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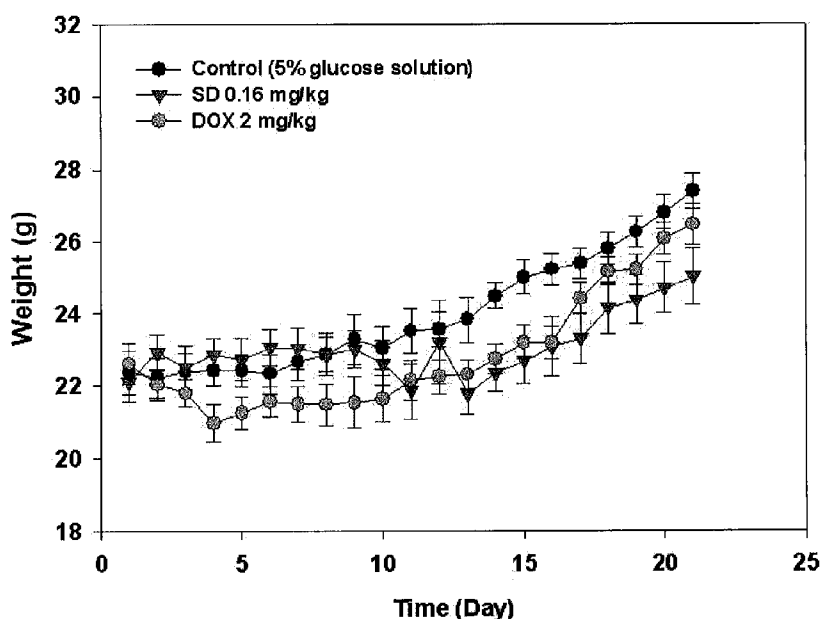
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[Continued on next page]

(54) Title: MULTI-FUNCTIONAL COMPLEX FOR IMAGING AND DRUG DELIVERY

Fig. 10



(57) Abstract: The present invention relates to a multi-functional complex for imaging and drug delivery comprising a plurality of nanoparticles, wherein the nanoparticles comprise: (a) a signal generating core; and a water soluble polymeric outer shell coated on the signal generating core, comprising a surface-anchoring site containing silyl-, hydroxysilyl- or alkoxy-silyl-functionalized groups and a drug-binding site.



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MULTI-FUNCTIONAL COMPLEX FOR IMAGING AND DRUG DELIVERY

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

5 The present invention relates to a multi-functional complex for imaging and drug delivery.

BACKGROUND OF TECHNIQUE

10 Superparamagnetic iron oxide nanoparticles (SPION) has currently been an emerging technique in biomedical applications such as magnetic resonance imaging (MRI) diagnosis (Baghi, M. *et al.*, *Anticancer Res.* 2005, 25, 3665-3670; Martina, M. *et al.*, *J. Am. Chem. Soc.* 2005, 127, 10676-10685; Blasberg, R. G. *Mol. Cancer Ther.* 2003, 2, 335-343; Artemov, D. J. *Cell Biochem.* 2003, 90, 518-524; Schmitz, S. A. *Rofo* 2003, 175, 469-476; Kroft, L. J. *et al.*, *J. Magn. Reson. Imaging* 1999, 10, 395-403; Bonnemain, B. J. *Drug Target* 1998, 6, 167-174; Bellin, M. F. *et al.*, *Eur. J. Radiol.* 2000, 34, 257-264), drug delivery (Kohler, N. *et al.*, *Langmuir* 2005, 21, 8858-8864; Gupta, A. K *et al.*, *J. Mater. Sci. Mater. Med.* 2004, 15, 493-496) and therapy (hyperthermia, Gupta, A. K.; Gupta, M. *Biomaterials* 2005, 26, 3995-4021). SPION (Ito, A. *et al.*, *Cancer Lett.* 2004, 212, 167-175) may potentially provide
20 higher contrast enhancement in MRI than in conventional paramagnetic Gd-based contrast agents due to its superparamagnetic property.

 Although many researches for use as contrast agents or drug delivery of nanoparticles have been made, single multi-functional particles to enable both imaging and drug delivery have not practically developed yet.

25

 Throughout this application, various publications and patents are referred and citations are provided in parentheses. The disclosures of these publications and patents in their entities are hereby incorporated by references into this application in

order to fully describe this invention and the state of the art to which this invention pertains.

DETAILED DESCRIPTION OF THE INVENTION

5 The present inventors have made intensive studies to develop a multi-functional complex to simultaneously enable imaging and drug delivery by a single nanoparticle. As results, we have discovered that both imaging and drug delivery could be successfully achieved by fabricating the multi-functional complex through loading of drug onto nanoparticles comprising a signal generating core and a
10 suitable water-soluble polymeric outer shell coated on the core.

 Accordingly, it is an object of this invention to provide a multi-functional complex for imaging and drug delivery.

 It is another object of this invention to provide a simultaneous method for imaging and drug delivery.

15

 Other objects and advantages of the present invention will become apparent from the following detailed description together with the appended claims and drawings.

20 In one aspect of this invention, there is provided multi-functional complex for imaging and drug delivery comprising a plurality of nanoparticles, wherein the nanoparticles comprise:

- (a) a signal generating core; and
- (b) a water soluble polymeric outer shell coated on the signal generating
25 core, comprising a surface-anchoring site containing silyl-, hydroxysilyl- or alkoxysilyl-functionalized groups and a drug-binding site.

 The present inventors have made intensive studies to develop a multi-

functional complex to simultaneously enable imaging and drug delivery by a single nanoparticle. As results, we have discovered that both imaging and drug delivery could be successfully achieved by fabricating the multi-functional complex through loading of drug onto nanoparticles comprising a signal generating core and a
5 suitable water-soluble polymeric outer shell coated on the core.

In the present nanoparticles, various signal generating cores may be used. Preferably, the signal generating core is a paramagnetic, a superparamagnetic or a proton density signal generating core and most preferably a superparamagnetic signal generating core.

10 The illustrative paramagnetic signal generating core suitable in the present invention includes stable free radicals (*e.g.*, stable nitroxides), transition elements, lanthanoids and actinoids. Preferable element includes Gd(III), Mn(II), Cu(II), Cr(III), Fe(II), Fe(III), Co(II), Er(II), Ni(II), Eu(III) and Dy(III).

The illustrative superparamagnetic signal generating core suitable in the
15 present invention includes ferro- or ferrimagnetic compounds, *e.g.*, pure iron, magnetic iron oxide (*e.g.*, magnetite, Fe_3O_4), $\gamma\text{-Fe}_2\text{O}_3$, manganese ferrite, cobalt ferrite and nickel ferrite. Preferably, the present superparamagnetic signal generating core is Fe_3O_4 or $\gamma\text{-Fe}_2\text{O}_3$ and most preferably Fe_3O_4 .

The illustrative proton density signal generating core suitable in the present
20 invention is a perfluorocarbon.

The water soluble outer shell is coated on the signal generating core. The water soluble outer shell allows the present contrast agent to possess solubility in water and anti-biofouling property and to exhibit enhanced stability and imaging ability as compared with uncoated nanoparticles.

25 The outer shell consists of water soluble polymers containing silicons. The water soluble polymers containing silicons used herein include various water soluble polymers covalently bound to silicons. Preferably, the water soluble polymers as main backbones include poly(acrylic acid) or its derivatives, poly((meta)acrylic acid) or

its derivatives, poly(acrylic amide) or its derivatives, poly(undecenoic acid) or its derivatives, copolymers thereof, chitosan or its derivatives, dextran or its derivatives (*e.g.*, carboxymethyl dextran), cellulose or its derivatives (*e.g.*, carboxymethyl cellulose), heparin or its derivatives, alginate or its derivatives. More preferably, the
5 water-soluble polymer as main backbones has poly(acrylic acid) or its derivatives, poly((meta)acrylic acid) or its derivatives or copolymers thereof.

Silicon may be linked to the water-soluble polymer described above through various forms or manners, for example, the linkage via monoester bond. When silicon may be linked to the water-soluble polymer via monoester bond, it may be
10 directly linked to the water-soluble polymer or indirectly through hydrocarbon moiety of C₁-C₅ (*e.g.*, propyl group).

According to a preferable embodiment, the water soluble polymer containing silicons may be bound covalently to the signal generating core via silicon atom. That is, covalent bond between silicon and functionalized group on the signal generating
15 core may be formed. There was a problem in stability of coating because conventional coating was composed of non-covalent bonds. The present covalent bond between the outer shell and the signal generating core via silicon may remarkably enhance stability of the outer shell coating.

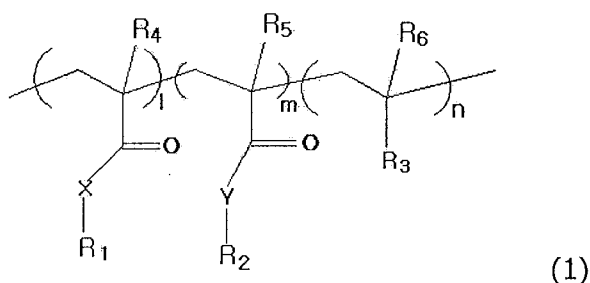
According to a preferable embodiment, the water soluble polymer containing
20 silicons may be crosslinked through bonds between silicon atoms. The crosslinking may be made by heating a synthetic polymer (*e.g.*, 80°C). The crosslinking may much more significantly enhance stability of the silicon-contained water soluble polymer coating.

According to a preferable embodiment, a polymer with anti-biofouling
25 property may be linked to the water soluble polymer containing silicons. Preferably, the polymer with anti-fouling property is PEG (polyethylene glycol), polyalkylene oxide [*e.g.*, polyoxyethylene, polyoxypropylene or their copolymers (*e.g.*, polyethylene oxide-polyoxypropylene oxide-polyethylene oxide copolymer)],

polyphenylene oxide, copolymer of PEG and polyalkylene oxide, poly(methoxyethyl methacrylate), poly(methacryloyl phosphatidylcholine), perfluoro-polyether, dextran or polyvinylpyrrolidone, more preferably PEG, polyalkylene oxide or copolymer of PEG and polyalkylene oxide and most preferably PEG. The further polymers have not only
 5 solubility in water but also anti-biofouling property which prevents proteins and cells in body from binding to nanoparticles. The further polymer, *e.g.*, PEG may be linked to the water soluble polymer containing silicons via various forms or manners, for example, the linkage by monoester bond. When PEG may be linked to the water-soluble polymer through monoester bond, silicon may be linked directly to
 10 monoester bond or indirectly through hydrocarbon moiety of C₁-C₅.

Where PEG is further linked to the water-soluble polymer, hydrophilic PEG layer may contribute to excellent water dispersibility due to exposure on the surface of nanoparticle.

According to a preferable embodiment, the water soluble polymer is
 15 represented by the following Formula 1:



wherein R₁ represents silylalkyl, (alkoxysilyl)alkyl or (hydroxysilyl)alkyl; R₂ represents PEG (polyethylene glycol), polyalkylene oxide, polyphenylene oxide, copolymer of PEG and polyphenylene oxide, poly(methoxyethyl methacrylate),
 20 poly(methacryloyl phosphatidylcholine), perfluoro-polyether, dextran or polyvinylpyrrolidone; R₃ represents aldehyde, epoxy, haloalkyl, primary amine, thiol, maleimide, ester, carboxyl or hydroxyl; R₄, R₅ and R₆ independently represent H or C₁-C₅ alkyl; X, Y and Z independently represent oxygen, sulfur or nitrogen atom; l, m and n independently represent an integer of 1-10,000.

As used herein, the term "silylalkyl" refers to an alkyl group substituted with silyl group. For example, the silylalkyl includes silylmethyl, silylethyl, silylpropyl and silylbutyl groups. The term "(alkoxysilyl)alkyl" means alkoxy-substituted silylalkyl groups. For example, the (alkoxysilyl)alkyl includes trimethoxysilylethyl, trimethoxysilylpropyl, trimethoxysilylbutyl, trimethoxysilylpentyl, triethoxysilylethyl, triethoxysilylpropyl, triethoxysilylbutyl, methyldimethoxysilylethyl, methyldimethoxysilylpropyl, dimethylmethoxysilylethyl and dimethylmethoxysilylpropyl. The term "(hydroxysilyl)alkyl" refers to hydroxyl-substituted silylalkyl groups including *e.g.*, trihydroxysilylethyl, trihydroxysilylpropyl, trihydroxysilylbutyl and trihydroxysilylpentyl.

Preferably, R_1 in the Formula 1 represents (alkoxysilyl)alkyl or (hydroxysilyl)alkyl, more preferably $(C_{1-6} \text{ alkoxysilyl})C_{1-10}$ alkyl or $(\text{hydroxysilyl})C_{1-10}$ alkyl and most preferably $(C_{1-3} \text{ alkoxysilyl})C_{1-5}$ alkyl or $(\text{hydroxysilyl})C_{1-5}$ alkyl.

R_3 in the Formula 1 represents a functionalized group bound to drug. The functionalized group includes, but not limited to, the groups activated by aldehyde; epoxy; haloalkyl; primary amine; thiol; maleimide; ester (preferably, N-hydroxysuccinamide ester functionalized group); and carboxyl (activated by hydroxyl-succinamide ester) and hydroxyl (activated by cyanogen bromide). Most preferably, the functionalized group includes carboxy groups. When a carboxyl group is included as the functionalized group, it may be activated by succinamide, succinamidyl ester, sulfo-succinamidyl ester, 2,3,5,6-tetrafluorophenol ester, 4-sulfo-2,3,5,6-tetrafluorophenol ester, aldehyde, acidic anhydride, azide, azolid, carboimide, epoxide, ester, glycidyl ether, halide, imidazole or imidate. Most preferably, the functionalized carboxyl group may be activated by succinamide, succinamidyl ester.

