin was injected prior to anesthesia to prevent coagulation of blood in the coronary arteries. The hearts were harvested, and cleaned by clamping and ligating the aorta to a 22G needle cannula, and running cold PBS solution through the cannula. The hearts were then fixed in 4% paraformaldehyde overnight at 4°C, followed by dehydration with
20 sucrose solution for 24 hours. The hearts were then mounted in OCT embedding compound (Tissue-Tek), and frozen at -80°C for 48 hours. Sections of 6-8 μ m thick tissue in a transverse orientation were then cut with a cryostat, and mounted on standard histological slides. Analysis of sections was as protocol below.

In vivo study of OH-Tam-loaded nanoparticle uptake in injured myocardium

25 [0325] The distribution and accumulation of OH-Tam-loaded nanoparticles in the injured and non-injured regions of the heart after ischemia-reperfusion injury was studied in the Rosa26^{CreER};tdT^{F/F} transgenic mouse model. Briefly, OH-Tam nanoparticles were first generated as described above. Rosa26^{CreER};tdT^{F/F} mice then underwent ischemia-reperfusion cardiac injury as described above. 400 μ g of nanoparticles were injected into the lateral tail
30 vein at either 1, 4, or 24 hours after reperfusion. As a control, 2 mice underwent direct injection of nanoparticles into the border zone of the injured region. After 7 days, the mice were euthanized by cervical dislocation after anesthesia with 5% isoflurane. Heparin was

injected prior to anesthesia to prevent coagulation of blood in the coronary arteries. For each mouse, the heart was harvested. The heart was then fixed in 4% paraformaldehyde overnight at 4°C, followed by dehydration with sucrose solution for 24 hours. The organs were then mounted in OCT embedding compound, and frozen at -80°C for 48 hours. Sections of 6-8µm thick tissue were then cut in a transverse orientation with a cryostat, and mounted on standard histological slides. Imaging was then performed under fluorescence microscopy (AF6000LX, Leica Microsystems, Wetzlar, Germany)

In vivo study of anti-CD11b OH-Tam-loaded nanoparticle targeting of ischemic myocardium

[0326] Targeting of ischemia by anti-CD11b OH-Tam-loaded nanoparticles was studied in the Rosa26^{CreER};tdT^{F/F} transgenic mouse model. Briefly, anti-CD11b-conjugated nanoparticles were prepared as described above. As a control, OH-Tam-loaded nanoparticles that did not undergo any antibody conjugation were also prepared. Rosa26^{CreER};tdT^{F/F} mice then underwent ischemia- reperfusion cardiac injury as described above. For each of these conditions, 900 µg of conjugated nanoparticles (300 µg for 3 consecutive days) were injected into the lateral tail vein of the mice. After the fourth day, the mice were then euthanized by cervical dislocation after anesthesia with 5% isoflurane. Heparin was injected prior to anesthesia to prevent coagulation of blood in the coronary arteries. For each mouse, the heart, one lung, one kidney, the liver, and the spleen, were harvested. The organs were then fixed in 4% paraformaldehyde overnight at 4°C, followed by dehydration with sucrose solution for 24 hours. The organs were then mounted in OCT embedding compound, and frozen at -80°C for 48 hours. Sections of 7-8µm thick tissue were then cut with a cryostat, and mounted on standard histological slides. Immunofluorescent staining on the frozen sections was performed using a primary antibody to CD68, CD31, PDGFR-α, α-actinin, and the appropriate AlexaFluor 647-conjugated secondary antibody (Life). Imaging was then performed under fluorescence microscopy (AF6000LX, Leica Microsystems, Wetzlar, Germany) as well as confocal microscopy (TCS SP5-STED, Leica Microsystems, Wetzlar, Germany).

Results.

In vivo study of OH-Tam-loaded nanoparticle uptake in injured myocardium

[0327] The ability to distinguish areas of injury was first assessed by subjecting a mouse to LAD ligation ischemia-reperfusion injury, followed by cross-sectioning of the heart

at the area of LAD ligation (Figure 30). It is readily apparent from fluorescent microscopy that areas of injury lose their natural auto-fluorescence and thus are easily identified. This gives us then the ability to separate study and analyze the regions of injury in comparison to control regions.

5 [0328] The accumulation of OH-Tam-loaded PLGA(50:50) in areas of ischemia-reperfusion injury was then investigated in the CreER-tdTomato transgenic mouse model (Figure 31). After tail vein injection of the nanoparticles in mice that had been subjected to LAD ligation and release, the areas of ischemia-reperfusion injury were analyzed under fluorescence microscopy for tdTomato expression, and compared to control regions in the heart that did not show signs of injury. It can be seen that many positive cells are present in the areas of injury, whereas no positive cells can be seen in the control non-injured regions. This would indicate that more nanoparticle uptake and accumulation occurred in the areas of ischemia-reperfusion injury, resulting in more cell recombination and expression of tdTomato.

15 **In vivo study of anti-CD11b OH-Tam-loaded nanoparticle targeting of ischemic myocardium**

[0329] The ability to further increase nanoparticle drug delivery to regions of cardiac ischemic injury through attachment of a targeting ligand was investigated using anti-CD11b PLGA(50:50) OH-Tam-loaded nanoparticles in the Rosa26CreER-tdTomato transgenic mouse model (Figure 32).

[0330] It was hypothesized that the accumulation of macrophages and neutrophils as part of the standard inflammatory response to ischemia-reperfusion injury would provide a natural epitope-rich target for the nanoparticles. In targeting circulating monocytes and neutrophils (in addition to macrophages already present in the injury region), the nanoparticles could potentially be picked up by cells while in the bloodstream, and carried to the site of injury by the inflammatory cell.

[0331] Figure 33, panel a shows a cross section under fluorescence microscopy. Even under low magnification it is apparent that the injured region contains a great deal of positive recombined cells. These cells, specifically in the border region with uninjured tissue, were analyzed under Confocal microscopy. This revealed that the tdTomato positive cells (Figure 32, panel c) co-localized with Coumarin 6 (Figure 32, panel b), and stained positive for CD68

(Figure 32, panel d), indicating successful targeting of macrophages/monocytes by the nanoparticles.

[0332] In another cross section (Figure 33), there appeared to be a large cluster of cells that on fluorescence microscopy resembled cardiomyocytes rather than macrophages.

- 5 This was further analyzed on Confocal microscopy, and confirmed to be cardiomyocytes by staining for cardiomyocyte marker α -actinin (Figure 33, panel d). In addition, these cells also showed co-localization of Coumarin 6 (Figure 33, panel c), indicating that the nanoparticles, while targeted to macrophages, actually did eventually reach the cardiac parenchymal tissue.

Discussion

- 10 **[0333]** These results demonstrate for the first time that nanoparticle accumulation and drug delivery to cardiac tissue (of OH-Tam, as evidenced by reporter protein expression) is increased in regions of ischemia-reperfusion injury. This may be a result of increased nanoparticle extravasation into the sites of injury due to damage of the capillary endothelium, a known pathological complication of ischemia-reperfusion injury. In addition, it was shown
- 15 for the first time that nanoparticle accumulation and drug delivery to cardiac tissue can be increased by targeting the nanoparticles to macrophages, a cell type that is well known to accumulate in regions of inflammation and injury. Furthermore, it was shown that targeting the nanoparticles to macrophages can actually enable nanoparticle uptake and drug delivery into cardiomyocytes at the region of injury. This was an unexpected result, and may be due
- 20 to increase in the local concentration of nanoparticles from macrophage accumulation/processing and exocytosis into the surrounding environment. Taken together, these findings offer a roadmap for rational development of improved nanoparticle systems for delivery of therapeutic agents to areas of ischemia-reperfusion injury.

- [0334]** It is understood that the examples and embodiments described herein are for
- 25 illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

30

CLAIMS

What is claimed is:

1. A method for conditional and target-specific recombination, said method comprising:
 - 5 providing a mammal comprising cells that express an ER-ligand-inducible CRE recombinase under the control of a promoter, and that comprises a nucleic acid sequence flanked by a pair of *loxP* sequences; and
 - administering to said mammal biodegradable polymer nanoparticles containing an estrogen receptor ligand where said nanoparticles provide specific delivery to a
 - 10 target tissue of said estrogen receptor ligand thereby activating said CRE recombinase in said target tissue which performs target specific recombination of the nucleic acid between the *loxP* sequences in said target tissue.
2. The method of claim 1, wherein said mammal comprises cells that express an ER-ligand-inducible CRE recombinase under the control of a non-tissue specific
- 15 promoter.
3. The method according to any one of claims 1-2, wherein said *loxP* sequences are inverted in orientation with respect to each other.
4. The method according to any one of claims 1-2, wherein said *loxP* sequences are in the same orientation with respect to each other.
- 20 5. The method according to any one of claims 1-4, wherein said nanoparticles comprise a polymer selected from the group consisting of Poly-D,L-lactide-co-glycolide (PLGA), Poly(L-lactic acid) (PLA), Poly-ε-caprolactone (PCL), chitosan, and gelatin.
6. The method of claim 5, wherein said nanoparticles comprise Poly-D,L-lactide-co-glycolide (PLGA).
- 25 7. The method of claim 6, wherein said nanoparticles comprise Poly-D,L-lactide-co-glycolide (PLGA) lactide:glycolide 50:50 (50:50 PLGA).

8. The method of claim 6, wherein said nanoparticles comprise Poly-D,L-lactide-co-glycolide (PLGA) lactide:glycolide 85:15 (85:15 PLGA).

9. The method of claim 5, wherein said nanoparticles comprise poly(L-lactic acid).

5 10. The method according to any one of claims 1-9, wherein said nanoparticles range in size from about 50 nm, or from about 100 nm, or from about 150 nm, up to about 500 nm, or up to about 400 nm, or up to about 300 nm, or up to about 200 nm.

11. The method of claim 10, wherein said nanoparticles range in size from about 150 nm up to about 200 nm.

10 12. The method according to any one of claims 1-11, wherein the zeta potential of said nanoparticles is at least about -10 mV, or at least about -15 mV, or at least about -20 mV, or at least about 24 mV.

15 13. The method according to any one of claims 1-12, wherein said estrogen receptor ligand is selected from the group consisting of tamoxifen, 4-hydroxy-tamoxifen, and a tamoxifen analogue.

14. The method of claim 13, wherein said estrogen receptor ligand is ICI 182,780.

15. The method according to any one of claims 1-14, wherein tissue specificity of said nanoparticles is by preferential passive accumulation in a target tissue.

20 16. The method of claim 15, wherein tissue specificity of said nanoparticles is by preferential passive accumulation in myocardium with damaged endothelium.

25 17. The method according to any one of claims 1-14, wherein tissue specificity of said nanoparticles is provided by tissue-specific binding moieties disposed on the surface of said nanoparticles.

18. The method of claim 17, wherein said tissue-specific binding moieties comprise targeting peptides.

19. The method of claim 18, wherein said targeting peptides comprise a peptide having an amino acid sequence selected from the group consisting of erythropoietin sequence, AGTFALRGDNPQG (SEQ ID NO:13), CDCRGDCFC (SEQ ID NO:14), NGRAHA (SEQ ID NO:15), SIGYPLP (SEQ ID NO:16), MTPFPTSNEANLGGGS (SEQ ID NO:17), EYHHYNK (SEQ ID NO:18), CNHRYMQMC (SEQ ID NO:19), QPEHSST (SEQ ID NO:20), VNTANST (SEQ ID NO:21), ASSLNIA (SEQ ID NO:22), RSNVAVP (SEQ ID NO:23), NRTWEQQ (SEQ ID NO:24), NQVGWS (SEQ ID NO:25), EARVRPP (SEQ ID NO:26), NSSRDLG (SEQ ID NO:27), NDVRAVS (SEQ ID NO:28), NDVRSAN (SEQ ID NO:29), VTAGRAP (SEQ ID NO:30), DLSNLTR (SEQ ID NO:31), RGDVGV (SEQ ID NO:32), RGDGLS (SEQ ID NO:33), PRSVTV (SEQ ID NO:34), DLGSARA (SEQ ID NO:35), ESGLSQS (SEQ ID NO:36), PRSTSDP (SEQ ID NO:37), NSSRSLG (SEQ ID NO:38), and MVNNFEW (SEQ ID NO:39).

20. The method of claim 17, wherein said tissue-specific binding moieties comprise antibodies.

21. The method of claim 20, wherein said tissue-specific binding moiety comprises an antibody that specifically binds a target selected from the group consisting of CD3, CD31, CD11b CD45, CD3, erbB2, Her2, CD22, CD74, CD19, CD20, CD33, CD40, MUC1, IL-15R, HLA-DR, EGP-1, EGP-2, G250, prostate specific membrane antigen (PSMA), prostate specific antigen (PSA), prostatic acid phosphatase (PAP), and placental alkaline phosphatase.

22. The method of claim 21, wherein said tissue-specific binding moiety comprises an anti-CD3 antibody.

23. The method of claim 21, wherein said tissue-specific binding moiety comprises an anti-CD11b antibody.

24. The method of claim 21, wherein said tissue-specific binding moiety comprises an anti-CD31 antibody.

25. The method of claim 20, wherein said tissue-specific binding moiety comprises an antibody that specifically binds a target that is a cancer marker selected from the cancer markers shown in Table 3.

26. The method according to any one of claims 20-25, wherein said antibody is a full-length immunoglobulin.

27. The method according to any one of claims 20-25, wherein said antibody is selected from the group consisting of Fv, Fab, (Fab')₂, (Fab')₃, IgGΔCH2, a unibody, and a minibody.

28. The method according to any one of claims 20-25, wherein said antibody is a single chain antibody.

29. The method of claim 28 wherein said antibody is an scFv.

30. The method according to any one of claims 20-29, wherein said antibody is a human antibody.

31. The method according to any one of claims 17-30, wherein said tissue-specific binding moiety is adsorbed onto the surface of said nanoparticle.

32. The method according to any one of claims 17-30, wherein said tissue-specific binding moiety is attached to said nanoparticle by a non-covalent interaction.

33. The method of claim 32, wherein said non-covalent interaction comprises a biotin/avidin interaction.

34. The method of claim 32, wherein said tissue-specific binding moiety is an antibody and said non-covalent interaction comprises an interaction between an antibody-binding peptide and said antibody.

35. The method of claims 34, wherein said non-covalent interaction comprises an interaction between said antibody and a protein selected from the group consisting of Protein A, Protein G, Protein L, Protein Z, Protein LG, Protein LA, and Protein AG.

36. The method of claim 34, wherein said non-covalent interaction comprises an interaction between said an antibody and a moiety selected from the group consisting of PAM, D-PAM, D-PAM-θ, TWKTSRISIF (SEQ ID NO:40), FGRLVSSIRY (SEQ ID NO:41, Fc-III, EPIHRSTLTALL (SEQ ID NO:42), HWRGWV (SEQ ID NO:43),

HYFKFD (SEQ ID NO:44), HFRRHL (SEQ ID NO:45), NKFRGKYK (SEQ ID NO:46), NARKFYKG (SEQ ID NO:47), KHRFNKD (SEQ ID NO:48).

37. The method of claim 34, wherein said non-covalent interaction comprises an interaction between said tissue-specific binding moiety and FcB6.1 peptide.

5 38. The method according to any one of claims 17-30, wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle.

39. The method of claim 38, wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle via a non-cleavable linker.

10 40. The method of claim 39 wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle via carbodiimide chemistry utilizing EDC and sulfo-NHS.

41. The method of claim 38, wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle via a cleavable linker.

15 42. The method of claim 38, wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle via a cleavable linker comprising a disulfide linker or an acid-labile linker.

20 43. The method of claim 38, wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle via an acid label linker comprising a moiety selected from the group consisting of a hydrazone, an acetal, a cis-aconitate-like amide, a silyl ether.

44. The method of claim 38, wherein said tissue-specific binding moiety is chemically conjugated to said nanoparticle via a non-amino acid, non-peptide linker shown in Table 5.

25 45. The method according to any one of claims 1-44, wherein said mammal comprises cells that express an ER-ligand-inducible CRE recombinase under the control of a constitutive promoter.

46. The method according to any one of claims 1-44, wherein said mammal comprises cells that express an ER-ligand-inducible CRE recombinase under the control of an inducible promoter.

47. The method according to any one of claims 1-44, wherein said
5 mammal comprises cells that express an ER-ligand-inducible CRE recombinase under the control of a CMV, Rosa26, or β -actin promoter.

