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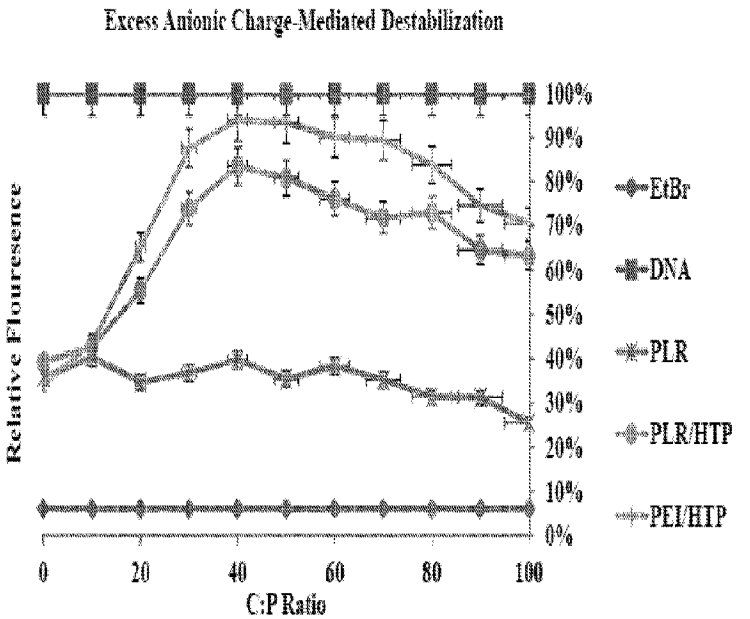
(54) Title: NANOPARTICLE-MEDIATED GENE DELIVERY, GENOMIC EDITING AND LIGAND-TARGETE D MODIFICATION IN VARIOUS CELL POPULATIONS



(57) Abrégé/Abstract:
An improved nanoparticle for transfecting cells is provided. The nanoparticle includes a core polyplex and a silica coating on the core polyplex and, optionally, a polymer attached to an outer surface of the silica coating, where the polyplex includes an anionic polymer, a cationic polymer, a cationic polypeptide, and a polynucleotide. Also provided is an improved method of modifying intracellular polynucleotides. The method includes contacting a cell with a nanoparticle that includes a core polyplex and a silica coating on the core polyplex and, optionally, a polymer attached to an outer surface of the silica coating, where the polyplex includes an anionic polymer, a cationic polymer, a cationic polypeptide, and a polynucleotide.

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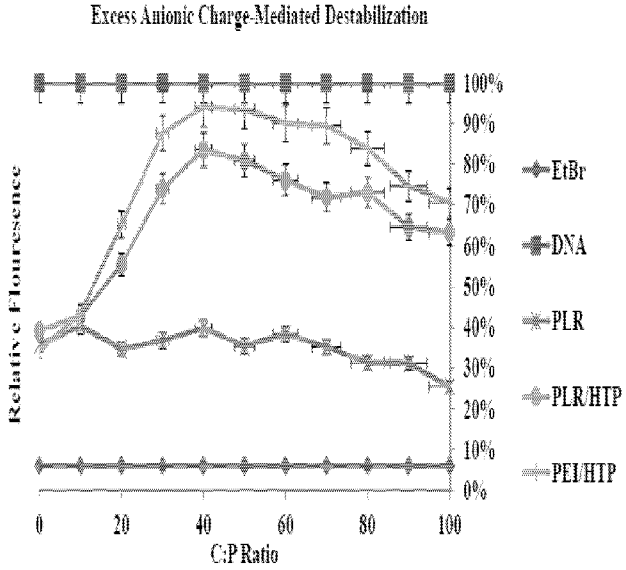

FIGURE 5

(57) Abstract: An improved nanoparticle for transfecting cells is provided. The nanoparticle includes a core polyplex and a silica coating on the core polyplex and, optionally, a polymer attached to an outer surface of the silica coating, where the polyplex includes an anionic polymer, a cationic polymer, a cationic polypeptide, and a polynucleotide. Also provided is an improved method of modifying intracellular polynucleotides. The method includes contacting a cell with a nanoparticle that includes a core polyplex and a silica coating on the core polyplex and, optionally, a polymer attached to an outer surface of the silica coating, where the polyplex includes an anionic polymer, a cationic polymer, a cationic polypeptide, and a polynucleotide.

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NANOPARTICLE-MEDIATED GENE DELIVERY, GENOMIC EDITING AND LIGAND-TARGETED MODIFICATION IN VARIOUS CELL POPULATIONS

[0001]

[0002]

BACKGROUND OF THE INVENTION

Technical Field

[0003] The present invention generally relates to use of nanoparticles to transfect cells. More particularly, the present invention relates to coated nanoparticles with a polyplex core for intracellular delivery of ploynucleotides to modify gene expression.

Background Information

[0004] Introducing polynucleotides into cells to alter gene expression requires appropriate packaging of the polynucleotides to protect them from degradation before cell entry, to permit entry into cells, and to direct delivery to the appropriate subcellular compartment. Effectiveness in altering expression may also depend on time-frames of release of polynucleotides from packaging after cellular entry. Available nanoparticle-based technologies for modifying gene expression suffer from low levels of cellular transfection and limited effectiveness upon transfection, at least in part because of their limitations in satisfying the foregoing requirements. It is therefore desirable to obtain a nanoparticle-based transfection agent and method of use thereof that addresses all of these requirements to enhance effectivenes

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SUMMARY OF THE INVENTION

[0005] The shortcomings of the prior art are overcome, and additional advantages are provided, through the provision, in one aspect, of a nanoparticle. The nanoparticle includes a core polyplex and a silica coating on the core polyplex, and the polyplex includes an anionic polymer, a cationic polymer, a cationic polypeptide, and a polynucleotide. In another aspect, the nanoparticle may also include a polymer attached to an outer surface of the silica coating.

[0006] A method of modifying intracellular polynucleotides is also provided. The method includes contacting a cell with a nanoparticle that includes a core polyplex and a silica coating on the core polyplex, and the polyplex includes an anionic polymer, a cationic polymer, a cationic polypeptide, and a polynucleotide. In another aspect, the nanoparticle may also include a polymer attached to an outer surface of the silica coating.

[0006a] According to one particular aspect, the invention relates to a nanoparticle comprising:

a core polyplex and a silica coating thereon;

wherein said core polyplex comprises:

(i) one or more anionic polymers selected from the group consisting of poly(D-glutamic acid), a glycosaminoglycan, a glycoprotein, a polysaccharide, poly(mannuronic acid), poly(guluronic acid), heparin, heparin sulfate, chondroitin, chondroitin sulfate, keratan, keratan sulfate, aggrecan, poly(glucosamine), and any combination of two or more of the foregoing,

(ii) a cationic polymer,

(iii) a cationic polypeptide, and

(iv) a polynucleotide,

wherein the cationic polypeptide is a histone tail peptide.

[0006b] According to another particular aspect, the invention relates to an An *in vitro* method of modifying intracellular polynucleotides, the method comprising:
contacting *in vitro* a cell with a nanoparticle, wherein said nanoparticle comprises a core polyplex and a silica coating thereon; wherein the core polyplex comprises:

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(i) one or more anionic polymers selected from the group consisting of poly(D-glutamic acid), a glycosaminoglycan, a glycoprotein, a polysaccharide, poly(mannuronic acid), poly(guluronic acid), heparin, heparin sulfate, chondroitin, chondroitin sulfate, keratan, keratan sulfate, aggrecan, poly(glucosamine), and any combination of two or more of the foregoing,
(ii) a cationic polymer,
(iii) a cationic polypeptide, and
(iv) a polynucleotide,
wherein the cationic polypeptide is a histone tail peptide.

[0007] Additional features and advantages are realized through the techniques of the present invention. These, and other objects, features and advantages of this invention will become apparent from the following detailed description of the various aspects of the invention taken in conjunction with the accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0008] One or more aspects of the present invention are particularly pointed out and distinctly claimed as examples in the claims at the conclusion of the specification. The foregoing and other objects, features, and advantages of the invention are apparent from the following detailed description taken in conjunction with the accompanying drawings in which:

[0009] FIGs. 1A-1B are diagrammatic representations of some embodiments of a nanoparticle and components thereof in accordance with an aspect of the present invention;

[0010] FIGs. 2A is a diagrammatic representation of how a nanoparticle may be manufactured in accordance with an aspect of the present invention;

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[0011] FIG. 2B is a diagrammatic representation of means by which a cell may uptake and intracellularly process a nanoparticle in accordance with an aspect of the present invention;

[0012] FIG. 3 is a graph illustrating the effects on polyplex complexation of including different ratios of various charged polymers and polynucleotides in accordance with an aspect of the present invention;

[0013] FIG. 4 is a graph illustrating the effects on polyplex complexation of including different ratios of various charged polymers and polynucleotides, with or without including an anionic polymer in the polyplex, in accordance with an aspect of the present invention;

[0014] FIG. 5 is a graph illustrating the destabilizing effect on a polyplex of including increasing amounts of an anionic polymer in the presence or absence of cationic polypeptides in accordance with an aspect of the present invention;

[0015] FIG. 6 is a graph illustrating sizes of nanoparticles possessing various layers in accordance with an aspect of the present invention;

[0016] FIG. 7 is photomicrographs of cells transfected with various nanoparticles demonstrating cellular uptake and subcellular localization of nanoparticles following transfection in accordance with an aspect of the present invention;

[0017] FIG. 8 is photomicrographs of cells transfected with nanoparticles showing duration of residence of nanoparticles in cells following transfection in accordance with an aspect of the present invention;

[0018] FIGs. 9A-B is photomicrographs showing cellular uptake of nanoparticles possessing a layer of polymers attached to the outside of a silica coating of a polyplex in accordance with an aspect of the present invention;

[0019] FIG. 10 is a diagrammatic representation of TALEN peptides encoded for by a nucleic acid included in a nanoparticle that cause knockdown of expression of sclerostin in accordance with an aspect of the present invention;

[0020] FIGs. 11A-11C are graphs illustrating the effects transfecting cells with different amounts of nanoparticles that target sclerostin expression on sclerostin and β-catenin expression in accordance with an aspect of the present invention;

[0021] FIGs. 12A-12F are graphs illustrating the effects of transfecting cells with different amounts of nanoparticles that target sclerostin expression on expression levels of various cellular signaling peptides in accordance with an aspect of the present invention;

[0022] FIG. 13 is photomicrographs demonstrating effects of transfecting cells with nanoparticles that target sclerostin expression on expression of a co-transfected reporter gene that is responsive to transcription factors whose activity is inhibited by sclerostin-mediated signalling in accordance with an aspect of the present invention;

[0023] FIGs. 14A-14C are photomicrographs demonstrating effects of transfecting cells with nanoparticles that target sclerostin expression on mineralization in accordance with an aspect of the present invention.

DETAILED DESCRIPTION OF THE INVENTION

[0024] Aspects of the present invention and certain features, advantages, and details thereof, are explained more fully below with reference to the non-limiting embodiments illustrated in the accompanying drawings. Descriptions of well-known materials, fabrication tools, processing techniques, etc., are omitted so as to not unnecessarily obscure the invention in detail. It should be understood, however, that the detailed description and the specific examples, while indicating embodiments of the invention, are given by way of illustration only, and are not by way of limitation. Various substitutions, modifications, additions and/or arrangements within the spirit and/or scope of the underlying inventive concepts will be apparent to those skilled in the art from this disclosure.

[0025] The present disclosure provides, in part, a multilayered nanoparticle for transfecting cells with agents to modify gene expression. Nanoparticles designed for improved serum stability, targetted delivery to specific cell types, greater nuclear specificity and compartment-specific unpackaging, improved ability to retain significant payload levels during initial stages of internalization, and ability to

maintain release of payload for a various durations following internalization, and methods of use thereof, are provided.

[0026] In one aspect, complexes of polynucleotides with polymers, or polyplexes, created by condensation of cationic polymers and polynucleotides in the presence of anionic polymers may mediate increased transfection efficiency over polynucleotide-cationic polymer conjugates. Though this process may produce more particles and increase the net surface area of nanoparticles exposed for cellular uptake, an improved electrostatic repulsory element may also be at play in releasing nucleic acids through this technique. Surprisingly, in contrast to a more rapid disaggregation of nucleotides from nanoparticle polyplexes that include anionic polymers as would have been predicted on the basis of existing literature, in one aspect of the present invention, including an anionic polymer in a nanoparticle polyplex core may prolong the duration of intracellular residence of the nanoparticle and release of agents that affect gene expression or otherwise regulate cellular function, or payloads.

[0027] In another aspect, the presence of a cationic polypeptide in a nanoparticle may mediate stability, subcellular compartmentalization, and payload release. As one example, fragments of the N-terminus of histone peptides, referred to generally as histone tail peptides, within various polyplexes are not only capable of being deprotonated by various histone modifications, such as in the case of histone acetyltransferase-mediated acetylation, but may also mediate effective nuclear-specific unpackaging as components of polyplexes. Their trafficking may be reliant on alternative endocytotic pathways utilizing retrograde transport through the Golgi and endoplasmic reticulum, and the nature of histones existing inside of the nuclear envelope suggests an innate nuclear localization sequence on histone tail peptides. In one aspect of the present invention, including a histone tail peptide may promote nuclear localization of nanoparticles and result in enzyme-mediated release of polynucleotide payload therefrom.

[0028] In another aspect, silica coatings of polyplexes may seal their payloads before and during initial cellular uptake. Commonly used polyplexes consisting of poly(ethylenimine) and DNA have a tendency to shed the majority (~ 90%) of their payloads during cellular internalization, with the remaining payload often remaining bound to its cationic nanocarrier's polymeric remains. With transiently stabilizing

interlayers of silica, greater intracellular delivery efficiency may be observed despite decreased probability of cellular uptake. In another aspect of the present invention, coating a nanoparticle polyplex with a silica coating may seal the polyplex, stablizing it until its release upon processing in the intended subcellular compartment.

[0029] In another aspect of the present invention, transfection efficiency may be further increased by adding another layer of cationic polymer, making the delivery efficiency as much as two orders of magnitude greater than a bare or silica-coated polyplex, presumably due to the anionic nature of an oligomeric silica coating being cell repulsive. In a further aspect, silica-coated polyplexes and their further-layered derivatives are stable in serum and are suitable for in vivo experiments unlike cationic polymer/nucleic acid conjugates on their own.

[0030] FIGs. 1A-1B show examples of components of a nanoparticle in accordance with the present invention. In accordance with the present invention, a nanoparticle polyplex core may include a polynucleotide, an anionic polymer, a cationic polymer, and a cationic polypeptide. A silica coating may then be applied to the polyplex core, and polymers may then be attached to an outer surface of the silica coating. The polynucleotide may be a DNA vector for driving intracellular expression of a nucleic acid sequence it contains. However, a nanoparticle may also comprise other types of polynucleotides, such as linear DNA or various types of RNA, including dsDNA, ssDNA, mRNA, siRNA, or CRISPR RNA sequences, or others, or any combination of the foregoing. A nanoparticle may also include, in addition to or in place of any of the foregoing examples of polynucleotides, a peptide nucleic acid, other charged or polar small molecules between 50 and 1000 Da, or alternatively between 200 and 10 kDa, in size, such as cyclic nucleotides such as cAMP, DNA origami templates, aptamers, charged polypeptides, proteins or protein fragments between 2 and 100 kDa, peptoids, phosphorylated or sulfated constituents, anionically modified constituents, and multimeric or oligomeric combinations of the foregoing. A person of ordinary skill would understand any of the foregoing, or any combination thereof, as being included within the present invention.

[0031] Continuing with FIG 1A, in one aspect of the invention, a cationic polymer within the polyplex may be a polypeptide containing cationic amino acids and may be, for example, poly(arginine), poly(lysine), poly(histidine), poly(ornithine),

poly(citrulline), or a polypeptide that comprises any combination of more than one of the foregoing. A nanoparticle may also include, in addition to or in place of any of the foregoing examples of cationic polymers, poly(ethylenimine), poly(aspartamide), polypeptoids, a charge-functionalized polyester, a cationic polysaccharide, an acetylated amino sugar, chitosan, or a variant or variants that comprise any combination of more than one of the foregoing, in linear or branched forms.

[0032] In one example, a cationic polymer may comprise a poly(arginine), such as poly(L-arginine). A cationic polymer within the polyplex may have a molecular weight of between 1 kDa and 200 kDa. A cationic polymer within the polyplex may also have a molecular weight of between 10 kDa and 100 kDa. A cationic polymer within the polyplex may also have a molecular weight of between 15 kDa and 50 kDa. In one example, a cationic polymer comprises poly(L-arginine) with a molecular weight of approximately 29 kDa, as represented by SEQ ID NO: 1 (PLR). In another example, a cationic polymer may comprise linear poly(ethylenimine) with a molecular weight of 25 kDa (PEI). In another example, a cationic polymer may comprise branched poly(ethylenimine) with molecular weight of 10 kDa. In another example, a cationic polymer may comprise branched poly(ethylenimine) with a molecular weight of 70 kDa. In another example, a cationic polymer may comprise a D-isomer of poly(arginine) or of any of the foregoing polymers such as polypeptides, which may be particularly advantageous because polymers such as polypeptides containing a D-isomer may be less susceptible to degradation within a cell and therefore have a prolonged effect on influencing payload release and the rate thereof over time.

[0033] Continuing with FIG 1A, in a further aspect of the invention, an anionic polymer within the polyplex may be a polypeptide containing anionic amino acids, and may be, for example, poly-glutamic acid or poly-aspartic acid, or a polypeptide that comprises any combination of the foregoing. A nanoparticle may also include, in addition to or in place of any of the foregoing examples of anionic polymers, a glycosaminoglycan, a glycoprotein, a polysaccharide, poly(mannuronic acid), poly(guluronic acid), heparin, heparin sulfate, chondroitin, chondroitin sulfate, keratan, keratan sulfate, aggrecan, poly(glucosamine), or an anionic polymer that comprises any combination of the foregoing. In one example, an anionic polymer

may comprise poly-glutamic acid. An anionic polymer within the polyplex may have a molecular weight of between 1 kDa and 200 kDa. An anionic polymer within the polyplex may also have a molecular weight of between 10 kDa and 100 kDa. An anionic polymer within the polyplex may also have a molecular weight of between 15 kDa and 50 kDa. In one example, an anionic polymer is poly(glutamic acid) with a molecular weight of approximately 15 kDa. Polymers consisting of or including a D-isomer of glutamic acid may be particularly advantageous because they may be less susceptible to degradation within a cell and therefore have a prolonged effect on influencing payload release and the rate thereof over time. For example, the anionic polymer within the polyplex may have the sequence represented by SEQ ID NO: 2 (PDGA). In another example, an anionic polymer may comprise a D-isomer of any of the foregoing polymers or polypeptides, which may be particularly advantageous because polymers such as polypeptides containing a D-isomer may be less susceptible to degradation within a cell and therefore have a prolonged effect on influencing payload release and the rate thereof over time.

Continuing with FIG. 1A, in another aspect of the invention, a cationic peptide in a nanoparticle's polyplex core may be a fragment of a histone peptide, such as of the H1, H2, H3, or H4 proteins. The fragment may include amino acids whose sequence corresponds to the N-terminus of a histone protein. For example, the fragment may comprise up to the first 5 (SEQ ID NO: 9), 10 (SEQ ID NO: 10), 15 (SEQ ID NO: 11), 20 (SEQ ID NO 12), 25 (SEQ ID NO: 13) or more N-terminal amino acids of a histone protein. The fragment may also be amidated on its C-terminus. The fragment may also have been modified such that one or more lysine residue is methylated, one or more histidine, lysine, arginine, or other complementary residues are acetylated or susceptible to acetylation as a histone acetyltransferase or acetyl CoA substrate, or any combination of the foregoing. For example, a cationic peptide in a nanoparticle polyplex core may have the sequence as represented by SEQ ID NO: 3, which comprises the first 25 amino acids of the human histone 3 protein, amidated on its C-terminus, and tri-methylated on the lysine 4 in accordance with the present invention (HTP).