Preferably, R_4 , R_5 and R_6 in the Formula 1 independently represent H or C_1-C_3 alkyl, more preferably H or C_1-C_2 alkyl and most preferably H or methyl group. According to a preferable embodiment, X, Y and Z represent oxygen atom.

According to a preferable embodiment, the present contrast agent may be used in magnetic resonance imaging (MRI). The contrast agent could be used in MRI for various organism tissues and particularly exhibit excellent imaging ability in MRI for *in vivo* cancer imaging. It is one of most features of this invention that MRI for *in vivo* cancer imaging could be efficiently used even in the absence of cancer-specific targeting ligand (*e.g.*, antibody).

According to a preferable embodiment, the average diameter of polymer-coated nanoparticle is in a range of 5-50 nm and more preferably 10-50 nm. The size of the nanoparticles is much smaller value than that of conventional dextran-coating SPIONs such as CLION and MION. The present polymer-coated nanoparticle also exhibits a narrow range of distribution. The small size may allow the present nanoparticle to easily penetrate tissue of interest (*e.g.*, tumor tissue), resulting in enhancing accumulation of nanoparticles within tissue. As result, imaging ability of the present nanoparticle may be improved due to penetration easiness. The small size and narrow distribution of the present nanoparticle may contribute to strengthen magnetic signals and their homogeneity.

The present nanoparticles have saturation magnetization (M_s) values in a range of preferably 20-100 emu/g Fe, more preferably 40-90 emu/g Fe, much more preferably 60-85 emu/g Fe and most preferably 75-85 emu/g Fe. Given the M_s value of conventional polymer-coated SPION to be 30-50 emu/g Fe (48), it could be appreciated that the M_s value of this invention may be excellently improved. The large M_s value of the present nanoparticle may have an excellent advantage in generating signals, resulting in enhancement of imaging ability.

According to a preferable embodiment, the drug is further bound to the reactive site of the water soluble polymeric outer shell. The linkage between the drug and the reactive site may be not particularly restricted and be formed, for example, by an ionic, a covalent, a coordinate and a non-covalent bond, preferably an ionic and a covalent bond.

The drug bound to the present multi-functional complex is, for example, chemical drugs, proteins, peptides or nucleotides (DNA or RNA).

Proteins or peptides bound to the present multi-functional complex may not be particularly restricted and include, but not limited to, hormone, hormone analog, enzyme, enzyme inhibitor, signal transduction protein or its part, antibody or its part, single-chain antibody, binding protein or its binding domain, antigen, adhesion protein, structural protein, regulatory protein, toxin protein, cytokine, transcriptional factor, blood coagulation factor and vaccine. In more detail, proteins or peptides delivered by the present multi-functional complex include insulin, IGF-1 (insulin-like growth factor 1), growth hormone, erythropoietin, G-CSFs (granulocyte-colony stimulating factors), GM-CSFs (granulocyte/macrophage-colony stimulating factors), interferon α , interferon β , interferon γ , interleukin-1 α and β , interleukin-3, interleukin-4, interleukin-6, interleukin-2, EGFs (epidermal growth factors), calcitonin, ACTH (adrenocorticotrophic hormone), TNF (tumor necrosis factor), atobisban, buserelin, cetorelix, deslorelin, desmopressin, dynorphin A (1-13), elcatonin, eleidosin, eptifibatide, GHRH-II (growth hormone releasing hormone-II), gonadorelin, goserelin, histrelin, leuprorelin, lypressin, octreotide, oxytocin, pitressin, secretin, sincalide, terlipressin, thymopentin, thymosine α 1, triptorelin, bivalirudin, carbetocin, cyclosporin, exedine, lanreotide, LHRH (luteinizing hormone-releasing hormone), nafarelin, parathyroid hormone, pramlintide, T-20 (enfuvirtide), thymalfasin and ziconotide.

Chemical drugs bound to the present multi-functional complex may not be particularly restricted and for example, include anti-inflammatory agents, analgesics, antiarthritic agents, antispasmodics, antidepressants, anti-psychotic agents, tranquilizer, antianxiety agents, drug antagonists, anti-parkinson drugs, choline agonists, anti-cancer agents, anti-angiogenesis inhibitors, immunosuppressants, anti-virus agents, antibiotics, anorexing agents, anticholinergics, antihistamine agents, antimigraine agents, hormones, coronary, cerebral or peripheral vasodilators,

contraceptives, antithrombotic agents, diuretics, antihypertensive agents and cardioprotective agents, but not limited to. More substantially, chemical drugs bound to the present multi-functional complex include, but not limited to, asibycin, aclarubicin, acodazole, acronycin, adogelecin, alanoycin, aldesrukin, alopurinol sodium, altretamine, aminoglutetimide, amonafide, ampligen, amsacrin, androgens, anguidin, apidicholine glycinate, asaray, 5-azacitidin, azathioprin, baker's antipol, β -2-deoxyguanosine, bisantrene HCl, bleomycine sulfate, bulserphan, buthionin, sulfoxymine, BWA 773U82, BW 502U83/HCl, BW 7U85 mesylate, serasemide, carbetimer, carboplatin, carmustine, chlorambusil, chloroquinoxaline-sulfonamide, chlorozothocine, chromomycin A3, cisplatin, cladribin, corticosteroid, corinerbacterium parboom, CPT-11, crysnatol, cyclocytidine, cyclophosphamide, cytarabin, cytembena, davis maliate, decarbazin, doctinomycin, daunorubicin HCl, diazyridin, dexrazoic acid, diunhydrogalactitol, diazikuon, dibromodulcitol, didemin B, diethyldithiocarbamate, dichlycoaldehyde, dihydro-5-azacytin, doxorubicin, echinomycin, dedatrexate, edelpocin, eplolniten, elsamitruzin, epirubicin, ethorubicin, ethramustin phosphate, ethanidazole, ethiofos, etophocide, fadrazole, fazarabin, penretinide, philgrastim, pinasteride, flabone acetic acid, ploxyridin, fludarabin phosphate, 5-fluorouracil, Fluosol™, flutamide, gallium nitrate, gemthytabin, gothererin acetate, halfsulpham, hexamethylene biacetamide, homoharingtonin, hydragin sulfate, 4-hydroxyandrostenedion, hydrozyurea, idarubicin HCl, iphosphamide, 4-ipomeanol, iproplatin, isothretinoin, leukoborin calcium, leuproride acetate, levamisol, liposome daunorubicin, liposome capturing doxorubicin, romustatin, rhodamine, mytancin, mechlorethamin, hydrochloride, melphalan, menogaryl, merbaron, 6-mercaptopurine, methna, methanol extract in Bacillus Caltehe-Guarin, methotrexate, N-methylformamide, mipepristone, mitoguazone, mytomycin-C, mitotan, mitoxantron hydrochloride, nabylon, napoxydin, neocarzinostatin, octreotide acetate, ormaplatin, oxalyplatin, pacclitaxel, pala, pentostatin, piperazindion, pipobroman, pirarubicin, piritrexim, piroxantron hydrochloride, PIXY-321,

plicarmycin, forpimer sodium, prenimustin, procarbazine, pyrazofurin, lazoic acid, sargramostim, semustin, spirogermanium, spiromustin, streptonygrin, streptozosin, sulofener, suramin sodium, tapoxipen, taxorere, tegafur, teniposide, terephthalamidine, teroxyrone, thioguanine, thiotepa, thymidine injection, thidzofurin, topotecan, toremiphen, trethinoine, trifluorazirin hydrochloride, trifluridin, trimetrexate, uracil mustard, vinblastin sulfate, vincristin sulfate, vinorelbine, vinorelbine, vinsolidine, Yoshi 864 and zorubicin.

The drugs may be linked covalently or non-covalently to the drug-binding site. According to a preferable embodiment, the drug is linked non-covalently to the drug-binding site. Preferably, the non-covalent bond is ionic bond, coordinate bond, hydrophobic interaction, Van der Waals bond or combinations thereof and most preferably ionic bond, hydrophobic interaction, Van der Waals bond or combinations thereof.

According to a preferable embodiment, the present nanoparticle further comprises a targeting molecule. The targeting molecule includes, *e.g.*, antibodies, aptamers or cell adhesive peptides and most preferably cell adhesive peptides.

When the cell adhesive peptides are linked to the multi-functional complex of this invention, cellular uptake may be more efficiently generated. Consequently, both therapy effect of drug and imaging effect of contrast agent in the multi-functional complex may be much more excellently enhanced.

When the targeting molecule is introduced to the present nanoparticle, it may be introduced to the water soluble outer shell. The targeting molecule may be linked to the present nanoparticle by further introducing functionalized group to combine it with the water soluble outer shell.

The suitable cell adhesive peptides in this invention include RGD(Arg-Gly-Asp), RGDS(Arg-Gly-Asp-Ser), RGDC(Arg-Gly-Asp-Cys), RGDV(Arg-Gly-Asp-Val), RGES(Arg-Gly-Glu-Ser), RGDSPASSKP(Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro), GRGDS(Gly-Arg-Gly-Asp-Ser), GRADSP(Gly-Arg-Ala-Asp-Ser-Pro), KGDS(Lys-Gly-Asp-Ser),

GRGDSP(Gly-Arg-Gly-Asp-Ser-Pro), GRGDTP(Gly-Arg-Gly-Asp-Thr-Pro), GRGES(Gly-Arg-Gly-Glu-Ser), GRGDSPC(Gly-Arg-Gly-Asp-Ser-Pro-Cys), GRGESP(Gly-Arg-Gly-Glu-Ser-Pro), SDGR(Ser-Asp-Gly-Arg), YRGDS(Tyr-Arg-Gly-Asp-Ser), GQQHHLGGAKQAGDV (Gly-Gln-Gln-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val),
 5 GPR(Gly-Pro-Arg), GHK(Gly-His-Lys), YIGSR(Tyr-Ile-Gly-Ser-Arg), PDSGR(Pro-Asp-Ser-Gly-Arg), CDPGYIGSR(Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg), LCFR(Leu-Cys-Phe-Arg), EIL(Glu-Ile-Leu), EILDV(Glu-Ile-Leu-Asp-Val), EILDVPST(Glu-Ile-Leu-Asp-Val-Pro-Ser-Thr), EILEVPST(Glu-Ile-Leu-Glu-Val-Pro-Ser-Thr), LDV(Leu-Asp-Val) and LDVPS(Leu-Asp-Val-Pro-Ser), more preferably RGD(Arg-Gly-Asp), RGDS(Arg-Gly-Asp-Ser),
 10 RGDC(Arg-Gly-Asp-Cys) or RGDV(Arg-Gly-Asp-Val), and most preferably RGD(Arg-Gly-Asp).

In another aspect of this invention, there is provided a simultaneous method for imaging (particularly, MR imaging) and drug delivery comprising administering to
 15 a subject in need a pharmaceutical composition which comprises the multi-functional complex described above as an effective ingredient.

The present multi-functional complex may be administrated with the pharmaceutically acceptable carrier. The pharmaceutically acceptable carrier may be conventional one for formulation, including lactose, dextrose, sucrose, sorbitol, mannitol, starch, rubber arable, potassium phosphate, arginate, gelatin, potassium silicate, microcrystalline cellulose, polyvinylpyrrolidone, cellulose, water, syrups, methyl cellulose, methylhydroxy benzoate, propylhydroxy benzoate, talc, magnesium stearate, and mineral oils, but not limited to. Details of suitable pharmaceutically acceptable carriers and formulations can be found in *Remington's Pharmaceutical Sciences* (19th ed., 1995), which is incorporated herein by reference.
 25

The pharmaceutical composition according to the present invention may be administered via parenterally. When the pharmaceutical composition of the present invention is administered parenterally, it can be done by intravenous, intramuscular, intra-articular, intra-synovial, intrathecal, intrahepatic, intralesional or intracranial

injection. A suitable dose of the pharmaceutical composition of the present invention may vary depending on pharmaceutical formulation methods, administration methods, the patient's age, body weight, sex, severity of diseases, diet, administration time, administration route, an excretion rate and sensitivity for a used pharmaceutical composition.

The method to obtain MR image using the present composition may be carried out according to conventional methods. For example, method and device for MR imaging was disclosed in D. M. Kean and M. A. Smith, Magnetic Resonance Imaging: Principles and Applications (William and Wilkins, Baltimore 1986), US Pat. No. 6,151,377, No. 6,144,202, No. 6,128,522, No. 6,127,825, No. 6,121,775, No. 6,119,032, No. 6,115,446, No. 6,111,410 and No. 6,028,891, the disclosures of which are incorporated herein by reference.

As above described in detail, the invention may carry out imaging and drug delivery in a single system, thus enabling simultaneous diagnosis and treatment. The multi-functional complex of the present invention could stably load drugs as well as MR contrast agents.

Interestingly, the present multi-functional complex has markedly improved a pharmaceutical efficacy at a lower dose as compared to bare drug.

