Research Article

Predictive Models of Diffusive Nanoparticle Transport in 3-Dimensional Tumor Cell Spheroids

Yue Gao,¹ Mingguang Li,² Bin Chen,¹ Zancong Shen,¹ Peng Guo,¹ M. Guillaume Wientjes,^{1,2} and Jessie L.-S. Au^{1,2,3}

Received 4 January 2013; accepted 19 March 2013; published online 20 April 2013

Abstract. The rapidly evolving nanotechnology field highlights the need of better understanding the relationship between nanoparticle (NP) properties and NP transport in solid tumors. The present study tested the hypothesis that the diffusive transport and spatial distribution of NP can be predicted based on the following parameters: interstitial NP diffusivity, NP–cell interaction parameters (cell surface binding capacity, rate constants of association, dissociation, and internalization). We (a) established the models and equations; (b) experimentally measured, in monolayer pharynx FaDu cells, the model parameters for three NP formulations (negatively charged polystyrene beads, near-neutral liposomes, and positively charged liposomes, with respective diameter of 20, 110, and 130 nm); and (c) used the models and parameters to simulate NP diffusion in 3-dimensional (3D) systems. We next measured the NP concentration–depth profiles in tumor cell spheroids, an avascular 3D system, and found good agreement between model-simulated and experimental data in spheroids for the negative and neutral NP (>90% predicted data points at three NP concentrations and three treatment times were within the 95% confidence intervals of experimental data). Model performance was inferior for positive liposomes containing a fusogenic lipid. The present study demonstrated the possibility of using *in vitro* NP–cell biointerface data in monolayer cultures with *in silico* studies to predict the NP diffusive transport and concentration–time–depth profiles in 3D systems, as functions of NP concentrations and treatment times. Extending this approach to include convective transport may yield a cost-effective means to predict the NP delivery and residence in solid tumors.

KEY WORDS: 3-dimensional tumors; computational models; diffusional transport; nanoparticle; solid tumors.

INTRODUCTION

Nanotechnology has emerged as an important tool in cancer translational research. Nanoparticles (NP) offer a

suitable means to deliver diagnostics and therapeutics including small and large molecules, gene vectors, and biosensors. NP pose several advantages: (a) improve the solubility of hydrophobic compounds, (b) protect a molecule from undesirable interactions with biological milieu components (*e.g.*, reduce its metabolism or degradation), and (c) favorably alter the distribution and provide passive targeting of solid tumors (due to the permeable/leaky blood vessels and the impaired lymphatic drainage in solid tumors) (1–3).

We recently reviewed the determinants of NP delivery to their intended target sites (4). NP transport to tumor interstitium, cell membrane, and intracellular compartments is determined by tumor pathobiological properties (*e.g.*, tumor cell density, extracellular matrix materials, perfusion, vessel leakiness), physicochemical properties of NP (*e.g.*, size, surface properties, aggregation), mechanisms of NP transport (*e.g.*, diffusion, convection), and interactions between NP and biological matrices (*e.g.*, binding to cell surface or extracellular proteins, internalization in cells). On one hand, these various factors can exert opposite effects on different transport processes. For example, NP are frequently surface-modified with targeting ligands to improve targeting and internalization, but binding of ligands to cell surface receptors limits NP transport. Similarly, larger NP with sizes between the capillary pore size in normal tissues (<10 nm in most tissues) and the pore size in tumor vasculature (typically between 100 and 200 nm) provides

Yue Gao and Mingguang Li contributed equally to this work.

¹ Division of pharmaceuticals, College of Pharmacy, The Ohio State University, Columbus, Ohio 43210, USA.

² Optimum Therapeutics LLC, 9363 Towne Centre Drive, San Diego, California 92121, USA.

³ To whom correspondence should be addressed. (e-mail: jau@optimumtx.com)

ABBREVIATIONS: 2D and 3D, 2- and 3-Dimensional; AUC, Area under curve; $B_{\max}$, Cell surface binding capacity; BS, Binding sites on cell surface; C, Nanoparticle concentration; D, Diffusion coefficient; DAPI, 4', 6-Diamidino-2-phenylindole dihydrochloride; DOPE, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine; DOTAP, 1,2-Dioleoyl-3-trimethylammonium propane; DPPC, 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine; ECM, Extracellular matrix; HPLC, High-performance liquid chromatography; HSPC, Hydrogenated soy phosphatidylcholine; NP, Nanoparticles; PBS, Phosphate-buffered saline; PEG-DSPE 1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000]; Rhod-DOPE, 1,2-Dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) ammonium salt; SDS, Sodium dodecyl sulfate; TEM, Transmission electron microscopy; $W_{1/2}$, Half-width.

passive tumor targeting but also retards the transport. On the other hand, as NP is versatile and can be made of different types of materials, and can have different sizes, surface charges, and modifications, there is the potential to tailor the NP design to its intended function. We propose the latter can be facilitated by computational tools to predict the NP transport and delivery to target sites and interactions with targets as functions of NP properties.

After systemic administration, the delivery of particles to cells in solid tumors involves three processes, *i.e.*, transport to tumors *via* blood perfusion, extravasation, and transport through interstitial space (4). These processes are mainly driven by diffusion that depends on diffusivity and concentration gradient and by convection that depends on hydraulic conductivity and pressure gradient.

The goal of the present study was to test the hypothesis that the diffusive transport of NP in tumor interstitium, as functions of interstitial NP concentration and time, can be predicted based on interstitial NP diffusivity, and NP–cell interactions (*i.e.*, cell surface binding capacity, rate constants of association, dissociation, and internalization). Models and equations were established to describe diffusive NP transport. The NP–cell biointerface data needed to parameterize the models were obtained experimentally using monolayer cultured cells. Because NP–cell biointerface depends on NP properties, we studied three types of NP: negatively charged polystyrene beads, near-neutral zwitterionic hydrogenated soy phosphatidylcholine (HSPC) liposomes sterically stabilized with pegylation, and pegylated cationic liposomes with fusogenic properties; the respective initial particle diameters were 20, 110, and 130 nm. Cationic liposomes are a popular choice for the delivery of oligonucleotides (*e.g.*, RNA, DNA). Fusogenic lipids are used to fuse with the endosomal membrane to facilitate endosomal escape (5). We recently showed that surface charge and pegylation have opposing quantitative effects on NP binding to cell surface and internalization such that the effect of one parameter is offset by the other (6).

Model performance was evaluated by comparing model-simulated results to the experimental results obtained with 3D multicellular tumor cell spheroids. Spheroids are aggregates of tumor cells without blood vessels, retain many properties of solid tumors (*e.g.*, multicellular structures, extracellular matrix, tight junctions between cells, gradients of nutrient and oxygen concentrations, and heterogeneous cell proliferation rate). The absence of vasculature in spheroids ascertains that the transport was due to diffusion and not confounded by convection. Tumor cell spheroids are considered a surrogate for solid tumors and are commonly used to study drug delivery and patient tumor sensitivity to specified drugs (7–13).

MATERIALS AND METHODS

Overview

The research plan comprised the following five steps.

- (a) Model development. The diffusive flux in 3-dimensional (3D) tumor interstitium is calculated from the

free NP concentration at a radial position r in the interstitium which, in turn, is determined by several concentration- and time-dependent processes including NP binding and internalization in cells. These various kinetic components are described by partial differential equations.

- (b) Obtain model parameters. The kinetics of interactions between NP and individual cells, which are specific to the NP and the cell used, were experimentally determined using 2-dimensional (2D) monolayer cultures. Other model parameters not dependent on NP properties were obtained or calculated from literature data.
- (c) *In silico* studies. The above models and model parameters were used with computation software to simulate NP diffusive transport and spatial distribution in 3D tumor interstitium.
- (d) Experimentally determine the concentration–depth profiles of fluorescence-labeled NP in 3D tumor cell spheroids (three initial NP concentrations and three treatment times).
- (e) Evaluate model performance by comparing the model-simulated NP concentration–depth profiles in a 3D system to the experimental data in 3D spheroids.