[0034] In another embodiment, a nanoparticle may include or contain, in addition to or in place of any of the foregoing cationic polypeptides, a nuclear localization

sequence. A cationic polypeptide may comprise a nuclear localization sequence on its N- or C-terminus. A nuclear localization sequence may comprise an importin or karyopherin substrate, or may have or contain a sequence corresponding to SEQ ID NO: 8. In another embodiment, a nanoparticle may include, in addition to or in place of any of the foregoing cationic polypeptides, a mitochondrial localization signal or a peptide fragment of mtHSP70.

[0035] Continuing with FIG. 1B, in another aspect of the invention, the nanoparticle may comprise a reversible coating that provides stability to the polyplex core prior to cellular or compartmental internalization, preventing premature degradation or destabilization. For example, a silica coating may be applied to the polyplex core. In another example, calcium phosphate or hydroxyapatite may be applied to a polyplex core. In another example, a branched cationic polymer, polypeptide, or peptoid may be applied to a a polyplex core, with an anionic charge excess. A coating, such as a silica coating, may protect the polyplex from degradation before exposure to the endosomal microenvironment.

[0036] In another aspect, a nanoparticle may comprise a layer of polymers attached to or electrostatically bound with the external surface of coated polyplex, such as to or with the external surface of a silica coating. Such external polymers may serve to prevent cellular repulsion of the coated polyplex so as to promote contact with and uptake by a cell. An external polymer layer may also serve to promote internalization by specific cell types, such as if the externally attached polymer is or mimics a ligand to a receptor expressed by a cell type of which transfection is desired. A polymer in a polymer layer attached to the outer surface of coating on a polyplex may be from between 0.1 to 20 kDa in size, or may be up to 40 or 50 kDa in size.

[0037] Examples of polymer comprising a polymer layer attached to the external surface of the coated core polyplex include those represented by SEQ ID NO: 4, which is an approximately 10 kDa poly(arginine) polymer, and SEQ ID NO: 5, which is human vasoactive endothelial growth factor protein, in accordance with the present invention. In another example, a polymer comprising a layer attached to the external surface of the coated core polyplex may comprise an anchor substrate of from between 1 to 25 repeating anionic or cationic moieties at the N-terminus, C-terminus, 5', or 3' end of a polymer, polypeptide, or polynucleotide to provide electrostatic

conjugation of a targeting motif contained in the polymer, polypeptide, or polynucleotide to the coated polyplex core. In another example, a polymer comprising a layer attached to the external surface of the coated core polyplex may comprise a polymer, polypeptide, or polynucleotide sequence that exhibits base pair complementarity or binding affinity for an amino acid sequence binding motif to bind additional layers that may be added thereupon.

[0038] In another aspect of the present invention, illustrated in FIG. 2A, a cationic polyplex is created, then coated with a silica coating. Polyplex cores of nanoparticles may be created via electrostatic interactions leading to condensation. Two equal-volume solutions may be created, one with pH-unadjusted 40 mM HEPES (pH ~5.5) combined with 0.1% w/v a cationic polymer and a cationic polypeptide in water and the other with 30 mM Tris-HCl (pH ~7.4) combined with 0.1% w/v anionic polymers and a polynucleotide in water. In one embodiment, the cationic polymer comprises SEQ ID NO: 1, the anionic polymer comprises SEQ ID NO: 2, and the cationic polypeptide comprises SEQ ID NO: 3. These solutions may be combined via dropwise addition of the cationic solution to the anionic one with no stirring. After 30 minutes of incubation at room temperature, a 200 uL solution containing 10 ug of nucleic acids within polyplexes may be added dropwise to a 45 mM sodium silicate (Sigma) solution in Tris-HCl (pH = 7.4) and allowed to incubate for between 8 and 24 hours at room temperature. Silica-coated polyplexes may be isolated via centrifugation with a 300 kDa Nanosep® filter (Pall, Port Washington, NY) at 3000g in order to isolate complexes from unbound silica species and polymers. Nanoparticles may further be resuspended in a solution containing a polymer to be attached to the external surface of the silica coating. For example, they may be resuspended in a solution comprising a polymer represented by SEQ ID NO: 4 or SEQ ID NO: 5 at 0.1% w/v for one hour. Nanoparticles may then be centrifuged again before resuspension in transfection medium. This method is but one example of manufacturing nanoparticles in accordance with the present invention.

[0039] FIG. 2B is a diagrammatic representation of contacting a cell with a nanoparticle in accordance with the present invention leading to cellular internalization of the nanoparticle, such as by caveolae-mediated endocytosis or macropinocytosis. Nanoparticles may further be retrogradely transported through the

Golgi and endoplasmic reticulum or processed through lysosomal pathways, resulting in loss of the coating, such as a silica coating, and exposure of the polyplex core. The polyplex core may further be translocated into the cell nucleus, where enzymatic processing my degrade the cationic polymer, such as through activity of arginases, or otherwise promote unpackaging of the polyplex core, such as through acetylation of a histone tail peptide within the polyplex, leading to release of polynucleotides such as plasmid DNA from the polyplex core, in accordance with the present invention. Other intracellular processing steps modifying the constituents of a nanoparticle and its polyplex core or coating thereof or polymer layer attached to the coating may also occur in accordance with the present invention.

[0040] In a further aspect, the present invention includes optimized ratios of anionic and cationic polymers, cationic polypeptides, and polynucleotides for complexation of a polyplex core as part of a nanoparticle. In one example, plasmid DNA was fluorescently tagged with ethidium bromide (40 ng EtBr / ug DNA) before addition of various polymeric constituents in molar [1(positive)]:[1(negative)] ratios of [amine (n)]:[phosphate (p) + carboxylate (c)], or of c:p in the instance of poly(D-glutamic acid) (PDGA; SEQ ID NO: 2) addition. Addition of linear poly(ethylenimine) (PEI, 25 kDa) was compared to addition of poly(L-arginine) (PLR, 29 kDa; SEQ ID NO: 1) independently, as well as in conjunction with a H3K4(Me3) histone tail peptide (HTP; SEQ ID NO: 3), in order to quantify similar complexation behaviors between the two polymers as part of a binary complex (i.e., PEI + DNA or PEI + DNA) or ternary complexes (HTP + PEI + DNA or HTP + PLR + DNA). Where a cationic polymer and cationic polypeptide were both present, the relative molar ratio of each component was 60%:40%, respectively. A Zeiss filter and spectrophotometer were used to excite EtBr-tagged DNA at 510 nm for an emission at 595 nm, and results were compared amongst various formulations with unbound EtBr as a negative control.

[0041] FIG. 3 is a graph showing the effects of varying the ratio of anionic or cationic polymers or polypeptides to polynucleotides. The X axis shows charged polymer-to-phosphate ratio and the Y axis shows relative fluorescence following combination of indicated constituents. A decrease in relative fluorescence indicates displacement of EtBr from DNA and polyplex formation. Ratios of cationic polymer,

or of cationic polymer and cationic polypeptide, to DNA of approximately 5:1 and higher exhibited an approximately 40% decrease in fluorescence indicating complexation of DNA and polymers into polyplexes. Addition of PDGA in the absence of cationic polymers or cationic polypeptides did not affect complexation.

[0042] After complexing PLR-HTP-DNA, PEI-HTP-DNA, PLR-DNA and PEI-DNA polyplexes and determining that PDGA possesses no ability to cause complexation of polynucleotides, PDGA's influence on formation kinetics was established by comparison of [5.5(positive)]:[1(negative)] and [10(positive)]:[1(negative)] molar ratios of [amine (n)]:[phosphate (p)] and [amine (n)]:[phosphate (p) + carboxylate (c)] on complexation efficiencies in order to determine effects of excess cationic and equalized charge ratios on nanoparticle complexation. Inclusion of carboxylate groups from PDGA was expected to have effects on overall formation kinetics comparable to inclusion of phosphate groups from DNA. Relative fluorescence was compared to DNA without addition of polymers or polypeptides or EtBr in the absence of DNA as controls.

[0043] FIG. 4 indicates the effects of adding PDGA to cationic polymers and cationic polypeptides on polyplex complexation kinetics. DNA was complexed with HTP, PLR or PEI, with or without addition of PDGA. Shown are experiments using cationic polymer (PLR or PEI)-to-polynucleotide molar ratios of 5.5:1 (as shown in the bars labeled n/p = 5.5) and cationic polymer (PLR or PEI)-to-polynucleotide plus anionic polymer molar ratios of 5.5:1 and 10:1 (as shown in the bars labeled n/(p+2c) = 5.5 or 10), with or without addition of HTP. Addition of PDGA did not impair complexation kinetics at any of the molar ratios tested.

[0044] Effects of including a cationic polymer and cationic polypeptide on polyplex destabilization were also determined, as shown in FIG. 5. Polyplex nanoparticles of DNA and cationic polypeptides (PLR with or without HTP, or PEI with HTP) with [(PDGA) carboxylate(c):(DNA) phosphate(p)] molar ratios varying from 0 to 100 were complexed as described, compared to DNA or EtBr alone as controls, and the effects of destabilization (as indicated by increased fluorescence) was determined. In the absence of HTP, addition of PDGA did not lead to polyplex destabilization. However, in the presence of HTP, adding molar ratios of PDGA to DNA of 20 and above led to polyplex destabilization. These results indicate a surprising synergistic

effect of cationic polypeptide and anionic polymer on complex destabilization. Cationic polypeptide incorporation, and/or inclusion of cationic constituents of disparate molecular weights or sizes, into a nanoparticle polyplex core may beneficially enhance the ability of a cationic polymer to promote dissociation and release of the polynucleotide payload from the polyplex and its other constituents.

[0045] Dynamic light scattering (BRAND) was used to determine the hydrodynamic radii of nanoparticles at various stages of formation. Nanoparticles containing core polyplexes with plasmid DNA, PLR, PDGA, and HTP, at a molar ratio of [amide]:[(phosphate)] of 5.5:1 were complexed as described. Some polyplex cores were further coated with silica as described. And some silica-coated polyplexes were further layered with cationic polymer (SEQ ID NO: 4) as described. 30 – 60 minutes of measurements were obtained following initial core formation of ternary complexes, silica coating of cores, and cationic polymer-coating of silica-coated cores. FIG. 6 is a graph showing diameters of nanoparticles. Uncoated polyplex cores and polyplex cores coated with silica were approximately 70-150 nm in diameter on average. In other embodiments, polyplex cores and silica-coated polyplex cores may be within a range of 100-170 nm in average diameter. Adding a cationic polymer coating to the silica coating yielded a nanoparticle with an average diameter of approximately 170 nm. In other embodiments, silica-coated polyplex cores with an additional layer of cationic polymer attached to the outer layer of silica may be within a range of approximately 80-200 nm in average diameter.

[0046] Cellular uptake of nanoparticles was also determined. Fluorescein isothiocyanate (FITC) was covalently conjugated to amines of PEI (25 kDa linear) and PLR (29 kDa) such that the molar ratio of amines to FITC was 100:1. The reaction was performed in darkness at room temperature for four hours in equal volumes of water and DMSO. In order to establish conjugation, a 0.05% w/v 500 uL solution of each fluorescently modified polymer was centrifuged in a 10 kDa Nanosep® filter and the eluate's fluorescence intensity (485 ex./520 em.) was compared to the unfiltered polymer solution as well as water. mCherry plasmid (Addgene) was included in nanoparticles to permit fluorescent detection of plasmid-driven expression.

[0047] MC3T3 murine osteoblasts were cultured on polystyrene T-75 tissue culture plastic flasks (Corning, CA, USA). Dulbecco's modified eagle medium supplemented with 10% Fetal Bovine Serum (Thermo Fisher Scientific, VA, USA) was used for osteoblasts along with 1% penicillin/streptomycin (Invitrogen, NY, USA). Xylenol orange was added to the cell culture media from day 15 to day 25 after initiation of cell culture. At day 25 cells were fixed and assayed for mineralization. For mCherry plasmid delivery using FITC-modified nanoparticles, osteoblasts were plated at 1000 cells/well in 96-well plates and allowed to adhere for 12 - 16 hours in antibiotic-free DMEM containing 10% FBS. Immediately before transfection, medium was replaced with equal volumes of OptiMEM-suspended nanoparticles and DMEM containing 10% FBS.

[0048] All complexes were FITC-labeled and subjected to qualitative observation of fluorescence intensity (488/520 ex./em.) before transfection. 96-well-plated osteoblasts (1000 cells/well) were transfected with 200 ng of plasmids in triplicates for each binary (plasmid and cationic polymer), ternary (plasmid and cationic polymer, plus anionic polymer or cationic polypeptide), and quaternary (plasmid, cationic polymer, anionic polymer, and cationic polypeptide) complex as well as its silica-coated counterpart, with 1 control and 8 experimental sets (n = 3) in total. 5% serum was used in order to study effects of serum on extracellular properties of aggregation.

[0049] At 30-hours post-transfection, bimodal fluorescent imaging allowed for simultaneous observation of FITC-labeled nanoparticles (488 ex./520 em.) and the mCherry gene expression that they were responsible for (633 ex./680 em.). A minimum of 20 cells were observed at different locations in each well and representative images were obtained. ImageJ was used to process the overlaid images and combine phase-contrast, 488/520 and 633/680 channels.

[0050] Photomicrographs demonstrating cellular uptake are shown in FIG. 7. Circles in FIG. 7 indicate where high levels of nuclear localization is apparent. Silica-coated binary nanoparticles show burst release properties (i.e., nuclear localization is not apparent in the DNA-PLR + silica samples). Inclusion of PDGA in polyplex cores causes prolonged release of plasmid within cell nuclei. This effect of PDGA to cause prolonged release was surprising in light of literature suggesting the opposite:

that including cationic polymers in nanoparticle polyplexes would hasten, and shorten the duration of, dissociation of polynucleotide payload from other polyplex constituents. Addition of HTP also causes extensive nuclear localization.

[0051] Further coating of silica-coated nanoparticles (DNA-HTP-PDGA-PLR + Si) with poly(arginine) (SEQ ID NO: 4) causes nanoparticles to be stable in serum and causes extended residence of nanoparticle payload within cells. FIG. 8. is photomicrographs showing cellular uptake and retention of silica-coated FITC-conjugated polyplex cores, to which an additional layer of poly(L-arginine) (SEQ ID NO: 4) has been added, by MC3T3 murine osteoblasts, in accordance with the present invention. Unlike for silica-coated nanoparticles shown in FIG. 7, no aggregation of nanoparticles containing an additional layer of cationic polymers on the outside of the silica coating is observable in FIG. 8, indicating that such nanoparticles remain stable in serum. Furthermore, these nanoparticles are observed to display extended residence within the cell nucleus such that fluorescence qualitatively peaks within approximately 1.5 days and detectable fluorescence was sustained through 14 days.

[0052] Layering silica-coated polyplex cores with polymers specifically directed to bind to particular cell types can further enhance uptake. Associating ligands for cellular receptors with the surface of a nanoparticle can enhance affinity of the nanoparticle for cells that express such receptors and increase transfection of such cells. As one example in accordance with the present invention, silica-coated polyplexes were coated with VEGF (SEQ ID NO: 5), a high-affinity ligand for VEGF receptors, which are expressed at high levels by human umbilical vein endothelial cells (HUVECs). HUVECs were incubated with silica-coated FITC-conjugated polyplexes with poly(L-arginine) (SEQ ID NO: 4) or human VEGF (SEQ ID NO: 5) attached to the outer surface of the silica coating for 40 min before being washed twice with PBS then resuspended in DMEM (10% FBS). Cells were imaged 4 hrs later. After this short incubation period, only low levels of transfection with nanoparticles containing a poly(L-arginine) layer attached to the external silica surface (FIG. 9A) was observed, whereas coating with VEGF instead of poly(L-arginine) resulted in significantly greater cellular internalization at this four-hour time point. A skilled artisan would recognize that virtually any other cell type may also be transfected by nanoparticles in accordance with the present invention, and that a layer

of polymers may be attached to the outer layer of silica-coated polyplex cores to promote or otherwise influence this effect. Such a person would also comprehend that other means of contacting cells with nanoparticles to effect such outcomes, such as i.p., i.v., i.m. or s.c. or other injection or transdermal administration or via suppository to, or ingestion or oral or nasal inhalation by, a human or animal, or contact with explanted tissue or cells or stem cells, would also be included within the present invention.

[0053] In another aspect of the invention, a polynucleotide encoding a nuclease may be incorporated into the nanoparticle polyplex core. As one nonlimiting example, a polynucleotide that encodes and drives expression of a TALEN (Transcription Factor-Like Effector Nucleases) may be included in the nanoparticle. Like Zinc Finger Nucleases, TALENs utilize a modular DNA binding motif (TALE) that can be modified to introduce new DNA binding specificities and even nucleases (TALEN). TALEs consist of multiple repeat variable diresidues (RVDs) which each specify binding to a single nucleotide. TALE arrays are made by stringing together RVDs in a specific order to provide specificity and binding affinity to desired DNA sequences. Commonly, these genome-splicing tools are engineered by fusing non-specific cleavage domains, such as FokI nucleases, to TALEs. TALEN assembly protocols are available that allow assembly of these repetitive sequences, including an open source assembly method known as Golden Gate.

[0054] In another aspect of the present invention, nanoparticles may be designed and used in a manner to regulate expression of signaling molecules to alter cellular function. For example, sequences of chromosomal DNA may be deleted or altered to generate cellular or animal models of disease states or treatments therefor, or to treat disease states or otherwise enhance human health. One nonlimiting example of a protein whose expression may be modified in accordance with the present invention is sclerostin (SOST). SOST binding to the LRP5/6 receptor inhibits Wnt signaling, perhaps via feedback systems between Wnt3A, Wnt7B, Wnt10A, sclerostin, β-catenin, LEF1, and TCF1. Desuppressing these cascades via removal of sclerostin may result in significantly increased mineralization activity.

[0055] Osteoprogenitor (OPG) and RANKL are also expected to play a responsive role to SOST deletion, where RANKL expresses itself as a receptor for promoting

osteoclastogenesis via osteoclast-linked RANK or ODF (osteoclast differentiation factor) binding, and OPG binds antagonistically to RANKL. Thus, the ratio between OPG and RANKL is a determinant of the relationship between bone formation and resorption. However, single cultures of osteoblasts will communicate through other forms of paracrine signaling and this ratio should be more reflective of behavior of altered cells in co-culture with osteoclasts or in vivo.

[0056] In another aspect of the present invention, a nanoparticle may be designed so as to allow transfection with a TALEN that may disrupt expression of SOST and consequently generate a high bone-mass phenotype. As one example, TALENS may be engineered to specifically bind to loci in the SOST gene and create double-stranded breaks in the genome to disrupt transcription or translation and reduce SOST expression. As a further example, a nanoparticle may contain plasmids that encode two TALENs that create double-stranded breaks on either side of the chromosomal locus of the start codon for SOST. Repair of endogenous genomic DNA following excision of the sequence encoding the start codon may result in transcription of sclerostin mRNA lacking the start codon that cannot be properly translated into SOST protein, thereby driving down SOST expression and activity. A diagrammatic representation of this model is shown in FIG. 10, where a "left" TALEN and "right" TALEN bind to and cleave sites on opposite sides of the SOST start codon locus. As one example, a left TALEN may have the sequence represented by SEQ ID NO: 6, and a right TALEN may have the sequence represented by SEQ ID NO: 7. A nanoparticle may comprise an expression plasmid, such as pUC19 (Genbank Accession Number L09137 X02514), into which a nucleotide sequence that encodes a right or left TALEN, such as those represented by SEQ ID NO: 6 and SEQ ID NO: 7, has been subcloned so as to drive cellular expression of the encoded TALEN. A nanoparticle may also include combinations of expression plasmids that comprise sequences that encode left and right TALENs.