The present invention will now be described in further detail by examples. It would be obvious to those skilled in the art that these examples are intended to be more concretely illustrative and the scope of the present invention as set forth in the appended claims is not limited to or by the examples.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 schematically represents preparation of the polymer in the present invention.

Fig. 2 schematically represents the multi-functional complex coated by the polymer.

Fig. 3 represents a fluorescent graph measuring amount that anticancer agent, doxorubicin is loaded. Each uppermost and lowest graph is represented by the following: 4 μ g doxorubicin only and DOX@TCL-SPION of the indicated amount.

Fig. 4 is a dissociation constant (K_d) of doxorubicin in DOX@TCL-SPION.

5 Fig. 5 represents a particle distribution of the multi-functional complex.

Fig. 6 shows the patterns releasing doxorubicin in the multi-functional complex.

Fig. 7 represents MRI contrast ability of DOX@TCL-SPION in *in vivo* tumor tissues.

10 Figs. 8a-8b represent results for accumulation of DOX@TCL-SPION in *in vivo* tumor tissues and for biodistribution of DOX@TCL-SPION for each organ. SD indicates DOX@TCL-SPION.

Fig. 9 represents growth inhibition of tumor size in DOX@TCL-SPION. SD indicates DOX@TCL-SPION and DOX is doxorubicin *per se*.

15 Fig. 10 shows changes of weight in mouse administrated with DOX@TCL-SPION. SD indicates DOX@TCL-SPION and DOX is doxorubicin *per se*.

Fig. 11 schematically represents preparation of cRGD-TCL-SPION.

Figs. 12a-12b represent results measuring an absorbance to analyze an amount of cRGC conjugated with cRGD-TCL-SPION.

20 Fig. 13 represents analysis of size and zeta-potential to Carboxyl TCL-SPION, Amine TCL-SPION and cRGD-TCL-SPION.

Fig. 14 exhibits images analyzing cellular uptake of TCL-SPION and cRGD-TCL-SPION.

25 Fig. 15 is results measuring ICP-AES (Inductively Coupled Plasma-Atomic Emission Spectrometry) to analyze cellular uptake of cRGD-TCL-SPION.

Fig. 16 represents a fluorescent graph measuring amount that anticancer agent, doxorubicin is loaded in cRGD-TCL-SPION.

Fig. 17 is a cytotoxicity analysis in U87MG cell line of Dox@cRGD-TCL-SPION

that doxorubicin is loaded.

Figs. 18a and 18b represent MRI contrast ability in *in vivo* tumor tissue of Dox@cRGD-TCL-SPION and DOX@TCL-SPION, respectively.

5

EXAMPLES

EXAMPLE 1: Synthesis of PEG/Silicon/COOH Copolymer

TMSMA (3-(trimethoxysilyl)propyl methacrylate, Sigma-Aldrich Chemical Co., 2.24 mmol, 0.56 g), PEGMA (poly(ethylene glycol)methyl ether methacrylate, Sigma-Aldrich, 2.24 mmol, 1.06 g) and NAS (N-acryloxysuccinimide, Acros Co., 1.92 mmol, 0.32 g) were dissolved in 8 ml of THF (tetrahydrofuran, Sigma-Aldrich). This mixture was degassed using N₂ streaming for 20 min. After adding 0.1 mmol of AIBN (2,2'-azobisisobutyronitrile, Sigma-Aldrich, 10 mg) as a radical initiator, the polymerization reaction was carried out at 70°C for 24 hrs. As results, the water soluble polyethylene glycol-silicon-N-acryloxysuccinimide (PEG-Silicon-NAS) copolymer was synthesized. Where THF was eliminated by evaporating in vacuum and NAS was dispersed in pure distilled water, polyethylene glycol-silicon-carboxyl (PEG-Silicon-COOH) copolymer was formed by easily converting NAS into carboxyl group in aqueous solution. It was confirmed using nuclear magnetic resonance (NMR) analysis that each ratio of polyethylene glycol and silicon and carboxyl group was 0.85: 1: 0.71 as shown in Fig. 1.

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EXAMPLE 2: Magnetic Nanoparticle Coated with Polyethylene Glycol/Silicon/Carboxyl Copolymer

0.5 g of FeCl₃ · 6H₂O (Sigma-Aldrich) and 0.184 g of FeCl₂ · 4H₂O (Sigma-Aldrich) were added in 30 ml of distilled water and mixed by stirring. The pH of the mixture was changed from approximately 1.8 to above 10 by adding 7.5 ml of NH₄OH (Fluka) while stirring vigorously for 30 min, resulting in synthesis of black iron oxide particle. The synthetic compound was exposed to magnetic field using

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rare-earth magnet (Daehan-magnet Co.) and the supernatant was discarded after most black particles sank down toward the magnet. The black precipitates were added and slightly stirred in 30 ml of distilled water to redisperse. The supernatant was removed by re-exposure to magnetic field. Polymer was coated on magnetic particles by stirring for 1 hr after 30 ml of distilled water containing 250 mg of THF-removed water-soluble polyethylene glycol-silicon-carboxyl (PEG-Silicon-COOH) copolymer was added. The compounds of polymer-coated black particles were treated with high frequency for 30 min. Black supernatant was thoroughly collected after fewer sank particles were excluded in the aqueous solution by exposure to magnetic field for 12 hrs. This solution was centrifuged at 6,000 rpm for 10 min and subsequently at 10,000 rpm for 10 min to further remove very small aggregates that might exist in the solution. The aqueous solution was heated at 80°C for 2 hrs so that polymers were strongly combined with particles. Final products were separated, generating carboxyl-TCL-SPION (carboxyl Thermally Cross Linked-SPION). As shown in Fig. 2, the polyethylene portion of polymer-coated magnetic nanoparticles administered into body permits to prevent absorption of proteins or cells, blocking their phagocytosis by macrophage. The silicon portion would bind more covalently to the Fe portion of magnetic nanoparticles, enabling thermal crosslinking of nanoparticles to generate much more stable coating. The carboxyl portion as a functional part allows ligands or anti-cancer agents (e.g. doxorubicin) to be bound to nanoparticles.

EXAMPLE 3: Loading of Doxorubicin on Carboxyl-TCL-SPION

0.25 mg of carboxyl-TCL-SPION was dissolved in 5% glucose solution (pH 7.4) and serially diluted by two-fold to 0.18 µg, giving 17 mass samples. Each mass sample was prepared in the total volume of 10 µl. Four µg of doxorubicin was dispersed in 300 µl of 5% glucose solution (pH 7.4) and its fluorescence was measured at 480 nm excitation wavelength and at 500-640 nm emission wavelength.

The carboxyl-TCL-SPION prepared thus was added to doxorubicin solution in the order of from the most diluted (0.18 μg) to concentrated (0.25 mg) samples and then fluorescence for doxorubicin was measured at each concentration. The amine group of doxorubicin in 5% glucose solution is charged positively. The carboxyl group exhibiting negative charge exposed on the surface of carboxyl-TCL-SPION could bind to the amine group of doxorubicin through ion-ion bonds, resulting in loading of doxorubicin onto carboxyl-TCL-SPION. As shown in Fig. 3, the decrease in the fluorescence intensity in proportion to increase in doxorubicin loading amount on carboxyl-TCL-SPION results from extinguish of the fluorescence of doxorubicin by electron exchange due to closer distance from each other by ionic bonds. In analyzing the amount of carboxyl-TCL-SPION for extinguishing the fluorescence of 4 μg doxorubicin, the present inventors observed that 0.2 mg of carboxyl-TCL-SPION eliminated fluorescence intensity of 4 μg doxorubicin. Accordingly, the inventors understood that the amount of carboxyl-TCL-SPION eradicating fluorescence intensity of doxorubicin could be considered as the maximum loading amount of doxorubicin. Afterwards, the present inventors prepared doxorubicin-loaded carboxyl-TCL-SPION by dissolving 4 μg of doxorubicin in 5% glucose solution and adding 0.2 mg of carboxyl-TCL-SPION. Based on the fluorescence intensity of doxorubicin at 555 nm, the dissociation constant K_d , was calculated as 56.46 μg of carboxyl-TCL-SPION (Fig. 4).

EXAMPLE 4: Analysis of Size and Zeta-Potential of Doxorubicin-Loaded Carboxyl-TCL-SPION

The hydrodynamic size of the carboxyl-TCL-SPION was determined by measuring dynamic light scattering (DLS) using ELS 8000 (Otsuka Electronics Korea). As shown in Fig. 5, the average size of each carboxyl-TCL-SPION and DOX@TCL-SPION was exhibited at 22.1 ± 5.0 nm and 21.3 ± 6.4 nm, and narrow size distribution was found, suggesting that loading of doxorubicin has no significant

effect on size of carboxyl-TCL-SPION. The particle size of the present nanoparticles is much smaller than those of conventionally dextran-coating SPIONs such as CLION and MION. Because the DLS measurement provides information on the hydrodynamic particle size of the total cluster (*i.e.*, magnetic core and coating polymer), the size of only iron oxide core was additionally investigated by TEM. The size of carboxyl-TCL-SPION and DOX@TCL-SPION was measured using TEM (Philips TECNAI F20). For microscope analysis, DW-diluted carboxyl-TCL-SPION and DOX@TCL-SPION was dropped and deposited on carbon-coated copper grid and allowed to be air-dried for 24 hrs. As described in Fig. 4, iron oxide nanoparticles of carboxyl-TCL-SPION and DOX@TCL-SPION had a diameter in a range of 4-10 nm.

For zeta-potential measurement, carboxyl-TCL-SPION and DOX@TCL-SPION were diluted with distilled water to prepare at a concentration of 1 mg/ml. It could be observed by zeta-potential analysis whether the carboxyl group on the surface of magnetic nanoparticle was exposed and whether electrical potential was altered upon loading of doxorubicin.

Table 1

Item	Size (nm)	Zeta-potential (mV)	Loading efficiency (%)
Carboxyl-TCL-SPION	22.1 ± 5.0	-37.26 ± 1.73	-
DOX@TCL-SPION	21.3 ± 6.4	-25.10 ± 2.24	54.33 ± 3.2

As shown in Table 1, each zeta-potential of carboxyl-TCL-SPION and DOX@TCL-SPION was -37.26 ± 1.73 mV and -25.10 ± 2.24 mV. It could be appreciated that carboxyl-TCL-SPION exhibits strong negative charge due to exposed carboxyl group and binds ionically to amine group of doxorubicin having a positive charge, increasing zeta-potential of carboxyl-TCL-SPION to about 8 mV.

EXAMPLE 5: Analysis of Drug Release Rate of TCL-SPION-DOX

Time-course experiment was carried out to determine how fast doxorubicin

was released from DOX@TCL-SPION in aqueous solution. 4 µg of doxorubicin was dissolved in 300 µl of 5% glucose solution and 0.2 mg of carboxyl-TCL-SPION was added to prepare DOX@TCL-SPION. DOX@TCL-SPION was put in dialysis sac having a pore in a range of 50 K and then both ends were sealed by dialysis forceps.

5 Dialysis sacs containing DOX@TCL-SPION were taken in 30 ml of phosphate buffer saline (pH 7.4) and acetate buffer (pH 5.1), respectively. The container was shaken at 50 rpm in 37°C water bath. After 30 min, 1 hr, 3 hrs, 6 hrs, 12 hrs and 24 hrs, 1 ml of each solution was collected and stored. In every collection step, remainder of each solution in container was discarded and newly replaced with phosphate buffer

10 saline and acetate buffer. The doxorubicin in the collected solution was measured at 480 nm excitation wavelength and at 500-640 nm emission wavelength. To prepare standard solution of doxorubicin, 1 µg/ml of the doxorubicin solution was serially diluted by two-fold to obtain 7 ng/ml solution, preparing 8 samples and their fluorescence values were measured at 480 nm excitation wavelength and at 500-640

15 nm emission wavelength. The fluorescence intensity at 555 nm was also determined for each sample to plot a standard linear graph. The amount of doxorubicin in solutions collected at each time point was determined using the standard linear graph. As results, it was observed that doxorubicin was released from DOX@TCL-SPION in acetate buffer much faster than in phosphate buffer saline. More than 50%

20 of doxorubicin was released within 2 hrs in phosphate buffer saline and within 45 min in acetate buffer. It was also observed that more than 80% of doxorubicin in both solutions was released within 24 hrs.

EXAMPLE 6: *In Vivo* MRI Imaging Potential of DOX@TCL-SPION for Tumor

25 **Tissue**

The present inventors investigated whether DOX@TCL-SPION as MRI contrast agents can image tumor tissues. Male C57BL/6 mice with transplanted LLC cell line (Lewis lung carcinoma cell line, ATCC) on their back were used as test animals. Mice

were anesthetized for imaging according to a general inhalation anesthesia (1.5% isoflurane in a 1:2 mixture of O₂/N₂). Each of the DOX@TCL-SPION (13 mg Fe/kg, 0.16 mg dox eq./kg) and 4 µg of doxorubicin (0.16 mg/kg) in 5% glucose solution was injected intravenously through the tail vein. MR imaging was performed with a
5 1.5 T imager (GE Signa Exite Twin-speed, GE Health Care) using an animal coil (4.3 cm Quadrature Volume Coil, Nova Medical System). For MR imaging of mice, T2-weighted fast-spin echo (repetition time ms/echo time ms of 4,200/102, flip angle 90°, echo train length of 10, 5cm field of view, 2 mm section thickness, 0.2-mm intersection gap, 256×160 matrix) and T1-weighted spoiled gradient echo
10 (185/minimum, 60° flip angle, 2 mm section thickness, 0.2-mm intersection gap, 256×160 matrix) sequences were carried out.