48. The method according to any one of claims 1-47, wherein said inducible Cre recombinase comprises CreER^{T2}.

49. The method according to any one of claims 1-47, wherein said
10 inducible Cre recombinase comprises an iCre.

50. The method according to any one of claims 1-49, wherein said *loxP* sites comprise at least 2 pairs of *loxP* sites.

51. The method according to any one of claims 1-49, wherein said *loxP* sites comprise at least 3 pairs of *loxP* sites.

52. The method according to any one of claims 1-51, wherein said *loxP*
15 site(s) comprise a wild-type *loxP* site.

53. The method according to any one of claims 1-52, wherein said *loxP* site(s) comprise a *loxP* variant selected from the group consisting of lox 511, lox 5171, lox 2272, M2, M3, M7, M11, lox 71, and lox 66.

54. The method according to any one of claims 1-53, wherein said nucleic
20 acid flanked by said pair of *loxP* sequences comprises a gene or a portion of a gene and said recombination inhibits or blocks expression of a functional product from said gene.

55. The method according to any one of claims 1-53, wherein said nucleic
acid flanked by said pair of *loxP* sequences comprises a transcriptional stop element where
25 said stop element is operably linked to a gene and said recombination removes said stop element allowing expression said gene.

56. The method of claim 55, wherein said gene is an oncogene.

57. The method of claim 56, wherein said gene is *K-ras*.

58. The method according to any one of claims 1-57, wherein said mammal is a non-human mammal.

59. The method of claim 58, wherein said mammal is a rat or mouse.

5 60. A transgenic murine, said murine comprising cells that express an ER-ligand-inducible CRE recombinase under the control of a non-tissue specific promoter.

61. The transgenic murine of claim 60, wherein said promoter is a constitutive promoter.

10 62. The transgenic murine of claim 60, wherein said promoter is an inducible promoter.

63. The transgenic murine of claim 60, wherein said promoter is a CMV, Rosa26, or β -actin promoter.

64. The transgenic murine according to any one of claims 60-63, wherein said inducible Cre recombinase comprises CreER^{T2}.

15 65. The transgenic murine according to any one of claims 60-63, wherein said inducible Cre recombinase comprises an iCre.

66. The transgenic murine according to any one of claims 60-65, wherein said murine comprises a nucleic acid sequence flanked by a pair of *loxP* sequences.

20 67. The transgenic murine of claim 66, wherein said *loxP* sites comprise at least 2 pairs of *loxP* sites.

68. The transgenic murine of claim 66, wherein said *loxP* sites comprise at least 3 pairs of *loxP* sites.

69. The transgenic murine according to any one of claims 66-68, wherein said *loxP* site(s) comprise a wild-type *loxP* site.

70. The transgenic murine according to any one of claims 66-69, wherein said *loxP* site(s) comprise a *loxP* variant selected from the group consisting of lox 511, lox 5171, lox 2272, M2, M3, M7, M11, lox 71, and lox 66.

5 71. The transgenic murine according to any one of claims 66-70, wherein said nucleic acid flanked by said pair of *loxP* sequences comprises a gene or a portion of a gene and said recombination inhibits or blocks expression of a functional product from said gene.

10 72. The transgenic murine according to any one of claims 66-71, wherein said nucleic acid flanked by said pair of *loxP* sequences comprises a transcriptional stop element where said stop element is operably linked to a gene and said recombination removes said stop element allowing expression said gene.

73. The transgenic murine of claim 72, wherein said gene is an oncogene.

74. The transgenic murine of claim 73, wherein said gene is *K-ras*.

15 75. A nanoparticle for cell- or tissue-specific or preferential delivery of a pharmaceutical, said nanoparticle comprising a biodegradable polymer containing said pharmaceutical.

76. The nanoparticle of claim 75, wherein said nanoparticle comprises a polymer selected from the group consisting of Poly-D,L-lactide-co-glycolide (PLGA), Poly(L-lactic acid) (PLA), Poly-ε-caprolactone (PCL), chitosan, and gelatin.

20 77. The nanoparticle of claim 76, wherein said nanoparticles comprise Poly-D,L-lactide-co-glycolide (PLGA).

78. The nanoparticle of claim 77, wherein said nanoparticles comprise Poly-D,L-lactide-co-glycolide (PLGA) lactide:glycolide 50:50 (50:50 PLGA).

25 79. The nanoparticle of claim 77, wherein said nanoparticles comprise Poly-D,L-lactide-co-glycolide (PLGA) lactide:glycolide 85:15 (85:15 PLGA).

80. The nanoparticle of claim 76, wherein said nanoparticles comprise poly(L-lactic acid).

81. The nanoparticle according to any one of claims 75-80, wherein said nanoparticle ranges in size from about 50 nm, or from about 100 nm, or from about 150 nm, up to about 500 nm, or up to about 400 nm, or up to about 300 nm, or up to about 200 nm.

82. The nanoparticle of claim 81, wherein said nanoparticle ranges in size
5 from about 150 nm up to about 200 nm.

83. The nanoparticle according to any one of claims 75-82, wherein the zeta potential of said nanoparticle is at least about -10 mV, or at least about -15 mV, or at least about -20 mV, or at least about 24 mV.

84. The nanoparticle according to any one of claims 75-83, wherein tissue
10 specificity of said nanoparticles is by preferential passive accumulation in a target tissue.

85. The nanoparticle of claim 84, wherein tissue specificity of said nanoparticles is by preferential passive accumulation in myocardium with damaged endothelium.

86. The nanoparticle according to any one of claims 75-83, wherein tissue
15 specificity of said nanoparticles is provided by tissue-specific binding moieties disposed on the surface of said nanoparticles.

87. The nanoparticle of claim 86, wherein said tissue-specific binding moieties comprise targeting peptides.

88. The nanoparticle of claim 87, wherein said targeting peptides comprise
20 a peptide having an amino acid sequence selected from the group consisting of erythropoietin sequence, AGTFALRGDNPQG, CDCRGDCFC, NGRAHA, SIGYPLP, MTPFPTSNEANLGGGS, EYHHYNK, CNHRYMQMC, QPEHSST, VNTANST, ASSLNIA, RSNVVP, NRTWEQQ, NQVGWS, EARVRPP, NSSRDLG, NDVRAVS, NDVRSAN, VTAGRAP, DLSNLTR, RGDVGV, RGDGLS, PRSVTP, DLGSARA,
25 ESGLSQS, PRSTSDP, NSSRSLG, and MVNNFEW.

89. The nanoparticle of claim 86, wherein said tissue-specific binding moieties comprise antibodies.

90. The nanoparticle of claim 89, wherein said tissue-specific binding moiety comprises an antibody that specifically binds a target selected from the group consisting of CD3, CD11b, CD31, CD45, CD3, erbB2, Her2, CD22, CD74, CD19, CD20, CD33, CD40, MUC1, IL-15R, HLA-DR, EGP-1, EGP-2, G250, prostate specific membrane antigen (PSMA), prostate specific antigen (PSA), prostatic acid phosphatase (PAP), and placental alkaline phosphatase.

91. The nanoparticle of claim 90, wherein said tissue-specific binding moiety comprises an anti-CD3 antibody.

92. The nanoparticle of claim 90, wherein said tissue-specific binding moiety comprises an anti-CD11b antibody.

93. The nanoparticle of claim 90, wherein said tissue-specific binding moiety comprises an anti-CD31 antibody.

94. The nanoparticle of claim 89, wherein said tissue-specific binding moiety comprises an antibody that specifically binds a target that is a cancer marker selected from the cancer markers shown in Table 3.

95. The nanoparticle according to any one of claims 89-94, wherein said antibody is a full-length immunoglobulin.

96. The nanoparticle according to any one of claims 89-94, wherein said antibody is selected from the group consisting of Fv, Fab, (Fab')₂, (Fab')₃, IgGΔCH2, a unibody, and a minibody.

97. The nanoparticle according to any one of claims 89-94, wherein said antibody is a single chain antibody.

98. The nanoparticle of claim 97, wherein said antibody is an scFv.

99. The nanoparticle according to any one of claims 89-98, wherein said antibody is a human antibody.

100. The nanoparticle according to any one of claims 75-99, wherein pharmaceutical comprise an estrogen receptor ligand.

101. The nanoparticle of claim 100, wherein said pharmaceutical comprises an estrogen receptor ligand selected from the group consisting of tamoxifen, 4-hydroxy-tamoxifen, and a tamoxifen analogue.

102. The nanoparticle of claim 101, wherein said estrogen receptor ligand
5 tamoxifen analog is ICI 182,780.

103. A method of delivering a pharmaceutical to a region of injury *in vivo*, said method comprising:

administering to a subject in need thereof a nanoparticle comprising a biodegradable polymer containing said pharmaceutical, wherein said nanoparticle selectively
10 or preferentially accumulates in said region of injury.

104. The method of claim 103, wherein said nanoparticle comprises a polymer selected from the group consisting of Poly-D,L-lactide-co-glycolide (PLGA), Poly(L-lactic acid) (PLA), Poly-ε-caprolactone (PCL), chitosan, and gelatin.

105. The method of claim 104, wherein said nanoparticle comprises Poly-
15 D,L-lactide-co-glycolide (PLGA).

106. The method of claim 105, wherein said nanoparticle comprises Poly-D,L-lactide-co-glycolide (PLGA) lactide:glycolide 50:50 (50:50 PLGA).

107. The method of claim 105, wherein said nanoparticle comprises Poly-D,L-lactide-co-glycolide (PLGA) lactide:glycolide 85:15 (85:15 PLGA).

20 108. The method of claim 104, wherein said nanoparticle comprise poly(L-lactic acid).

109. The method according to any one of claims 103-108, wherein said nanoparticle ranges in size from about 50 nm, or from about 100 nm, or from about 150 nm, up to about 500 nm, or up to about 400 nm, or up to about 300 nm, or up to about 200 nm.

25 110. The method of claim 109, wherein said nanoparticle ranges in size from about 150 nm up to about 200 nm.

111. The method according to any one of claims 103-110, wherein the zeta potential of said nanoparticle is at least about -10 mV, or at least about -15 mV, or at least about -20 mV, or at least about 24 mV.

112. The method according to any one of claims 103-111, wherein tissue
5 specificity of said nanoparticle is by preferential passive accumulation in a target tissue.

113. The method of claim 112, wherein tissue specificity of said nanoparticle is by preferential passive accumulation in myocardium with damaged endothelium.

114. The method according to any one of claims 103-111, wherein tissue
10 specificity of said nanoparticle is provided by tissue-specific binding moieties disposed on the surface of said nanoparticle.

115. The method of claim 114, wherein said binding moieties selected from the group consisting of an antibody, a DNA aptamer, an RNA aptamer, a peptide aptamer, a cell-binding peptide, an anticalin, a lectin, and a DARPIN.

116. The method according to any one of claims 114-115, wherein said
15 tissue-specific binding moieties specifically or preferentially bind to markers on macrophages.

117. The method of claim 116, wherein said tissue specific binding moieties bind to CD11b.

118. The method according to any one of claims 114-117, wherein said
20 tissue-specific binding moieties comprise antibodies.

119. The method of claim 118, wherein said antibody is a full-length immunoglobulin.

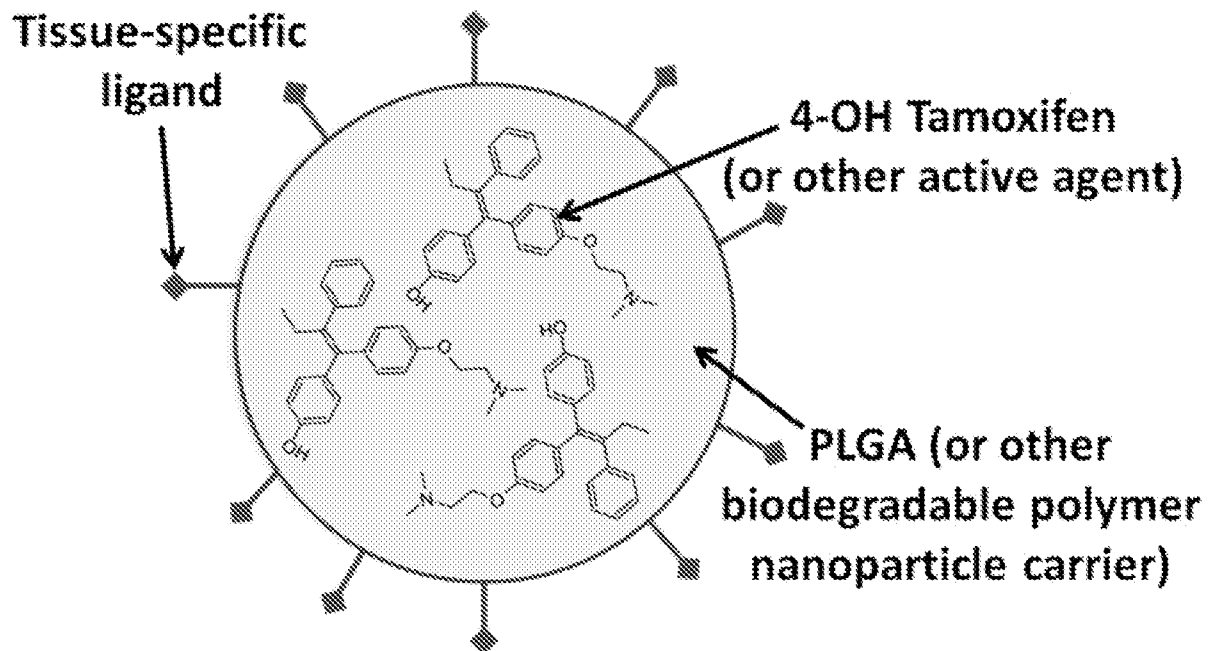
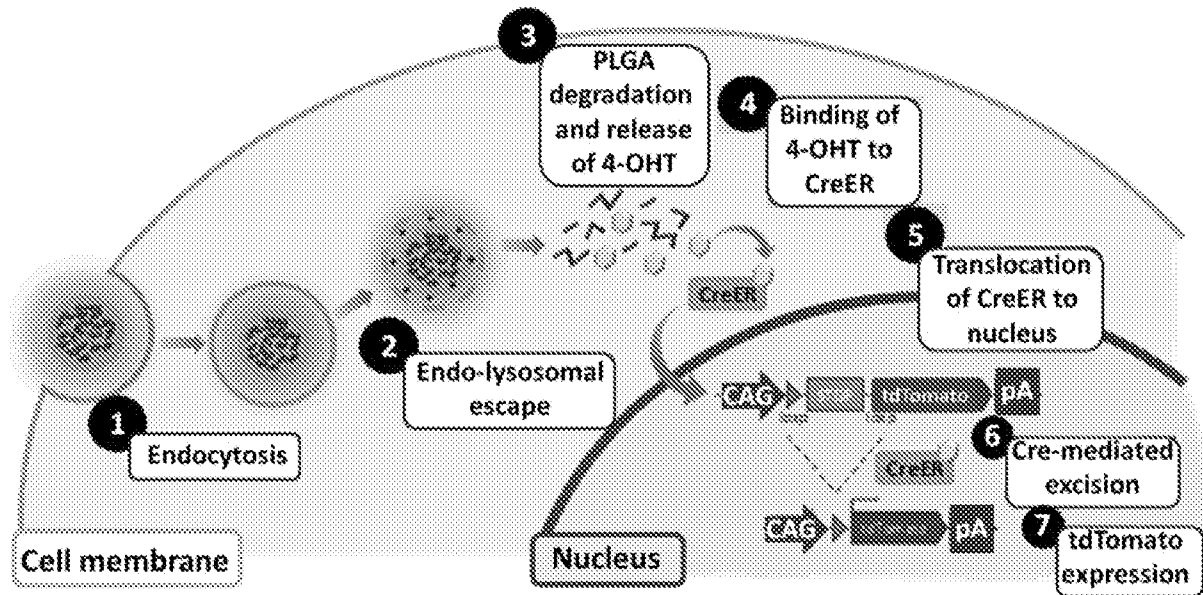
120. The method of claim 118 wherein said antibody is selected from the
25 group consisting of Fv, Fab, (Fab')₂, (Fab')₃, IgGΔCH2, a unibody, and a minibody.