Chemicals and Reagents

Phosphate-buffered saline (PBS, pH 7.4), and cell culture supplies (phenol red-free RPMI 1640 with L-glutamine, trypsin–EDTA, fetal bovine serum or FBS, antibiotic–antimycotic) were obtained from Invitrogen–GIBCO (Carlsbad, CA). Acridine orange, agarose, osmium tetroxide, glutaraldehyde, and other chemicals were purchased from Sigma Chemical Company (St. Louis, MO), sodium dodecyl sulfate (SDS) from Roche Diagnostics (Indianapolis, IN), 10% buffered formalin phosphate from Fisher Scientific Company (Fair Lawn, NJ), Triton-X 100 from RICCA Chemical Co. (Arlington, TX), and 4′-6-diamidino-2-phenylindole, dihydrochloride (DAPI) from Invitrogen Corp. (Eugene, OR). Red-fluorescent carboxylate-modified, negatively charged, spherical polystyrene NP (20 and 200 nm diameter) were purchased from Molecular Probes (Eugene, OR). Lipids (HSPC, 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-*N*-(lissamine rhodamine B sulfonyl) ammonium salt (Rhod-DOPE), 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-*N*-[methoxy(polyethylene glycol)-2000] (PEG-DSPE), cholesterol) were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). All other reagents and solvents were of analytical or HPLC grade and were purchased from Fisher Chemicals (Pittsburgh, PA). All materials were used as received.

Polystyrene Beads

The smaller polystyrene beads (20 nm, carboxylated, Invitrogen) were used for transport studies. In addition, we used larger beads (200 nm) to enable visualization in transmission electron microscopy. These beads are

manufacture labeled with proprietary fluorescence dye; a recent study showed minimal dye leakage (<0.05% in 24–72 h; (14)). The molar concentration of NP was calculated using Eq. 1.

$$\text{NP concentration (Mol/L)} = \frac{\text{NP conc. (number/L)}}{6 \times 10^{23} \text{ particle/mole}} = \frac{6C_{\text{stock}} \times 10^{12}}{N\rho\pi d^3} \quad (1)$$

where C_{stock} was the NP concentration in the manufacturer's stock solution (20 mg/ml), N is the Avogadro's constant, ρ was the density of polystyrene (1.055 g/cm³), and d was the NP diameter in meter. The stock solution was diluted 100-fold with serum-free DMEM medium, yielding a concentration of 200 g/ml (75 nM). NP suspension was sonicated in a bath sonicator for 5 min before use. Note that the actual size of the 20-nm beads in serum-free medium was about 55 nm, due to aggregation (see Results and Discussion).

Liposome Preparation

Two liposome formulations were prepared and both were labeled using Rhod-DOPE, where rhodamine was chemically conjugated to DOPE. The first formulation comprised HSPC, cholesterol, PEG-DSPE, and Rhod-DOPE (2:1:0.1:0.03 molar ratio, referred to as HSPC liposomes). The second formulation comprised DOTAP:DPPC:DOPE:Chol:PEG-DSPE:Rhod-DOPE (20:5:19:50:5:1 molar ratio, referred to as C20-5 liposomes). Liposomes were prepared using the standard thin-film hydration method (15), with some modifications. Briefly, lipids were dissolved in chloroform, and Rhod-DOPE was added. The solution was dried under nitrogen to form a thin film and then dried under vacuum overnight. The film was hydrated in an aqueous buffer (25 mM HEPES, and 140 mM NaCl, pH 7.4 for HSPC liposomes, and 10% sucrose for C20-5 liposomes). The suspension was extruded through a polycarbonate membrane (100-nm pore size) 11 times with an extruder (Avanti, Alabaster, AL).

Liposomes were diluted with serum-free DMEM; liposome concentration was represented as number of liposomes per unit volume. The number of lipid molecules per liposome for spherical unilamellar liposomes of radius R with bilayer thickness bt , the average head area A for the mixture of several lipids were calculated using a previously described method (Eq. 2) (16).

$$\text{Lipid molecules per liposome} = \frac{4\pi}{A} [R^2 + (R - bt)^2] \quad (2)$$

R was determined by dynamic light scatter. bt was set at 4 nm as previously reported (16). The respective values for head area A for the individual lipids in the liposomes were 0.72 nm² for DOTAP (17); 0.71 nm² for DPPC and HSPC, 0.41 nm² for DOPE, PEG-DSPE, and Rhod-DOPE; and 0.19 nm² for cholesterol (16); the calculated average A values for HSPC and C20-5 liposomes were 0.32 and 0.53 nm², respectively.

Monolayer and Spheroid Cultures

Human pharynx FaDu cells (ATCC, Manassas, VA) were maintained in RPMI 1640 medium supplemented

with 10% FBS and 1% antibiotic–antimycotic. Cells (50 × 10⁴ cells/ml in 10% FBS-containing RPMI medium, 2 ml per well in six-well plates) were incubated at 37°C and 5% CO₂ overnight.

Spheroids were prepared using the liquid overlay technique (18). Briefly, 96-well culture plates were coated with 1% agarose solution in PBS (pH 7.4, about 50°C, 50 µl per well) and placed at 4°C overnight for the coating to solidify. Cells (200 µl of 1 × 10⁴ cells/ml 10% FBS-containing medium) were added to each well. Spheroids were established after 4 days, and were collected and washed three times with serum-free medium before incubation with NP.

Determination of NP, Cell, and Spheroid Sizes

NP diameter was measured using a particle size analyzer (Brookhaven Instrument Co.) or Zetasizer Nano ZS90 (Malvern, Westborough, MA). Cell number was measured using a hemacytometer. The average cell size and radius were measured in three ways: (a) under microscope (total of ~300 cells), (b) using a coulter counter (four to five million cells), and (c) volume displacement by placing five to six million cells in a known volume of medium into a tubing with an internal radius of 0.29 mm (Dow Corning corporation, Midland, MI); the difference between the volumes of cell-containing and cell-free media equaled the volume of the cells. Volume of a cell was calculated using the equation for a sphere (volume = (4/3)πr³), assuming a spherical shape. Diameters of spheroids were measured under light and confocal microscopy. All studies used spheroids with 320–340 µm diameters.

Transmission Electron Microscopy

Cells or spheroids were processed for transmission electron microscopy (TEM) using the eight-step method described previously (19). Briefly, cells/spheroids were fixed in 2.5% glutaraldehyde in a 0.1 M PBS for 2 h at 4°C, rinsed three times in PBS for 5 min each, placed in 1% osmium tetroxide for 1 h, again rinsed three times, then dehydrated with 50%, 70%, 80%, 95%, and 100% ethanol and infiltrated with the transitional solvents (*i.e.*, propylene oxide), embedded in Spurr resin, and polymerized at 60°C for 16–24 h, and cut to 70–90-nm sections at room temperature using Leica EM UC6 ultramicrotome equipped with an FC6 cryochamber (Leica Microsystems Inc., Bannockburn, IL). The sections were observed under Tecnai G2 spirit TEM (FEI Co., Hillsboro, OR).

Confocal Laser-Scanning Microscopy

Confocal laser-scanning microscopy (Zeiss LSM 510; Carl Zeiss Inc., Oberkochen, Germany) was used to visualize the fluorescent signals. The three NP showed red fluorescence. The nuclear dye was acridine orange (green fluorescence) or DAPI (blue fluorescence). The respective excitation/emission wavelengths were 580/605 nm for polystyrene beads, 502/525 nm for acridine orange, 543/594 nm for Rhod-DOPE (fluorescent lipid in liposomes) and 401/421 nm for DAPI.

Monolayers cells (cultured on a glass cover slip in a six-well plate to about 50% confluency) and spheroids were incubated with polystyrene beads (75 nM), HSPC liposomes (1.13 nM), or C20-5 liposomes (0.55 nM), in serum-free medium; the plates were placed on an orbital shaker (50 rpm) in a CO₂ incubator at 37°C. At the end of experiments, cells or spheroids were washed three times with serum-free medium and fixed with 10% formalin for 20 min. Nuclei were stained with acridine orange (0.6 mg/ml, for monolayer study) for 10 min or DAPI (7.5 μ M, for spheroid study) for 5 min. Cell-coated coverslips were placed cell-side down onto glass microscope slides. Spheroids (three to eight per slide) were mounted onto a glass slide, covered with a cover slip, and examined under confocal microscopy. Micrographs of 3- μ m-thick optical sections were acquired near the center of monolayers/spheroids in each field of vision.