The quantitative analysis was performed by one radiologist for all MR imaging. The signal intensity (SI) was measured in defined regions of interest (ROI) which were in comparable locations within the tumor center. In addition, the SI in ROI of
15 back muscle adjacent to the tumor was measured. The size of ROI was chosen as two thirds the maximum diameter of tumor. Relative signal enhancement was calculated from SI measurements before (SI pre) and after (SI post) injection of the contrast agents by using the formula: $[(SI \text{ post} - SI \text{ pre}) / SI \text{ pre}] \times 100$, where SI pre: lesion signal intensity on pre-enhanced scan (control) and SI post: lesion signal
20 intensity on post-enhanced scan at 1 hr and 3 hrs.

Before injection of DOX@TCL-SPION, tumors are seen as hyperintensive areas in T2-weighted MR images (indicated by white arrows in Figure 7). The relative signal intensity (SI) on T2-weighted image was calculated as described above. At 1 hr post injection of DOX@TCL-SPION, some areas of darkening on T2-weighted MR
25 images were observed in the tumor area with a T2 signal drop of 40%, indicative of the accumulation of detectable amounts of DOX@TCL-SPION within the tumor. At 3 hrs post injection a T2 signal drop of 53% was observed, demonstrating that the DOX@TCL-SPION particles could be fast accumulated in tumor tissues due to their

resistance to recognition of macrophage and smaller sizes.

The prominent properties described above ensure DOX@TCL-SPION nanoparticles to become a promising diagnostic agent for cancer. In contrast, no signal changes were detected at 1 hr or 3 hrs post injection of doxorubicin.

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EXAMPLE 7: Accumulation of DOX@TCL-SPION in *In Vivo* Tumor Tissue and Biodistribution in Each Internal Organ

Male C57BL/6 mice with transplanted LLC cell line (Lewis lung carcinoma cell line, ATCC) on the back were used as test animals. Mice were anesthetized for
10 imaging according to a general inhalation anesthesia (1.5% isoflurane in a 1:2 mixture of O₂/N₂). Each of the DOX@TCL-SPION (13 mg Fe/kg, 0.16 mg dox eq./kg) and 4 µg of doxorubicin (0.16 mg/kg) in 5% glucose solution was injected intravenously through the tail vein. At each 1 hr or 3 hrs post-administration, all mice were sacrificed and their liver, lung, spleen, heart, kidney and tumor were extracted.
15 Optical images were observed using IVIS 100 imaging system (Xenongen Corp., Alameda, CA) after each internal organ was arranged at each time point. The accumulation amount of doxorubicin in each internal organ was determined by measuring the fluorescence of doxorubicin to be detectable in GFP/GFP channel. At 1 hr or 3 hrs post-administration of DOX@TCL-SPION, signals of doxorubicin were
20 observed strongly in all tumor tissues and weakly in liver, lung, heart and kidney not in spleen, as shown in Fig. 8. Unlikely, signals were detected weakly at 1 hr or 3 hrs post-administration of doxorubicin and relatively high signals were detected in liver at 1 hr post-administration. At least three regions of interest (ROI) in each internal organ were assigned and their measurement results were expressed as
25 pcs/sec/cm²/sr. The results were represented in Fig. 8. It could be appreciated that signals of DOX@TCL-SPION in tumor tissues were at least two-fold higher than those of doxorubicin at 1 hr and 3 hrs post-administration. Therefore, the results urge us to reason that the DOX@TCL-SPION nanoparticles with cancer targeting potential

provide more plausible therapeutic effects than doxorubicin and diagnosis applicability as well.

EXAMPLE 8: Inhibitory Effect on Growth of Tumor Size by DOX@TCL-SPION

Male C57BL/6 mice with transplanted LLC cell line (Lewis lung carcinoma cell line, ATCC) on the back were used as test animal. One week after tumor transplantation in which tumor size reached about 50 mm³, drug administration started. Mice were divided into three groups, *i.e.*, control (5% glucose solution), DOX@TCL-SPION (13 mg Fe/kg, 0.16 dox eq./kg) and doxorubicin (2 mg/kg). Each group consisted of 7-8 mice transplanted with lung cancer cells. Drug administration was carried out every two days for the total 6 times (administration days are indicated by arrow in Fig. 9). The changes of tumor size were continuously surveyed by measuring each short and long diameter of cancer using vernier calipers until 21 days after drug administration and tumor size was calculated. As shown in Fig. 9, the average size of tumor began to exhibit differences between three groups from 10 days after initial drug administration. The inhibition of tumor size growth was observed on 11 days of experiment (the day of the final drug administration) and successively observed on 17 days. It was also revealed that DOX@TCL-SPION as compared to control exhibited 57% of inhibitory effect at 21 days, *i.e.*, a final day to observe inhibitory growth effect to tumor size. In the group administered with 2 mg/kg of doxorubicin, although the dosage of doxorubicin was 12.5 fold higher than that of DOX@TCL-SPION, no drug effect was observed in which the growth rate of tumor size was as high as the control group. As represented in Fig. 10, the DOX@TCL-SPION showed little or no difference in mice weight from the control group during the period of time for drug administration and then exhibited doxorubicin cytotoxicity after the final drug administration due to its accumulation in body. On the other hand, doxorubicin showed cytotoxicity from the beginning of

drug administration but mice weight was continuously increased from the end of drug administration due to rapid excretion of drug. The weight recovery in the doxorubicin group at 18 days after drug administration was shown to be very similar to that in the control group. It could be appreciated that DOX@TCL-SPION serves as
5 inhibitors against cancers as well as MR imaging agents, demonstrating that TCL-SPION is an excellent candidate as a novel drug delivery system.

EXAMPLE 9: Preparation of cRGD-TCL-SPION

1) amine TCL-SPION

10 100 μ l of 2,2'-(ethylenedioxy) bis-(ethylamine) (1 M) and 100 μ l of N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (500 mM) were added to 1 ml of carboxyl TCL-SPION solution (15 mg/ml) and stirred for 6 hrs. This mixture was put in dialysis sac having a pore size of 50 K and then both ends were sealed by dialysis forceps. Dialysis sacs were stirred in distilled water. Fresh distilled water was
15 replaced at 3-6 hr intervals during stirring for 24 hrs and then the solution in dialysis sacs was harvested.

2) SPDP-TCL-SPION

Each 1 mg of 3-(2-pyridyl dithio) propionic acid, 1.4 mg of N-(3-
20 dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride, and 1 mg of hydroxysuccinimide was dissolved in DMSO, mixed and agitated for 2 hrs. 1 ml of the above amine-TCL-SPION solution was added to the solution and mixed for 18 hrs with rotation on intelli mixer. The resulting mixture was separated from non-reactive substances using a Sephadex G50 column and finally a black solution was collected
25 in vessels.

3) cRGD-TCL-SPION

NH₂OH (7 μ mol) was added to 100 μ l of cRGD stock solution (3 mg/ml) and

left for 1 hr at room temperature. 1 ml of the above SPDP-TCL-SPION solution was added to the mixture and mixed for 18 hrs with rotation on intelli mixer. The resulting mixture was separated from non-reactive substances using a Sephadex G50 column and finally a black solution was collected in vessels.

5 The conjugation of cRGD was determined by the following method. 10 μ l of solution was previously harvested prior to separation using Sephadex G50 column after synthesis of cRGD-TCL-SPION and centrifuged using spin-filter having a pore of 100 K to collect the solution to pass through. The OD value of the collective solution was measured under UV light (at 343 nm) and the amount of pyridine-2-thione
10 produced by cRGD was quantitated using a standard curve.

As shown in Figs. 12a-12b, the peak of pyridine-2-thione produced was observed at 343 nm, indicating that 40 μ g of cRGD (corresponding to about 4 wt (%)) per 1 mg of TCL-SPION was conjugated.

15 **EXAMPLE 10: Analysis of Size and Zeta-Potential of cRGD-TCL-SPION**

Size and zeta-potential of cRGD-TCL-SPION were analyzed according to the method described in Example 4. As shown in Fig. 13, the average particle size of each carboxyl-TCL-SPION, amine TCL-SPION and cRGD-TCL-SPION exhibited those of 28.2 ± 6.7 nm, 36.3 ± 8.3 nm and 33.9 ± 8.1 nm and narrow size distribution was
20 found. The size of TCL-SPION nanoparticle was increased to about 5-6 nm due to conjugation of cRGD.

In zeta-potential measurement, carboxyl-TCL-SPION, amine TCL-SPION and cRGD-TCL-SPION were diluted with distilled water, preparing at a concentration of 1 mg/ml. It might be determined by zeta-potential analysis whether the carboxyl
25 group on the surface of magnetic nanoparticle was changed to the amine group and whether cRGD was suitably conjugated. Each zeta-potential of carboxyl-TCL-SPION, amine TCL-SPION and cRGD-TCL-SPION was -24.07 ± 1.06 mV, -5.28 ± 0.8 mV and -29.33 ± 3.01 mV. The surface on carboxyl-TCL-SPION exhibited strong negative

charge due to the exposed carboxyl group and its carboxyl group was changed to neutral amine group, resulting in increase of zeta-potential of carboxyl-TCL-SPION to about 19 mV. It could be considered that cRGD-TCL-SPION also exhibited strong negative charge owing to conjugation of cRGD containing an aspartate having a form of COO⁻ in neutral solution.

EXAMPLE 11: Cellular Uptake Analysis of cRGD-TCL-SPION

200 µl of 2% gelatin solution was added to coverslip on 24-well and coated by incubation for about 4 hrs under UV and flow in clean bench. U87MG cells (glioblastoma cell line) expressing intensively alpha v beta 3 integrin were seeded to 50,000 cells per a well and cultured for 24 hrs in 5% CO₂ incubator. Each cRGD-TCL-SPION and TCL-SPION of a concentration of 0.1 mg/ml was seeded to wells and cultured for 12 hrs. Treated cells were washed 2 times by PBS and fixed with 4% formaldehyde for 10 min. The cells were re-washed 2 times by PBS and incubated with a 1:1 (v/v) mixture of 2% potassium ferrocyanide and 1% HCl for 10 min. After washing two-times with PBS, the coverslip on slideglass were observed using microscope. As shown in Fig. 14, cRGD-TCL-SPION and TCL-SPION as compared to untreated control displayed blue colors by cellular uptake, indicating that Fe is stained with ferrocyanide, and it was confirmed that the amount of cRGD-TCL-SPION was much higher than that of TCL-SPION.

To determine amount of Fe, ICP-AES (Inductively-Coupled Plasma-Atomic Emission Spectrometry) method was carried out. Each U87MG cells (glioblastoma cell line) and MCF-7 cells expressing slightly alpha v beta 3 integrin were seeded in 6 wells to 1,000,000 cells per well and cultured for 6 hrs in 5% CO₂ incubator.

cRGD-TCL-SPION and TCL-SPION of a concentration of 0.1 mg/ml and the solution containing 200 µM of free cRGD in cRGD-TCL-SPION were prepared. Each U87MG and MCF-7 cells was treated with the above solution and cultured for 3 hrs and 12 hrs. After two-times washing with PBS, cells were detached by trypsin and

after cells were harvested with centrifuge, ICP-AES was carried out. Fig. 15 clearly represented that the amount of Fe was higher uptake in cRGD-TCL-SPION than in TCL-SPION and TCL-SPION has no significant differences with control blocking an integrin with free cRGD, demonstrating that cRGD binds to intergrin, resulting in easily cellular uptake by receptor-mediated endocytosis. However, it could be revealed that cRGD conjugation with nanoparticles has no significant influence on cellular uptake in MCF-7 cells.

EXAMPLE 12: Loading of Doxorubicin in cRGD-TCL-SPION (DOX@cRGD-TCL-SPION)

According to the similar method described in Example 3, doxorubicin was loaded into cRGD-TCL-SPION and the fluorescence of loaded amount was measured. As shown in Fig. 16, amount of cRGD-TCL-SPION to eliminate all fluorescence intensity of 4 μ g doxorubicin was analyzed. As results, it could be observed that about 0.22 mg of cRGD-TCL-SPION eliminated the fluorescence intensity of doxorubicin. Based on this observation, the amount of cRGD-TCL-SPION eradicating fluorescence intensity of doxorubicin was considered as maximum amount to load doxorubicin and so doxorubicin-loaded cRGD-TCL-SPION (DOX@cRGD-TCL-SPION) was always prepared by dissolving 4 μ g of doxorubicin in 5% glucose solution and then adding about 0.22 mg of cRGD-TCL-SPION.