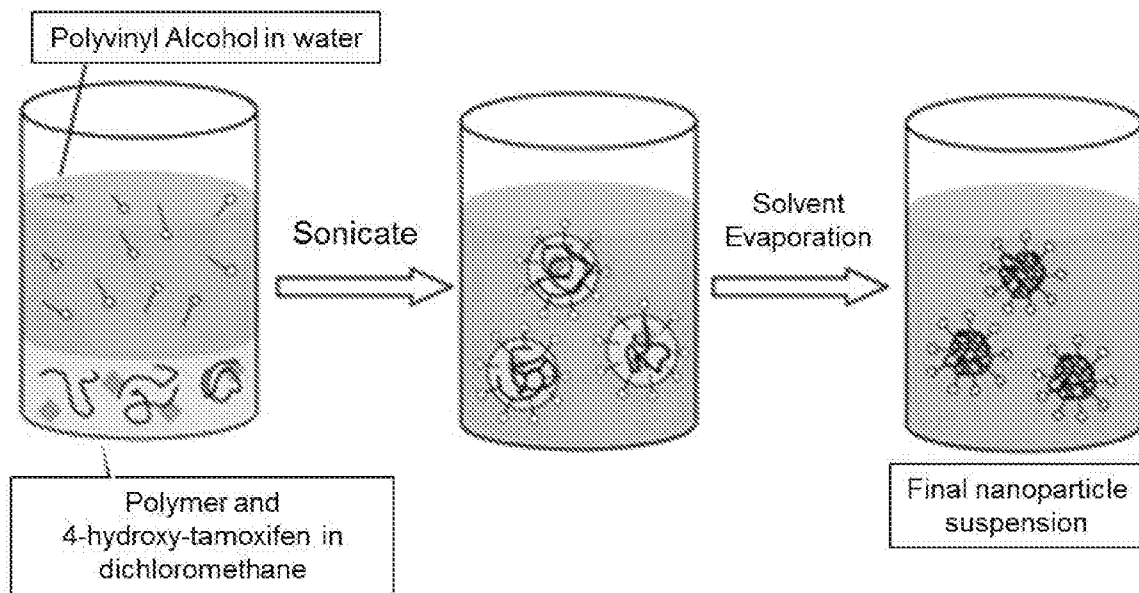
121. The method of claim 118, wherein said antibody is a single chain antibody.

122. The method according to any one of claims 103-121, wherein said pharmaceutical comprises an estrogen receptor ligand selected from the group consisting of tamoxifen, 4-hydroxy-tamoxifen, and a tamoxifen analogue.

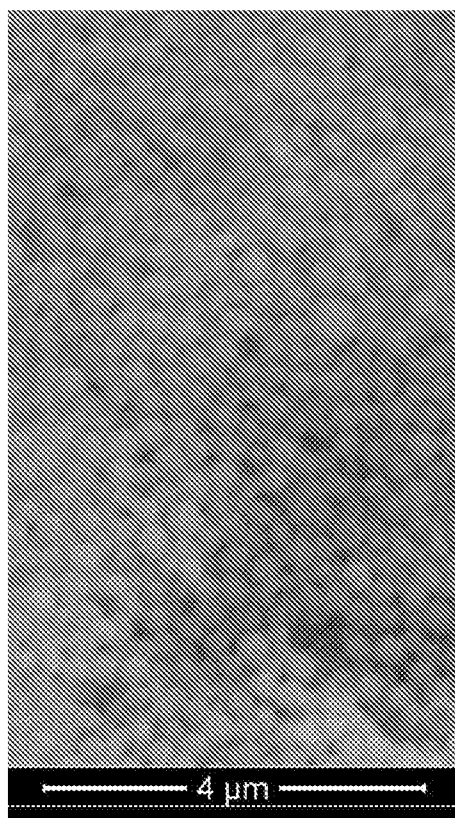
123. The method of claim 122, wherein said estrogen receptor ligand
5 tamoxifen analog is ICI 182,780.

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**Fig. 1A****Fig. 1B**

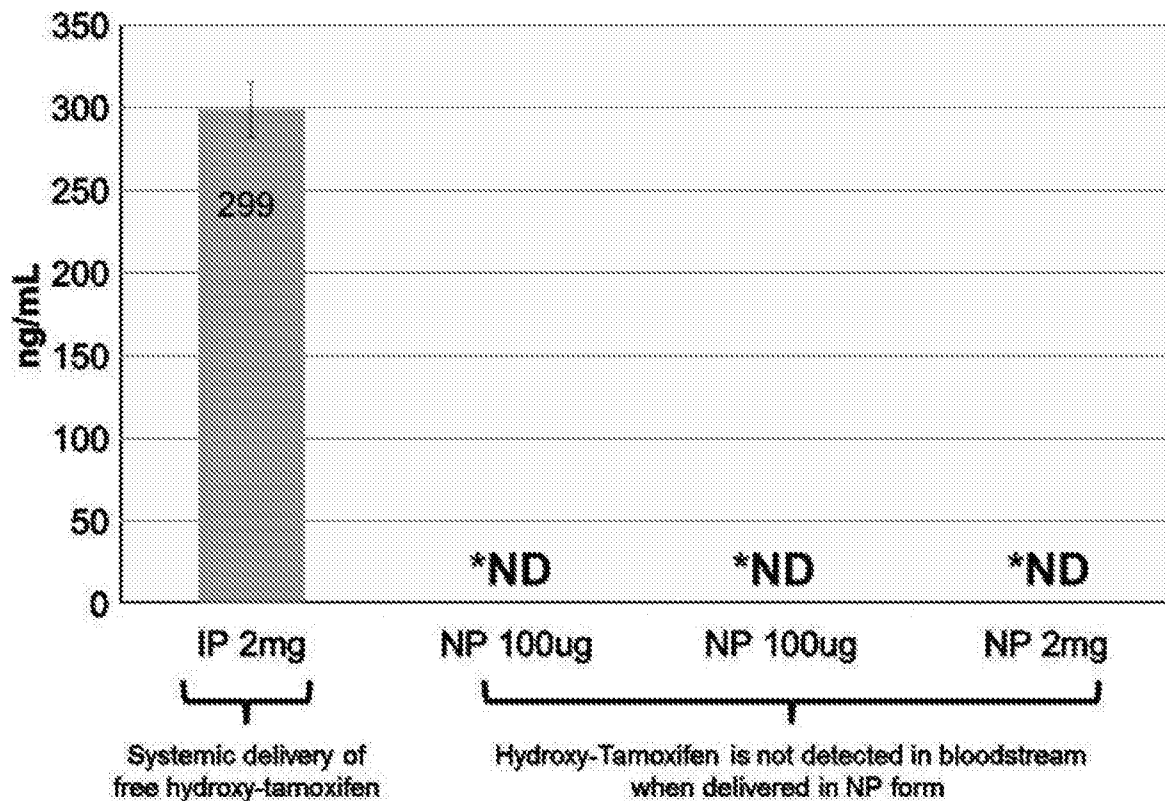
2/31**Fig. 2**

3/31



PLGA(50:50) -
4-hydroxy-tamoxifen
nanoparticles

Fig. 3

4/31**Fig. 4**

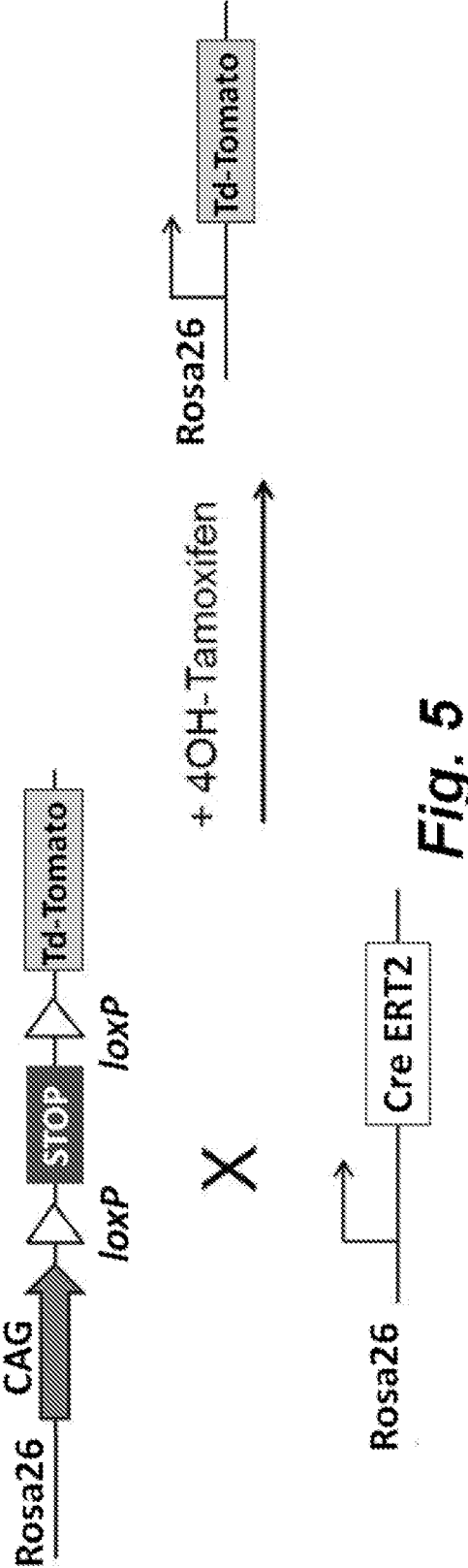


Fig. 5

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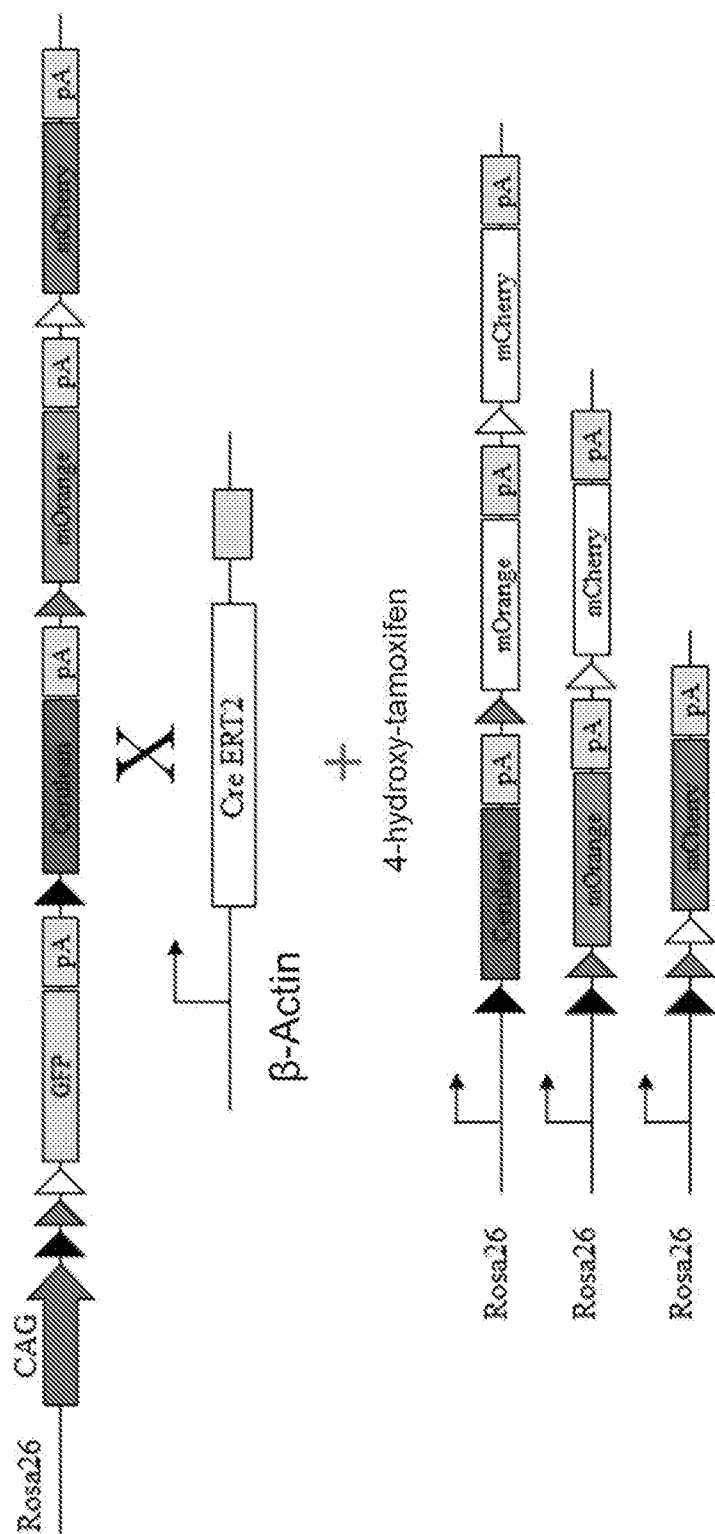
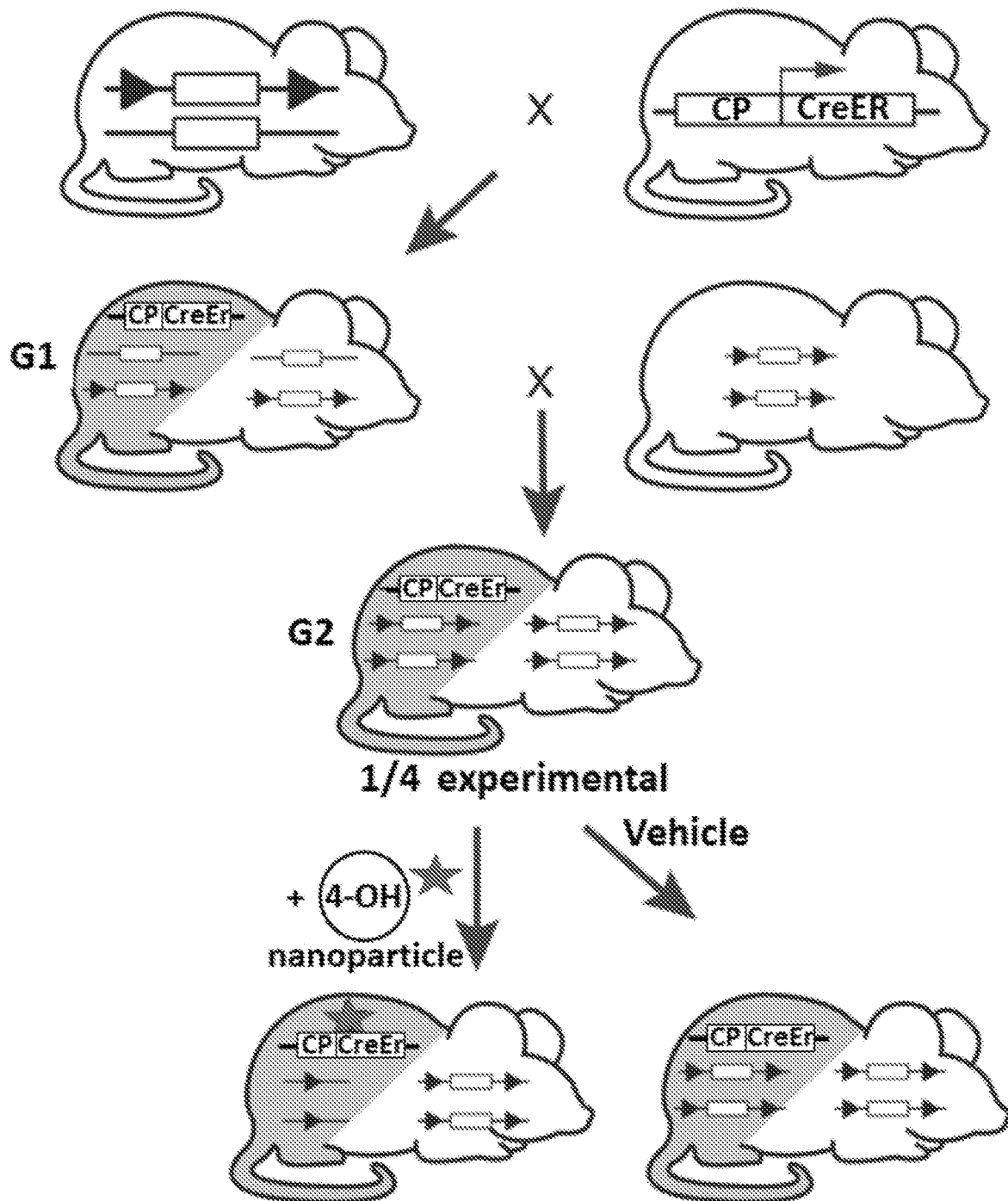
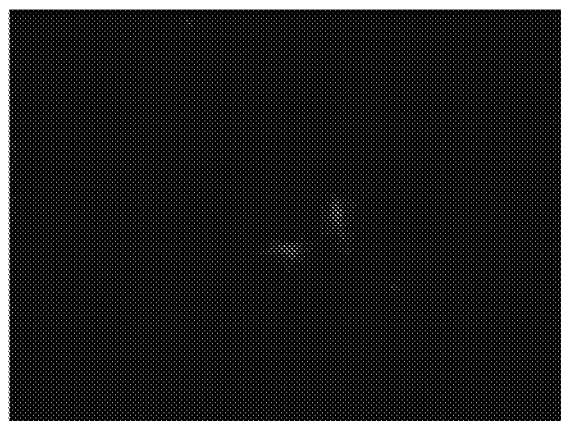
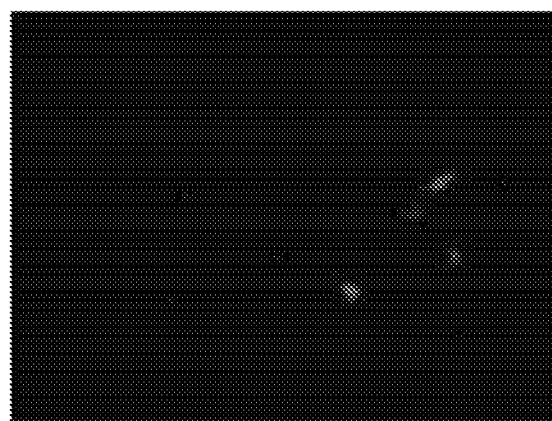
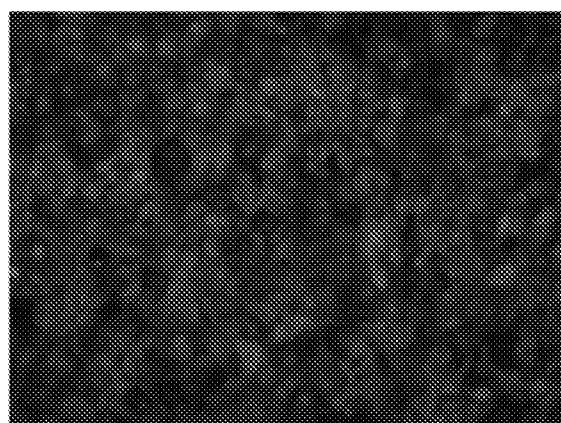
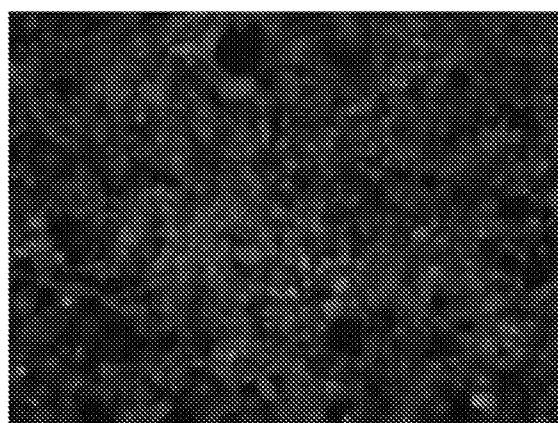
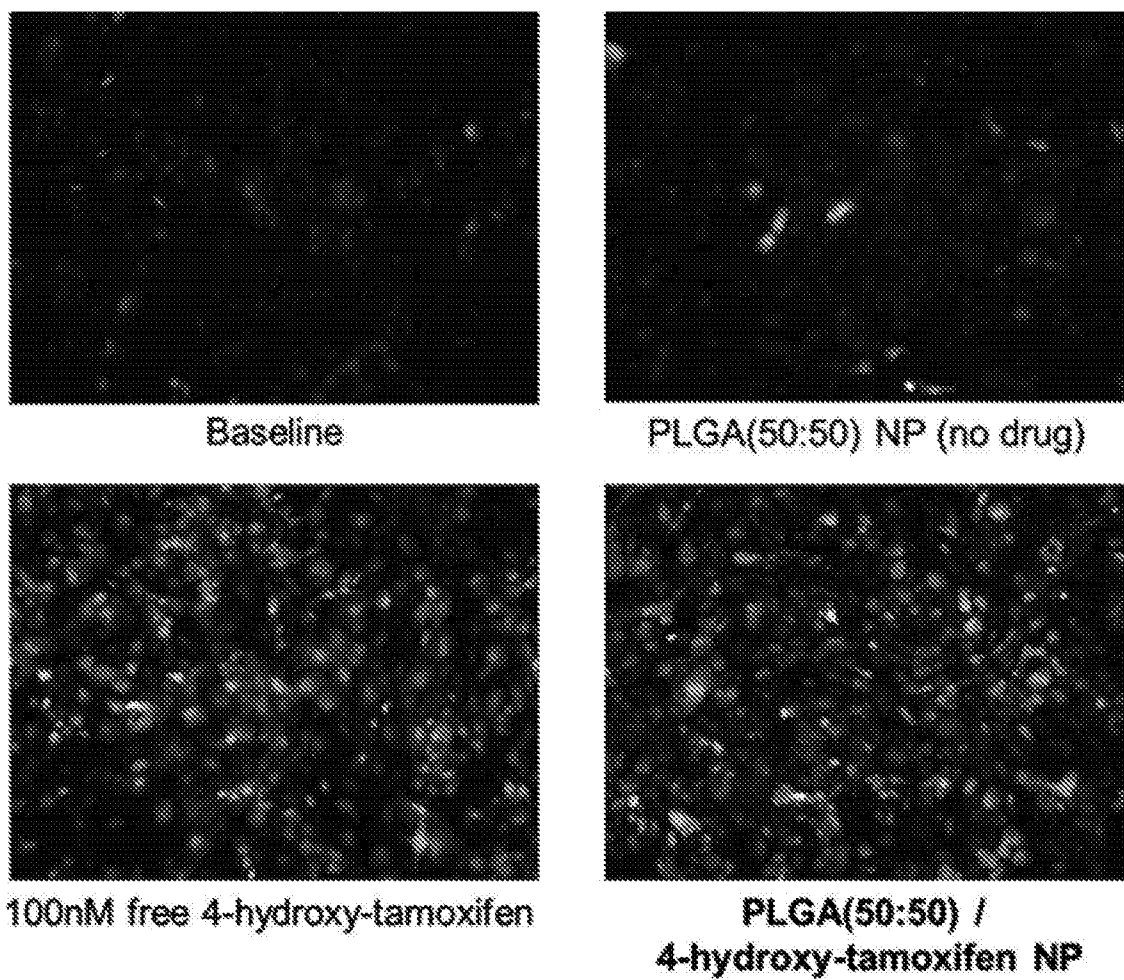