Quantitative Image Analysis of Confocal Microscopic Images

For each spheroid, a total of 20 lines were drawn from the center to the periphery using the Zeiss LSM META 510 software, with each line separated by 18° angles. The fluorescence intensity, as a function of distance from spheroid periphery, was quantified by averaging the intensities of the 20 lines; this procedure minimized the location-related variability within a spheroid. Only data points with fluorescence intensities that exceeded 2 standard deviations of the background fluorescence (of untreated spheroids) were included in the analysis. Changes in the intensity over distance were expressed as percentage of the intensity at the outer perimeter, and the average fluorescence–depth profiles of five spheroids per condition were used. The total amount or uptake of NP in the spheroids was quantified as the area under fluorescence intensity–penetration depth curve (AUC), calculated using the linear trapezoidal rule. Half-width $W_{1/2}$ was defined as the distance traveled by NP from the outer boundary to the depth in a spheroid where the total NP concentration (sum of NP in interstitial space or $C_{\text{interstitial}}$, NP bound to cell membrane or C_{bound} , plus NP internalized into cells or C_{in}) declined by 50%. A higher AUC value indicates an increase in the amount of NP whereas a longer $W_{1/2}$ indicates a deeper penetration and a more shallow concentration decline.

Methods to Measure Model Parameters in Monolayers

The binding and internalization parameters, *i.e.*, maximum binding sites B_{max} , and rate constants for NP internalization (k_{in}), binding (k_{on}), and dissociation (k_{off}), are functions of NP properties, tumor cell properties, and NP–cell interactions. These parameters were measured experimentally using monolayer cells. Their determination requires separating the three NP moieties, *i.e.*, free NP in the medium (C_{medium}), C_{bound} , and C_{in} ; these methodologies were developed using polystyrene beads, as follows. The total amount of cell-associated NP was measured at 37°C. We compared two methods described in the literature for separating the cell surface-bound NP from the internalized NP. The first method

uses a highly concentrated trypsin–EDTA solution ($\times 10$) to strip the bound NP after incubation for 30 min at 37°C (20). The NP removed by trypsin stripping represent the surface-bound NP and the remaining NP after stripping represent the internalized NP. The second method measures the total amount of cell-associated NP after incubation at 4°C which reflects the cell surface-bound NP, and the amount of cell-associated NP after incubation at 37°C which reflects the sum of surface-bound and internalized NP (21–23); the difference between these two values represents the internalized NP. We previously confirmed the 4°C measurement reflects the surface-bound positively charged liposomes (6). The effectiveness of the 10 $\times$ -trypsin stripping method was confirmed using confocal microscopy to compare the subcellular NP distribution before and after the treatment. As shown in Results, the two methods yielded comparable data. The first method was used for polystyrene beads, and the second method was used for the two liposome formulations.

The above processing steps were completed in less than 30 min. In order to establish the upper limit of potential errors that might have been caused by the procedures, we measured the extent of NP exocytosis. Briefly, cells were incubated with polystyrene beads (75 nM) for 5 h. The bead-loaded cells were washed, trypsinized, and placed into bead-free medium for 30 min at 37°C. Afterward, cells were collected and centrifuged; the amount of NP retained in the cell pellet was determined.

Measurement of Binding and Internalization Parameters in Monolayers

Monolayer cells (60–80% confluency) were incubated with NP at 4°C and/or 37°C for up to 6 h. For each preparation, studies used two or three initial NP concentrations and multiple time points. Additional NP concentrations were studied at the 6 h time point to obtain B_{max} . Experiments were repeated two or three times (at least three samples per experiment). After incubation, the culture medium containing the extracellular NP was collected. The remaining cells were washed gently three times with 1 ml ice-cold serum-free DMEM medium (for polystyrene beads) or PBS (for the two liposomes). The washings were completed in less than 5 min. The amounts of NP in the three successive washes were less than 1.2%, 0.3%, and 0.1% of the initial amount. The attached cells were collected after trypsinization (with 1 ml 0.5% 10 $\times$ trypsin–EDTA at 37°C for 30 min for cells treated with polystyrene beads and 0.5 ml 0.25% trypsin at 37°C for 5 min for cells treated with the two liposomes). For polystyrene beads, the resulting mixture was centrifuged at 1,000 $\times g$ at 4°C for 20 min, yielding the cell surface-bound NP in the supernatant and cells with the internalized NP in the pellet. The latter was solubilized by incubating with 1 ml 1% SDS at 37°C for 30 min. For the two liposomes, the resulting mixture was treated with 0.5 ml 0.5% Triton-X 100 at 37°C for 30 min.

The fluorescence signals in individual fractions were measured using a fluorescence spectrometer (PerkinElmer,

Waltham, MA) at excitation/emission wavelengths of 580/605 nm for polystyrene beads and 543/594 nm for Rhod-DOPE. The signals were corrected for the background signal in the control groups (*i.e.*, without NP), which were typically between 1% and 10% of the NP-treated groups. The three NP concentrations (C_{medium} , C_{bound} , C_{in}) were calculated from the fluorescence intensity using standard curves constructed with known concentrations of NP (1.88 to 37.5 nM for polystyrene beads, 0.01–5.4 nM for HSPC liposomes and 0.01–17.6 nM for the C20-5 liposomes; all standard curves were linear at the stated concentration ranges).

Effects of Treatment Time and NP Concentration on Diffusive NP Transport in 3D Tumor Cell Spheroids

Studies of the effect of treatment time on NP transport in spheroids used a fixed concentration (18.8 nM for polystyrene NP, 1.13 nM for HSPC liposomes, 0.55 nM for C20-5 liposomes) and several time points (up to 22 h). For the effect of NP concentration, we used a single time point and several concentrations (9.4–37.5 nM for polystyrene beads, 0.56–2.26 nM for HSPC liposomes, and 0.28–1.10 nM for C20-5 liposomes); these concentrations are within the range where the NP binding to cells was primarily saturable as described in the models. Fifty spheroids were used per condition. After incubation, spheroids were collected, washed, fixed, and analyzed with confocal microscopy and quantitative imaging as described above.

Computation Methods

Model parameters were obtained by fitting appropriate equations to experimental data using SigmaPlot (Systat Software, Inc.) or WinNonlin 6.0 (Pharsight Corporation). Partial differential equations describing diffusive NP transport in a 3D system were solved numerically with the finite element method, using the Multiphysics modeling software COMSOL 3.4 (Comsol Inc, Los Angeles, CA). For COMSOL simulations, we used the sphere geometry with a 330 μm diameter (equaled the average size of spheroids used in the experiments), and used the initial C_{medium} to generate the C_{total} –depth profiles in 3D spheroids as functions of various initial NP concentrations and treatment times. Note C_{medium} refers to NP concentration in culture medium in both monolayer and spheroid systems whereas $C_{\text{interstitial}}$ refers to concentration in interstitium in spheroids.

RESULTS

Model Development

Our goal was to develop a computational model that uses NP–cell biointerface data to predict the diffusive NP transport in 3D systems. The avascular tumor cell spheroids, devoid of convective transport, were used as the model system.

It has been shown that cell surface binding and internalization of liposomes involve both specific/saturable and nonsaturable kinetics (24). Receptors include Class B Scavenger SR-BI and CD36 receptors for anionic lipids (25) and Toll-like receptors (TLR4) for cationic lipids (26). The internalization of polystyrene beads is size-dependent, utilizing the clathrin-mediated endocytosis for small size (<200 nm) and caveolae-

mediated endocytosis for larger particles of 500 nm (27). For the first generation model, we considered only specific and saturable binding of NP to cell membrane.

Second, binding of nanoparticles to ECM (mainly collagen) is determined by nanoparticle properties (surface hydrophilicity, charge, size) and collagen (charge, diameter, amount). We assumed negligible nanoparticle binding to ECM, based on the following literature findings and our own unpublished results: (a) low ECM content in spheroids (less than 10% of the level in tumors) (28), (b) pegylation of liposomes diminishes interaction with ECM (29), (c) minimal interaction between positively charged liposomes and collagen (positively charge at physiological pH) (30), and (d) treatment of FaDu spheroids with collagenase did not significantly enhance the penetration of negatively charged polystyrene beads (20 nm; unpublished results), suggesting inconsequential interactions between beads and collagen in spheroids.

We made several additional assumptions: a spherical system with a radius R , negligible fluid phase endocytosis, negligible efflux from cells, and equal numbers of binding sites for cells in monolayers and in 3D structures as a previous study shows that the surface area of monolayer cells is indistinguishable for the same cells in suspension (31). We further assumed internalization of NP uses absorptive and receptor-mediated endocytosis, where NP first binds to cell surface binding sites prior to entering a cell.