[0057] A nanoparticle may also comprise other TALEN sequences, targeting SOST or any other gene of interest, and also may comprise other expression vectors, in accordance with the present invention. A nanoparticle may comprise other types of polynucleotides or analogs thereof, such as species of RNA or DNA including mRNA, siRNA, miRNA, aptamers, shRNA, AAV-derived nucleic acids, morpholeno

RNA, peptoid and peptide nucleic acids, cDNA, DNA origami, DNA and RNA with synthetic nucleotides, DNA and RNA with predefined secondary structures, CRISPR sequences, and multimers and oligomers, and any combination of the foregoing, in accordance with the present invention. In another example, a nanoparticle may comprise polynucleotides whose sequence may encode other products such as any protein or polypeptide whose expression is desired. A skilled artisan would recognize that the foregoing examples are in accordance with the present invention and may be encompassed by claims thereto.

[0058] Following transfection of MC3T3 murine osteoblasts with nanoparticles designed to knock down SOST expression in accordance with the present invention, ELISA and quantitative real-time PCR (qPCR) assays were performed on cell lysate and supernatant fractions. FIGs. 11A-11C are graphs demonstrating the effectiveness of different amounts (800 ng, 1600 ng, or 2500 ng) of nanoparticles (NP) containing expression plasmids comprising nucleotide sequences that encode left (SEQ ID NO: 6) and right (SEQ ID NO: 7) SOST TALENs, in accordance with the present invention, in modulating SOST expression and β-catenin expression over a period of up to over 20 days following transfection. For comparison, other cells were transfected with mRNA encoding the same TALENS using Lipofectamine, a known agent for cellular transfection. As shown in FIGs. 11A-11C, intracellular and extracellular SOST levels were suppressed for at least several weeks following transfection with nanoparticles in accordance with the present invention, whereas β-catenin expression was concomitantly up-regulated, signifying effectiveness of the nanoparticles in downregulating SOST expression and activity.

[0059] qPCR was also performed to determine whether down-regulation of SOST expression with nanoparticles in accordance with the present invention may have downstream effects on other components of the relevant signaling cascade. Cells were transfected as described above. Results on expression of numerous components of the signaling pathway (SOST, β-catenin, TCF1, LEF1, Wnt3A, Wnt7B, Wnt10b, OPG, and RANKL), at 5, 14, and 21 days after transfection with different amounts of nanoparticles as indicated, are shown in FIGs. 12A-12F. For comparison, other cells were transfected with mRNA encoding the same TALENS using Lipofectamine. The real time PCR results showed a greater up regulation of Wnt responsive genes in the

cell lines transfected with nanoparticles delivering SOST TALENS as compared to the SOST TALENS delivered by Lipofectamine by up to 2 to 6 times as a response to knockdown of the Wnt signaling inhibitor sclerostin.

[0060] TCF/LEF-1-mediated transcription may also be upregulated following knockdown of SOST expression in accordance with the present invention. MC3T3-E1 cells were transfected with TOPflash and control FOPflash luciferase reporter plasmid constructs (Addgene# 12456 and 12457) that contain TCF/LEF-1 binding sites. The cells were plated at the density of 5000 cells/well of the 8-well labtek chamber slides and transfected with 1 ug of TOPflash and FOPflash plasmid separately. To control for the efficiency of transfection a control plasmid Renilla (Promega) was used. FIG 13 is photomicrographs showing upregulation of TCF/LEF-1-mediated transcription for 21 days following tranfection with nanoparticles containing plasmids encoding SOST-directed TALENS, in accordance with the present invention, consistent with an upregulation of TCF/LEF-1 expression and activity following transfection with the invented nanoparticles.

[0061] Knockdown of SOST expression in accordance with the present invention may also increase mineralization in stromal bone marrow cells and osteoblasts. Mineralization was quantified by two separate methods, first based on image thresholding of xylenol-orange-labeled vital cultures using MATLAB (Mathworks, Natick, MA), and second by atomic absorption spectroscopy (AAS). For the xylenol orange threshold, images of both phase and fluorescence (with Texas Red Filter Set) were taken in five adjacent regions of wells, and then stitched into a larger 8-bit image (4x, Nikon Ti-100). The phase channel was subtracted from the fluorescence, and a threshold was set to half the level between the background and signal (-6dB). The number of pixels above the threshold were counted and used to express the percentage of mineralized area in each well. The combination of phase and fluorescence allowed for unbound xylenol orange to be distinguished, whereas the use of decibel levels allowed for correction of the varied background levels in each image.

[0062] Mineralization was also quantified by atomic absorption with an atomic absorption spectrometer (AA-Perkin Elmer, MA). Each well was prepared by adding 0.5 mL of 10% nitric acid, and the resultant calcium content was measured relative to a standard curve and compared between groups. Care was taken to minimize

interference due to ionized calcium precipitating with phosphate phases, so a large excess of potassium and lanthanum ions was added to each well.

[0063] FIGs 14A-14C show the effects of transfection with nanoparticles in accordance with the present invention on mineralization following SOST knockdown. FIG. 14A is photomicrographs of staining of the mineralized matrix formed 25 days after SOST knockdown. Stromal cells are shown in panels A-C, wherein panel A show control cells, panel B shows cells transfected via Lipofectamine, and panel C shows cells transfected with nanoparticles containing plasmids encoding SOST-directed TALENs as described and in accordance with the present invention. MC3T3-E1 osteoblast cells are shown in panels D-G, wherein panel D show control cells, and panels E-G show cells transfected with nanoparticles containing plasmids encoding SOST-directed TALENs as described at doses of 800 ng, 1600 ng, and 2500 ng, respectively, in accordance with the present invention. FIGs. 14B and 14C are graphs showing quantification of mineralization. FIGs 14A-C demonstrate increased calcium concentration in stromal bone marrow cells and osteoblasts following transfection with SOST-targetting TALENS via nanoparticles in accordance with the present invention, further confirming the effectiveness of this technique of modifying the cellular expression and activity of genes and downstream signaling pathways.

[0064] While several aspects of the present invention have been described and depicted herein, alternative aspects may be effected by those skilled in the art to accomplish the same objectives. Accordingly, it is intended by the appended claims to cover all such alternative aspects as fall within the true spirit and scope of the invention.

CLAIMS:

1. A nanoparticle comprising:

a core polyplex and a silica coating thereon;

wherein said core polyplex comprises:

(i) one or more anionic polymers selected from the group consisting of poly(D-glutamic acid), a glycosaminoglycan, a glycoprotein, a polysaccharide, poly(mannuronic acid), poly(guluronic acid), heparin, heparin sulfate, chondroitin, chondroitin sulfate, keratan, keratan sulfate, aggrecan, poly(glucosamine), and any combination of two or more of the foregoing,

(ii) a cationic polymer,

(iii) a cationic polypeptide, and

(iv) a polynucleotide,

wherein the cationic polypeptide is a histone tail peptide.

2. The nanoparticle of claim 1, wherein the anionic polymer is poly (D-glutamic acid).

3. The nanoparticle according to claim 1 or 2, wherein the cationic polymer is selected from the group consisting of poly (ethyleneimine) and poly (L-arginine).

4. The nanoparticle according to any one of claims 1 to 3, wherein the histone tail peptide is a human H3 histone tail peptide.

5. The nanoparticle of claim 1, wherein the anionic polymer is poly (D-glutamic acid), the cationic polymer is selected from the group consisting of poly (ethyleneimine) and poly (L-arginine), and the cationic polypeptide is a histone tail peptide.

6. The nanoparticle according to any one of claims 1 to 5, wherein the polynucleotide comprises a nucleotide sequence that encodes a nuclease.

7. The nanoparticle of claim 6, wherein the nuclease is a TALEN.

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8. The nanoparticle of claim 7, wherein the TALEN is capable of inducing a break at a site-specific locus of DNA, wherein the break results in a change of expression of a protein encoded by a gene.

9. The nanoparticle of claim 8, wherein the change is a decrease and the gene encodes a sclerostin protein.

10. The nanoparticle of claim 5, further comprising a polymer attached to an outer surface of said silica coating.

11. The nanoparticle of claim 10, wherein said polymer attached to an outer surface of said silica coating comprises poly(L-arginine) or a vasoactive endothelial growth factor peptide.

12. An *in vitro* method of modifying intracellular polynucleotides, the method comprising: contacting *in vitro* a cell with a nanoparticle, wherein said nanoparticle comprises a core polyplex and a silica coating thereon; wherein the core polyplex comprises:

(i) one or more anionic polymers selected from the group consisting of poly(D-glutamic acid), a glycosaminoglycan, a glycoprotein, a polysaccharide, poly(mannuronic acid), poly(guluronic acid), heparin, heparin sulfate, chondroitin, chondroitin sulfate, keratan, keratan sulfate, aggrecan, poly(glucosamine), and any combination of two or more of the foregoing,

(ii) a cationic polymer,

(iii) a cationic polypeptide, and

(iv) a polynucleotide,

wherein the cationic polypeptide is a histone tail peptide.

13. The method of claim 12, wherein the anionic polymer is poly (D-glutamic acid).

14. The method according to claim 12 or 13, wherein the cationic polymer is selected from the group consisting of poly (ethyleneimine) and poly (L-arginine).

15. The method according to any one of claims 12 to 14, wherein the histone tail peptide is a human H3 histone tail peptide.

16. The method of claim 13, wherein the anionic polymer is poly (D-glutamic acid), the cationic polymer is selected from the group consisting of poly (ethyleneimine) and poly (L-arginine), and the cationic polypeptide is a histone tail peptide.

17. The method according to any one of claims 12 to 16, wherein the polynucleotide comprises a nucleotide sequence that encodes a nuclease.

18. The method of claim 17, wherein the nuclease is a TALEN.

19. The method of claim 18, wherein the TALEN is capable of inducing a break at a site-specific locus of DNA, wherein the break results in a change of expression of a protein encoded by a gene.

20. The method of claim 19, wherein the change is a decrease and the gene encodes a sclerostin protein.

21. The method of claim 16, further comprising a polymer attached to an outer surface of said silica coating.

22. The method of claim 21, wherein said polymer attached to an outer surface of said silica coating comprises poly(L-arginine) or a vasoactive endothelial growth factor peptide.

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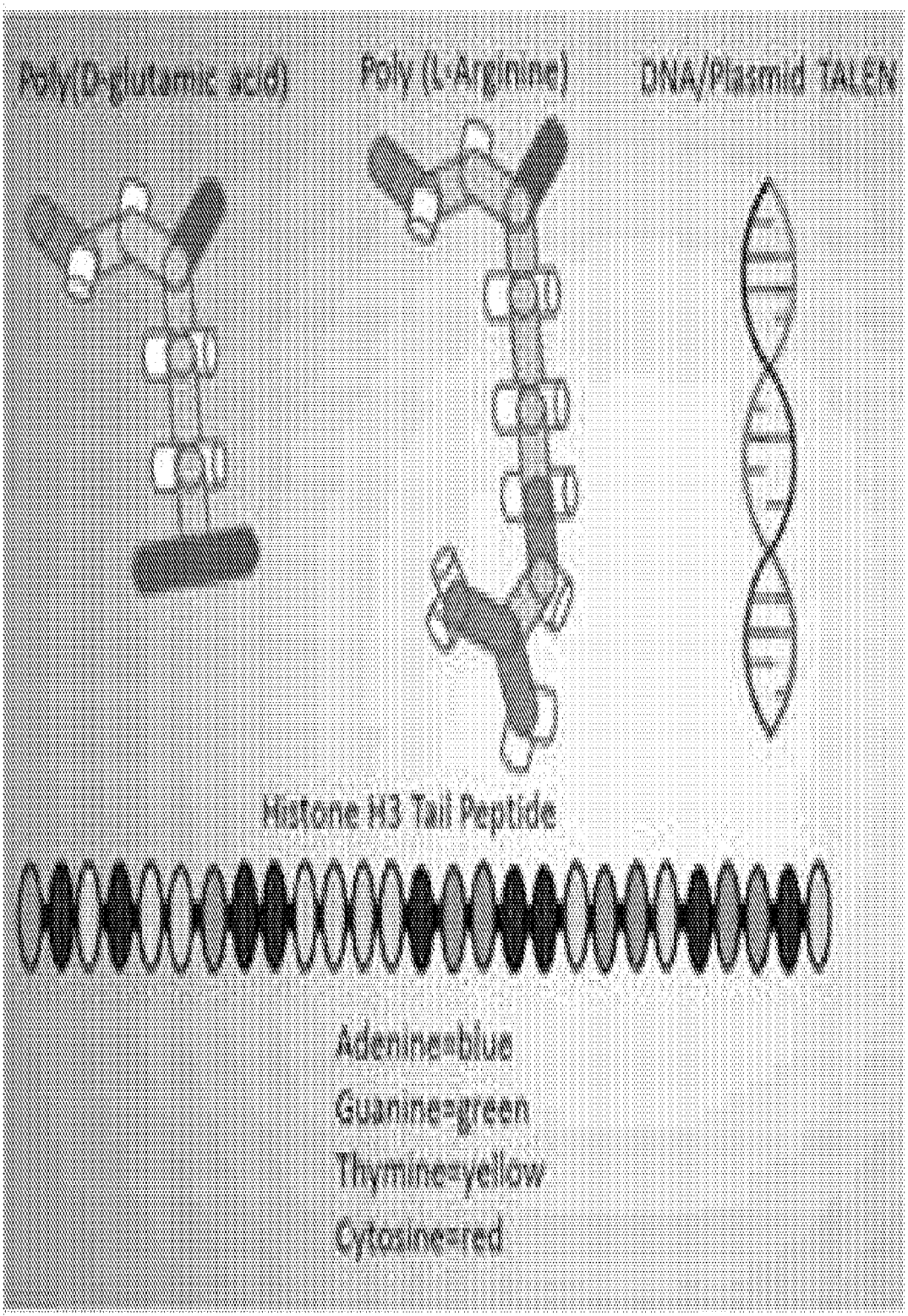

Poly(D-glutamic acid)
Poly (L-Arginine)
DNA/Plasmid TALEN
Histone H3 Tail Peptide
Adenine=blue
Guanine=green
Thymine=yellow
Cytosine=red

FIGURE 1A

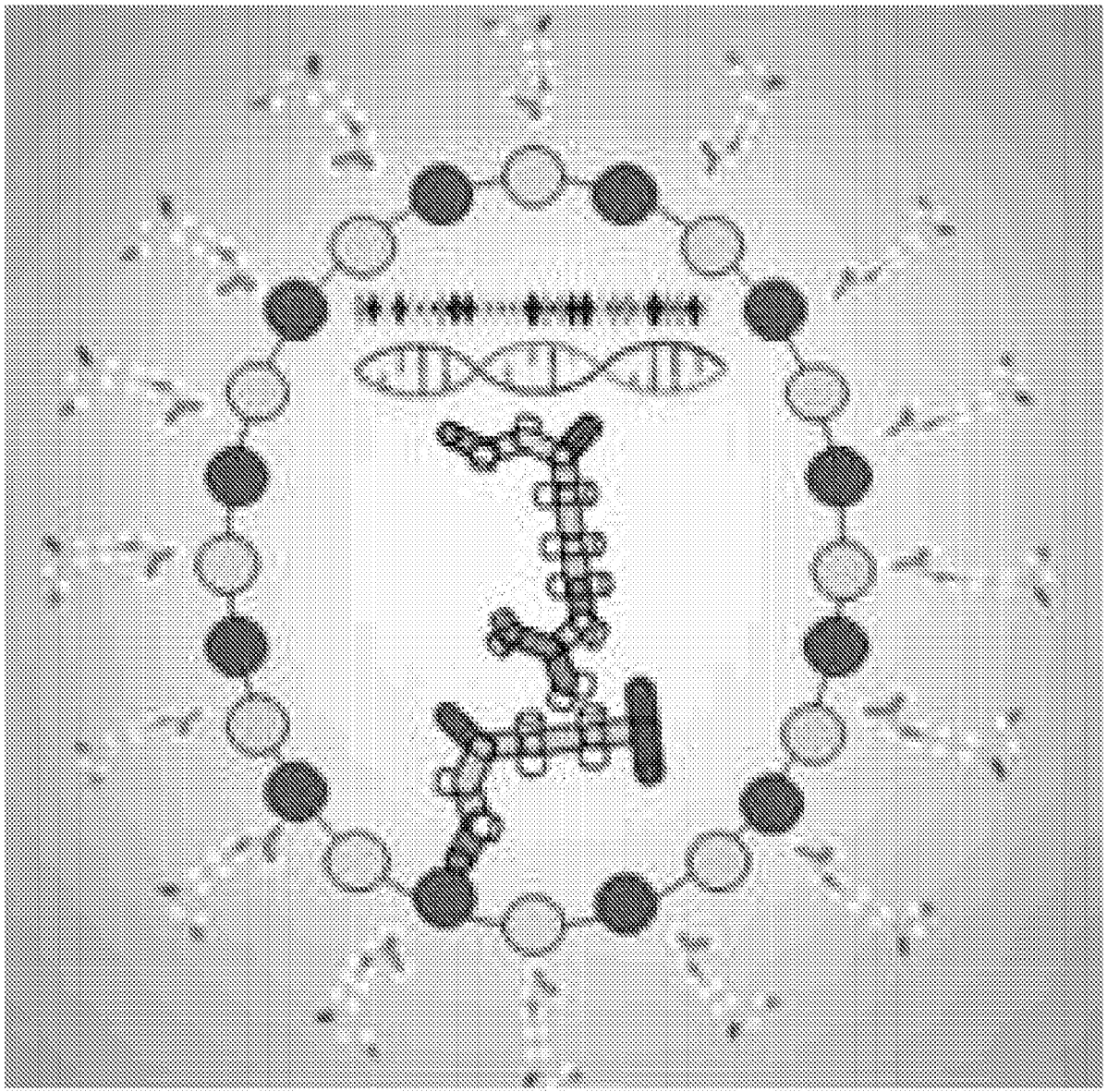


FIGURE 1B

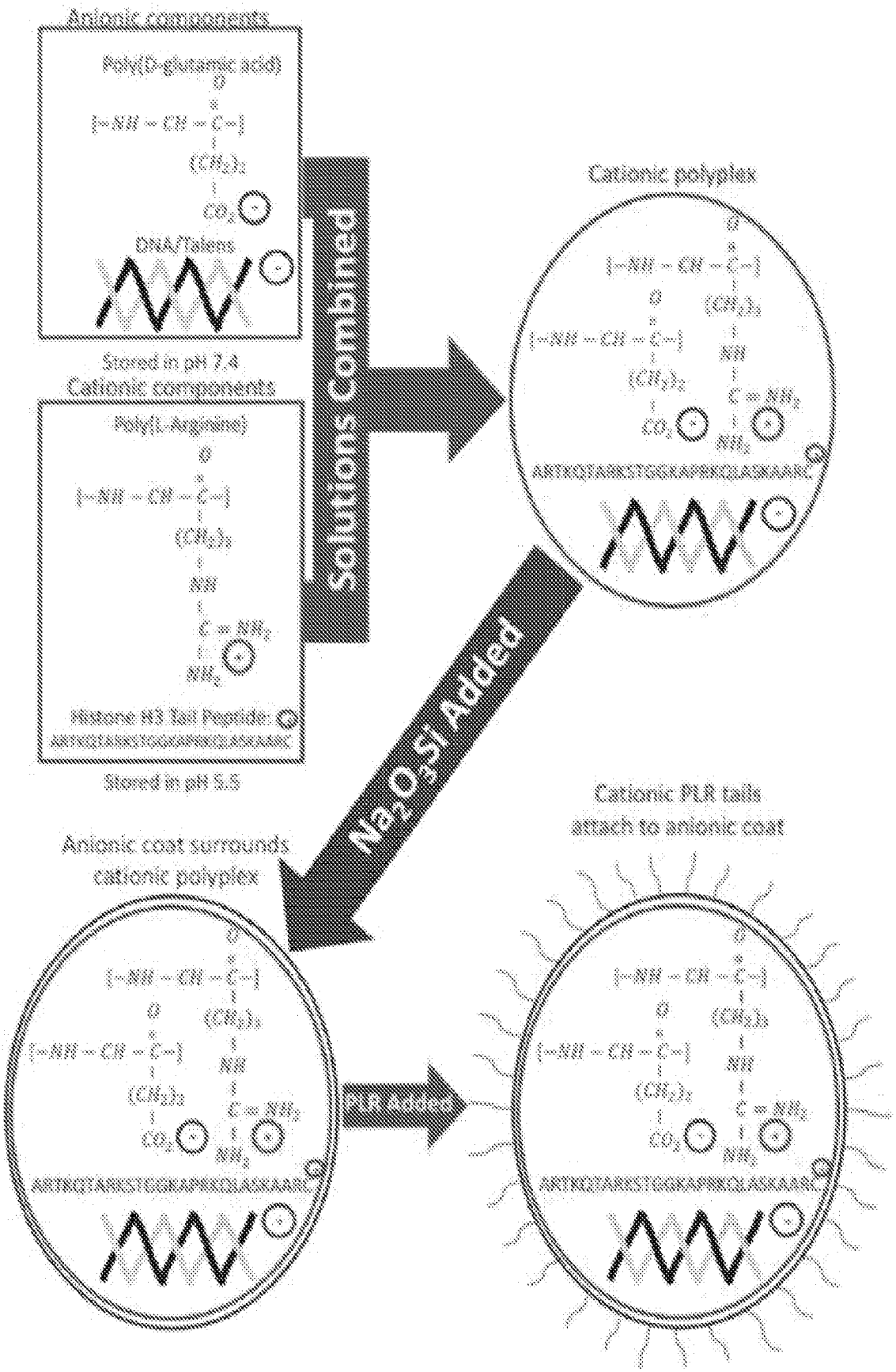

Anionic components
Poly(D-glutamic acid)
DNA/Talens
Stored in pH 7.4
Cationic components
Poly(L-Arginine)
Histone H3 Tail Peptide
Stored in pH 5.5
Solutions Combined
Cationic polyplex
Na_2O_3Si Added
Anionic coat surrounds cationic polyplex
Cationic PLR tails attach to anionic coat
PLR Added