Fig. 6

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**Fig. 7**

8/31**Baseline****PLGA(50:50) NP (no drug)****100nM free
4-hydroxy-tamoxifen****PLGA(50:50) NP /
4-hydroxy-tamoxifen NP*****Fig. 8***

9/31**Fig. 9**

10/31

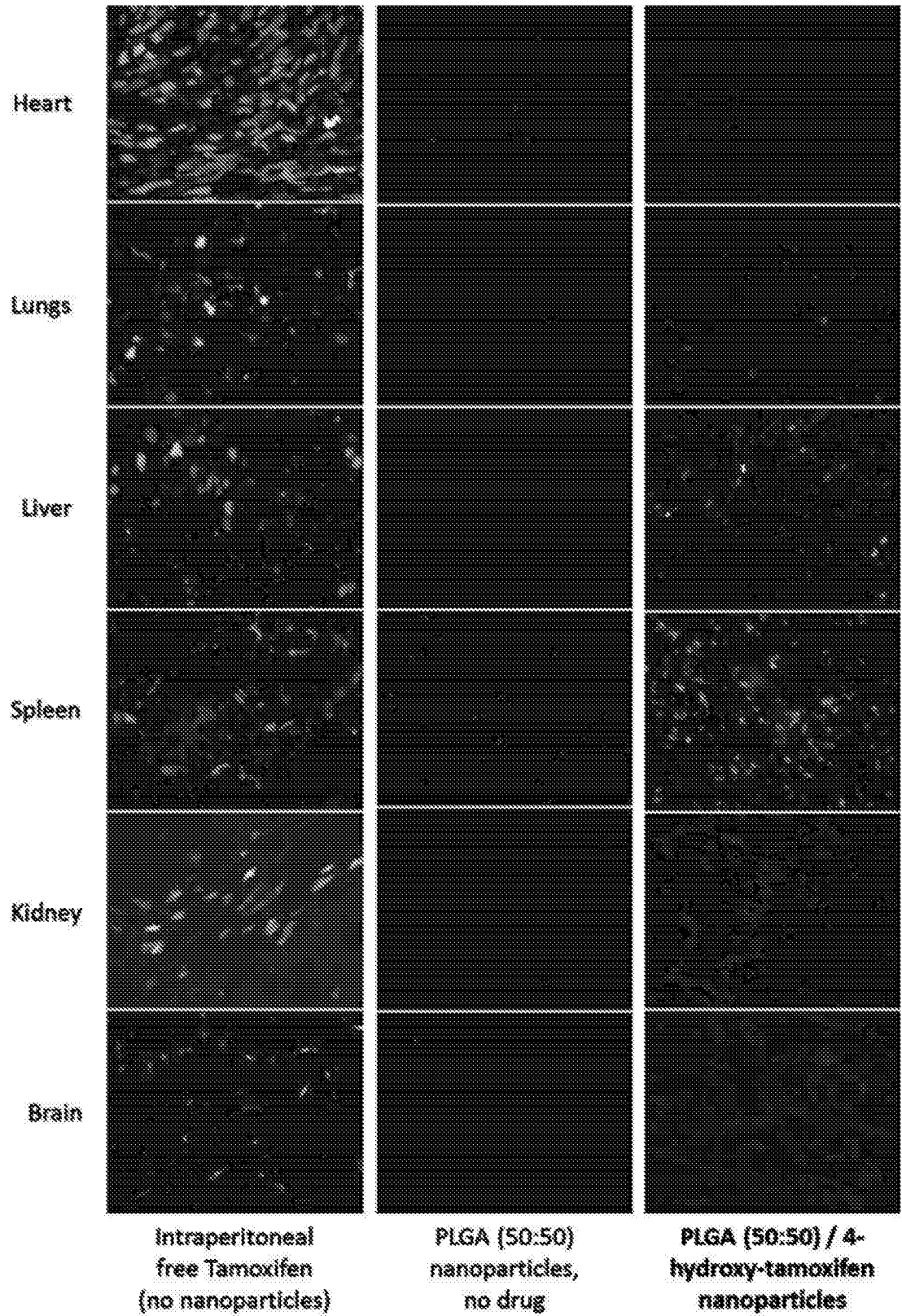
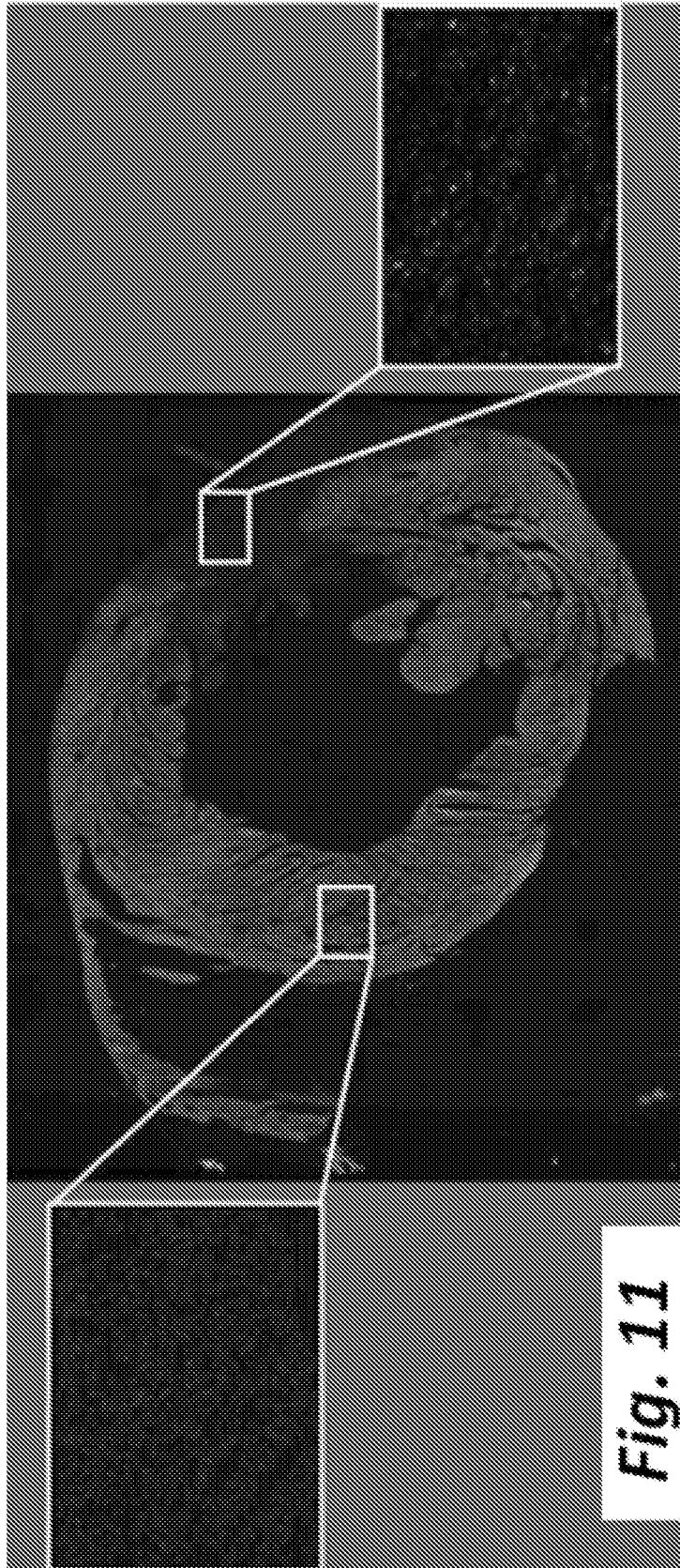
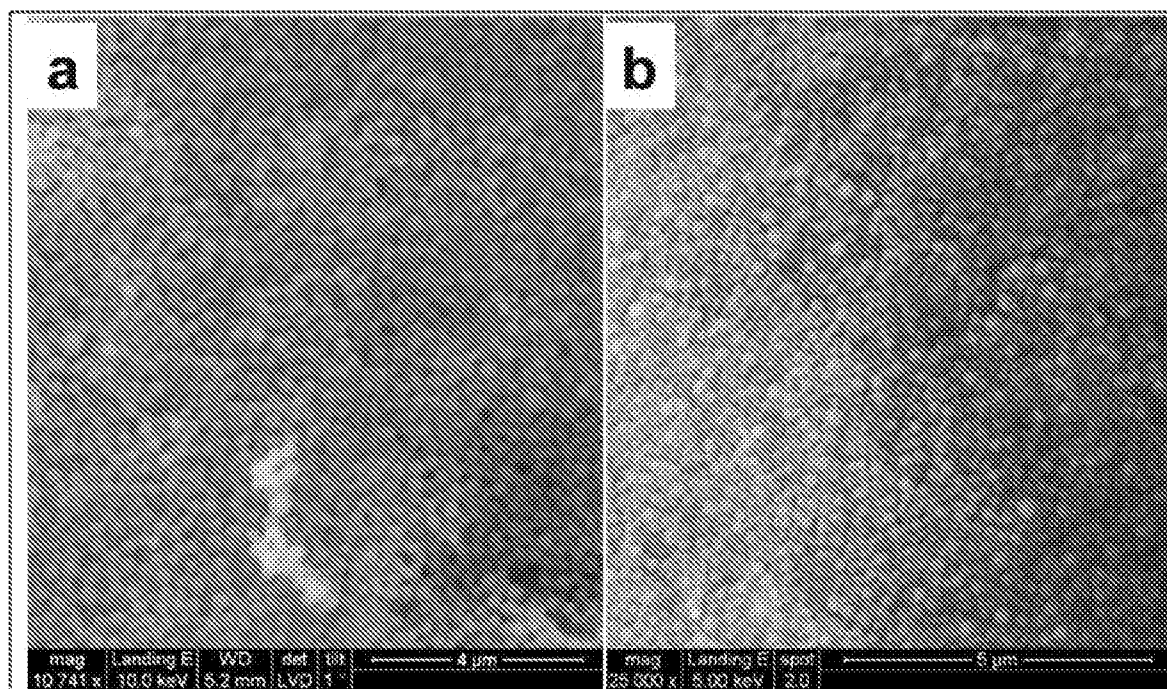
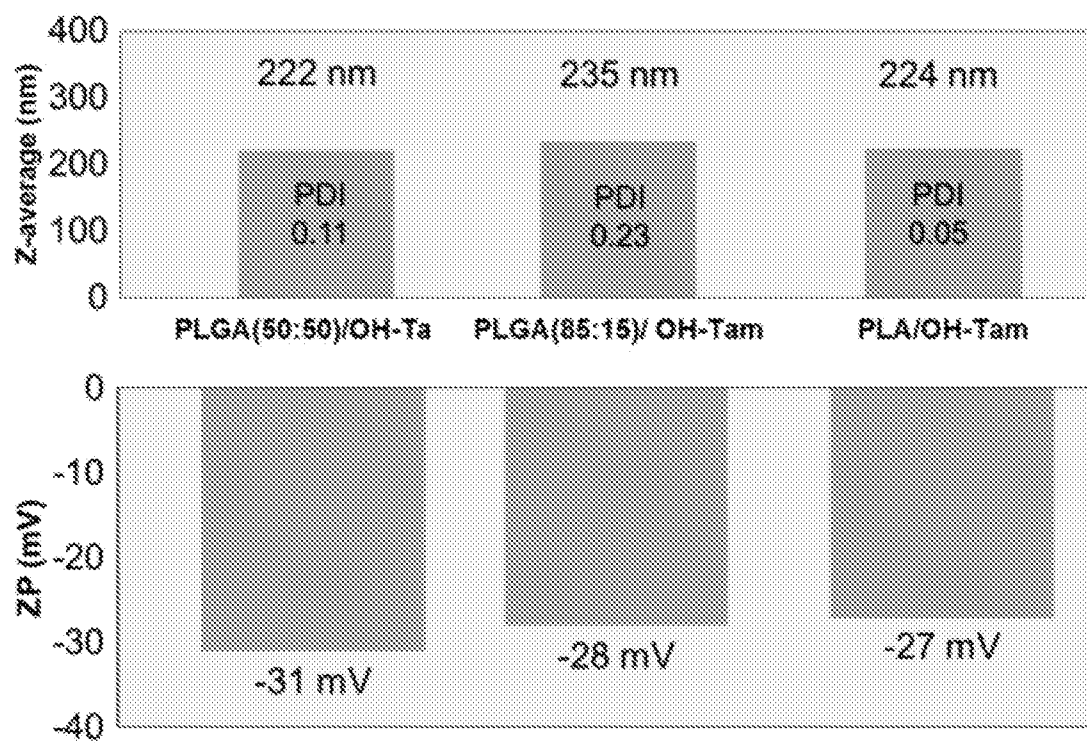