Figure 1a depicts the kinetic processes of NP diffusion in a 3D spheroid. NP in culture medium enter the interstitium *via* diffusive flux. The unbound or free NP in the interstitial space then bind to cell surface and undergo internalization. The corresponding respective NP concentrations are $C_{\text{interstitial}}$, C_{bound} , and C_{in} . Hence, diffusive NP transport models to depict the NP concentration–depth profile within a spheroid need to account for the diffusive flux (due to concentration gradient) and the depletion of the free NP due to cell binding and internalization. The kinetic rate constants, k_{on} and k_{off} for NP–cell interaction and k_{in} for NP internalization, and the number of binding sites on cell surface (BS) are functions of properties of NP and cells and hence could be determined in monolayers.

Note the fraction of extracellular NP removed due to binding or internalization in cells increased with time and accounted for up to 27% of the initial NP concentration (see below). Hence, the C_{medium} after incubation ($C_{\text{medium},t}$) was measured and used in the calculation.

Table I summarizes the values of NP-specific parameters and other model parameters, obtained as follows.

NP binding and internalization parameters from monolayer experiments are obtained. NP concentration in the monolayer cell uptake experiments was expressed in nanometer because the measurement (of total number of NP) was taken from cells solubilized with a fixed volume of lysate (1 ml of 1% SDS for polystyrene beads and 1 ml of Trypsin plus 0.5% Triton-X 100 for liposomes). The NP concentration was subsequently normalized to the cell number (by dividing with the number of cells in the sample and expressed as per 10^6 cells, *i.e.*, moles per 1 ml lysate per 10^6 cells).

Fig. 1. Diffusive NP transport in 3D tumor cell spheroids. **a** Schematic illustration of NP diffusion into spheroids (arrowed curves). *NP* nanoparticles. *BS* binding sites. **b–d** Respective confocal microscopic images of spheroids treated with polystyrene beads (20 nm), HSPC liposomes, and C20-5 liposomes for 12 h ($\times 20$ magnification). **e** Confocal microscopic image of cells incubated with polystyrene beads (200 nm) for 2 h ($\times 20$ magnification). Red color indicates NP formulations and green indicates nuclei. **f** Transmission electron microscopic image of cells in spheroids treated with polystyrene beads (200 nm) for 5 h ($\times 9,300$). Arrows indicate intracellularly located NP

Equations 3 and 4 describe the changes in C_{bound} and C_{medium} as a function of time. For B_{max} values, we first calculated B_{max} for the entire cell population ($B_{\text{max, total cells}}$), and then

divided this value with the cell number to calculate the B_{max} for a single cell ($B_{\text{max, single cell}}$). The latter was then used to calculate $B_{\text{max, spheroid}}$ (see below). Equation 5 describes C_{in} as a function

Table I. Values of Nanoparticle-Specific Parameters and Transport Model Parameters, Mean $\pm$ SD

Parameters	Polystyrene beads	HSPC liposomes	C20-5 liposomes
NP properties			
Diameter in cell-free medium, nm ^a	55 $\pm$ 0.3	131 $\pm$ 1.9	138 $\pm$ 0.5
After 2-h incubation with monolayer cells, nm	99 $\pm$ 1.2	129 $\pm$ 1.1	347 $\pm$ 8.6
After 6-h incubation with monolayer cells, nm	101 $\pm$ 0.4	129 $\pm$ 1.3	386 $\pm$ 4.9
After 12-h incubation with monolayer cells, nm	118 $\pm$ 1.1	130 $\pm$ 2.0	474 $\pm$ 5.0
Surface charge, mV	-49.1 $\pm$ 1.0	-9.7 $\pm$ 0.5	+37.7 $\pm$ 1.6
Model parameters measured in monolayers			
k_{off} , cell–NP dissociation rate constant, s ⁻¹ $\times 10^{-4}$	1.1 $\pm$ 0.3	3.4 $\pm$ 0.4	1.4 $\pm$ 0.3
k_{on} , cell–NP association rate constant, M ⁻¹ s ⁻¹ $\times 10^4$	0.5 $\pm$ 0.1	5.5 $\pm$ 0.6	20 $\pm$ 0.4
k_{in} , NP internalization rate constant, s ⁻¹ $\times 10^{-4}$	0.4 $\pm$ 0.04	0.4 $\pm$ 0.1	1.7 $\pm$ 0.1
$B_{\text{max, single cell}}$, number of binding sites/cell, $\times 10^6$	19.7 $\pm$ 6.6	0.29 $\pm$ 0.1	0.13 $\pm$ 0.03
Model parameters calculated for spheroids			
D_0 , diffusion coefficient in serum-free medium, mm ² s ⁻¹ $\times 10^{-6}$	7.27	4.22	4.00
D_{int} , diffusion coefficient in tumor interstitium, mm ² s ⁻¹ $\times 10^{-6}$	4.58	1.89	1.72
D_i , diffusion coefficient in spheroids, mm ² s ⁻¹ $\times 10^{-6}$			
Calculated using NP diameter in cell-free medium	0.89	0.37	0.33
Using NP diameter after 2-h incubation with monolayer cells	0.59	0.37	0.04
Using NP diameter after 6-h incubation with monolayer cells	0.57	0.37	0.03
Using NP diameter after 12-h incubation with monolayer cells	0.44	0.37	0.01
$B_{\text{max, spheroid}}$, cell surface binding capacity in spheroids, M $\times 10^{-7}$	94.1 $\pm$ 1.3	1.3 $\pm$ 0.1	0.56 $\pm$ 0.2
Boundary conditions for simulations			
C_{medium} , initial NP concentration in medium (nM)	9.5, 18.8, 37.5	0.56, 1.13, 2.26	0.28, 0.55, 1.10
t , treatment duration (h)	2, 5, 12	2, 6, 12	2, 6, 12

^a Initial diameters of NP suspended in water were 110 $\pm$ 1.4 and 130 $\pm$ 6.5 nm for HSPC and C20-5 liposomes, respectively. The size of polystyrene beads per manufacturer's label was 20 nm

of C_{bound} . At steady state and with the assumptions of only saturable binding, Eq. 3 becomes Eq. 6. Assuming C_{bound} is constant over time and C_{in} equals zero at time zero, Eq. 5 becomes Eq. 7.

$$\frac{\partial C_{\text{medium}}}{\partial t} = -k_{\text{on}} C_{\text{medium}} (B_{\text{max, total cells}} - C_{\text{bound}}) + k_{\text{off}} C_{\text{bound}} \quad (3)$$

$$\frac{\partial C_{\text{bound}}}{\partial t} = k_{\text{on}} C_{\text{medium}} (B_{\text{max, total cells}} - C_{\text{bound}}) - k_{\text{off}} C_{\text{bound}} - k_{\text{in}} C_{\text{bound}} \quad (4)$$

$$\frac{\partial C_{\text{in}}}{\partial t} = k_{\text{in}} C_{\text{bound}} \quad (5)$$

$$C_{\text{bound}} = \frac{B_{\text{max, total cells}} C_{\text{medium}}}{k_d + C_{\text{medium}}} \quad (6)$$

$$\frac{C_{\text{in}}}{t} = k_{\text{in}} C_{\text{bound}} \quad (7)$$

k_d is the C_{medium} at which C_{bound} equals 50% of $B_{\text{max, total cells}}$. Because our experimental results showed that steady state was not reached at the last time point (6 h), we used Eqs. 6 and 7 to obtain initial estimates of parameter values. Namely fitting Eq. 6 to the plot of C_{bound} vs. C_{medium} (32) using SigmaPlot yielded initial estimates for the values of k_d and $B_{\text{max, total cells}}$; fitting Eq. 7 to the plot of C_{bound} vs. C_{in} yielded the initial estimates of the multiplication product of $k_{\text{in}} \times t$ (equals slope of the best-fit line, t is the incubation time) (33,34) and hence the k_{in} value. Initial values of k_{on} and k_{off} were estimated from C_{bound} vs. time plot. The initial estimates were then used to obtain the final parameter values by fitting all experimental data using WinNonLin (and nonlinear regression) to the full dynamic model defined in Eqs. 3–5, to obtain the final parameter values ($B_{\text{max, total cells}}$, k_{in} , k_{on} , and k_{off}).

Determination of Cell Size

Cell volume is needed to calculate the interstitial void volume. The average cell radius measured by three methods was $7.46 \pm 1.05 \mu\text{m}$ (mean $\pm$ SD) by light microscopy ($n=300$ cells), $8.39 \pm 0.05 \mu\text{m}$ by Coulter counter ($n=4$ –5 million cells), and $7.44 \pm 0.26 \mu\text{m}$ by volume displacement ($n=5$ –6 million cells), respectively. The average value was $7.76 \pm 0.54 \mu\text{m}$. The average volume of a single FaDu cell was calculated to be $1.96 \pm 0.66 \times 10^{-6} \mu\text{l}$. This volume is comparable to our previous finding for MCF7 cells ($2.09 \pm 0.43 \times 10^{-6} \mu\text{l}$; 32).