FIGURE 2A

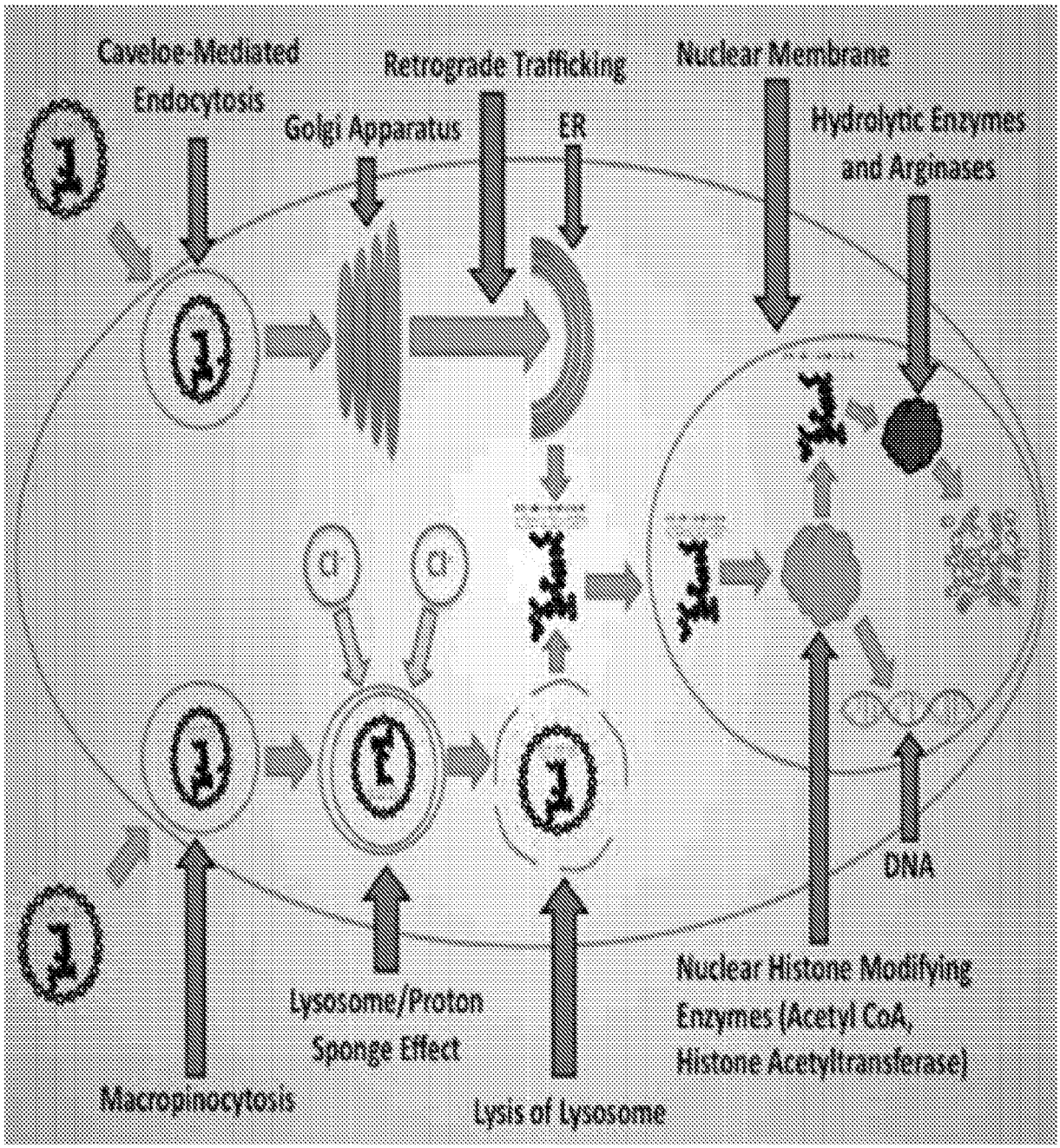

Caveloe-Mediated Endocytosis
Retrograde Trafficking
Nuclear Membrane
Golgi Apparatus
ER
Hydrolytic Enzymes and Arginases
DNA
Nuclear Histone Modifying Enzymes (Acetyl CoA, Histone Acetyltransferase)
Lysosome/Proton Sponge Effect
Macropinocytosis
Lysis of Lysosome

FIGURE 2B

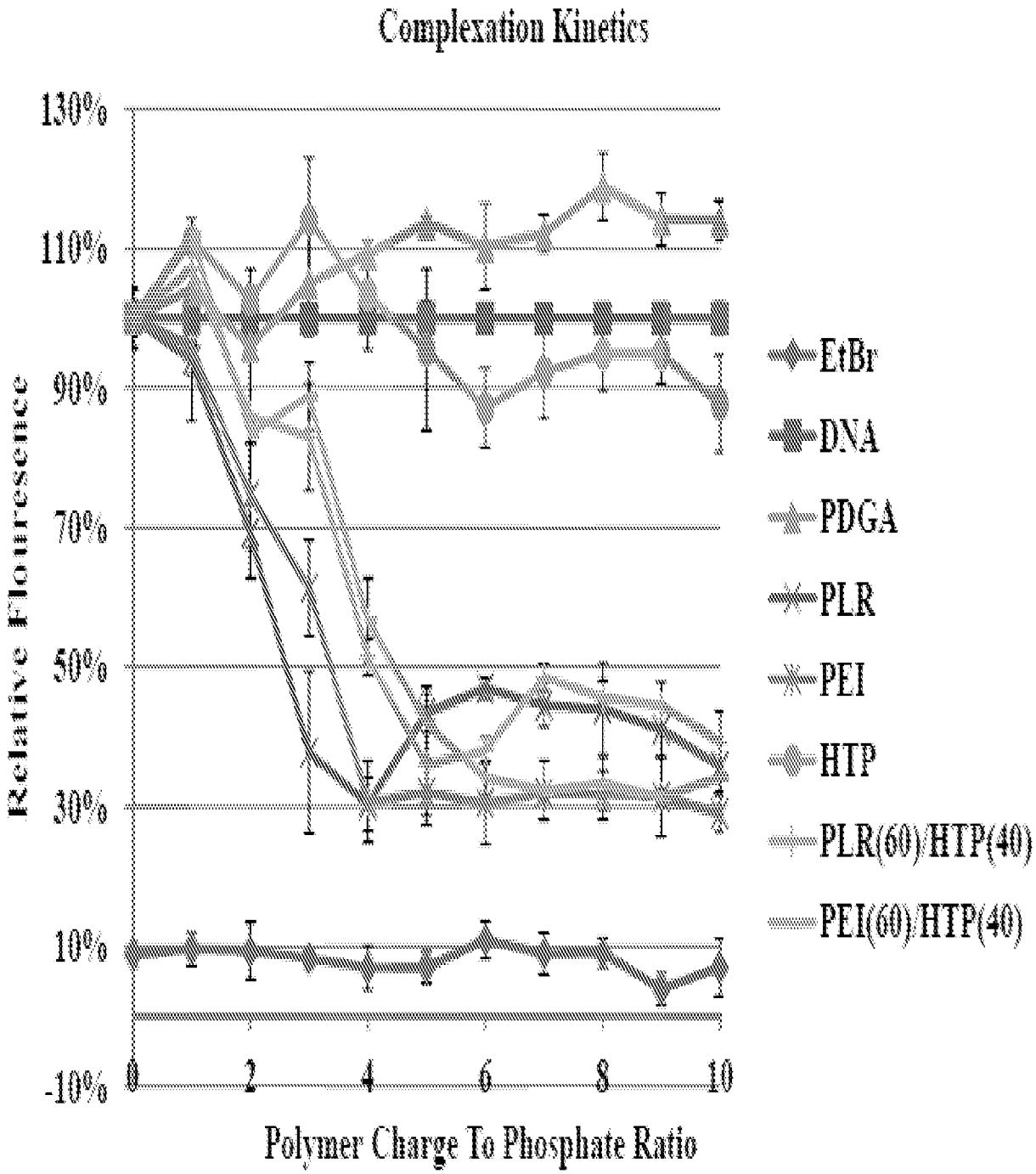

Complexation Kinetics
130%
110%
90%
70%
50%
30%
10%
-10%
Relative Flourescence
0
2
4
6
8
10
Polymer Charge To Phosphate Ratio
EtBr
DNA
PDGA
PLR
PEI
HTP
PLR(60)/HTP(40)
PEI(60)/HTP(40)

FIGURE 3

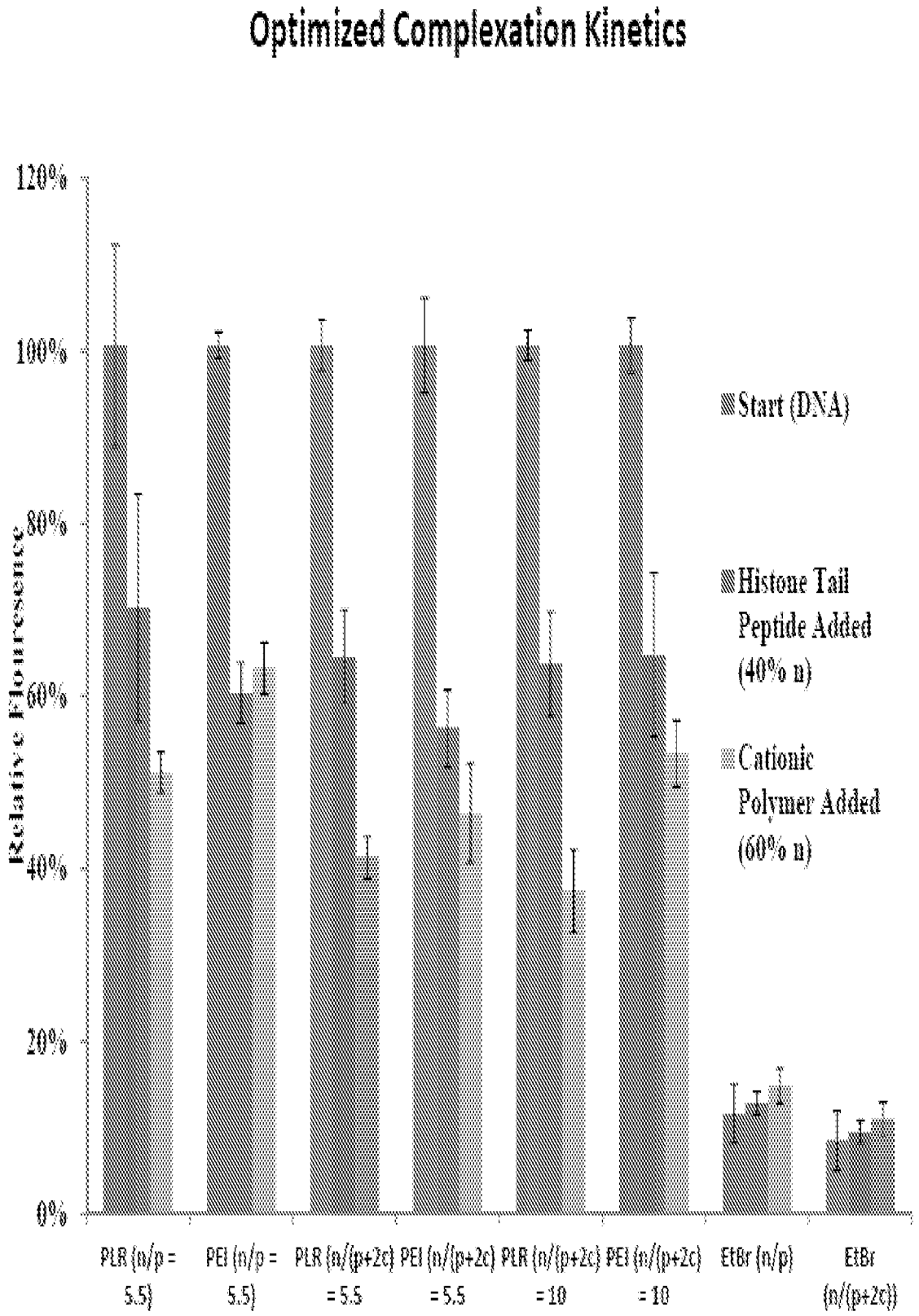

Optimized Complexation Kinetics
Relative Flourescence
120%
100%
80%
60%
40%
20%
0%
Start (DNA)
Histone Tail Peptide Added (40% n)
Cationic Polymer Added (60% n)
PLR (n/p = 5.5)
PEI (n/p = 5.5)
PLR (n/(p+2c) = 5.5
PEI (n/(p+2c) = 5.5
PLR (n/(p+2c) = 10
PEI (n/(p+2c) = 10
EtBr (n/p)
EtBr (n/(p+2c))

FIGURE 4

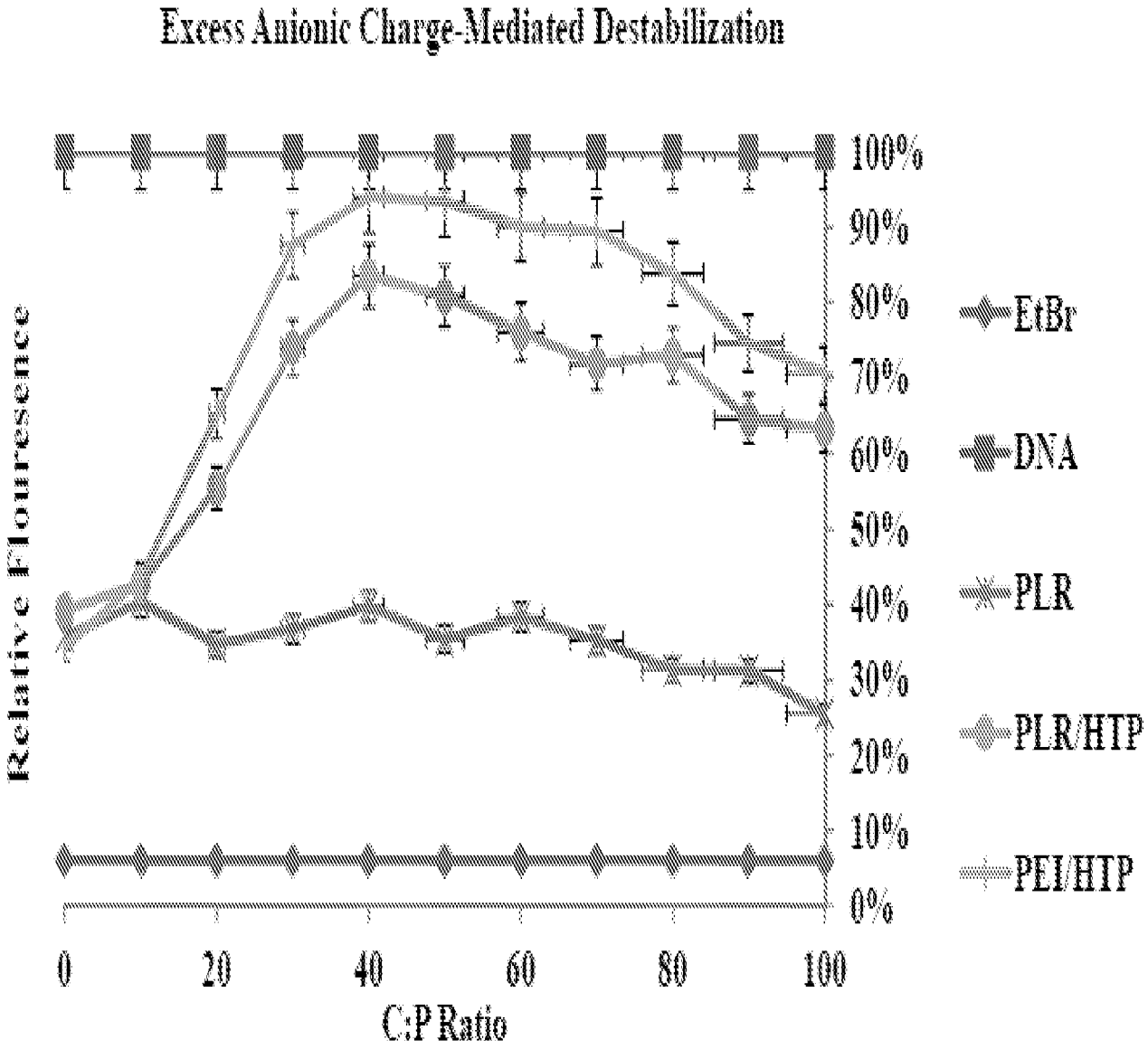

Excess Anionic Charge-Mediated Destabilization
Relative Flourescence
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
0
20
40
60
80
100
C:P Ratio
EtBr
DNA
PLR
PLR/HTP
PEI/HTP