Fig. 10

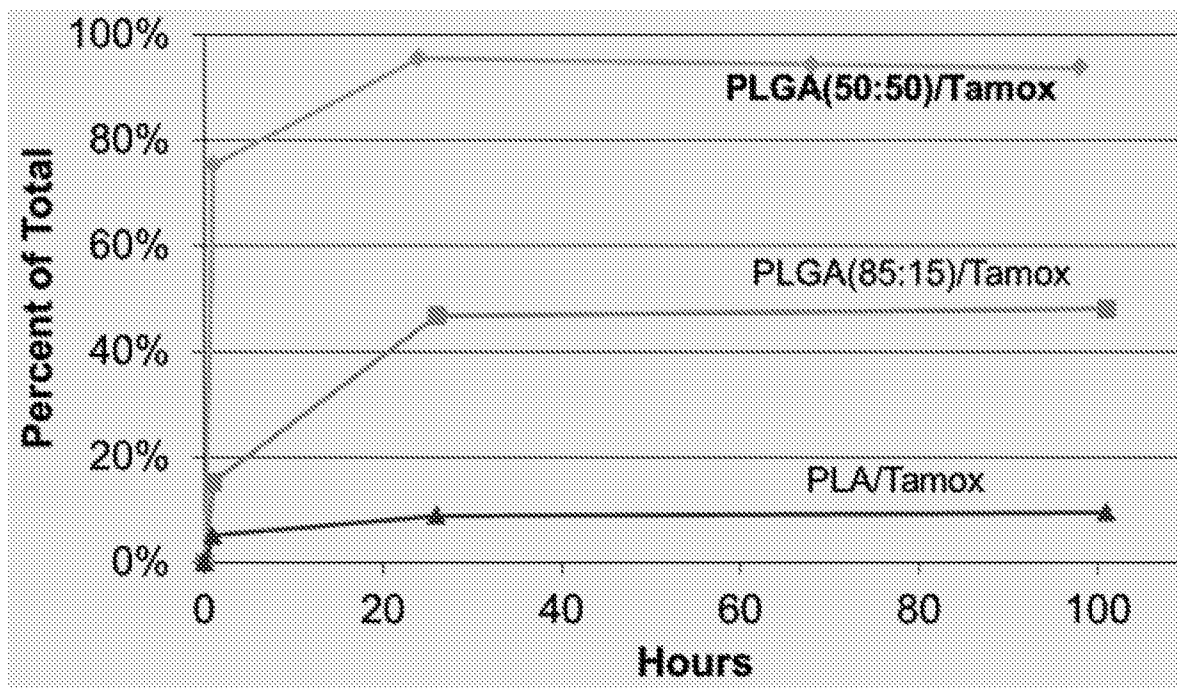
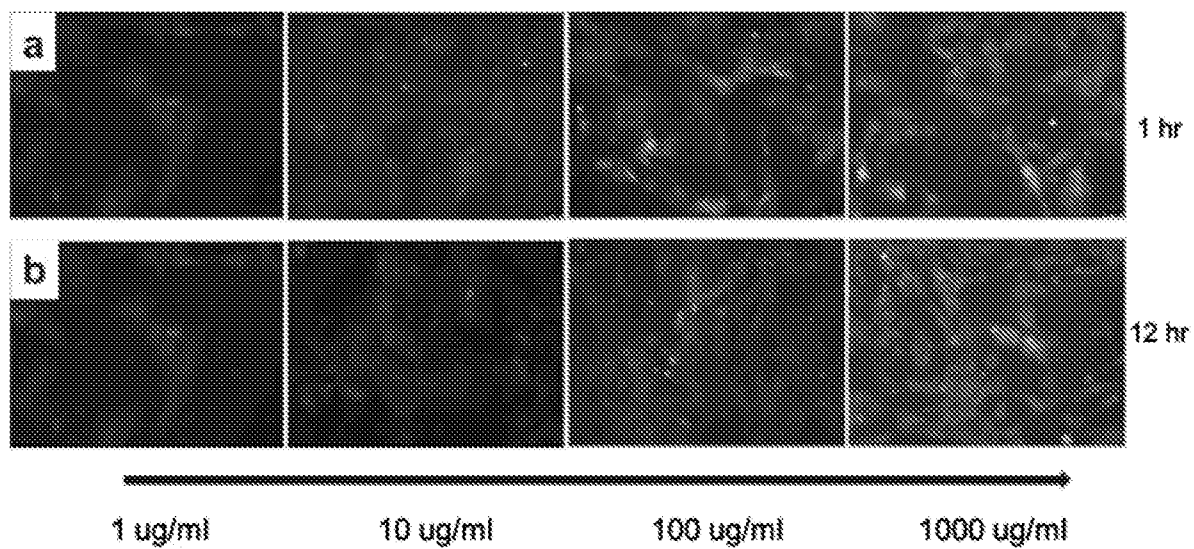
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*Fig. 12**Fig. 13*

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**Fig. 14****Fig. 15**

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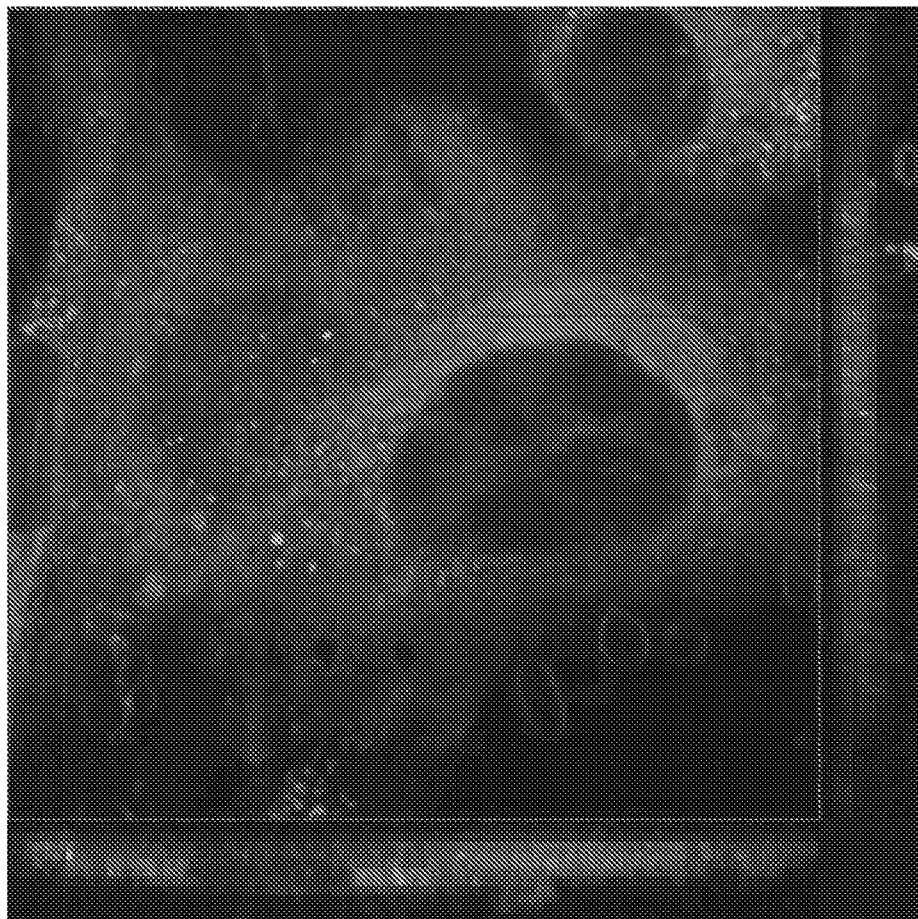