Validation of Methods to Separate Cell Surface-Bound NP from Internalized NP

This was accomplished with polystyrene beads; the confocal microscopy results showed that after 2 h incubation, polystyrene beads (20 nm) bound to cell surface and readily entered cells (Fig. 1e), and the transmission electron microscopic results similarly showed the beads in the cytoplasm of cells in spheroids (visualized using larger beads of 200 nm, Fig. 1f). These data confirmed the NP binding to cell surface

and internalization in cells. The confocal microscopic images of post-washing NP-treated spheroids showed that beads were located either on cell surface or inside cells, and were absent in the extracellular space (Fig. 1b–d), confirming their removal by the washing procedures.

Comparison of the cell surface-bound beads measured by the 10 $\times$ -trypsin stripping method and the 4 $^{\circ}$ C method confirms that the two methods yielded indistinguishable results (70.2 ± 5.5 vs. $69.7 \pm 3.2\%$ of total cell-associated NP).

Comparison of the bead-loaded cells, before and after incubation in bead-free medium, showed that 86.2% of the internalized NP was retained in cells after 30 min, the duration for the 10 $\times$ -trypsin stripping method. This indicates a potential maximum 14% loss of the internalized NP due to the processing. This value is likely an over-estimate because the actual 10 $\times$ -trypsin treatment was performed in the presence of beads (in excess of the internalized NP), which would reduce the concentration gradient and thereby reduce the efflux.

Diffusive Transport of NP into Spheroids

Equation 8 describes the relationship between the three NP moieties in a spheroid.

$$C_{\text{total}} = C_{\text{bound}} + C_{\text{in}} + C_{\text{interstitial}} \quad (8)$$

Equation 9 describes changes of $C_{\text{interstitial}}$ as functions of time t and position r in 3D spheroids. Replacing the term C_{medium} in Eq. 4 with $C_{\text{interstitial}}$ yielded Eq. 10 that describes changes in C_{bound} with time in spheroids. Changes in C_{in} with time in spheroids are a function of C_{bound} and are as described in Eq. 11. D_i is interstitial diffusivity and was calculated as described below. r is the radial position from the center of a spheroid, as used in polar coordinate systems. Because NP penetration is from the outer perimeter to the center of a spheroid, the NP penetration distance is defined as spheroid radius (R) minus r or $(R-r)$.

$$\begin{aligned} \frac{\partial C_{\text{interstitial}}}{\partial t} = D_i \frac{1}{r^2} \frac{\partial}{\partial r} \left(r^2 \frac{\partial C_{\text{interstitial}}}{\partial r} \right) \\ - k_{\text{on}} C_{\text{interstitial}} (B_{\text{max, spheroid}} - C_{\text{bound}}) \\ + k_{\text{off}} C_{\text{bound}} \end{aligned} \quad (9)$$

$$\begin{aligned} \frac{\partial C_{\text{bound}}}{\partial t} = k_{\text{on}} C_{\text{interstitial}} (B_{\text{max, spheroid}} - C_{\text{bound}}) \\ - k_{\text{off}} C_{\text{bound}} - k_{\text{in}} C_{\text{bound}} \end{aligned} \quad (10)$$

$$\frac{\partial C_{\text{in}}}{\partial t} = k_{\text{in}} C_{\text{bound}} \quad (11)$$

Solution of Eqs. 9–11 required definitions of initial conditions and boundary conditions. For initial conditions when t equals 0, $C_{\text{interstitial}}$, C_{bound} , and C_{in} inside a spheroid all equaled 0. For the boundary condition at the

outer perimeter of the spheroid when $r=R$, the boundary $C_{\text{interstitial}}$ (*i.e.*, concentration at the interface between outer spheroid perimeter and culture medium) equaled C_{medium} . In contrast to monolayers where the removal of NP due to binding and internalization in cells accounted for up to 27% of C_{medium} , the limited NP penetration into spheroids resulted in much smaller changes in the C_{medium} (<5% over 5–6 h). Hence, we used a constant C_{medium} value to analyze the spheroid data.

Calculation of NP Diffusion Coefficient in Spheroids

Calculation of the diffusion coefficient in 3D tumor cell spheroids D_i was performed in three steps. We first estimated the diffusion coefficient of NP in water at 37°C, D_0 , using the Stokes–Einstein equation (35).

$$D_0 = \frac{k_B T}{6\pi\eta r_p} \quad (12)$$

k_B is the Boltzman constant (equals $1.38 \times 10^{-23} \text{ m}^2 \text{ kg s}^{-2} \text{ K}^{-1}$). T is the body temperature (310 K). η is water viscosity at 310 K (equals $8.18 \times 10^{-4} \text{ N s m}^{-2}$). r_p is particle radius. As the apparent size of polystyrene beads and C20-5 liposomes increased over time, we used both the initial size (55 and 138 nm) and the final size at 12 h (117 and 474 nm) to calculate D_0 .

Next, the NP diffusion in interstitium was modeled as diffusion in a porous gel matrix (35). This required correcting D_0 to account for the presence of the matrix proteins, to obtain the diffusion coefficient in the interstitium D_{int} (Eq. 13).

$$D_{\text{int}} = D_0 e^{\left[(-\sqrt{\varphi_{\text{int}}}) \times \frac{r_p}{r_f} \right]} \quad (13)$$

φ_{int} is the volume fraction of tumor interstitial matrix. Because the major component of tumor matrix materials is collagen (36), we used values for collagen to calculate φ_{int} , which equals the multiplication product of (interstitial collagen concentration) and (effective specific volume of collagen fibers, which equals $1.89 \text{ cm}^3/\text{g}$) (37). The collagen concentration in the interstitium of a solid tumor is 0.35 g/cm^3 (37), and the collagen content in a spheroid is about one eleventh of the value in a tumor (28). Accordingly, the collagen concentration in a spheroid was 0.032 g/cm^3 and φ_{int} was 0.06. r_f is the radius of the tumor matrix proteins/fibers and equals 20 nm for collagen (37).

The third step was to adjust the diffusion coefficient of NP to account for porosity in solid tumor. D_{int} was converted to the interstitial diffusivity D_i using Eq. 14, to account for the diffusion in porous gel matrix with immobilized cells (38,39). ϕ is the cell density and $1-\phi$ equals the porosity in spheroids.

$$D_i = D_{\text{int}}(1 - \phi)^2 \quad (14)$$

Calculation of Maximum NP Binding Sites in Spheroids

Cell density in spheroids was calculated as the volume fraction occupied by cells, as follows. We measured the diameters of individual spheroids using confocal

microscopy, and selected 10 spheroids with similar sizes (less than 1% difference from the average size of 330 μm). Individual spheroids were incubated with $1 \times$ trypsin–EDTA for 20 min. The numbers of cells in the resulting single cell suspensions were counted, and the volume of a spheroid was calculated assuming a spherical shape. The volume of total cells in each spheroid was calculated as (total cell number in each spheroid) multiplied by (average volume of a single cell). The cell density for individual spheroids were calculated as (volume of total cells in each spheroid) divided by (spheroid volume); the average value was $56.0 \pm 7.3\%$ (range, 50.0–68.9%).

$B_{\text{max, single cell}}$ was used with Eq. 15 to calculate the molar concentration of cell surface binding sites in a spheroid, $B_{\text{max, spheroid}}$, at a cell density ϕ . r_{cell} was the radius of FaDu cells in suspension, in μm .

$$B_{\text{max, spheroid}} = \frac{\phi \times 10^{15}}{6 \times 10^{23} \times \frac{4}{3}\pi r_{\text{cell}}^3} B_{\text{max, single cell}} \quad (15)$$

Experimental Determination of NP Binding and Internalization in Monolayer Cells

Figure 2a shows the concentration-dependent uptake and accumulation of total, surface-bound, and internalized polystyrene beads, HSPC liposomes, and C20-5 liposomes in monolayer cells.

For polystyrene beads, all three moieties (C_{total} , C_{bound} , C_{in}) generally increased with extracellular NP concentrations (from 0 to 90 nM). The profiles show characteristics of saturable binding at lower C_{medium} (with C_{bound} approaching a plateau level at C_{medium} of ~ 40 nM), followed by nonsaturable binding (with C_{bound} increasing linearly at higher C_{medium}). Figure 2b shows the time-dependent uptake and accumulation; all three moieties increased with time and approaching plateau levels at 4–6 h. At 6 h, about 67% of the total NP was bound to cell surface and about 33% was internalized in cells.