EXAMPLE 13: Cytotoxicity Analysis of Dox@cRGD-TCL-SPION

Cytotoxicity of Dox@cRGD-TCL-SPION to human carcinoma cell line, U87MG cell line (ATCC) was analyzed by the following MTT method. U87MG cells were seeded on 96-well in a count of 5,000 cells and cultured for 24 hrs in 5% CO₂ incubator. The cells were treated with each of TCL-SPION, cRGD-TCL-SPION, Dox@TCL-SPION, Dox@cRGD-TCL-SPION and doxorubicin at concentrations ranging from 10⁻⁴ M to 10⁻¹¹ M (calculated based on doxorubicin concentration). That is, 10⁻⁴

M sample solutions (based on doxorubicin) were prepared by adding 58 μ l of doxorubicin to 3.2 mg/ml of each TCL-SPION and cRGD-TCL-SPION and then serially diluted by 10-fold to 10^{-11} M solutions for cell treatments. Each doxorubicin-unloaded TCL-SPION and cRGD-TCL-SPION was incubated with cells in amounts same as those of SPION for doxorubicin loading. In other words, 3.2 mg/ml of each TCL-SPION and cRGD-TCL-SPION was prepared and then serially diluted by 10-fold for cell treatments. After 24-hr treatment, cells were washed two-times with PBS. 5 mg/ml of MTT was treated in 20 μ l per each well and incubated for 4 hrs in 5% CO₂ incubator. The solution in wells was discarded and DMSO solution was added to well. The absorbance was measured at 570 nm.

As shown in Fig. 16, each IC₅₀ of doxorubicin, Dox@TCL-SPION and Dox@cRGD-TCL-SPION was 0.24 μ M, 0.14 μ M and 0.02 μ M, showing that the value of Dox@cRGD-TCL-SPION was the lowest. Doxorubicin-unloaded TCL-SPION and cRGD-TCL-SPION also exhibited cytotoxicity at concentrations of 3.2 mg/ml and 0.32 mg/ml, respectively.

EXAMPLE 14: Analysis of MR Imaging ability in *In Vivo* Tumor Tissue of Dox@cRGD-TCL-SPION

MR Imaging ability of Dox@cRGD-TCL-SPION was analyzed using male C57BL/6 mouse with transplanted U87MG cell line as a test animal. Experimental method was almost same to the method of Example 6. As shown in Fig. 18a, at 1 hr post injection of Dox@cRGD-TCL-SPION, a noticeable darkening appeared in the tumor area in the T2-weighted MR image. The mean decrease in T2 signal was 35-40% compared to pre-injection until 4 hrs post injection. In addition, the mean decrease in T2 signal was maintained in above 30% even after 12 hrs.

In MRI using Dox@TCL-SPION (Fig. 18b), The mean decrease in T2 signal was 32% compared to pre-injection at 1 hr post injection. Moreover, the mean decrease in T2 signal was 38% compared to pre-injection until 4 hrs post injection.

Having described a preferred embodiment of the present invention, it is to be understood that variants and modifications thereof falling within the spirit of the invention may become apparent to those skilled in this art, and the scope of this
5 invention is to be determined by appended claims and their equivalents.

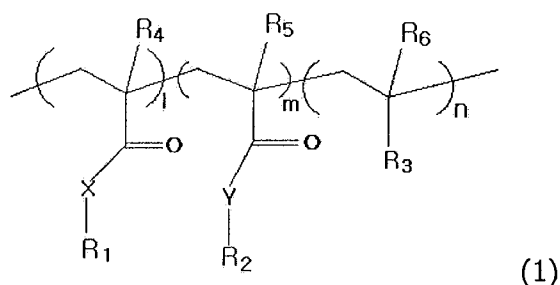
What is claimed is:

1. A multi-functional complex for imaging and drug delivery comprising a plurality of nanoparticles, wherein the nanoparticles comprise:
 - (a) a signal generating core; and
 - 5 (b) a water soluble polymeric outer shell coated on the signal generating core, comprising a surface-anchoring site containing silyl-, hydroxysilyl- or alkoxysilyl-functionalized groups and a drug-binding site.
- 10 2. The multi-functional complex according to claim 1, wherein the signal generating core is a paramagnetic, a superparamagnetic or a proton density signal generating core.
3. The multi-functional complex according to claim 1, wherein the signal generating core is a superparamagnetic signal generating core comprising an iron oxide.
- 15 4. The multi-functional complex according to claim 1, wherein the water soluble polymeric outer shell comprising silyl-, hydroxysilyl- or alkoxysilyl-functionalized group comprises poly(acrylic acid) or its derivatives, poly(meta)acrylic acid or its derivatives, poly(acrylic amide) or its derivatives, poly(undecenoic acid) or its derivatives, copolymers thereof, chitosan or its derivatives, dextran or its derivatives, cellulose or its derivatives, heparin or its derivatives, alginate or its derivatives, hyaluronate or its derivatives as main backbones.
- 20 5. The multi-functional complex according to claim 1, wherein the water soluble polymeric outer shell comprising silyl-, hydroxysilyl- or alkoxysilyl-functionalized group is crosslinked by binding between silicon atoms.
- 25 6. The multi-functional complex according to claim 1, wherein the water soluble

polymeric outer shell comprising silyl-, hydroxysilyl- or alkoxysilyl-functionalized group further comprises polyethylene glycol, dextran, polyvinylpyrrolidone, polypropylene glycol, copolymer of polyethylene glycol and polypropylene glycol, polyalkylene oxide or monoesterified derivatives thereof for anti-biofouling property.

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7. The multi-functional complex according to claim 1, wherein the water soluble polymeric outer shell is represented by the following Formula 1:



wherein R_1 represents silylalkyl, (alkoxysilyl)alkyl or (hydroxysilyl)alkyl; R_2 represents PEG (polyethylene glycol), polyalkylene oxide, polyphenylene oxide, copolymer of PEG and polyalkylene oxide, poly(methoxyethyl methacrylate), poly(methacryloyl phosphatidylcholine), perfluoro-polyether, dextran or polyvinylpyrrolidone; R_3 represents aldehyde, epoxy, haloalkyl, primary amine, thiol, maleimide, ester, carboxyl or hydroxyl; R_4 , R_5 and R_6 independently represent H or C_1 - C_5 alkyl; X, Y and Z independently represent oxygen, sulfur or nitrogen atom; l, m and n independently represent an integer of 1-10,000.

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8. The multi-functional complex according to claim 7, wherein the silicon within the polymer of the Formula 1 is thermally crosslinked to other silicon atom.

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9. The multi-functional complex according to claim 1, wherein the multi-functional complex is used in magnetic resonance imaging (MRI).

10. The multi-functional complex according to claim 1, wherein the multi-functional

complex is used in MRI for *in vivo* cancer imaging.

11. The multi-functional complex according to claim 1, wherein the nanoparticles coated with the polymer have average diameters in a range of 5-50 nm.

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12. The multi-functional complex according to claim 1, wherein the nanoparticles have saturation magnetization (M_s) values in a range of 20-100 emu/g Fe.

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13. The multi-functional complex according to claim 1, wherein the drug is further bound to the drug-binding site of the water soluble polymeric outer shell.

14. The multi-functional complex according to claim 13, wherein the drug is bound non-covalently to the drug-binding site.

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15. The multi-functional complex according to claim 14, wherein the non-covalent bond is ionic bond, coordinate bond, hydrophobic interaction, Van der Waals bond or combinations thereof.

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16. The multi-functional complex according to claim 15, wherein the non-covalent bond is ionic bond.

17. The multi-functional complex according to claim 1, wherein the drug comprises chemical drugs, proteins, peptides or nucleotides.

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18. The multi-functional complex according to claim 7, wherein the R_3 represents the carboxyl group and wherein the carboxyl group is activated by succinamide, succinamidyl ester, sulfo-succinamidyl ester, 2,3,5,6-tetrafluorophenol ester, 4-sulfo-2,3,5,6-tetrafluorophenol ester, aldehyde, acidic anhydride, azide, azolid, carboimide,

epoxide, ester, glycidyl ether, halide, imidazole or imidate.

19. The multi-functional complex according to claim 1, wherein the nanoparticle further comprises a targeting molecule.

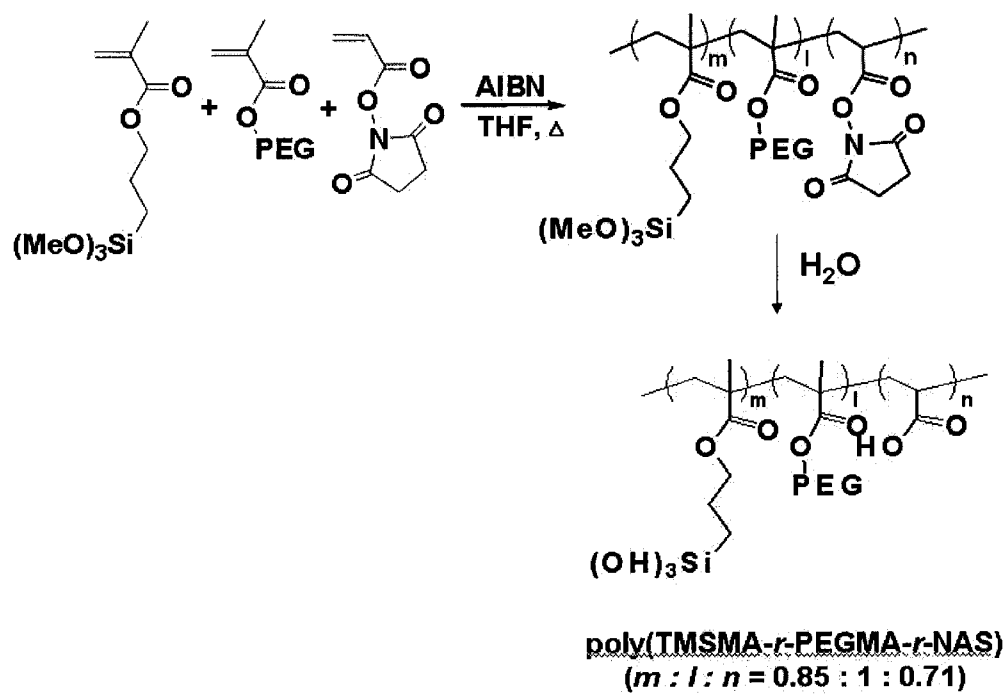
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20. The multi-functional complex according to claim 1, wherein the targeting molecule comprises antibodies, aptamers or cell adhesive peptides.

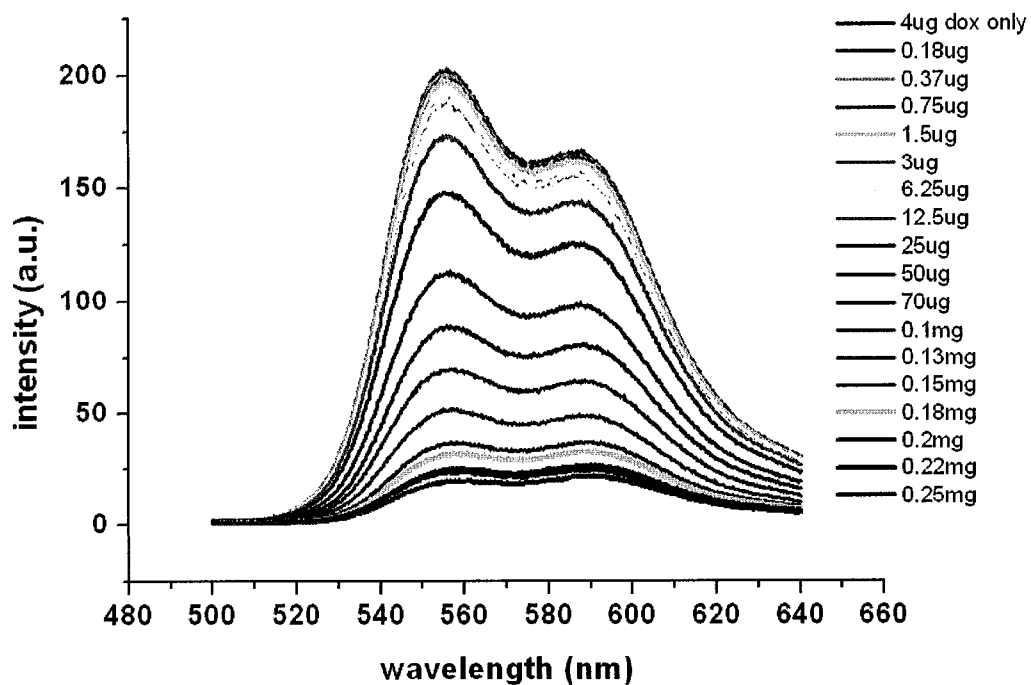
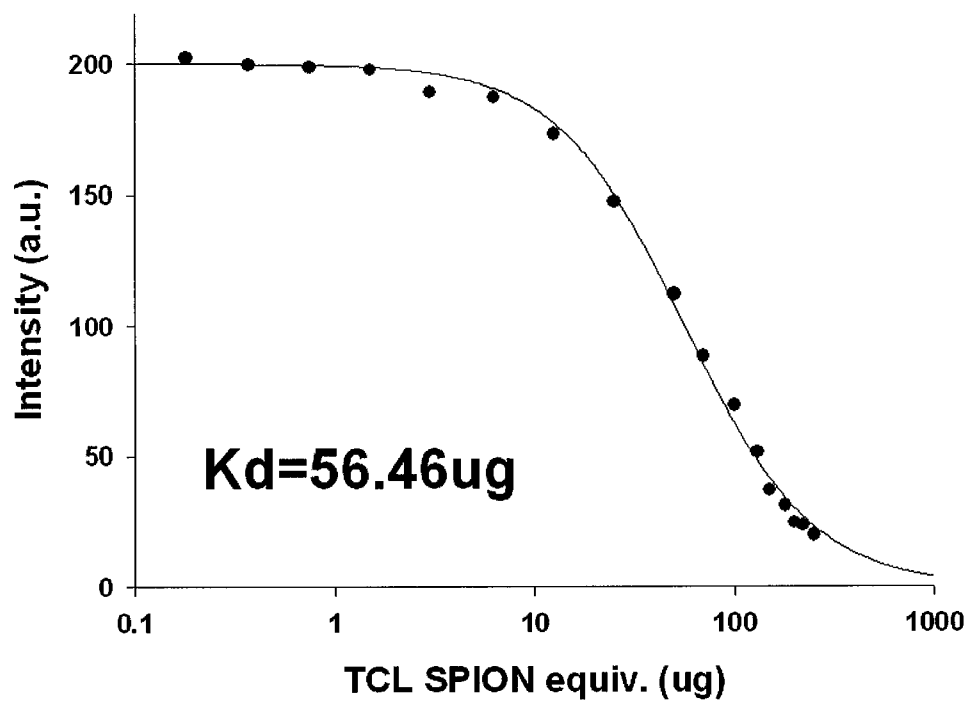
21. The multi-functional complex according to claim 20, wherein the cell adhesive peptide is RGD(Arg-Gly-Asp), RGDS(Arg-Gly-Asp-Ser), RGDC(Arg-Gly-Asp-Cys),
 10 RGDV(Arg-Gly-Asp-Val), RGES(Arg-Gly-Glu-Ser), RGDSPASSKP(Arg-Gly-Asp-Ser-Pro-Ala-Ser-Ser-Lys-Pro), GRGDS(Gly-Arg-Gly-Asp-Ser), GRADSP(Gly-Arg-Ala-Asp-Ser-Pro), KGDS(Lys-Gly-Asp-Ser), GRGDSP(Gly-Arg-Gly-Asp-Ser-Pro), GRGDTP(Gly-Arg-Gly-Asp-Thr-Pro), GRGES(Gly-Arg-Gly-Glu-Ser), GRGDSPC(Gly-Arg-Gly-Asp-Ser-Pro-Cys),
 15 GRGESP(Gly-Arg-Gly-Glu-Ser-Pro), SDGR(Ser-Asp-Gly-Arg), YRGDS(Tyr-Arg-Gly-Asp-Ser), GQQHHLGGAKQAGDV (Gly-Gln-Gln-His-His-Leu-Gly-Gly-Ala-Lys-Gln-Ala-Gly-Asp-Val), GPR(Gly-Pro-Arg), GHK(Gly-His-Lys), YIGSR(Tyr-Ile-Gly-Ser-Arg), PDSGR(Pro-Asp-Ser-Gly-Arg), CDPGYIGSR(Cys-Asp-Pro-Gly-Tyr-Ile-Gly-Ser-Arg),
 LCFR(Leu-Cys-Phe-Arg), EIL(Glu-Ile-Leu), EILDV(Glu-Ile-Leu-Asp-Val),
 20 EILDVPST(Glu-Ile-Leu-Asp-Val-Pro-Ser-Thr), EILEVPST(Glu-Ile-Leu-Glu-Val-Pro-Ser-Thr), LDV(Leu-Asp-Val) or LDVPS(Leu-Asp-Val-Pro-Ser).