FIGURE 5

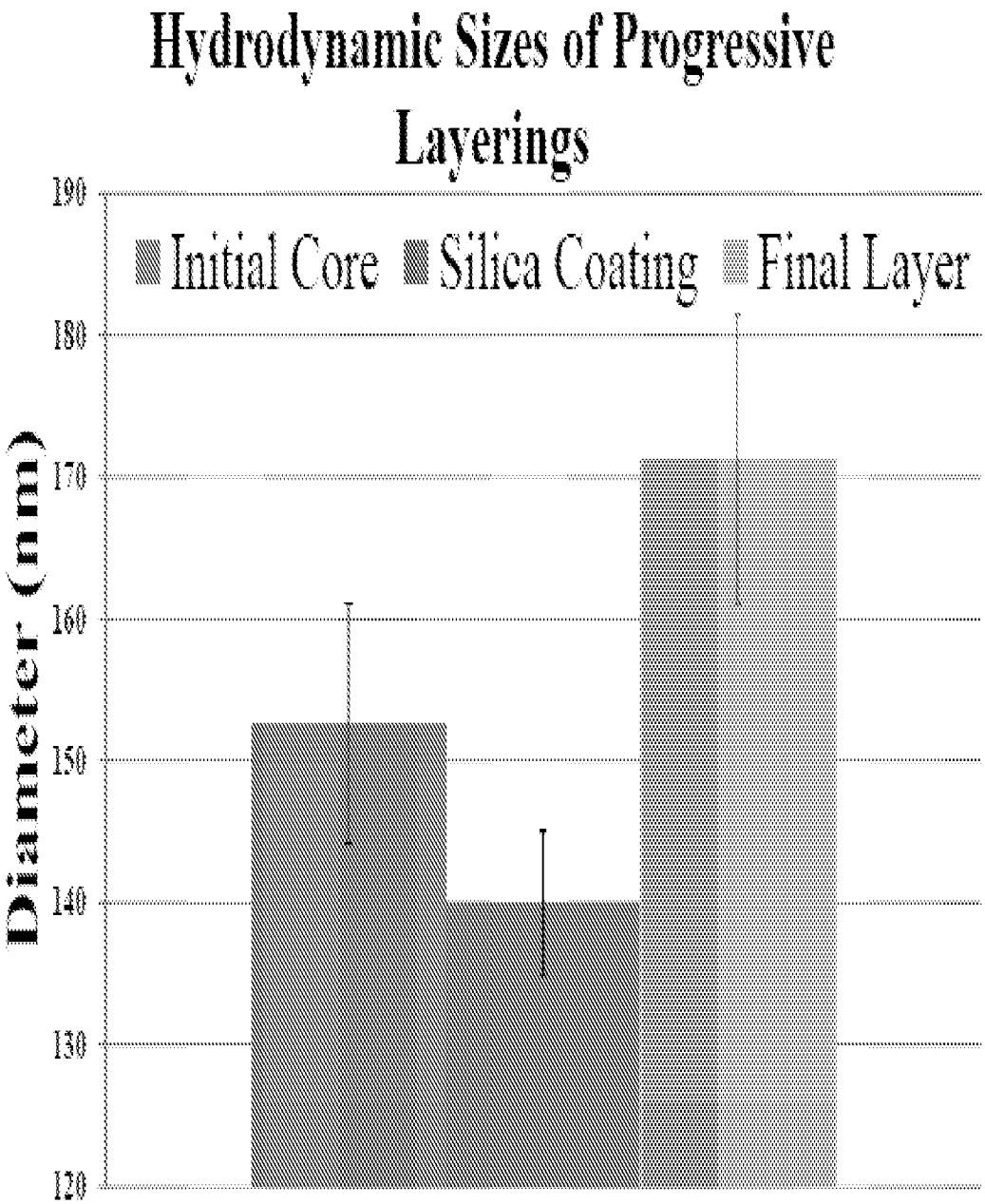

Hydrodynamic Sizes of Progressive Layerings
Initial Core
Silica Coating
Final Layer
Diameter (nm)
190
180
170
160
150
140
130
120

FIGURE 6

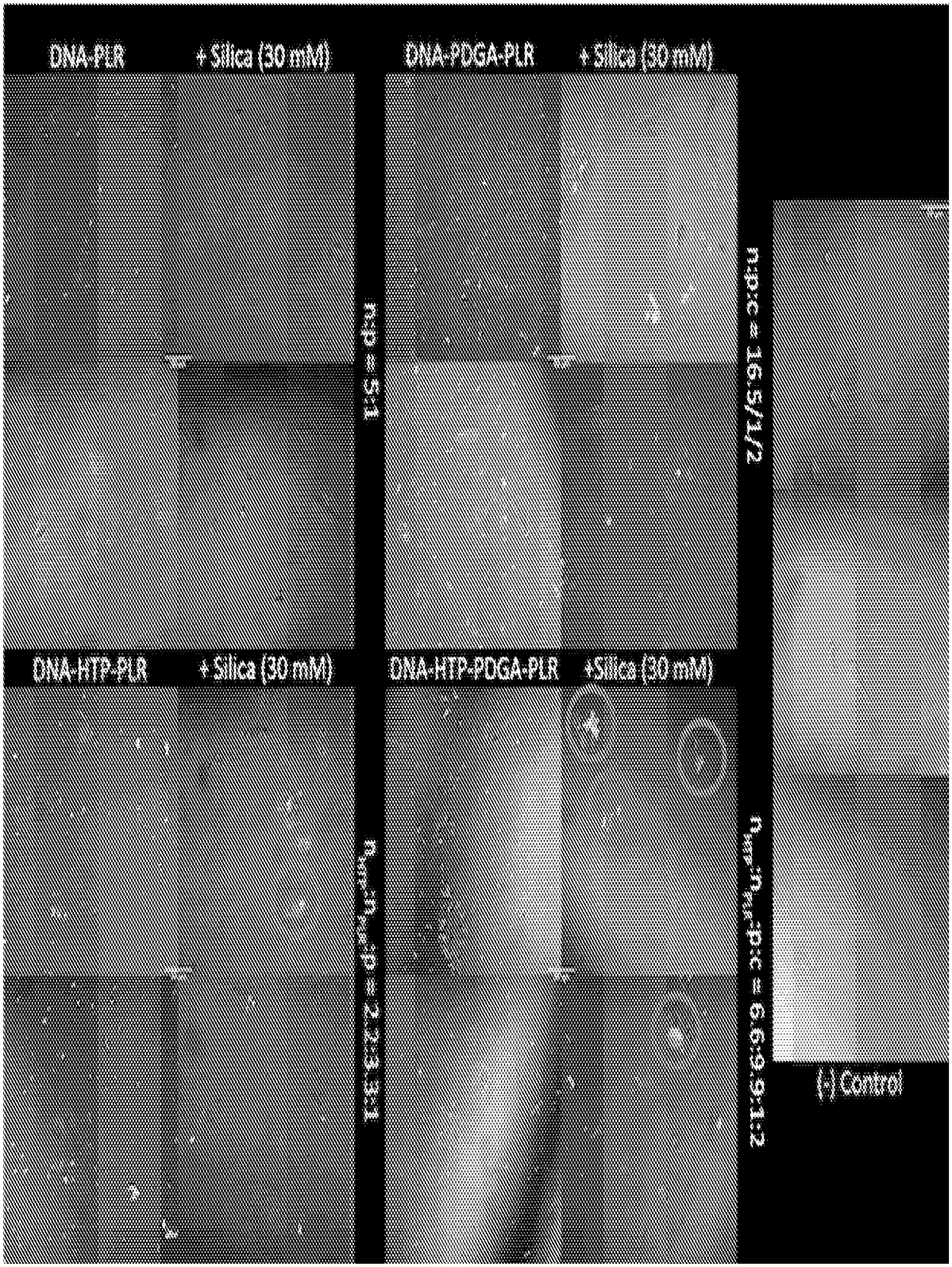

DNA-PLR
+ Silica (30 mM)
n:p:c = 5:1:1
DNA-PDGA-PLR
+ Silica (30 mM)
n:p:c = 16.5/1/2
DNA-HTP-PLR
+ Silica (30 mM)
DNA-HTP-PDGA-PLR
+Silica (30 mM)
(-) Control

FIGURE 7

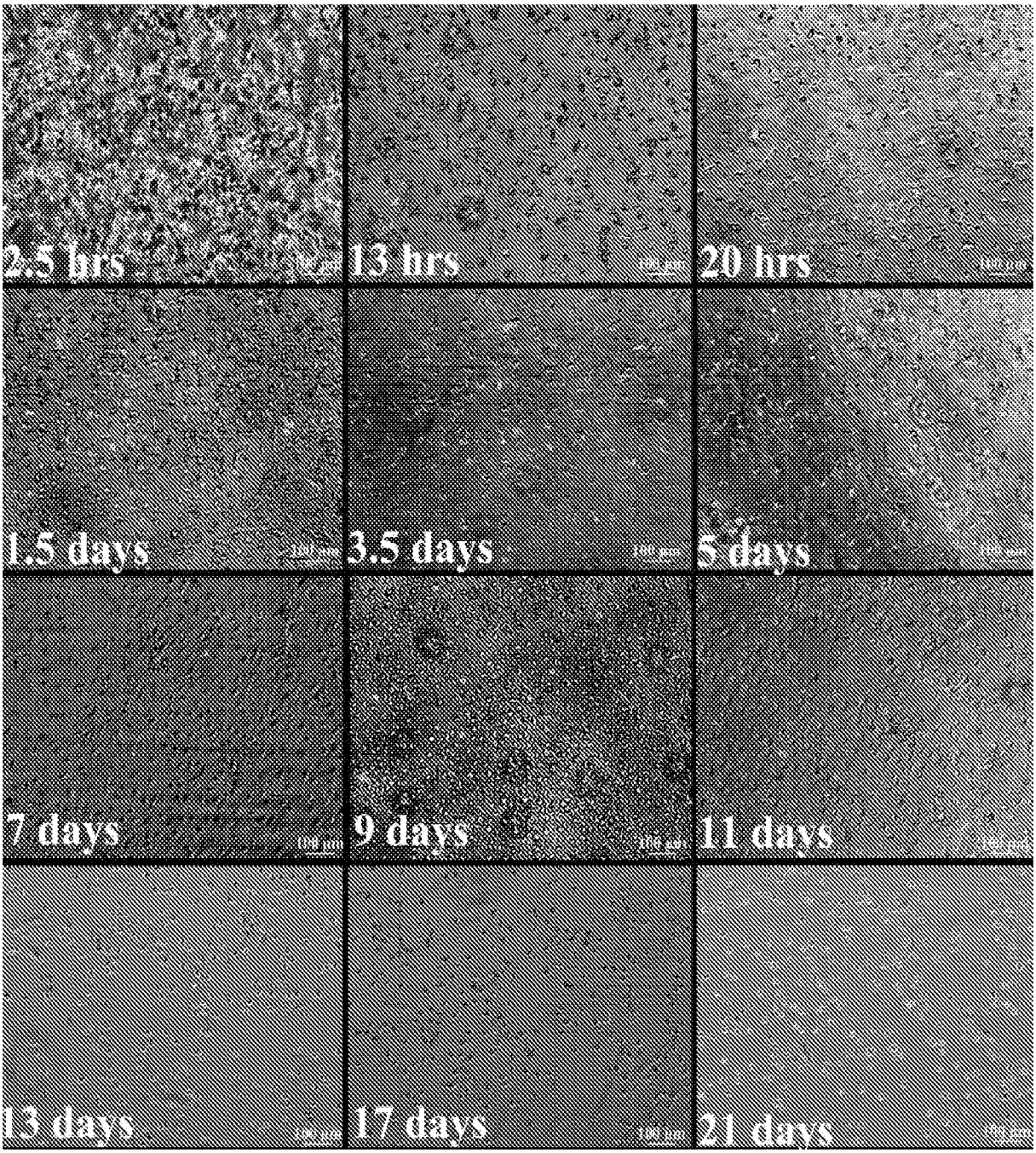

2.5 hrs
13 hrs
20 hrs
1.5 days
3.5 days
5 days
7 days
9 days
11 days
13 days
17 days
21 days

FIGURE 8

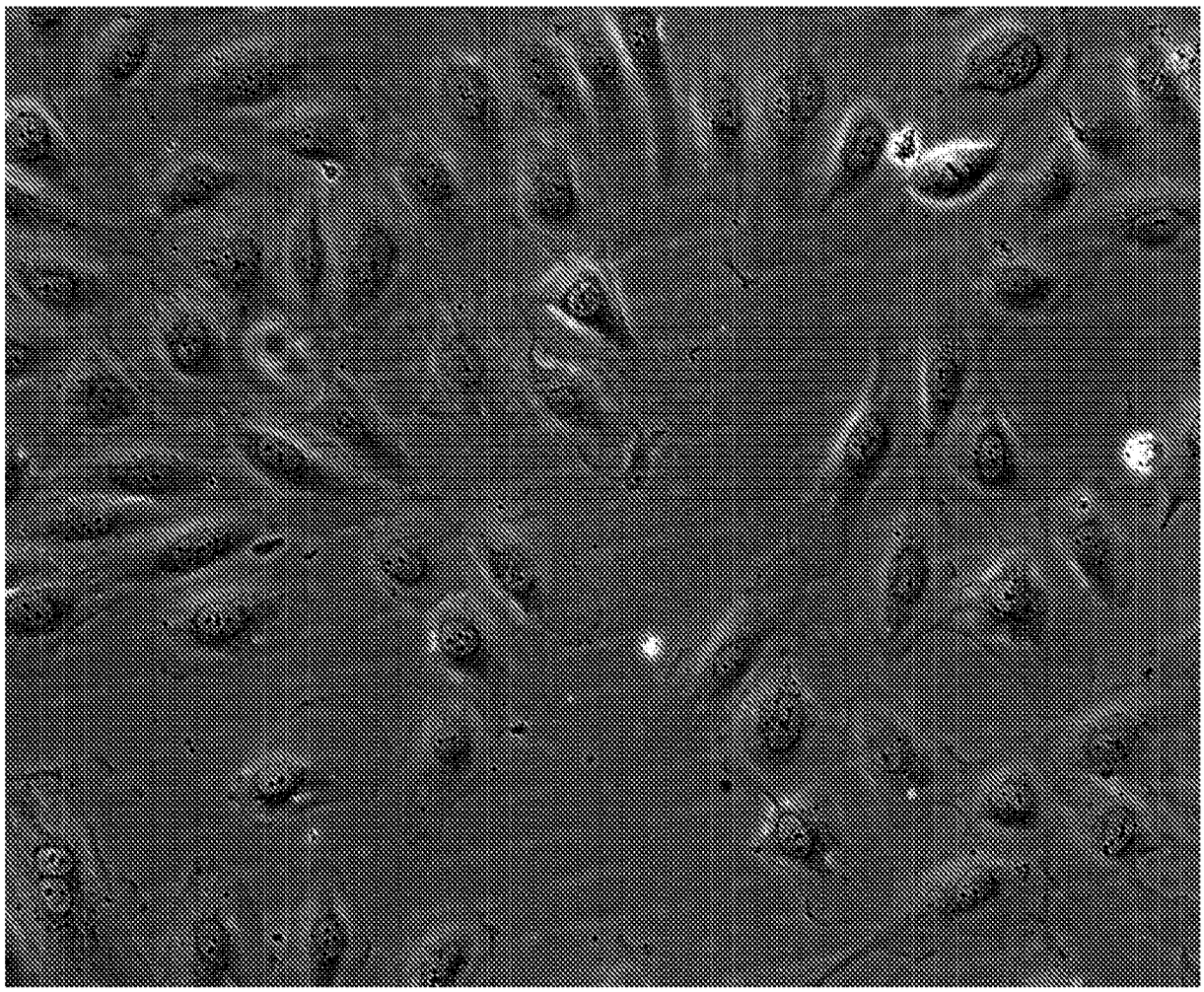


FIGURE 9A

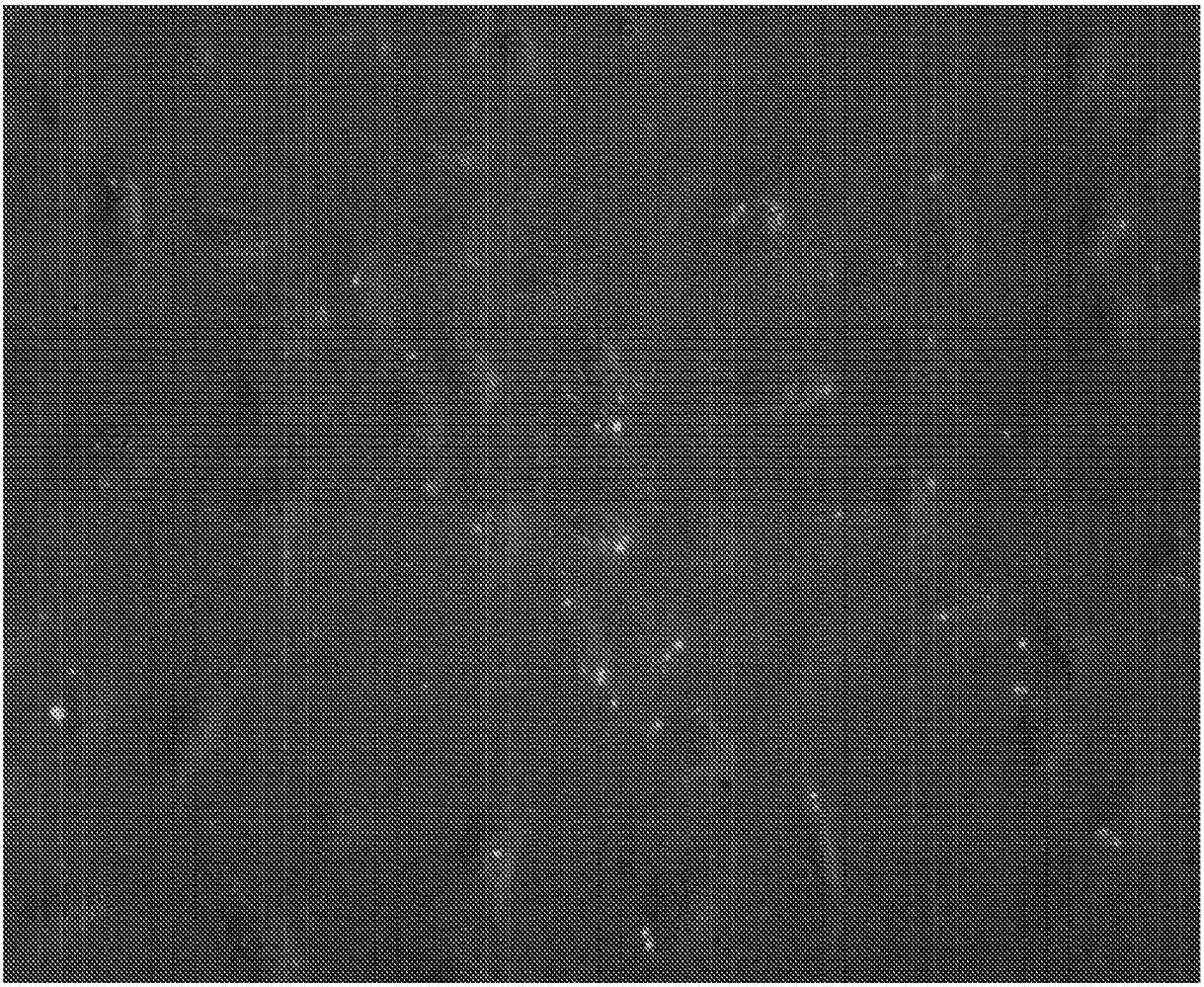


FIGURE 9B

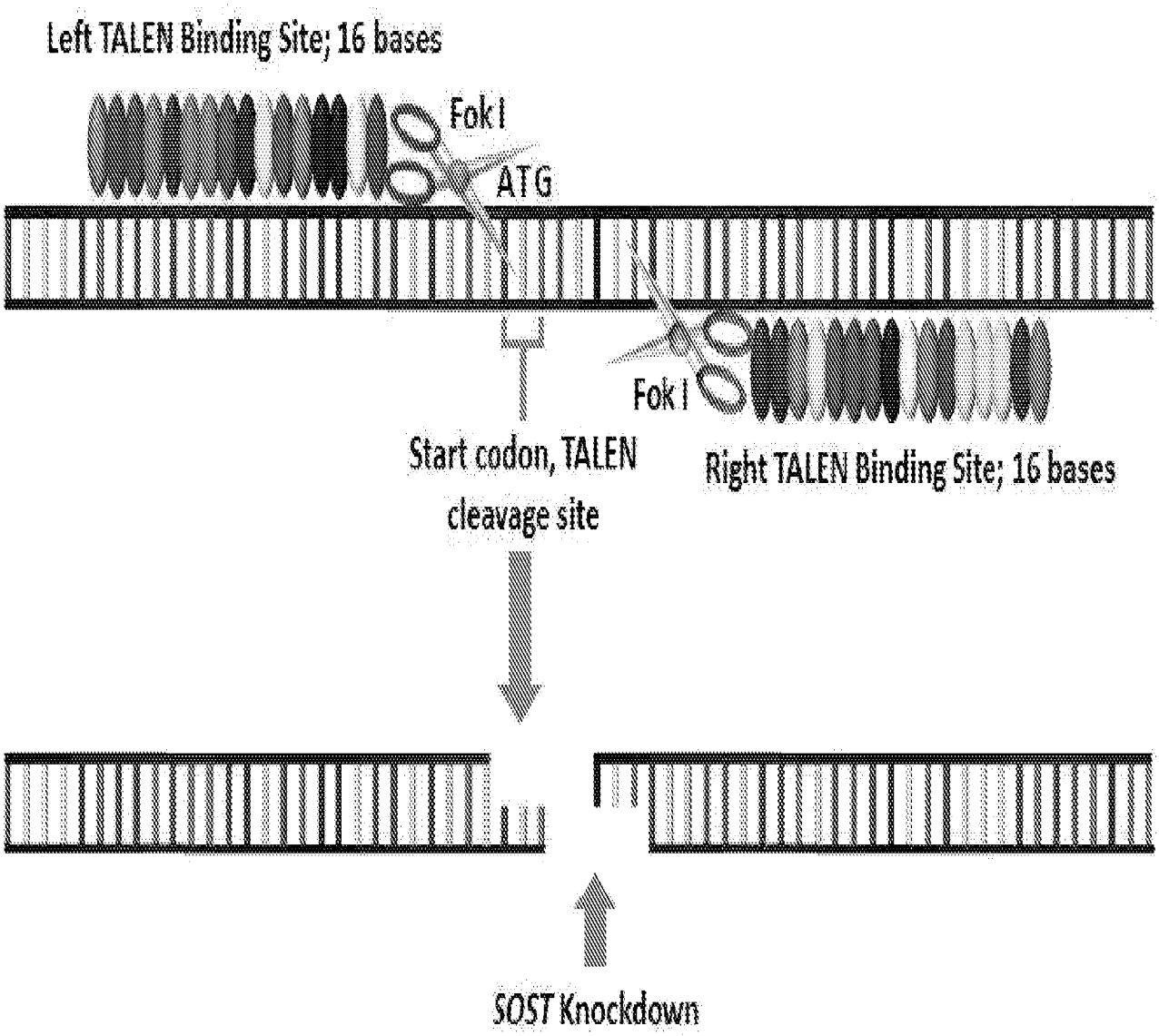

Left TALEN Binding Site; 16 bases
Fok I
ATG
Fok I
Start codon, TALEN cleavage site
Right TALEN Binding Site; 16 bases
SOST Knockdown