Similar findings were observed for the two liposomes, albeit with different bound-to-internalized ratios, *i.e.*, <30% internalization for HSPC and about 50% for C20-5, at 6 h (Fig. 2a, b).

A comparison of the total fluorescence intensity in the culture medium before and after incubation showed no loss when NP were incubated with cell-free medium whereas incubation with cells in monolayer cultures for 6 h reduced the total fluorescence to 77.4%, 90.1%, and 72.8% of the initial values for polystyrene beads, HSPC, and C20-5 liposomes, respectively. This data indicates that up to 10–30% of NP in extracellular medium were removed due to their interactions with cells. This data is in agreement with the amount of NP bound or internalized in cells, *i.e.*, C_{total} accounting for 21.4%, 10.0%, and 26.7% of the initial dose.

Calculation of Model Parameters

The diffusive transport models depicted in Eqs. 9–11 accounted for saturable NP binding to cells but not nonsaturable binding. Hence, we used only binding and internalization data at NP concentration range where saturable binding was apparent (*i.e.*, up to 45 nM C_{medium} for

Fig. 2. Kinetics of NP binding and internalization in cells: experimental results. **a** Effect of initial NP concentration. NP were incubated with cells for 6 h. **b** Effect of NP–cell interaction time. The initial NP concentrations (top curve to bottom curve) were 18.8 and 37.5 nM for polystyrene beads, 0.28, 0.56, and 1.13 for HSPC liposomes, and 0.11, 0.28, and 0.55 nM for C20-5 liposomes. **c** Determination of model parameter values. Initial estimates for $B_{\max}$ and k_{in} were obtained from C_{bound} vs. C_{medium} plot and C_{in} vs. C_{bound} plot, respectively. Initial values of k_{on} and k_{off} were estimated from C_{bound} vs. time plot. All data were used for final fitting with Eqs. 3–5 to determine the final parameter values. The NP concentrations were identical to (b). Note the different concentration scales for the three NP. The coefficient of determinations (r^2) for the linear regression lines on k_{in} were 0.838, 0.985, and 0.846 for polystyrene beads, HSPC liposomes, and C20-5 liposomes, respectively

Fig. 3. NP size change over time. Changes in NP size distribution upon incubation with cell-free culture medium, monolayer cells, or spheroids were monitored. Solid lines: 0 h. Dotted lines, from left to right: 2, 6, and 12 h. Note the different size scales for the three NP. The average sizes were between 55 and 67 nm in cell-free medium and 98–118 nm in monolayer and spheroid cultures for polystyrene beads. The corresponding sizes were 130–140 and 123–145 nm for HSPC liposomes, and 138–167 and 347–474 nm for C20-5 liposomes

Fig. 4. Change in NP transport barrier in spheroids over time. Penetration of the nucleus staining dye DAPI into spheroids after treatment with three NP for 2–22 h. DAPI penetration is indicated by *green fluorescence*. The concentrations of NP used were 75, 1.13, and 0.55 nM for polystyrene beads, HSPC liposomes, and C20-5 liposomes, respectively

polystyrene beads, 6.8 nM for HSPC liposomes, and 0.82 nM for C20-5 liposomes). Analysis of the experimental data in monolayer cultures using Eqs. 3–7 yielded the initial estimates and the final values of NP–cell biointerface parameters (Fig. 2c, Table I). The three NP showed ~ 3 -fold difference for k_{off} , 4-fold difference for k_{in} , but very different values for k_{on} (40-fold), and $B_{\text{max, single cell}}$ (150-fold). The rank orders of k_{on} and k_{in} were C20:5 liposomes > HSPC liposomes >

polystyrene beads, whereas the rank order for k_{off} was HSPC liposomes > C20-5 liposomes $\sim$ polystyrene beads.

As shown below, the differences in NP–cell biointerface parameters, as well as other NP properties (*e.g.*, size, charges), for the three NP resulted in differences in their diffusive transport in 3D tumor cell spheroids.

Particle Size Change. As diffusion coefficients are determined by particle size (Eqs. 12 and 13), we monitored the NP

Fig. 5. Evaluation of model performance in 3D spheroids: comparison of model-simulated NP spatial distribution to experimentally observed data. Study of effect of treatment time used a fixed initial NP concentration (18.8 nM for polystyrene beads, 1.13 nM for HSPC liposomes, 0.55 nM for C20-5 liposomes). Study of effect of initial NP concentration used a single treatment time (5 h for beads, 6 h for both liposomes). *Solid lines*: experimentally observed profiles. *Dashed lines*: model-simulated profiles. *Shaded areas*: 95% confidence intervals of experimental data. Relative C_{total} (sum of $C_{\text{interstitial}}$, C_{bound} , and C_{in}) normalized to the levels of reference treatment groups (18.8 nM and 5 h for polystyrene beads, 1.13 nM and 6 h for HSPC liposomes, 0.55 nM and 6 h for C20-5 liposomes)

Fig. 6. Evaluation of model performance in 3D spheroids: comparison of model-simulated NP transport parameters with experimentally observed parameters. AUC was calculated by linear trapezoidal rule. $C_{\max}$ is the highest fluorescence intensity in a concentration-depth profile. Half-width $W_{1/2}$ is the penetration depth corresponding to 50% NP concentration decline. AUC and $C_{\max}$ values are standardized to respective reference treatment groups (18.8 nM and 5 h for polystyrene beads, 1.13 nM and 6 h for HSPC liposomes, 0.55 nM and 6 h for C20-5 liposomes). *Symbols*: Experimentally observed data. *Dashed lines*: Model-simulated data

size over time. The results showed no size changes in cell-free medium for all three NP. Incubation with monolayer cultures or spheroids caused broadening of the size distribution histograms for all three NP. However, there was no change in the average size of HSPC liposomes whereas polystyrene beads and C20-5 liposomes showed progressive increases over time (Fig. 3, Table I). This data suggest interactions between cells or extracellular cellular components with beads and C20-5 liposomes caused particle size changes.

Changes in the particle sizes were calculated to result in 2- and 30-fold lower diffusion coefficients at 6 and 12 h for

polystyrene beads and C20-5 liposomes, respectively (Table I).

Experimental Determination of NP Diffusion into 3D Tumor Cell Spheroids

The typical size of FaDu spheroids after 4 days of incubation was about 250–350 μm . Larger spheroids of 500–700 μm were observed after 14 days. Because the larger spheroids contained necrotic regions in the center (not shown), we used smaller spheroids, with an average size of

Table II. Effects of NP–Cell Interaction Time and NP Concentration on NP Transport in 3D Spheroids

NP	Effect of NP–cell interaction time			Effect of concentration		
Polystyrene beads	2 h	5 h	12 h	9.4 nM	18.8 nM	37.5 nM
Relative AUC	0.52	1.00	1.75	0.61	1.00	1.57
Relative $C_{\max}$	0.67	1.00	1.54	0.66	1.00	1.48
$W_{1/2}$ (μm)	4.28	5.79	9.38	5.30	5.79	7.18
HSPC liposomes	2 h	6 h	12 h	0.56 nM	1.13 nM	2.26 nM
Relative AUC	0.63	1.00	1.31	0.35	1.00	1.65
Relative $C_{\max}$	0.63	1.00	1.69	0.59	1.00	2.28
$W_{1/2}$ (μm)	12.0	12.9	15.0	10.4	12.9	14.7
C20-5 liposomes	2 h	6 h	12 h	0.28 nM	0.55 nM	1.1 nM
Relative AUC	0.53	1.00	1.25	0.47	1.00	1.17
Relative $C_{\max}$	0.80	1.00	1.24	0.56	1.00	1.14
$W_{1/2}$ (μm)	5.88	15.2	15.2	13.0	15.2	17.4

Study of effect of treatment time used a fixed initial NP concentration (18.8 nM for polystyrene beads, 1.13 nM for HSPC liposomes, 0.55 nM for C20-5 liposomes). Study of effect of initial NP concentration used a single treatment time (5 h for beads, 6 h for both liposomes). AUC was calculated by linear trapezoidal rule. $C_{\max}$ is the highest fluorescence intensity in a C_{total} -depth profile. Half-width $W_{1/2}$ is the penetration depth corresponding to 50% NP concentration decline. AUC and $C_{\max}$ values are standardized to respective reference treatment groups (18.8 nM and 5 h for beads, 1.13 nM and 6 h for HSPC liposomes, 0.55 nM and 6 h for C20-5 liposomes)