22. A simultaneous method for imaging and drug delivery comprising administering to a subject in need a pharmaceutical composition which comprises the multi-
 25 functional complex as the effective ingredient according to any one of claims 1-21.

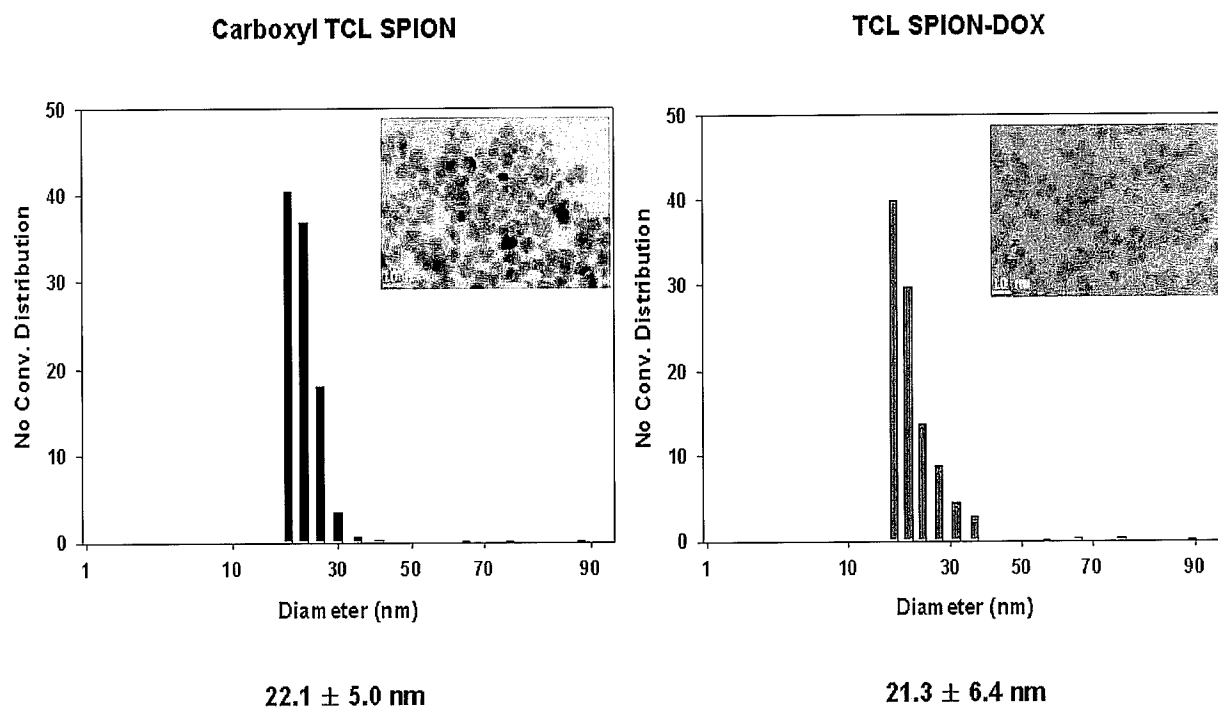
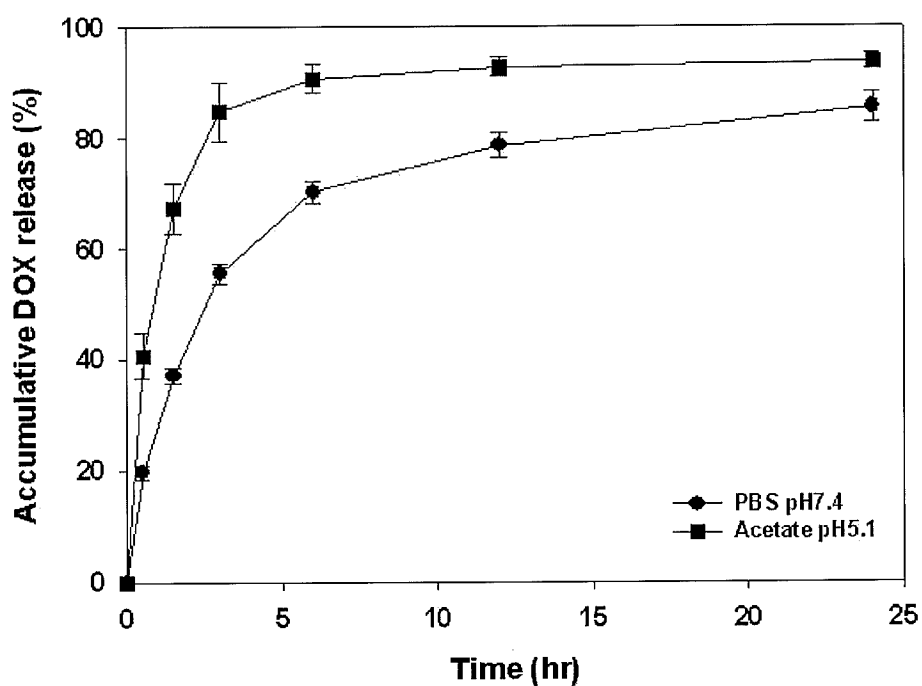
1/16

Fig. 1

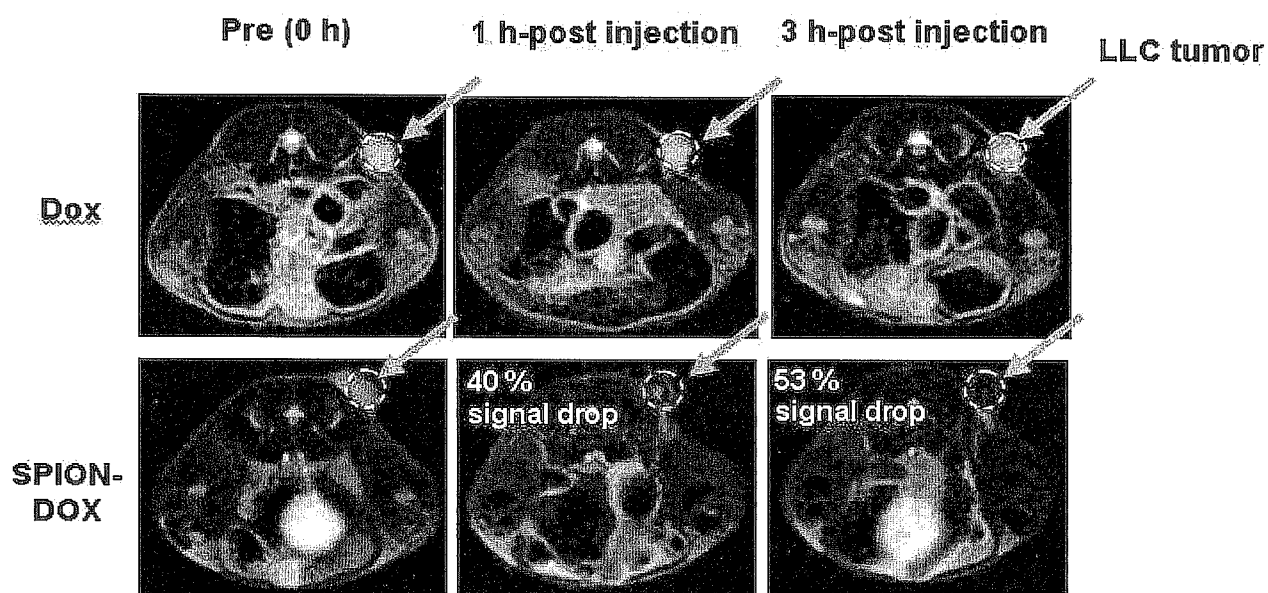
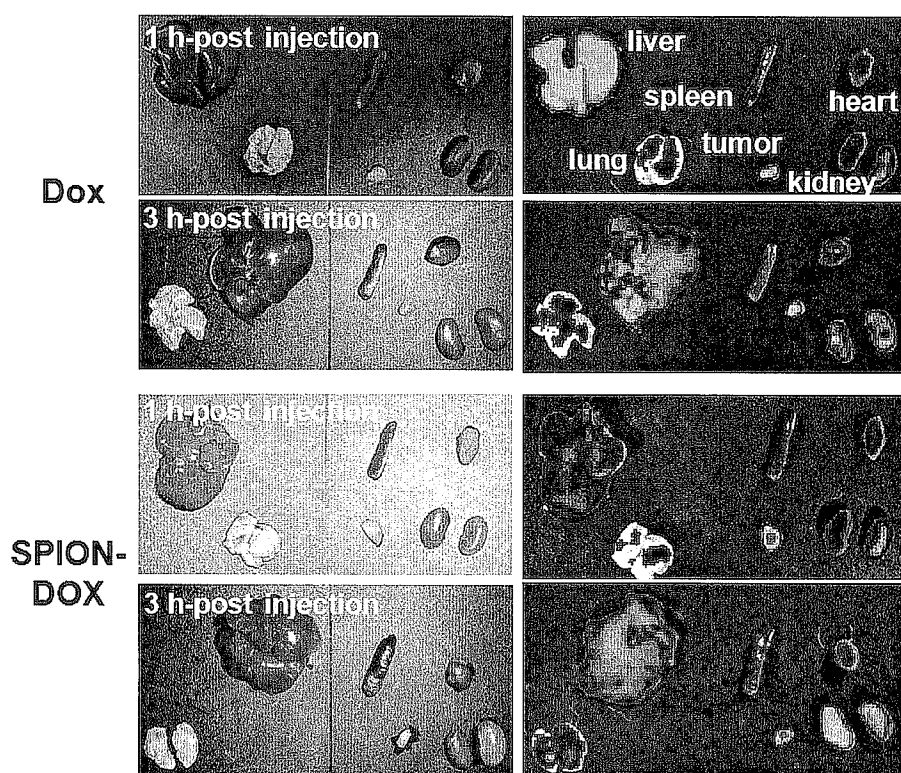
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Fig. 3**Fig. 4**