FIGURE 10

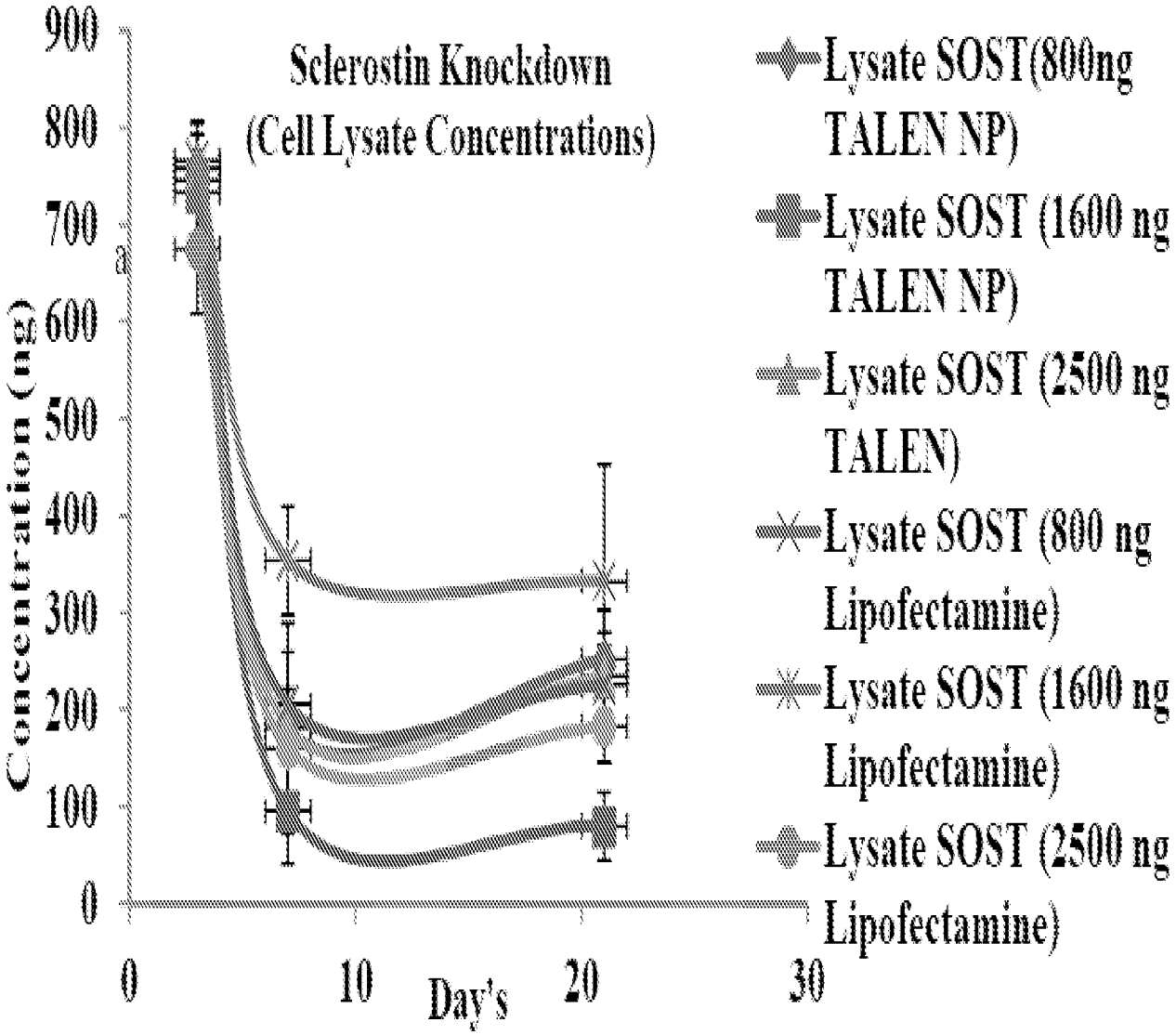

Sclerostin Knockdown
(Cell Lysate Concentrations)
Concentration (ng)
900
800
700
600
500
400
300
200
100
0
0
10
20
30
Day's
Lysate SOST(800ng TALEN NP)
Lysate SOST (1600 ng TALEN NP)
Lysate SOST (2500 ng TALEN)
Lysate SOST (800 ng Lipofectamine)
Lysate SOST (1600 ng Lipofectamine)
Lysate SOST (2500 ng Lipofectamine)

FIGURE 11A

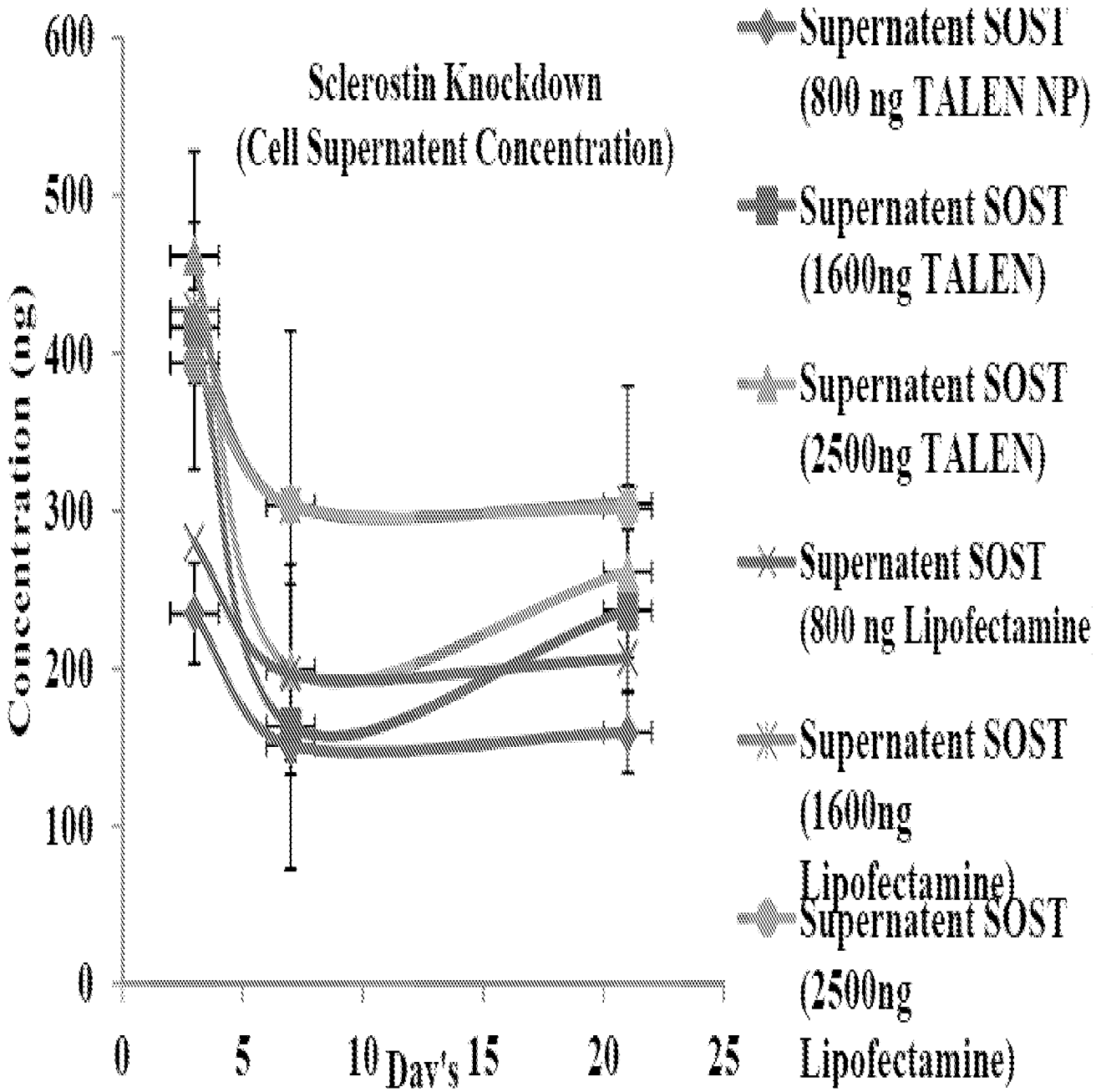

Sclerostin Knockdown
(Cell Supernatent Concentration)
Concentration (ng)
600
500
400
300
200
100
0
0
5
10
15
20
25
Day's
Supernatent SOST (800 ng TALEN NP)
Supernatent SOST (1600ng TALEN)
Supernatent SOST (2500ng TALEN)
Supernatent SOST (800 ng Lipofectamine
Supernatent SOST (1600ng Lipofectamine)
Supernatent SOST (2500ng Lipofectamine)

FIGURE 11B

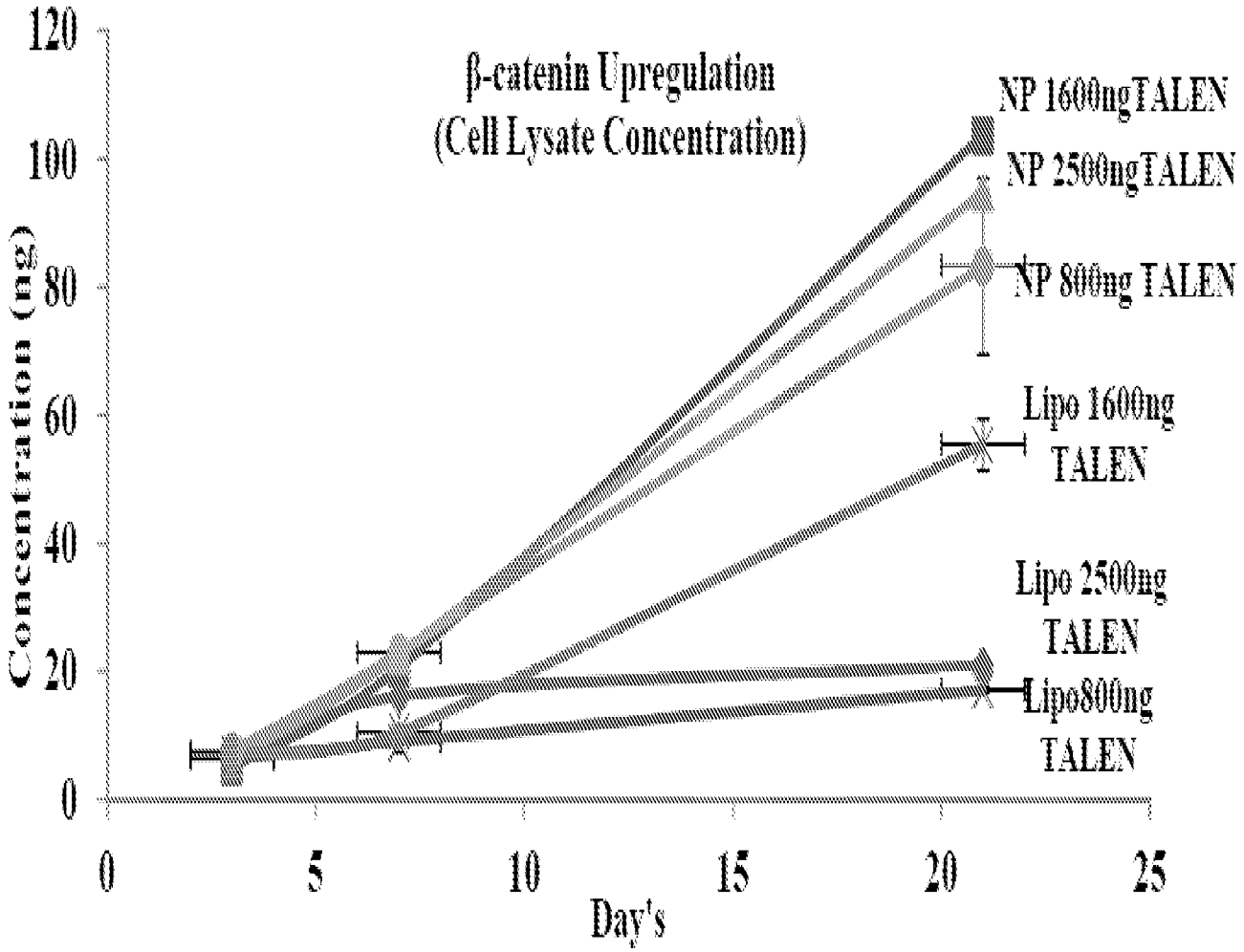

β-catenin Upregulation
(Cell Lysate Concentration)
Concentration (ng)
120
100
80
60
40
20
0
0
5
10
15
20
25
Day's
NP 1600ngTALEN
NP 2500ngTALEN
NP 800ng TALEN
Lipo 1600ng TALEN
Lipo 2500ng TALEN
Lipo800ng TALEN

FIGURE 11C

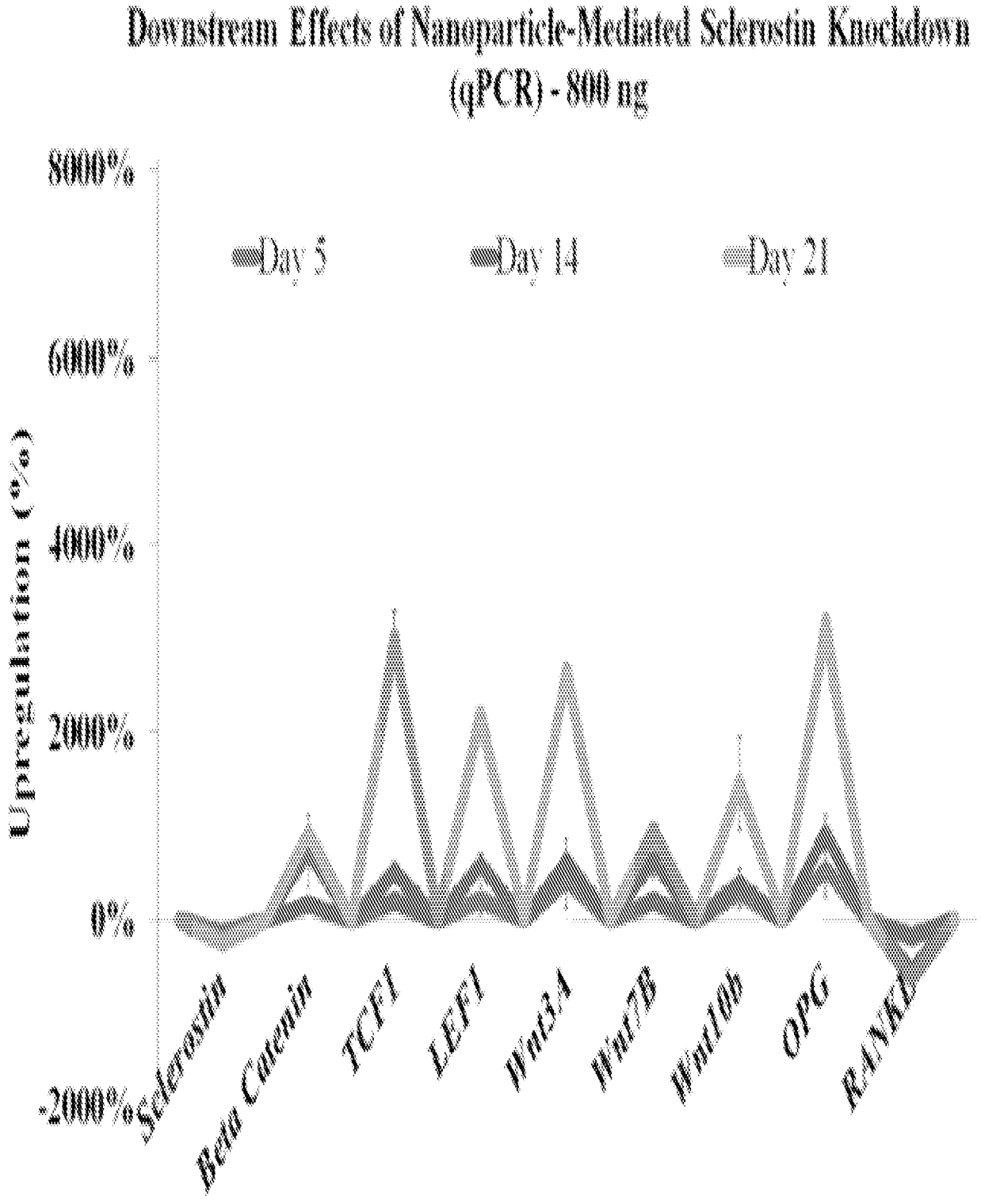

Downstream Effects of Nanoparticle-Mediated Sclerostin Knockdown (qPCR) - 800 ng
8000%
6000%
4000%
2000%
0%
-2000%
Upregulation (%)
Day 5
Day 14
Day 21
Sclerostin
Beta Catenin
TCF1
LEF1
Wnt3A
Wnt7B
Wnt10b
OPG
RANKL