Table III. Evaluation of Model Performance

Deviation between model-simulated data and experimental data, % of observed data				
NP and Parameter	Maximum under-estimation	Maximum over-estimation	Mean $\pm$ SD	Median
Polystyrene beads				
Individual data points	67.5	19.0	25.5 $\pm$ 41.6	16.8
AUC	12.9	16.7	8.53 $\pm$ 11.7	10.4
$C_{\max}$	7.15	28.9	13.9 $\pm$ 14.4	12.7
$W_{1/2}$	10.0	7.43	4.34 $\pm$ 6.38	3.86
HSPC liposomes				
Individual data points	25.5	67.6	15.1 $\pm$ 10.4	0.23
AUC	24.2	17.6	17.3 $\pm$ 23.4	16.8
$C_{\max}$	11.9	0.00	5.01 $\pm$ 5.26	3.96
$W_{1/2}$	22.4	21.5	10.5 $\pm$ 14.2	6.12
C20-5 liposomes				
Individual data points	70.1	94.0	36.2 $\pm$ 18.9	34.5
AUC	51.0	33.9	25.2 $\pm$ 30.1	21.6
$C_{\max}$	41.6	47.0	21.9 $\pm$ 20.9	32.7
$W_{1/2}$	50.8	4.62	41.7 $\pm$ 48.4	22.0
Frequency of simulated data points within the 95% confidence intervals of observed data				
	Effect of treatment time	Effect of concentration	Average, %	
Polystyrene beads	100% at 2 h	100% at 9.4 nM	99	
	100% at 5 h	100% at 18.8 nM		
	93% at 12 h	100% at 37.5 nM		
HSPC liposomes	96% at 2 h	93% at 0.56 nM	96	
	100% at 6 h	100% at 1.13 nM		
	92% at 12 h	100% at 2.26 nM		
C20-5 liposomes	23% at 2 h	85% at 0.28 nM	60	
	12% at 6 h	12% at 0.55 nM		
	92% at 12 h	85% at 1.1 nM		

Two methods were used. First, model-simulated concentration–depth profiles were compared with experimentally observed data, with respect to individual data points and penetration kinetic parameters (AUC, $C_{\max}$, and $W_{1/2}$). Absolute values were used to calculate mean, SD, and median values of deviations. The second method measured the fraction of model-simulated data points that fall within the 95% confidence intervals of experimental data

330 $\pm$ 8.6 μ m for beads, 325 $\pm$ 8.7 μ m for HSPC liposomes, and 327 $\pm$ 6.4 μ m for C20-5 liposomes (Fig. 1b–d).

The effect of NP treatment time on spheroid structure was evaluated by monitoring the nucleus dye penetration (Fig. 4). The confocal microscopy results show no changes in the depth of dye penetration over 22 h for polystyrene beads, indicating no substantial changes in the diffusive

barriers in spheroids over this duration. In contrast, the dye penetration for spheroids treated with HPSC and C20-5 liposomes remained constant for up to 12 h, followed by substantially deeper penetration at 22 h, indicating that liposome treatments altered the diffusion properties of spheroids. Based on these findings, the penetration was studied for up to 12 h.

Fig. 7. Effect of particle size change. Effect of NP size change on model-simulated spheroid penetration curves. Simulated curves obtained using either average NP size in cell-free medium (*solid lines*) or average size after incubation with monolayer cell cultures (*dashed lines*), for three NP–cell interaction times. Changes in NP size only altered the slope or $W_{1/2}$ and did not change the $C_{\max}$, resulting in a pair of two curves for each NP–cell interaction time. From *top to bottom*: simulated curves for incubation time of 2, 5, or 6 (5 for beads and 6 for HSPC and C20-5), and 12 h. HSPC liposomes did not display a size change and hence showed only one curve

We used two separate measurements to determine the amounts of NP that penetrated the spheroids. First, we compared the total fluorescence intensity in the extracellular culture medium before and after incubation with spheroids and found <5% loss from the medium. This finding is in agreement with the second measurement on the amount of NP recovered in the spheroids after 6 h incubation (1.3%, 2.0%, and 3.7% for polystyrene beads, HSPC liposomes, and C20-5 liposomes, respectively).

Figure 5 shows the C_{total} -spheroid depth profiles, and Fig. 6 shows the changes in total NP amount (expressed as AUC of the profile), maximum NP concentration in spheroids (C_{max}), and penetration depth ($W_{1/2}$). In general, increasing the NP-cell interaction time or C_{medium} resulted in higher AUC, C_{max} , and $W_{1/2}$ for all three NP. But, as summarized in Table II, there were qualitative and quantitative differences among the NP, e.g., different extents of changes in AUC, C_{max} , and $W_{1/2}$. The HSPC liposomes showed the most rapid initial rate of penetration (two to threetimes longer $W_{1/2}$ at 2 h). The slower penetration rates for polystyrene beads and C20-5 liposomes are probably due to interactions between charged particles with cells and/or extracellular components that retard interstitial transport (30).

In Silico Results and Comparison with Experimental Results

Figure 5 also compares the model-simulated profiles to the experimentally observed profiles for the three NP, whereas Fig. 6 compares the AUC, C_{max} , and $W_{1/2}$ values of the profiles. Table III shows the fractions of model-simulated data points that were within the 95% confidence intervals of the experimental results.

For polystyrene beads and HSPC liposomes, the model-simulated data closely aligned with the experimental results. For example, 92–100% (average of 99% for beads and 96% for HSPC) of individual data points of all six simulated profiles (three concentration and three treatment times) are within the 95% confidence intervals of the experimental results, indicating good accuracy. Similarly good agreements were observed for the model-simulated AUC, C_{max} , and $W_{1/2}$, with <17% deviations. The deviations for these four measurements are comparable among these two NP ($p > 0.05$, Student's t test). In comparison, the model for C20-5 liposomes showed inferior performance; only 3 of 6 treatment conditions showed good agreement among the simulated individual data points and the experiment results (85–100%), whereas the remaining 3 conditions showed inferior agreement (12–23%). In addition, the simulated AUC, C_{max} , and $W_{1/2}$ values for C20-5 liposomes showed significantly higher deviations from the experiment values compared to polystyrene beads and HSPC liposomes (>29% on average vs. <10%; $p < 0.05$; Student's t test).

We also investigated the effect of particle size increase during incubation with cells on model-predicted C_{total} -depth profiles. This was accomplished by substituting the D_i values calculated using the average particle size at the end of 2-, 6-, and 12-h incubation. HSPC liposomes, which showed constant size and D_i , displayed a single profile at each time point. In contrast, polystyrene beads and C20-5 liposomes, which showed progressively larger average size and smaller D_i over time, displayed diminished transport in proportion to the changes in D_i , and consequently increasingly inferior model performance (Fig. 7).

DISCUSSION

Our goal was to test the hypothesis that the diffusive transport and spatial distribution of NP in tumor interstitium, as functions of interstitial NP concentration and time, can be predicted using NP-cell biointerface data experimentally determined in cultured cells. The sizes of the three NP used in the current study covers the range of nano-size therapeutics, e.g., macromolecule drugs such as antibodies and proteins (5–20 nm) and nanoparticle drug carriers (e.g., 85 nm for Doxil and 130 nm for Abraxane; 1,40–42). Surface charges of the three NP are also within the range of nano-therapeutics –12 mV for Doxil, –30 mV for Abraxane, >40 mV for cationic liposomes (6,42–44). The different NP properties (charge, size, materials) led to differences in NP-cell interactions, as shown by their different k_{on} (>3-fold), k_{in} (>8-fold), and $B_{\text{max, single cell}}$ (>80-fold) values. These differences in turn led to differences in their diffusive transport in 3D tumor cell spheroids and penetration kinetic parameters (AUC, C_{max} , $W_{1/2}$) as functions of NP-cell interaction time and NP concentration.

The present study showed two notable experimental findings. First, while between 10% and 30% of the three NP were removed from the extracellular culture medium after 6-h incubation with cells in monolayer cultures, only 1–4% penetrated the spheroids. Because this difference far exceeds the difference in cell number in monolayers (10- vs. 3-fold) and because of the comparable surface area of cells in monolayers and suspension (31), we propose that slow diffusive is a significant rate-limiting step for the interstitial NP transport in avascular systems. Second, we observed significant size changes upon incubation with cells in serum-free medium for the negatively charged polystyrene beads and the positively charged C20-5 liposomes but not for the near-neutral HSPC liposomes; this data indicates the size change was caused by interactions of charged NP with cellular components excreted or shed in the extracellular medium. Ongoing studies using live cell confocal microscopy showed both polystyrene beads and C20-5 liposomes formed aggregates over time (as opposed to enlargement of individual NP).