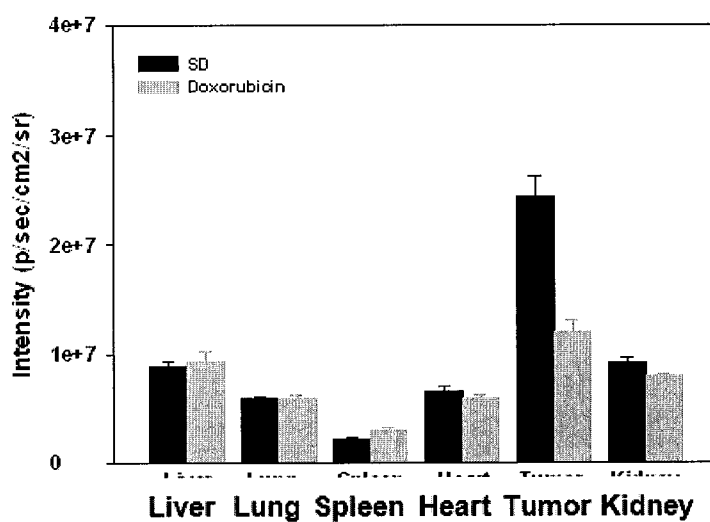
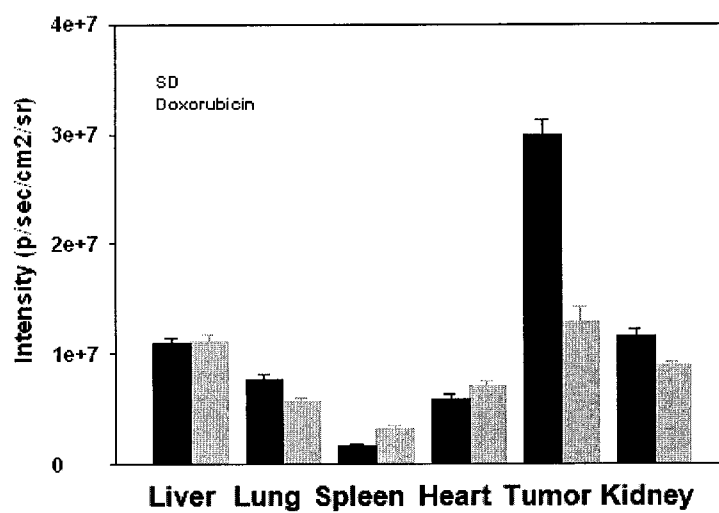
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Fig. 5**Fig. 6**

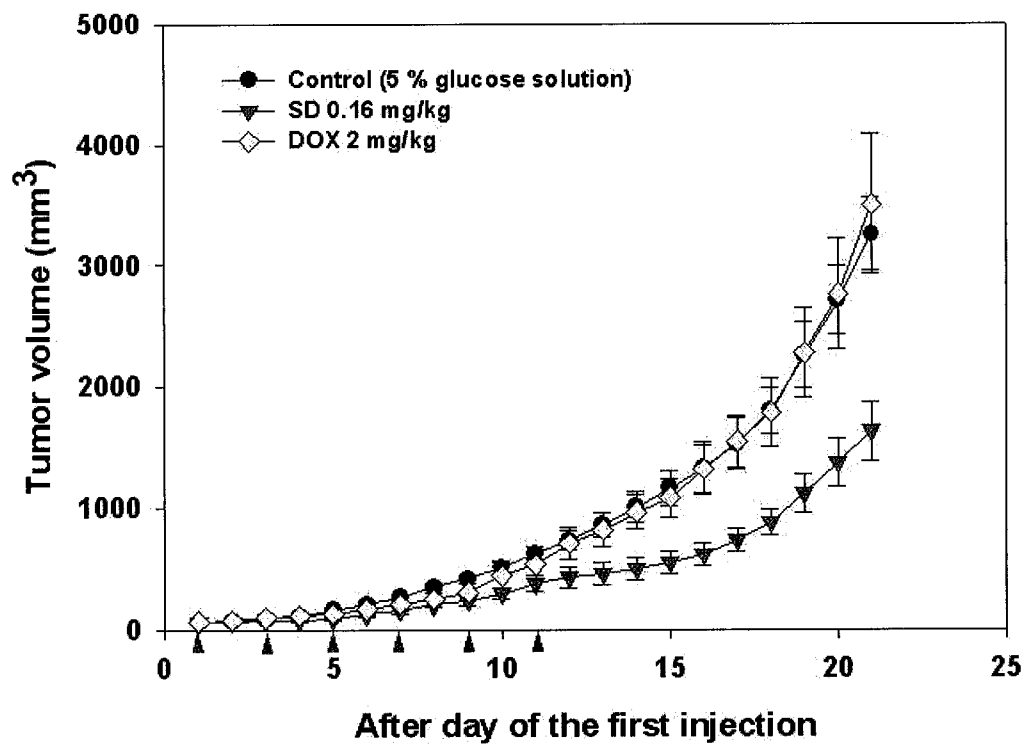
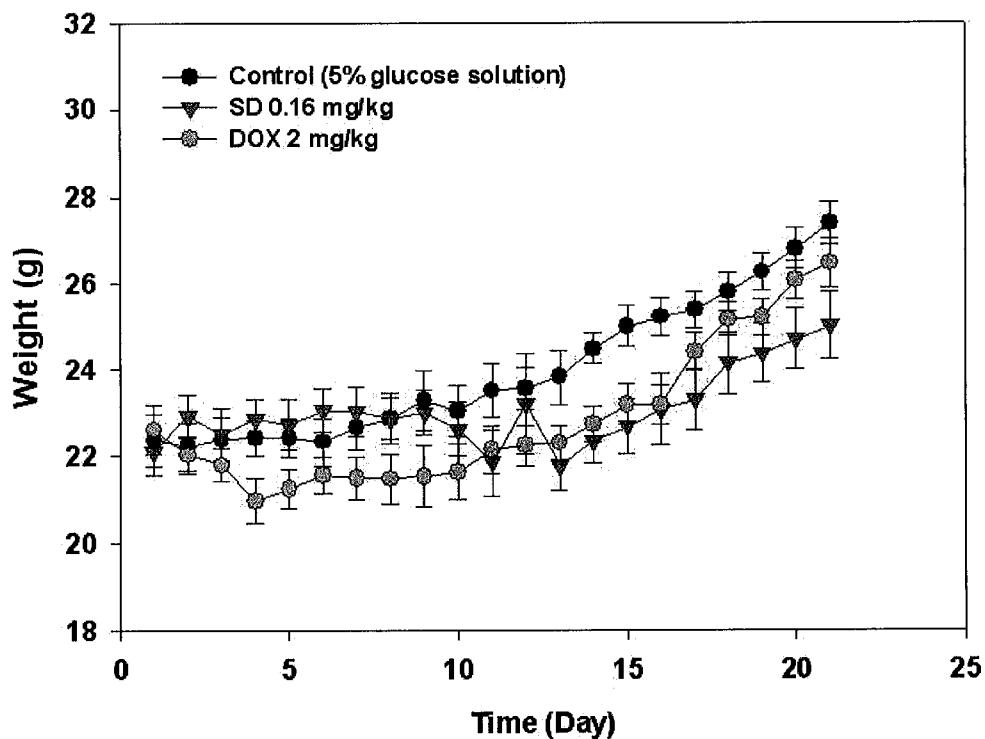
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Fig. 7**Fig. 8a**

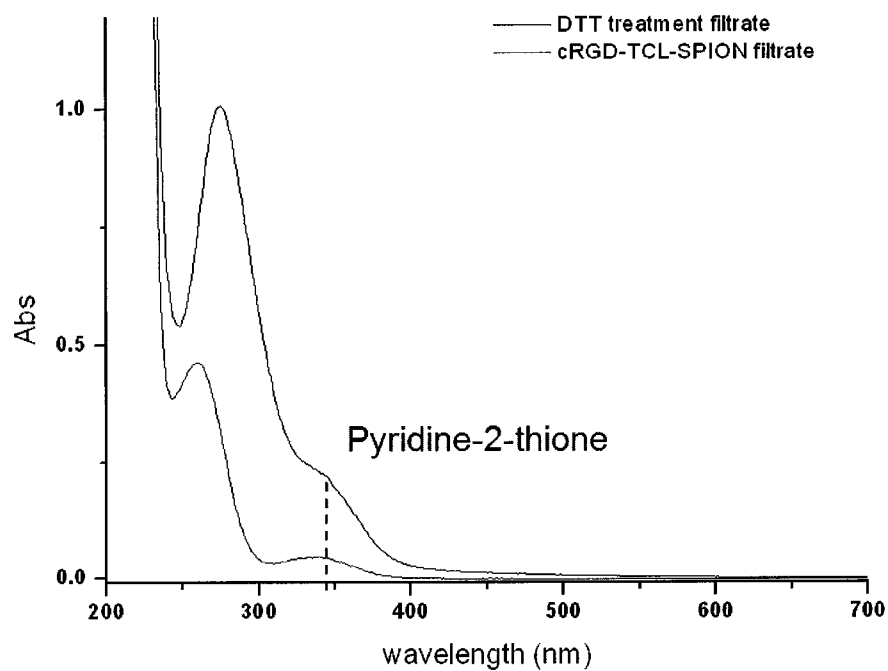
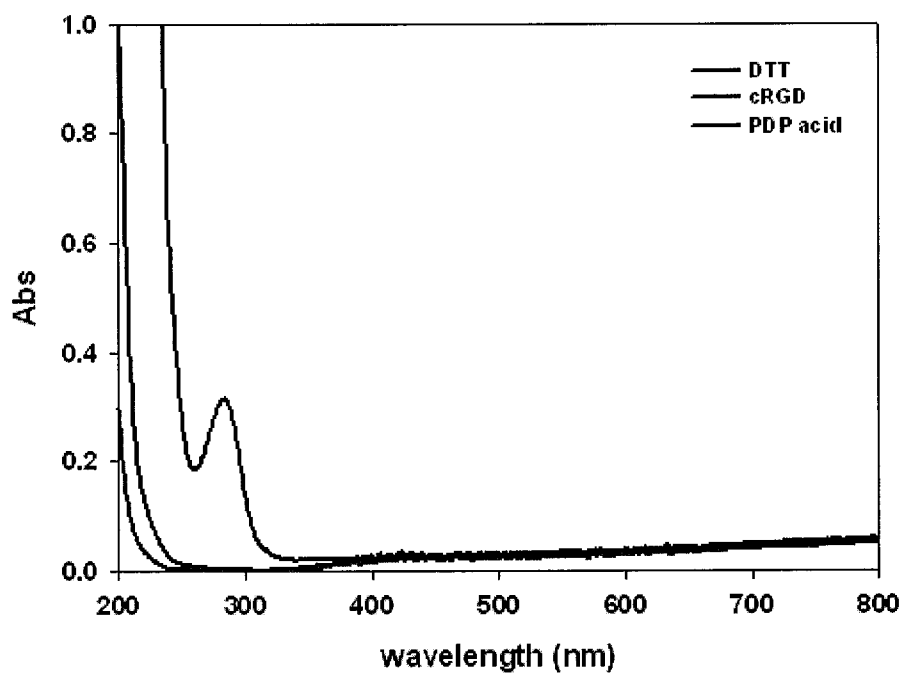
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Fig. 8b**1h post injection****3h post injection**

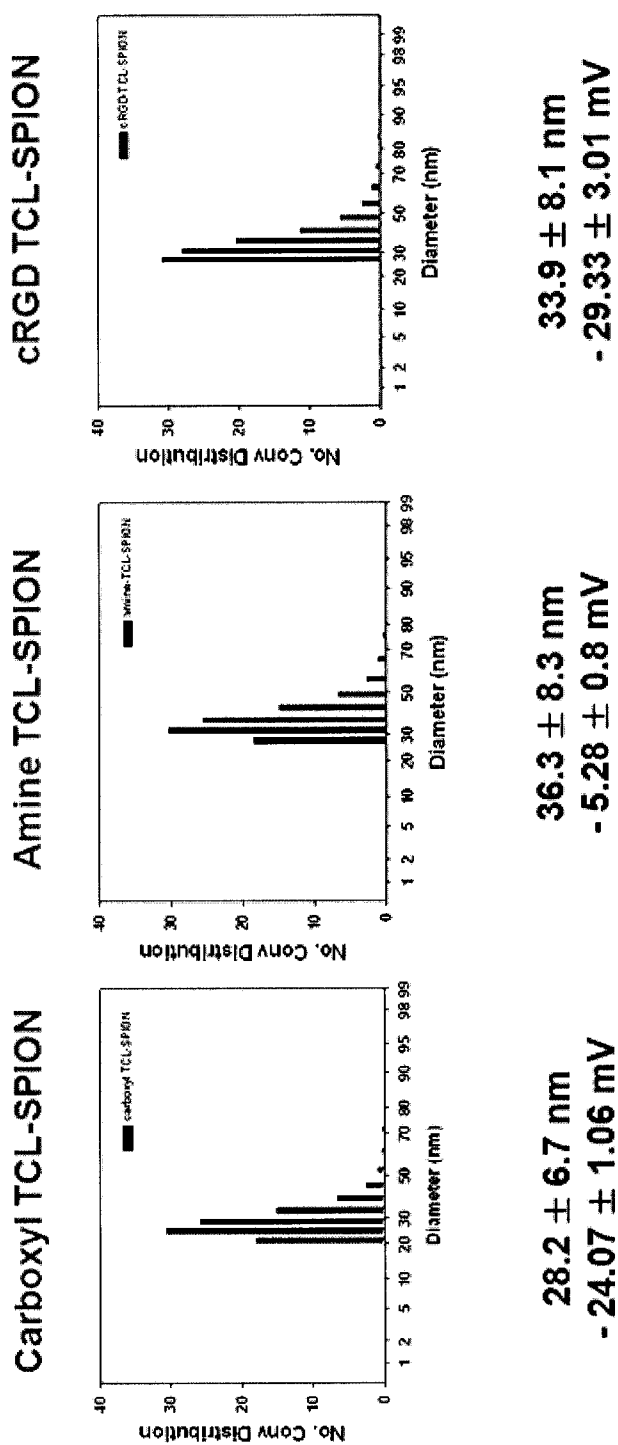
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Fig. 9**Fig. 10**