FIGURE 12A

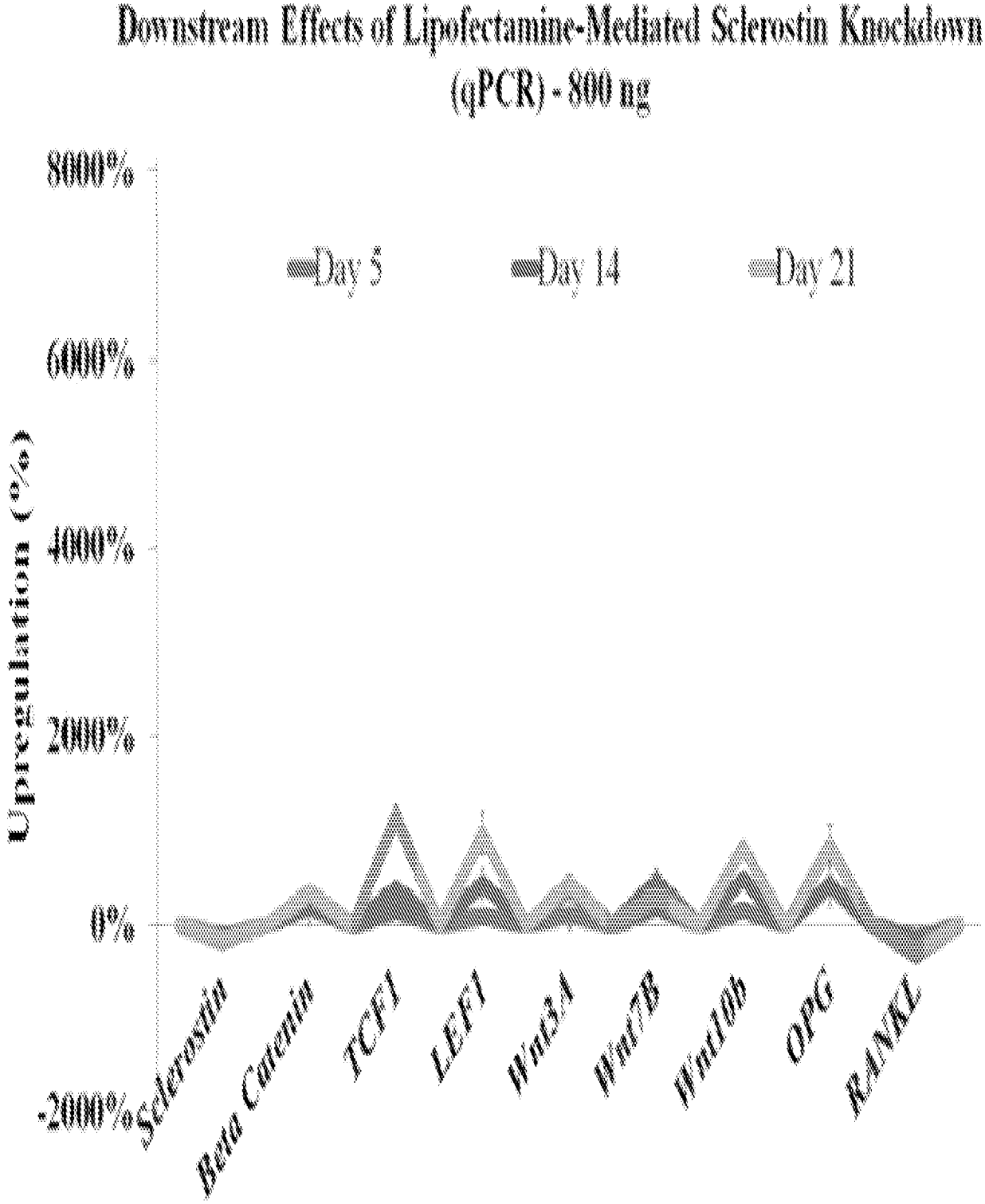

Downstream Effects of Lipofectamine-Mediated Sclerostin Knockdown (qPCR) - 800 ng
8000%
6000%
4000%
2000%
0%
-2000%
Upregulation (%)
Day 5
Day 14
Day 21
Sclerostin
Beta Catenin
TCF1
LEF1
Wnt3A
Wnt7B
Wnt10b
OPG
RANKL

FIGURE 12B

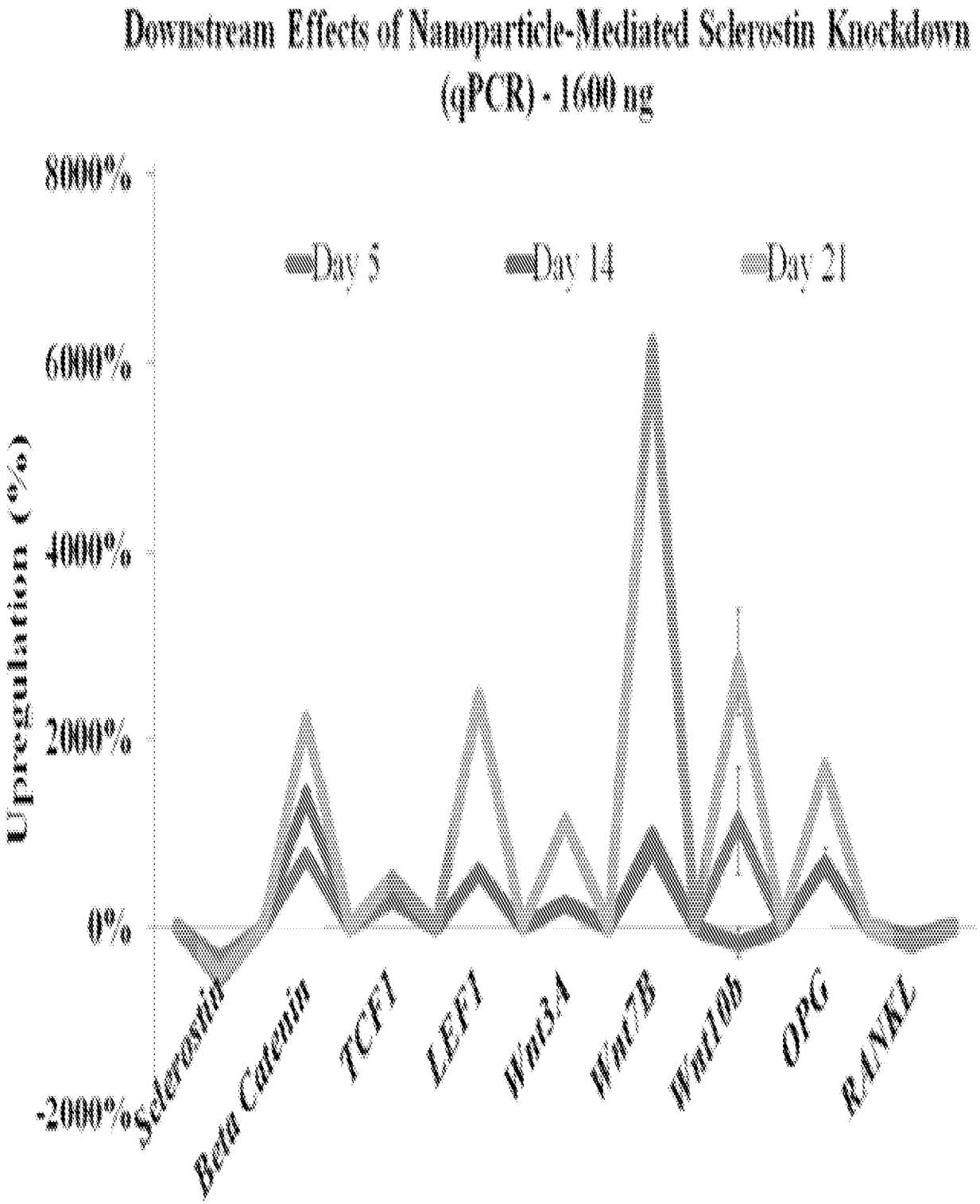

Downstream Effects of Nanoparticle-Mediated Sclerostin Knockdown (qPCR) - 1600 ng
Day 5
Day 14
Day 21
Upregulation (%)
8000%
6000%
4000%
2000%
0%
-2000%
Sclerostin
Beta Catenin
TCF1
LEF1
Wnt3A
Wnt7B
Wnt10b
OPG
RANKL

FIGURE 12C

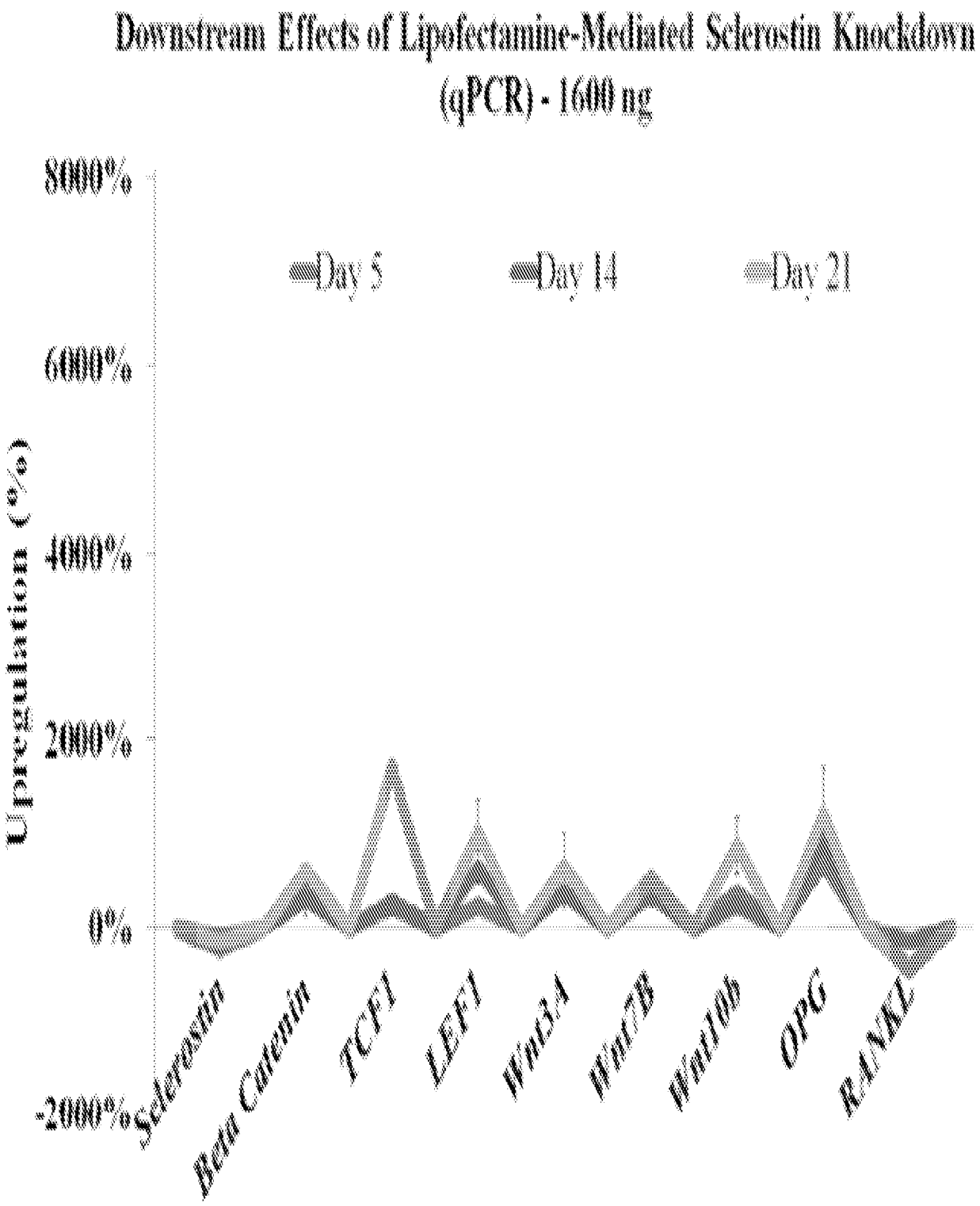

Downstream Effects of Lipofectamine-Mediated Sclerostin Knockdown (qPCR) - 1600 ng
8000%
6000%
4000%
2000%
0%
-2000%
Upregulation (%)
Day 5
Day 14
Day 21
Sclerostin
Beta Catenin
TCF1
LEF1
Wnt3A
Wnt7B
Wnt10b
OPG
RANKL

FIGURE 12D

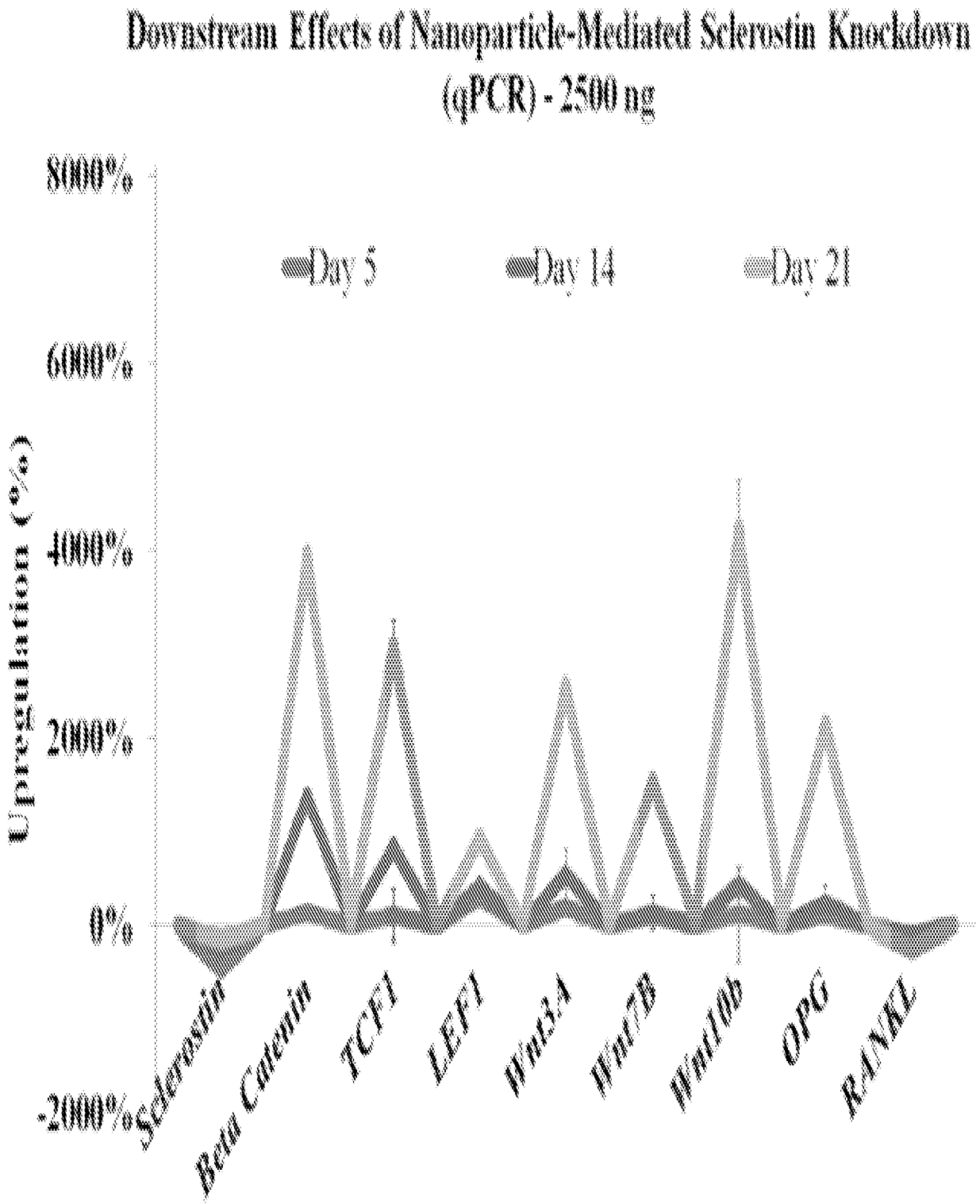

Downstream Effects of Nanoparticle-Mediated Sclerostin Knockdown (qPCR) - 2500 ng
8000%
6000%
4000%
2000%
0%
-2000%
Upregulation (%)
Day 5
Day 14
Day 21
Sclerostin
Beta Catenin
TCF1
LEF1
Wnt3A
Wnt7B
Wnt10b
OPG
RANKL

FIGURE 12E

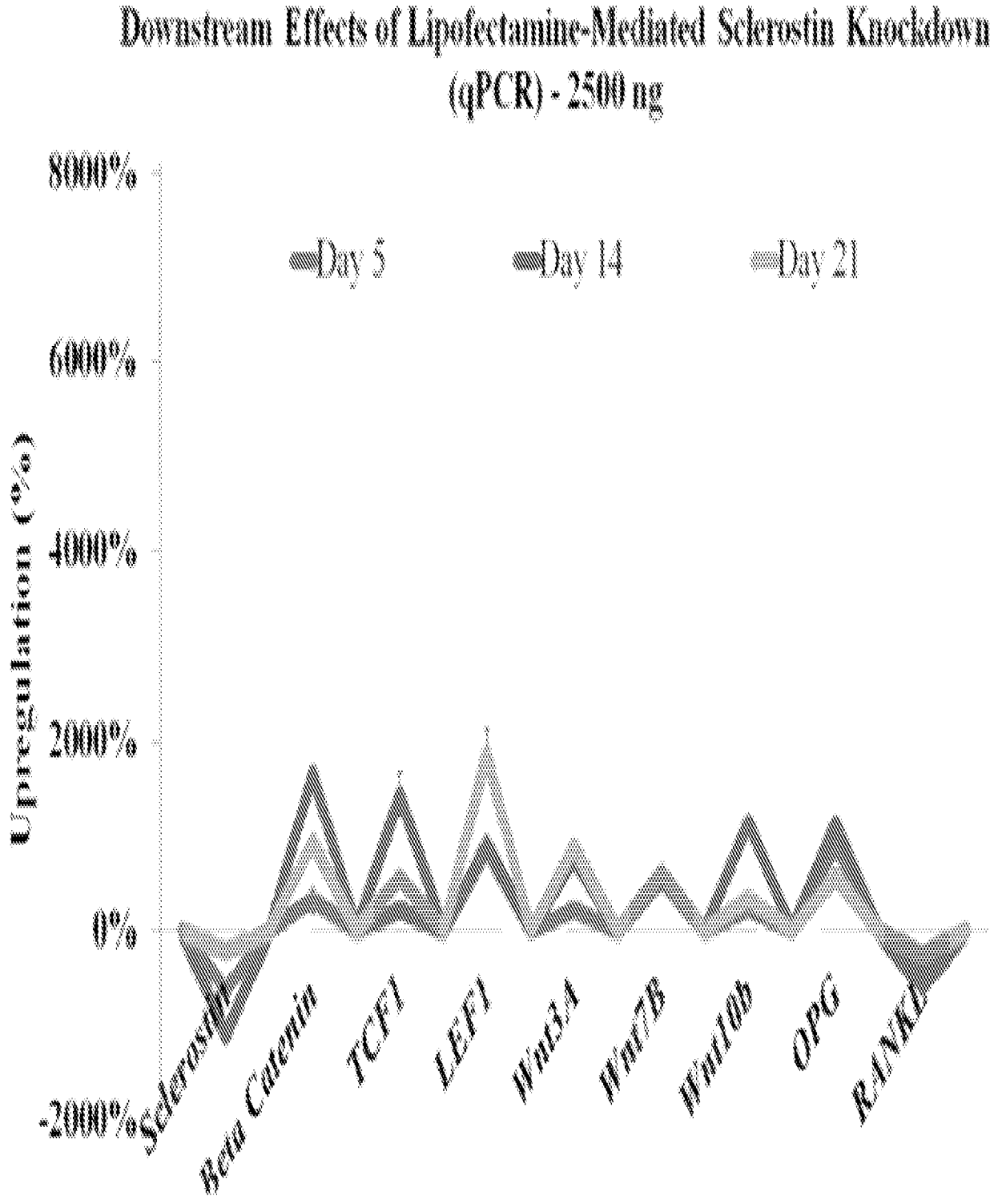

Downstream Effects of Lipofectamine-Mediated Sclerostin Knockdown (qPCR) - 2500 ng
8000%
6000%
4000%
2000%
0%
-2000%
Upregulation (%)
Day 5
Day 14
Day 21
Sclerostin
Beta Catenin
TCF1
LEF1
Wnt3A
Wnt7B
Wnt10b
OPG
RANKL