Fig. 16

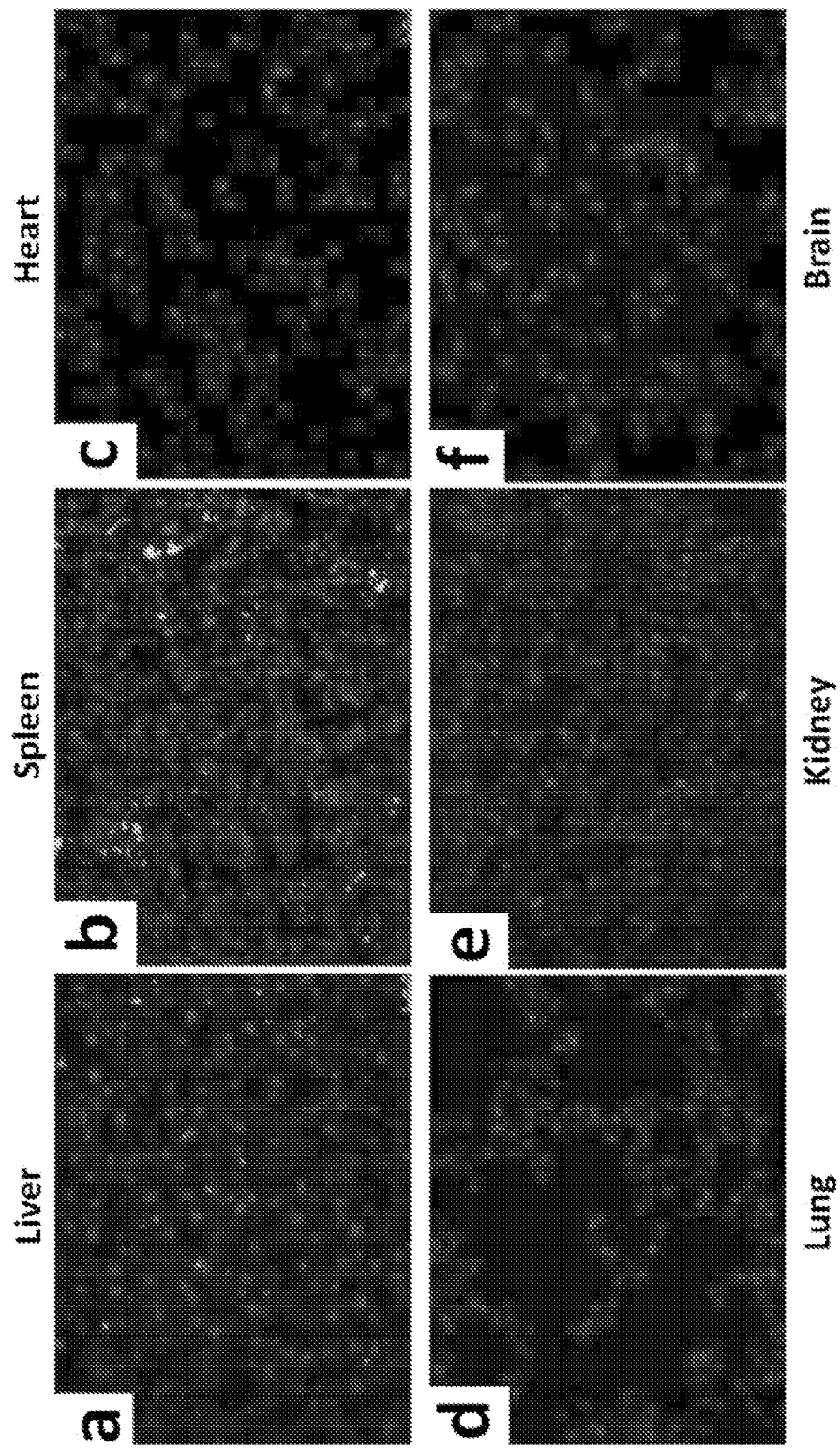


Fig. 17

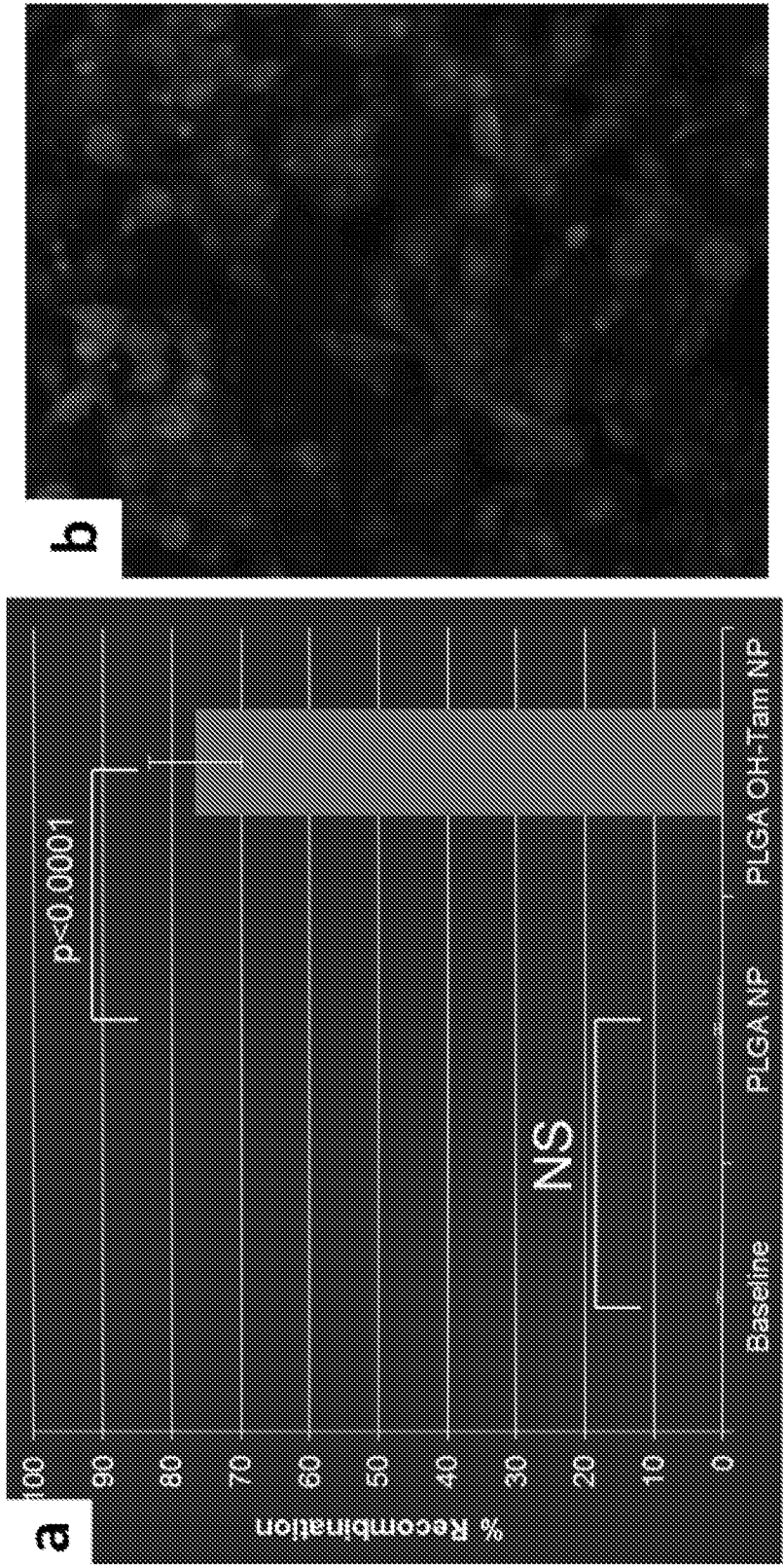


Fig. 18

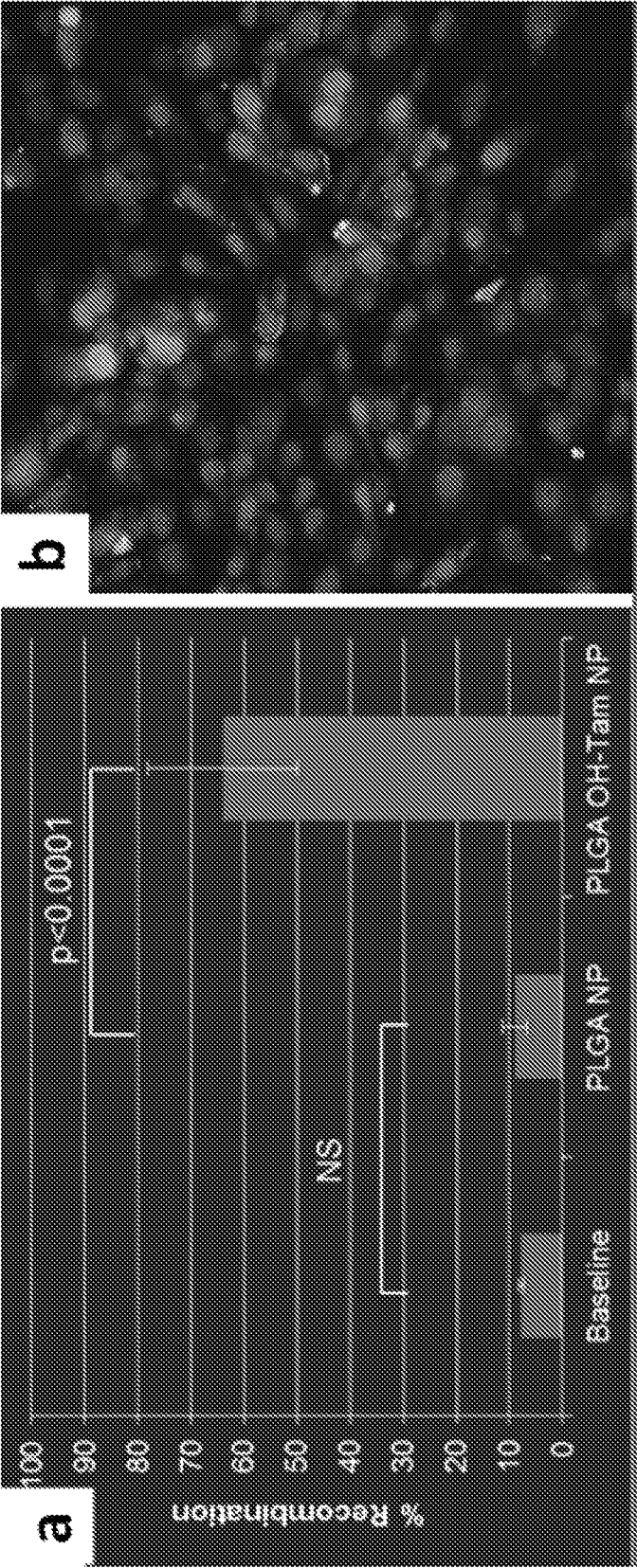


Fig. 19

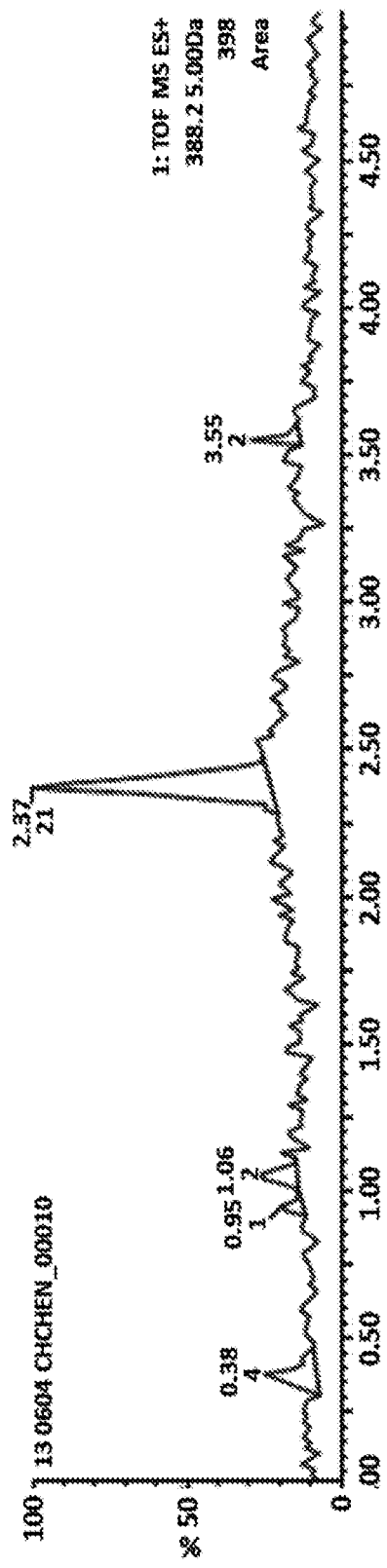
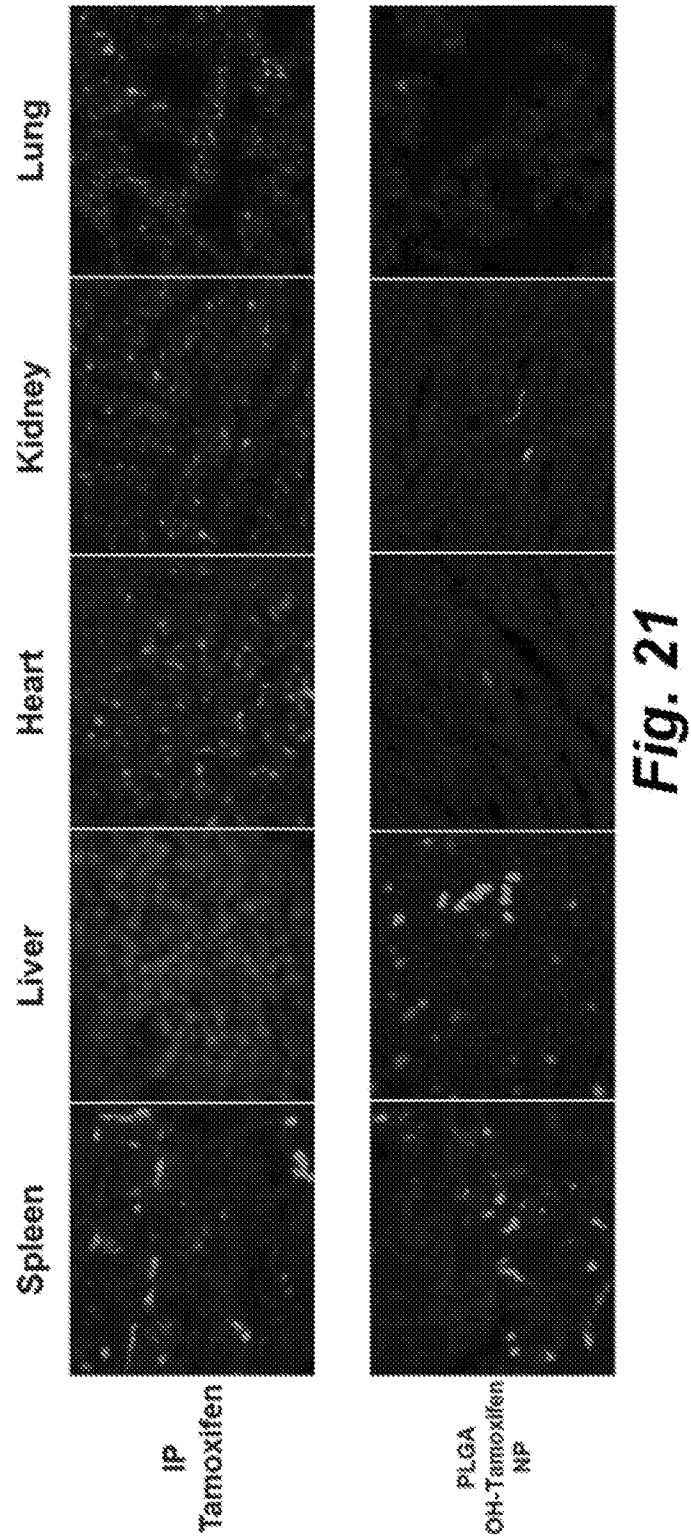
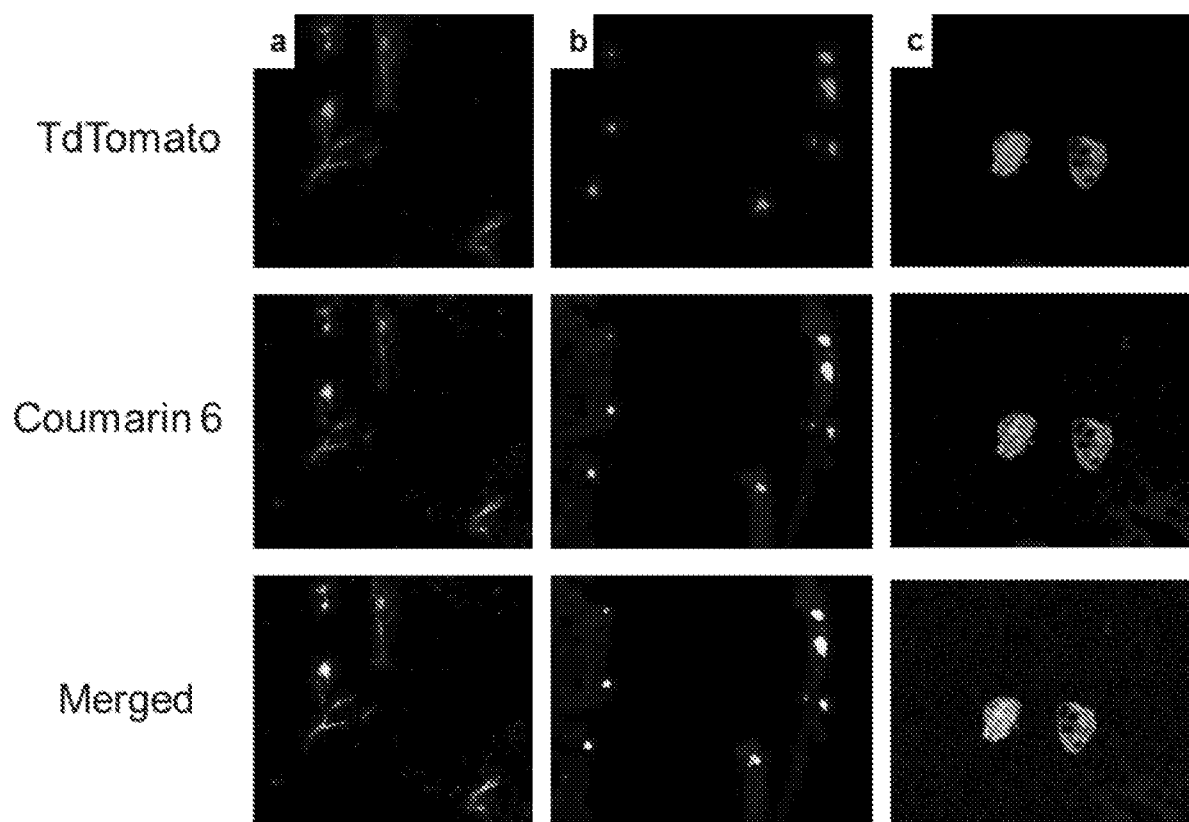