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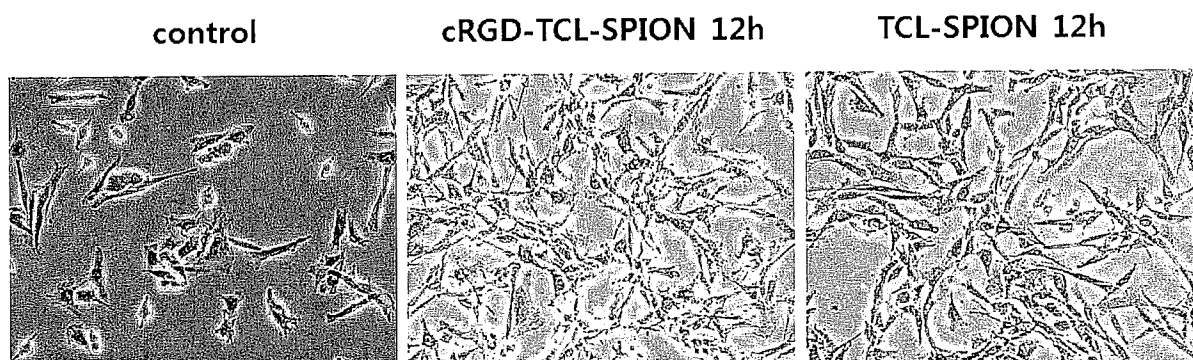
Fig. 12a**Fig. 12b**

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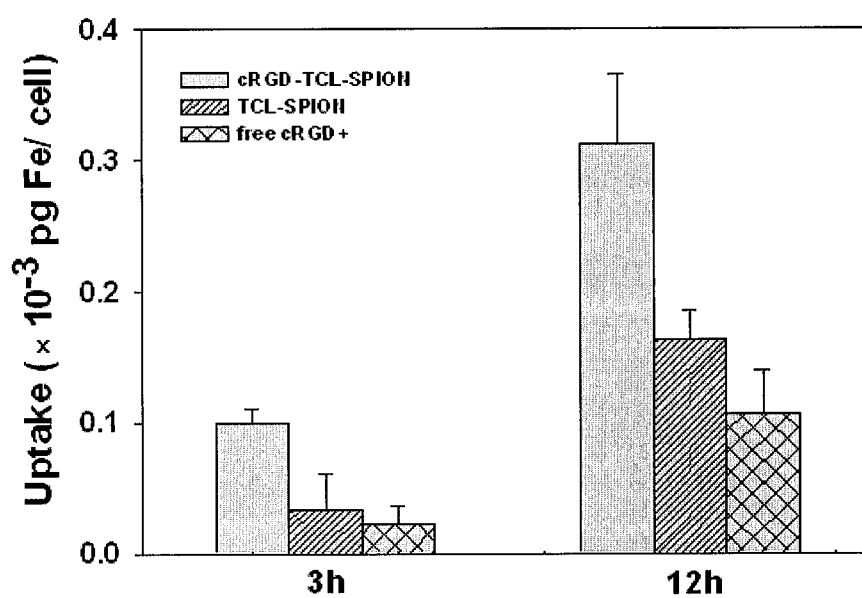
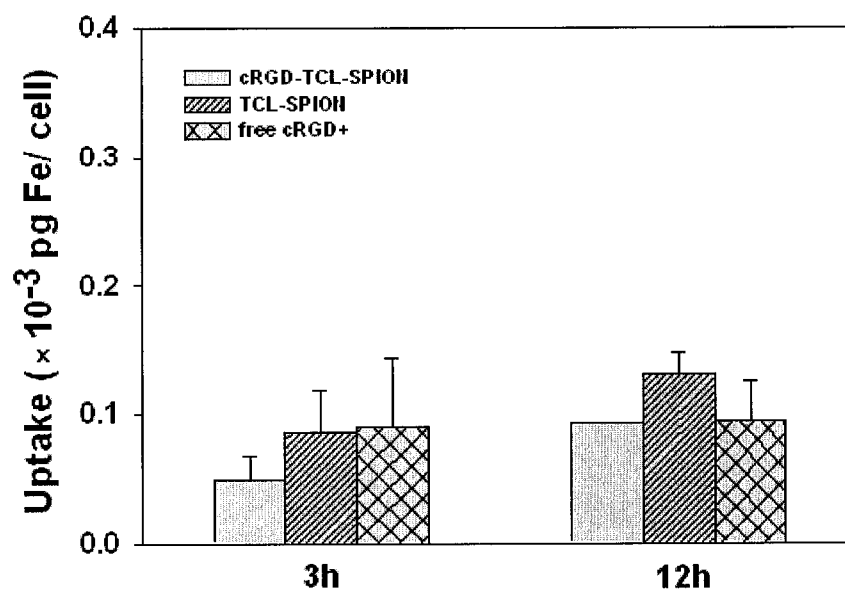
Fig. 13

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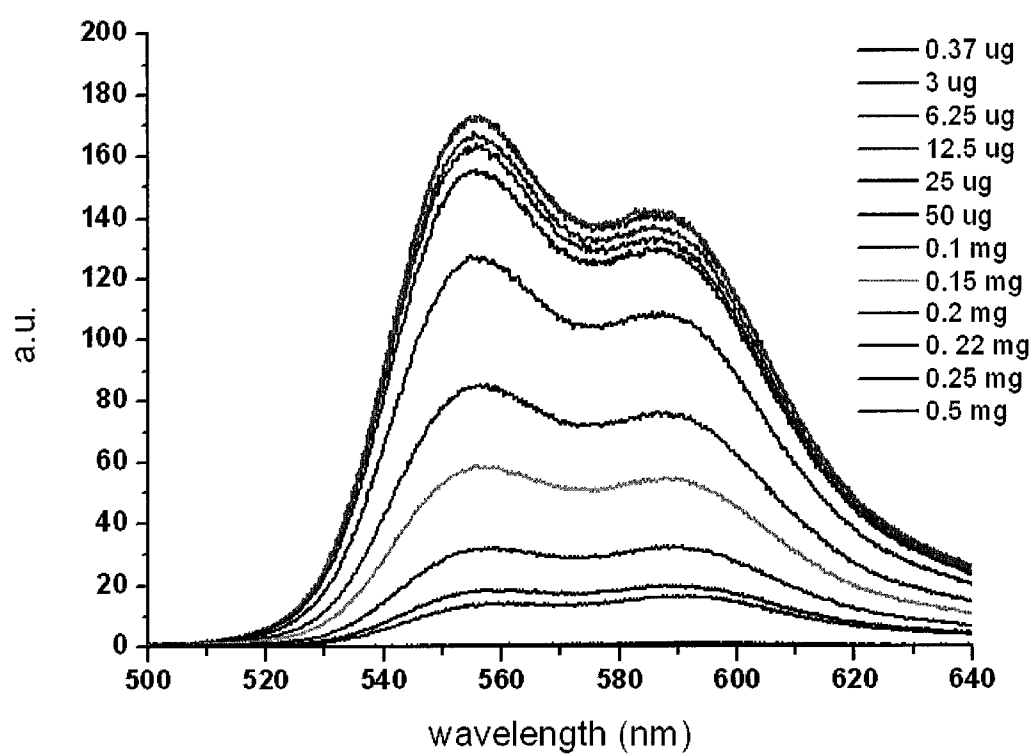
Fig. 14



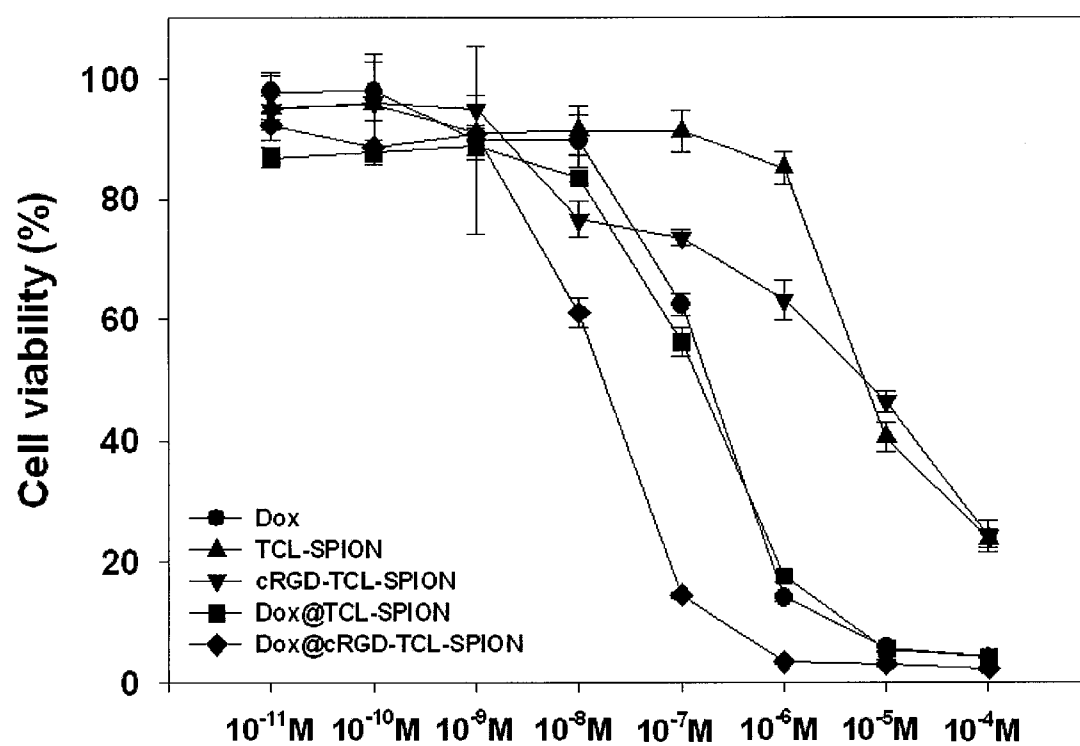
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Fig. 15**U87MG cell line****MCF-7 cell line**

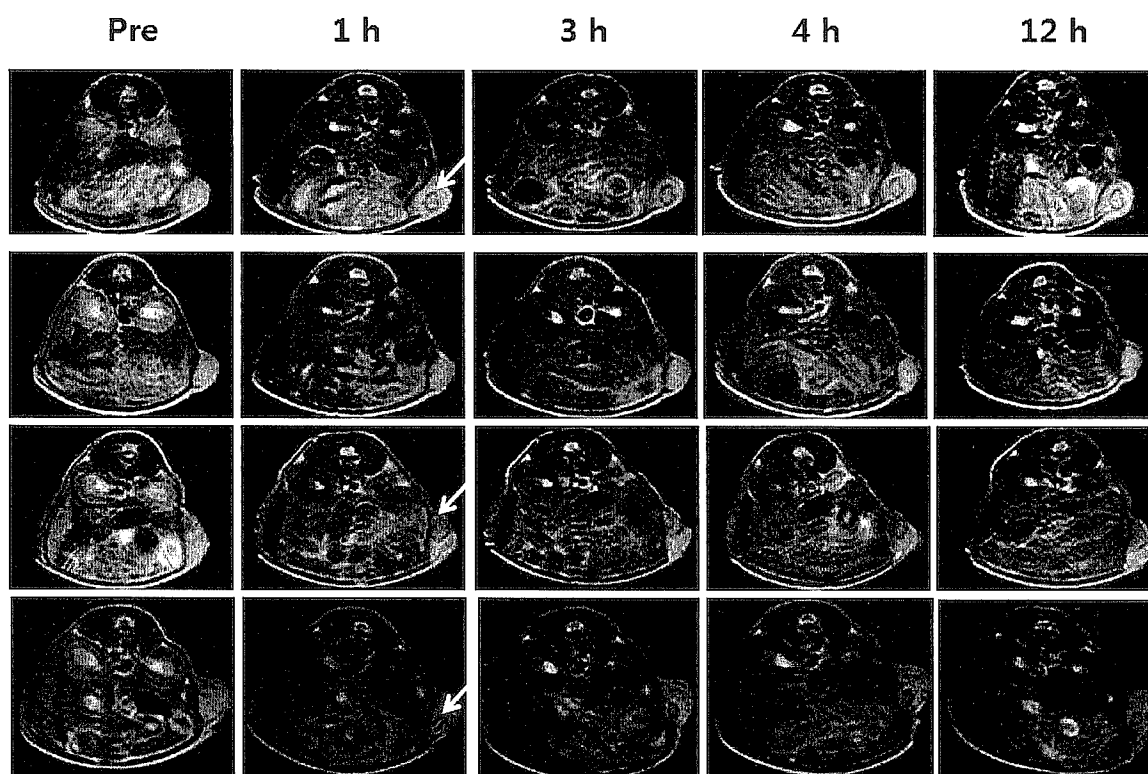
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Fig. 16

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Fig. 17

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Fig. 18a

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Fig. 18b