FIGURE 12F

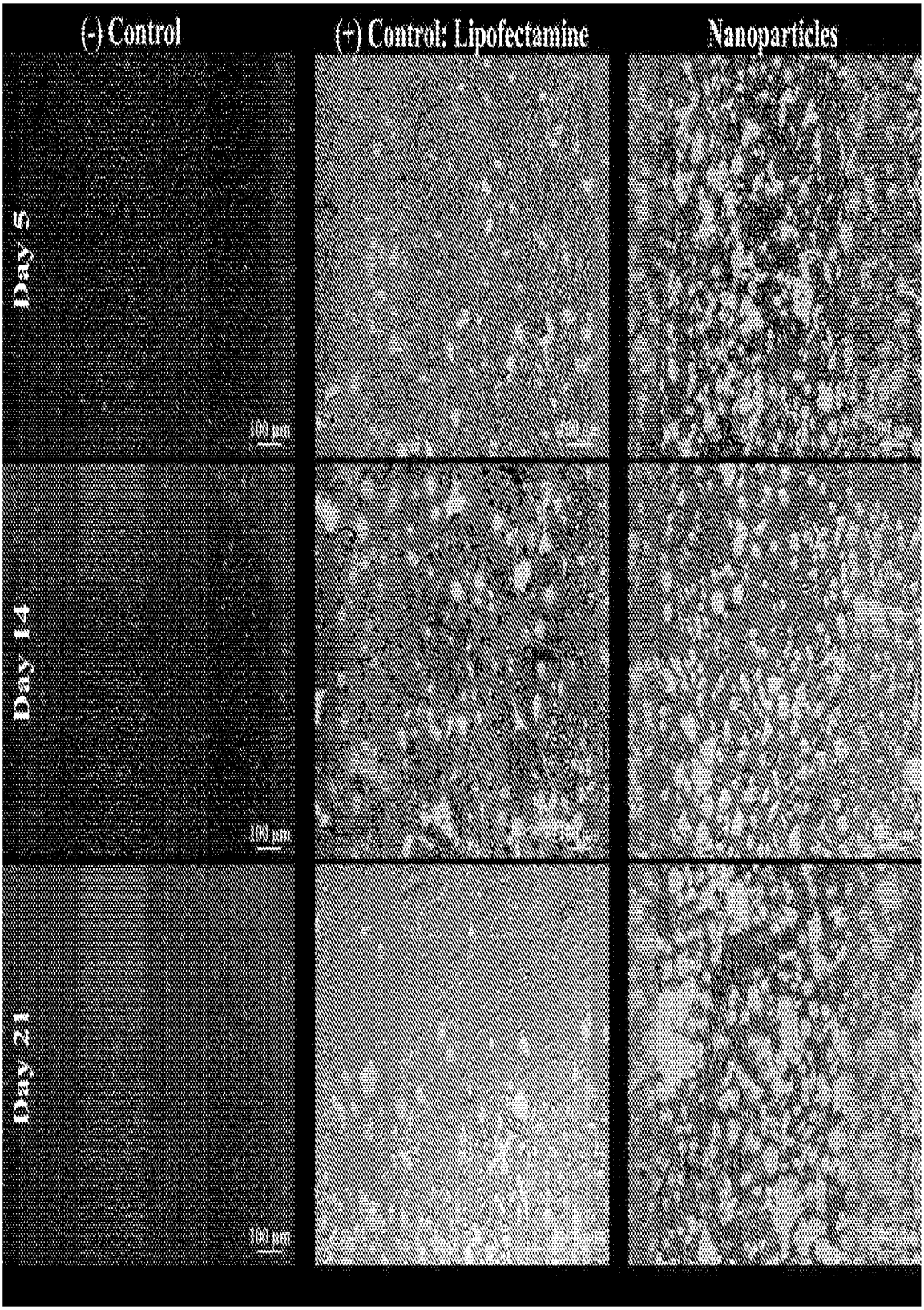

(-) Control
(+) Control: Lipofectamine
Nanoparticles
Day 5
Day 14
Day 21
100 μm

FIGURE 13

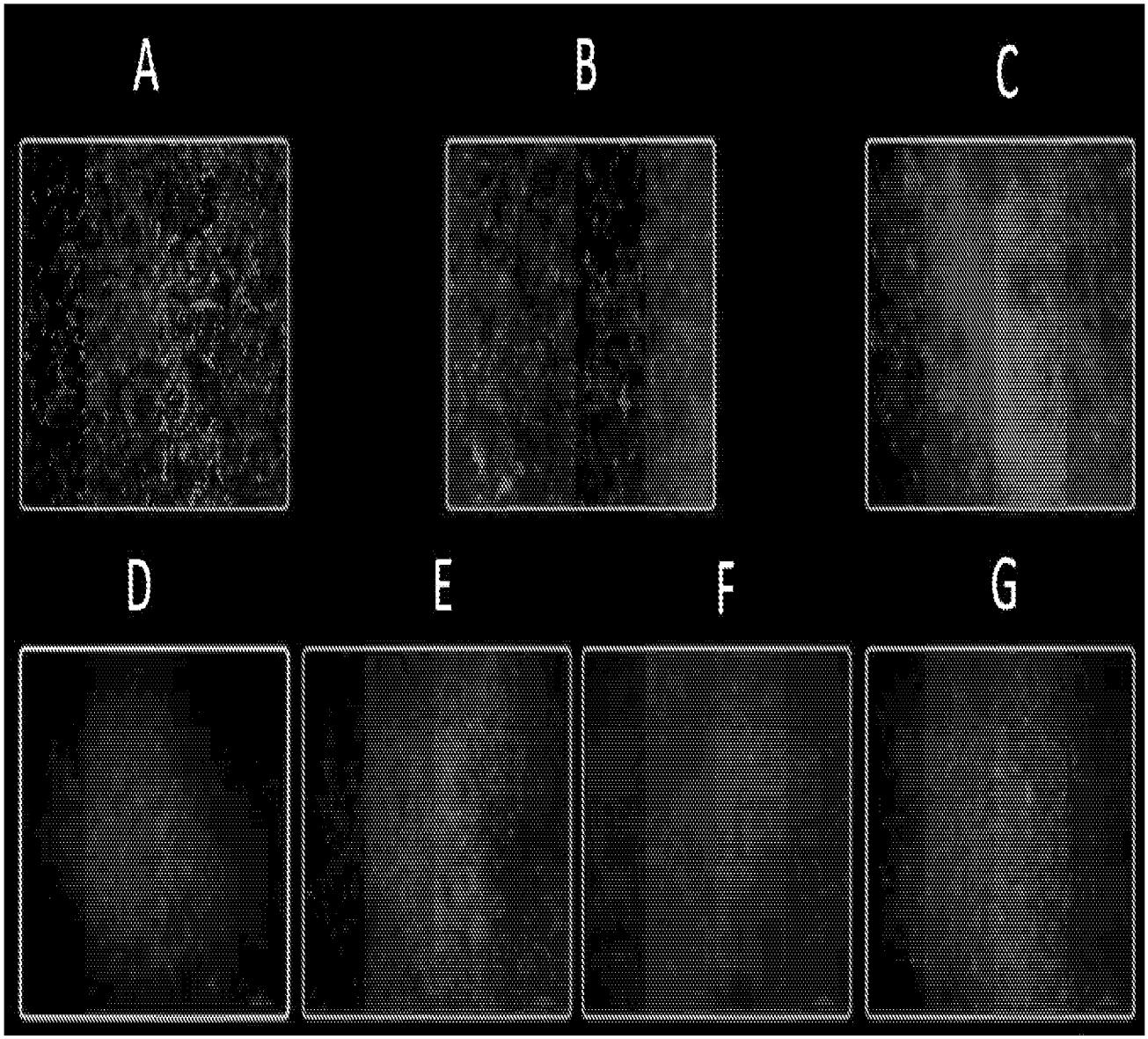


FIGURE 14A

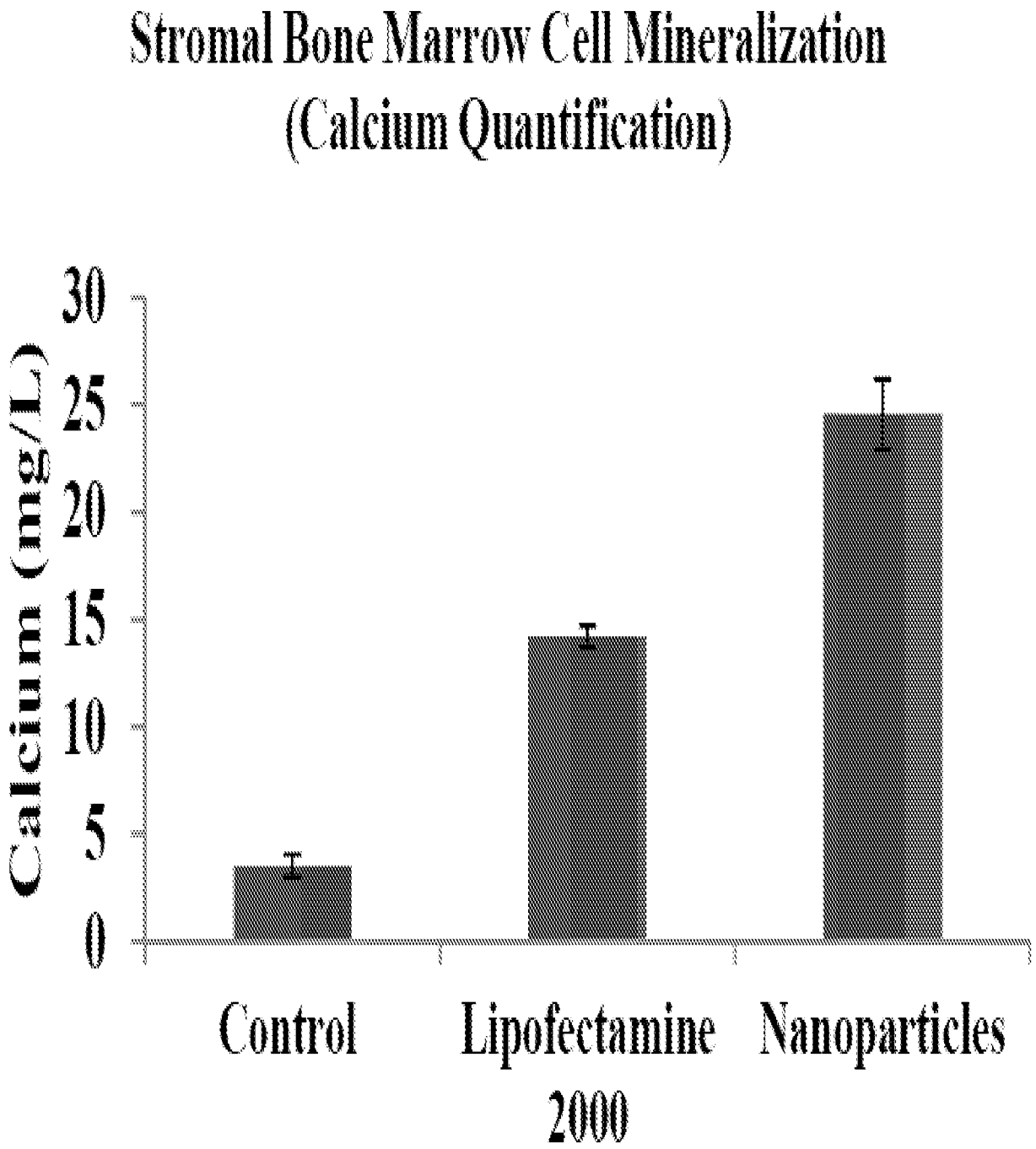

Stromal Bone Marrow Cell Mineralization
(Calcium Quantification)
Calcium (mg/L)
30
25
20
15
10
5
0
Control
Lipofectamine 2000
Nanoparticles

FIGURE 14B

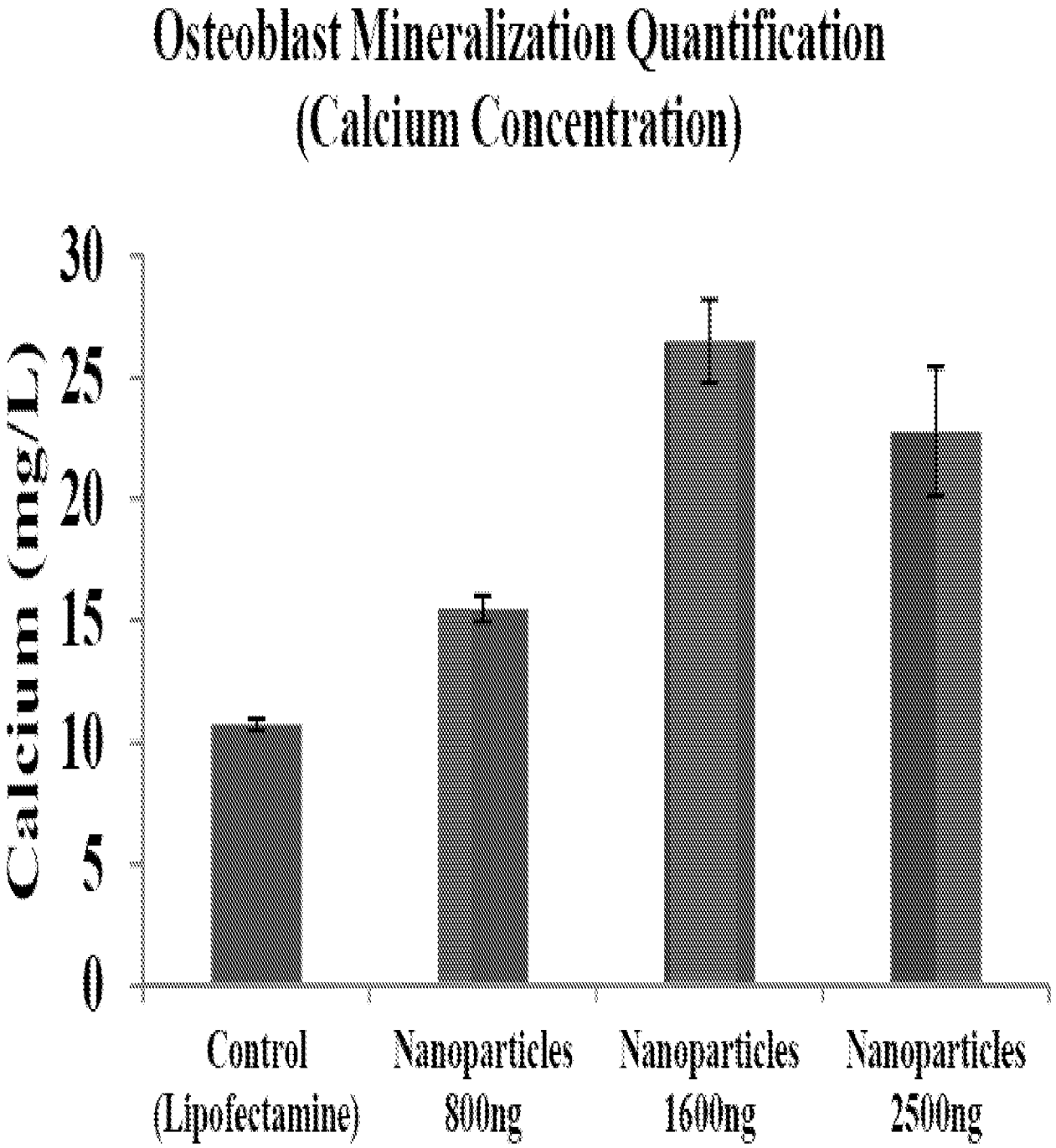
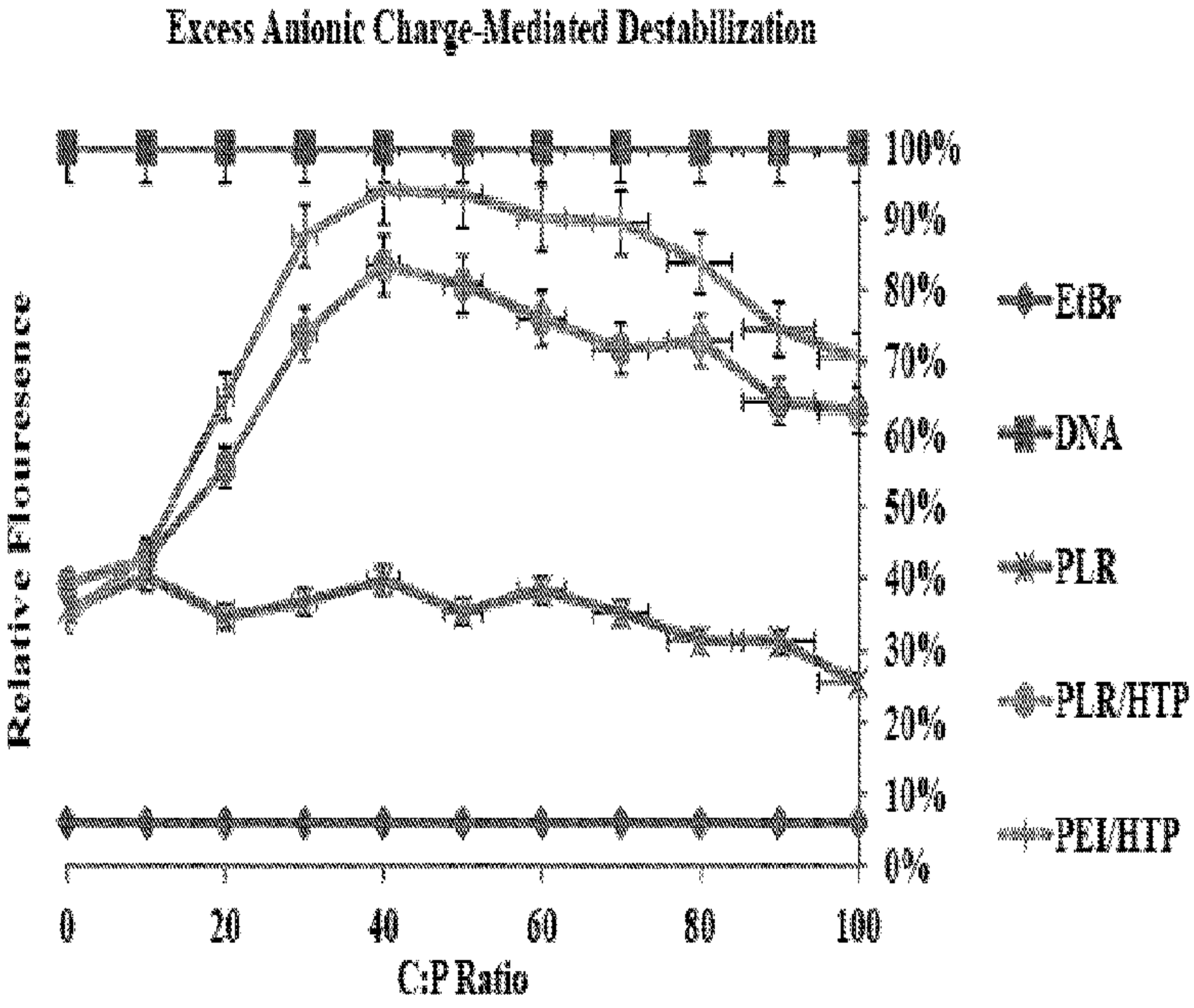

Osteoblast Mineralization Quantification
(Calcium Concentration)
Calcium (mg/L)
30
25
20
15
10
5
0
Control
(Lipofectamine)
Nanoparticles
800ng
Nanoparticles
1600ng
Nanoparticles
2500ng

FIGURE 14C


Excess Anionic Charge-Mediated Destabilization
Relative Flourescence
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
EtBr
DNA
PLR
PLR/HTP
PEI/HTP
0
20
40
60
80
100
C:P Ratio