Fig. 20



20/31**Fig. 22**

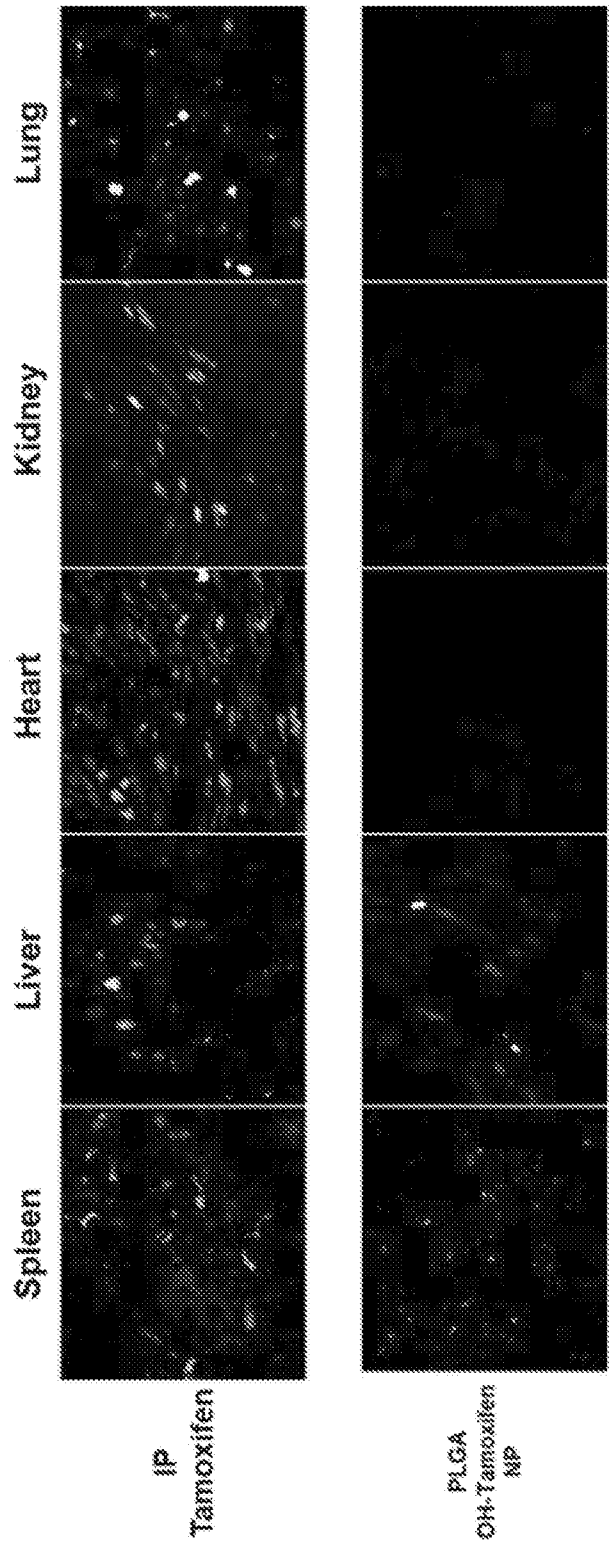
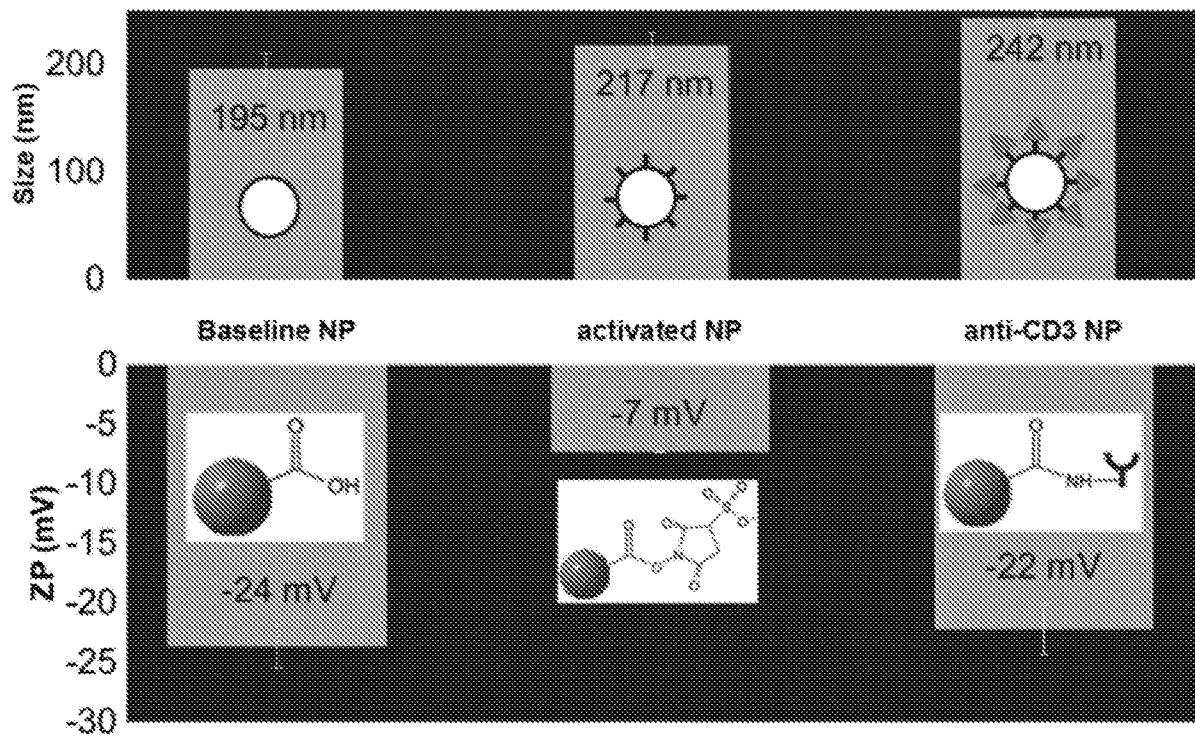
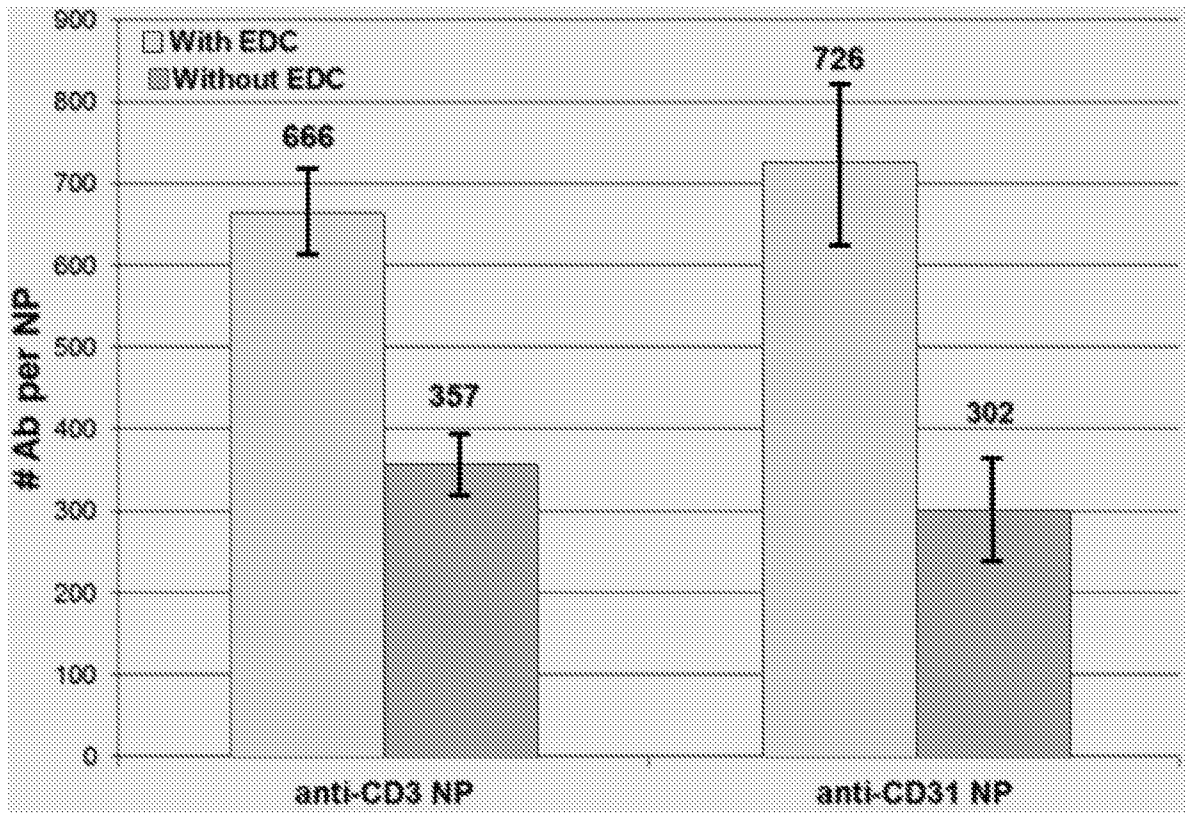
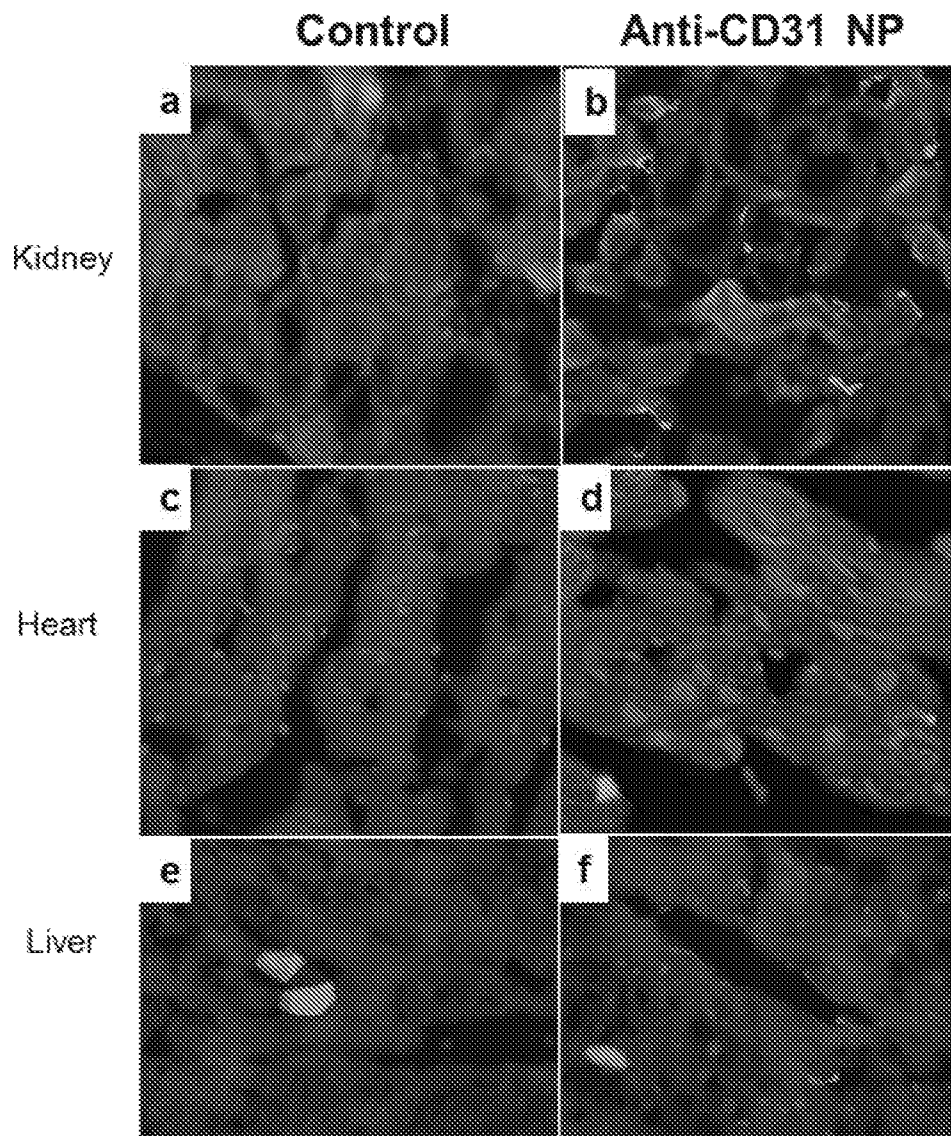


Fig. 23

22/31**Fig. 24**

23/31**Fig. 25**

24/31***Fig. 26***

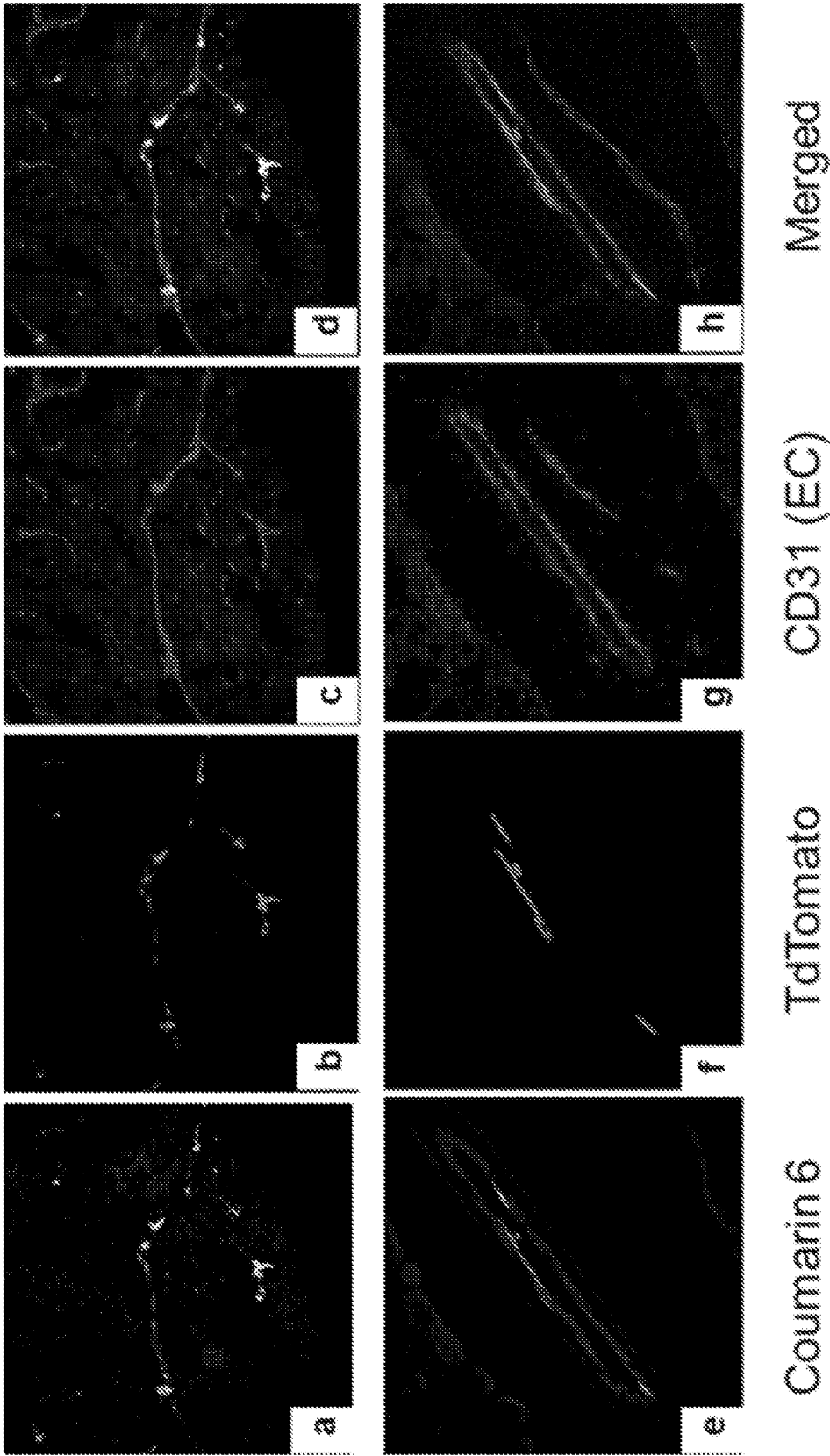


Fig. 27

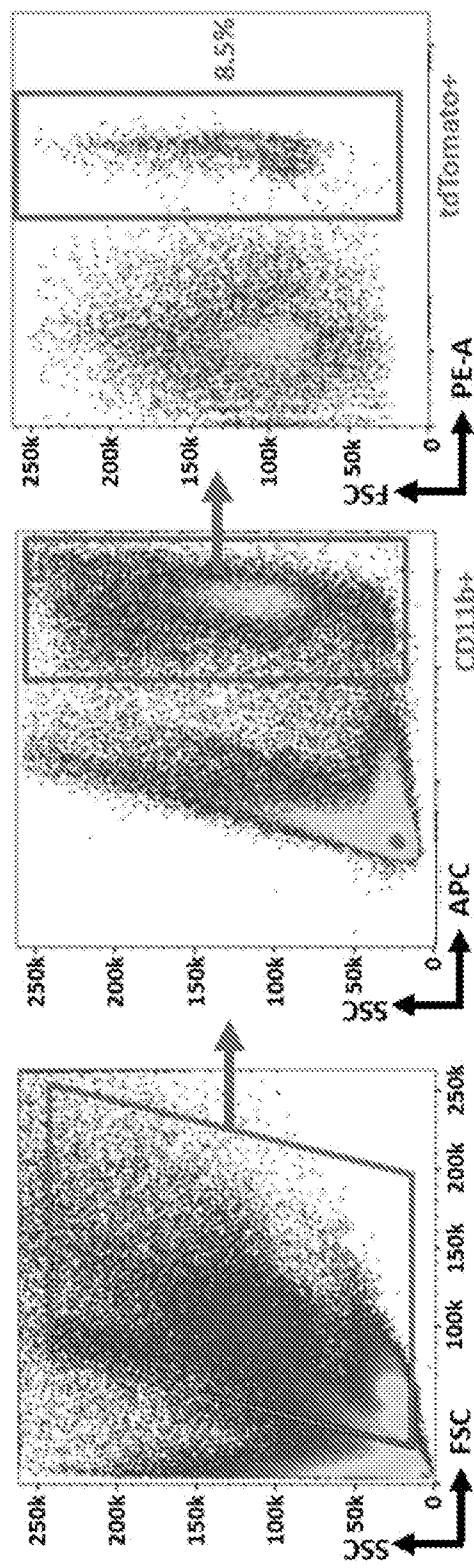
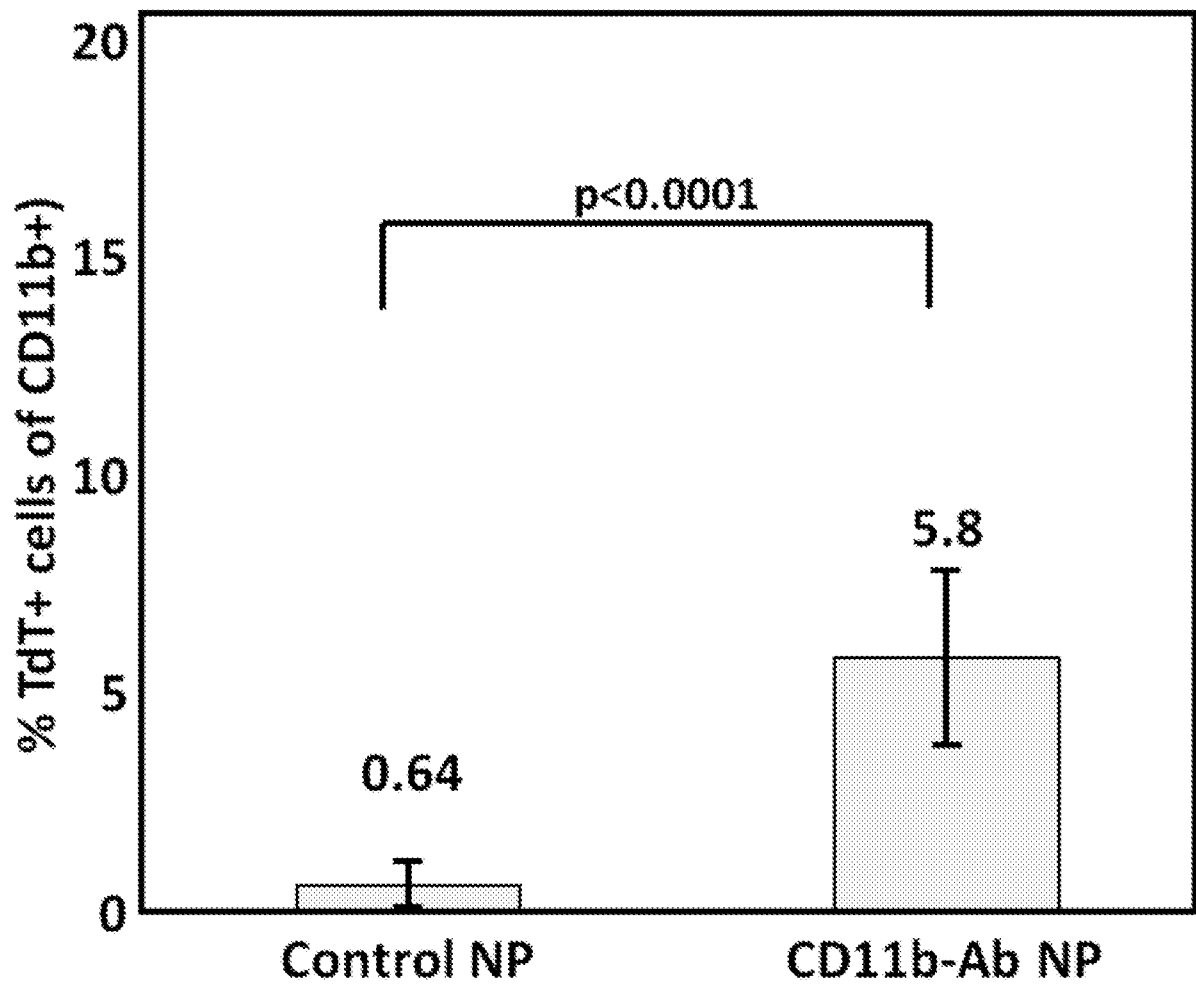


Fig. 28

27/31**Fig. 29**

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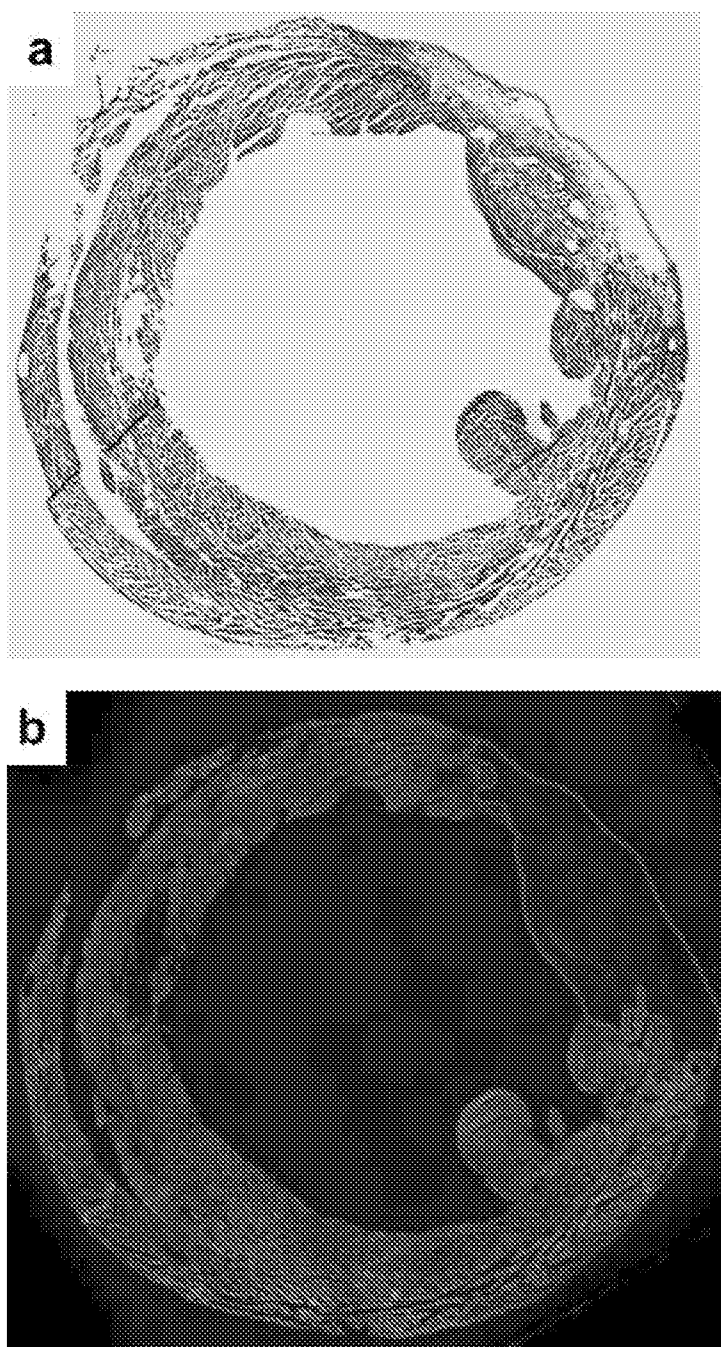
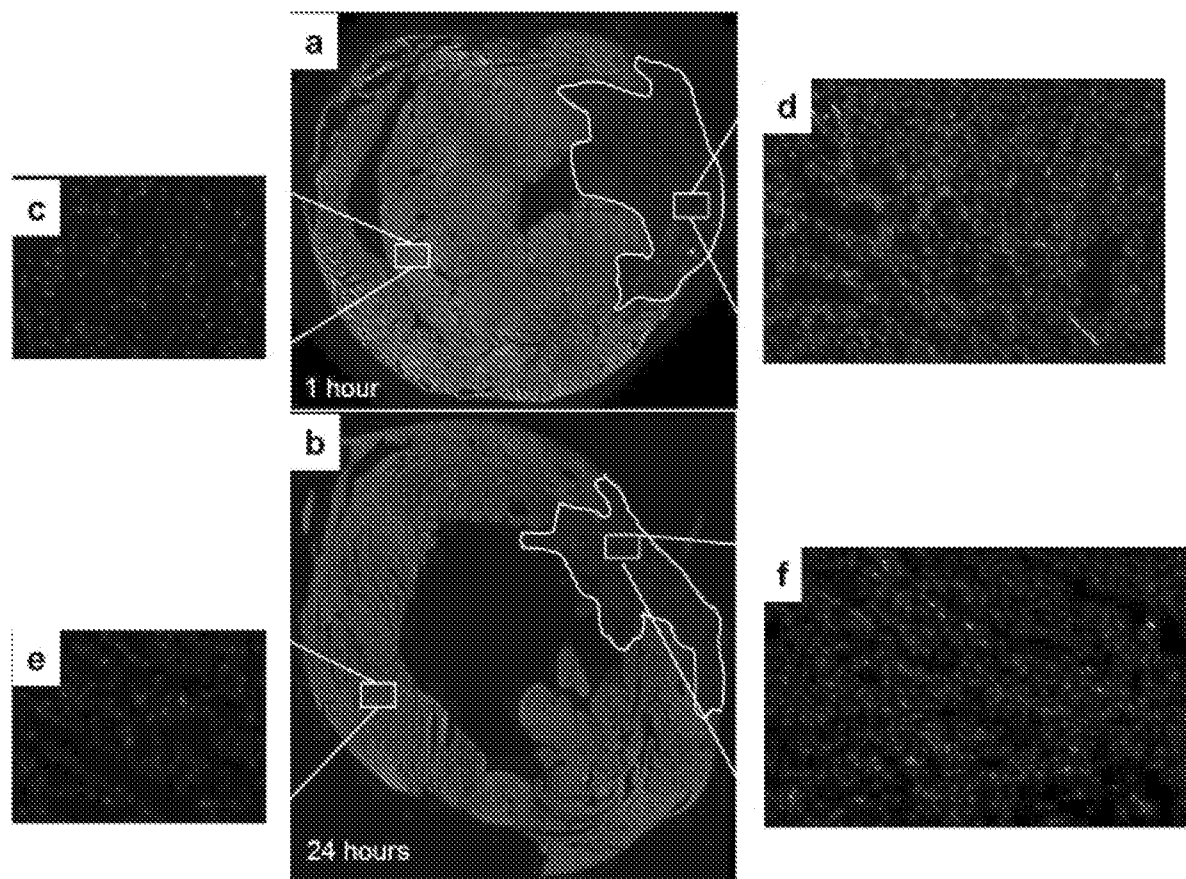


Fig. 30

29/31***Fig. 31***

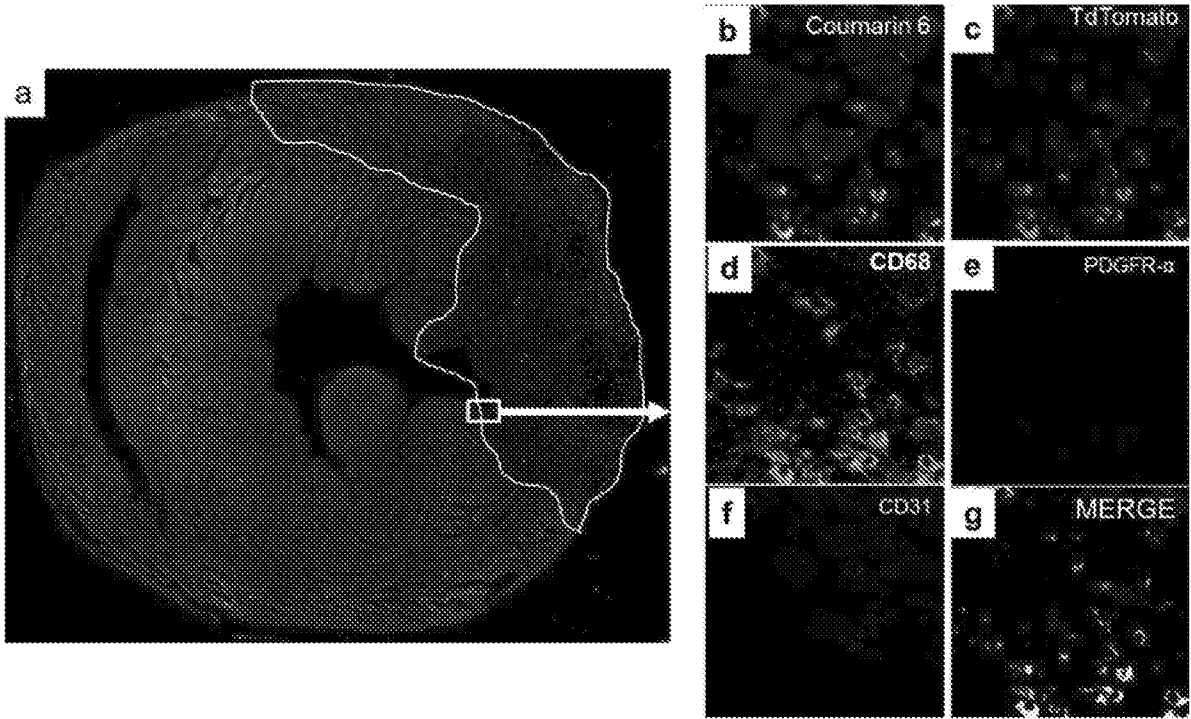
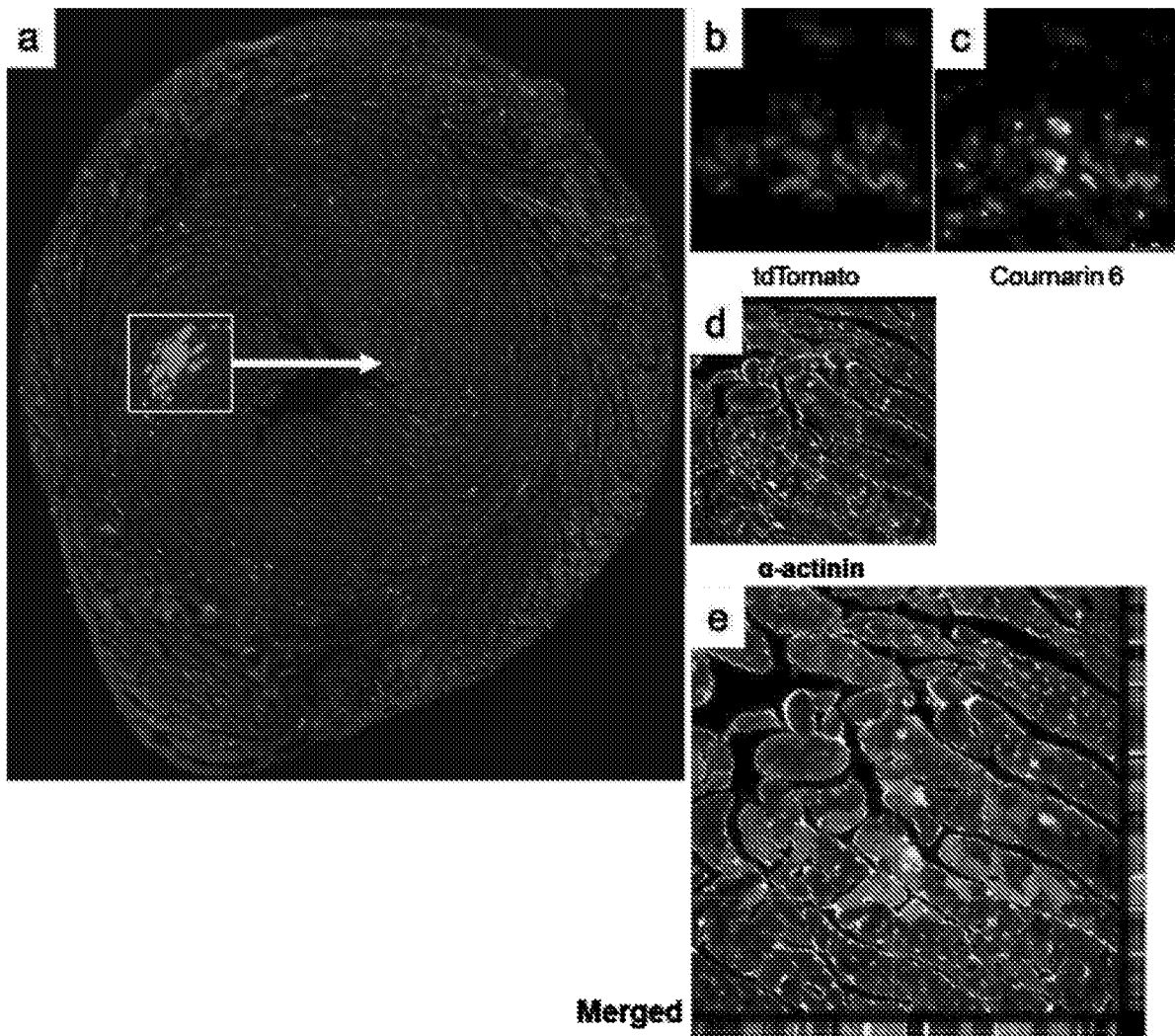


Fig. 32

31/31**Fig. 33**