The models in the present study yielded simulated data that closely aligned with the experimental data on the transport of negative-charged polystyrene beads and near-neutral liposomes in 3D spheroids; between 92% and 100% of model-predicted data points were within 95% confidence intervals of experimental results. That the models predicted the transport of these two NP with large differences in their interactions with tumor cells indicates the versatility of the models. On the other hand, model performance was inferior for the positively charged C20-5 liposomes containing fusogenic lipids. This was not due to the change in particle size over time as simulations using the D_i derived from the large final NP size did not improve the model performance. Another possibility is the inclusion of the fusogenic lipid DOPE known to fuse with cell membrane (45) might have altered the transport kinetics. Additional studies are needed to determine the cause of the inferior performance of the positively charged liposomes.

This study showed limited penetration of NP in FaDu spheroids. We propose this is in part due to the limited diffusive transport of nanoparticles (e.g., due to the large size) and in part due to binding of nanoparticles to cells. Such

“binding barrier” has been reported for small molecule drugs (46). Another likely cause is the presence of tight cell junctions creating a barrier to diffusion. Studies have been initiated to evaluate the transport model in other tumor cells that show lower levels of intercellular junction proteins and showed significantly deeper NP penetration.

Our models did not consider (a) convection, (b) charge effect, (c) efflux from cells which might have been negligible at earlier times but would become predominant at higher internalized NP concentrations, (d) processes known to play minor roles (*i.e.*, fluid phase endocytosis, extravasation by transcytosis across the vessel wall, electrochemical transport across the interstitium due to surface charge), (e) effect of NP shape (*e.g.*, angular instead of spherical), (f) nonsaturable binding of NP, which may become predominant with higher NP concentrations, (g) paracellular transport, (h) heterogeneous tumor interstitium due to nutrition gradient, and (i) potential role of intercellular junctions (*e.g.*, presence of tight junctions serves as a sieve to prevent NP transport). In addition, our models used the D_i values calculated with the average NP particle size to predict the diffusive transport. As shown in Figs. 3 and 7, particle size can change over time due to interaction with extracellular cell components and this size change can significantly alter the predictions. Furthermore, the total NP amount in spheroids was relatively small compared to the amount in the culture medium (1.3, 2.0, and 3.7% for polystyrene beads, HSPC, and C20-5 liposomes, respectively); based on the size distribution histograms, the corresponding sizes at these amounts were equal or smaller than 21, 72, and 77 nm. Hence, we cannot rule out the possibility that only the smaller particles penetrated the spheroids, in which case the D_i calculated for the average NP size would be an over-estimate. The above issues should be considered in future model development and refinement.

A recent study describes a model that depicts the diffusional transport of negatively charged polystyrene beads in tumor cell spheroids (47). This earlier model used similar governing equations and similar, carboxylated polystyrene beads as in the current study (both used negatively charged, 20 nm diameter in the current study and 40 nm in the earlier study), but has significant differences with respect to experimental design and results, model parameters calculation, and, most importantly, model performance. With respect to experimental model, the earlier study used cervical SiHa tumor cells and spin-flask method to form spheroids; the resulting spheroids were larger (500 μm) and showed a porous necrotic core. In contrast, we used pharyngeal FaDu tumor cells and liquid-overlay method to form smaller spheroids (330 μm) without necrotic cores. These differences might have contributed to the qualitative and quantitative differences in the two studies. For example, the NP concentration–depth profile in SiHa spheroids showed a V-shape, with high NP concentrations at the periphery and center, whereas our study showed continued concentration decline with increasing depth. NP penetration in SiHa spheroids was very rapid, reaching the center within 1 h. In contrast, NP penetration in FaDu spheroids was limited and proceeded at a much slower rate (less than 30% to the center in 22 h). A

second major difference between the two studies is the calculation of two key model parameters; the earlier study used a two-step approach. The first step was to measure the space between cells in spheroids and used the value to calculate the cell surface area for NP binding and the interstitial porosity. The porosity was used to calculate the diffusion coefficient. The next step was to use a correction factor to compensate for the 100-fold difference between the results in spheroids and monolayers, and a second correction factor (7.5-fold) to compensate for the tortuosity in spheroids. In contrast, we used the surface binding sites for cells in monolayer cultures and the experimentally measured cell density in spheroids to calculate the total binding sites within spheroids, and did not need to use correction factors. With respect to model performance, the earlier model was not able to predict the experimental results qualitatively or quantitatively. That is, compared to the experimental results, the model-predicted profiles of time-dependent NP penetration at spheroid periphery (radial position between 0.8 and 1) for collagenase-treated groups were in the reverse order and the model-predicted profiles of concentration-dependent NP penetration for collagenase-minus groups showed different shapes (highest levels at periphery/mid-way *vs.* highest at periphery/core) and several-fold lower NP concentrations.

In summary, the present report demonstrated the successful use of diffusive transport models plus NP–cell interaction parameters that are readily determined in monolayer cultures to predict the diffusion and spatial distribution of negative and neutral NP in 3D tumor cell spheroids as functions of NP concentrations and treatment times. As tumor cell spheroid is considered a good experimental model of solid tumors (7–11), it is reasonable to expect the current models can be applied to predict the diffusive transport in solid tumors. We propose that extending this approach to include the other major transport mechanism (convection) may yield a cost-effective means to predict the NP delivery and residence in solid tumors. Coupling such tumor transport models with other multi-scale transport kinetic models (*e.g.*, physiologic-based pharmacokinetic models) may enable an investigator to predict the delivery and residence of NP in tumors based on the NP properties and NP–cell biointerface parameters. Furthermore, because the models account for different NP moieties (free, membrane-bound, and internalized), it will be possible to predict the fates of NP at the desired target sites, and thereby enable the optimization of NP for their intended uses (*e.g.*, free NP for imaging agents, surface-bound NP for antibodies or internalized NP for RNAi).

CONCLUSIONS

The present study established a computational model to predict the time- and concentration-dependent diffusion of NP in 3D tumor cell spheroids. Comparison of model-simulated and experimental results indicate that diffusive transport of negative and neutral NP in 3D spheroids can be predicted using NP diffusivity, binding, and internalization in 2-D monolayers, with >90% accuracy.

ACKNOWLEDGMENTS

This work was supported in part by a research grant R01EB015253 from the National Institute of Biomedical Imaging and Bioengineering, DHHS, JLSA, and MGW have financial interests in Optimum Therapeutics LLC.

REFERENCES

- Jain RK, Stylianopoulos T. Delivering nanomedicine to solid tumors. *Nat Rev Clin Oncol*. 2010;7(11):653–64.
- Bhagat M, Halligan S, Sofou S. Nanocarriers to solid tumors: considerations on tumor penetration and exposure of tumor cells to therapeutic agents. *Curr Pharm Biotechnol*. 2012;13(7):1306–16.
- Bae KH, Chung HJ, Park TG. Nanomaterials for cancer therapy and imaging. *Mol Cells*. 2011;31(4):295–302.
- Li Y, Wang J, Wientjes MG, Au JL. Delivery of nanomedicines to extracellular and intracellular compartments of a solid tumor. *Adv Drug Deliv Rev*. 2012;64(1):29–39.
- Kono K, Iwamoto M, Nishikawa R, Yanagie H, Takagishi T. Design of fusogenic liposomes using a poly(ethylene glycol) derivative having amino groups. *J Control Release*. 2000;68(2):225–35.
- Li Y, Wang J, Gao Y, Zhu J, Wientjes MG, Au JL. Relationships between liposome properties, cell membrane binding, intracellular processing, and intracellular bioavailability. *AAPS J*. 2011;13(4):585–97.
- Jang SH, Wientjes MG, Lu D, Au JL. Drug delivery and transport to solid tumors. *Pharm Res*. 2003;20(9):1337–50.
- Minchinton AI, Tannock IF. Drug penetration in solid tumours. *Nat Rev Cancer*. 2006;6(8):583–92.
- Durand RE. Chemosensitivity testing in V79 spheroids: drug delivery and cellular microenvironment. *J Natl Cancer Inst*. 1986;77(1):247–52.
- Carlsson J, Nilsson K, Westermark B, Ponten J, Sundstrom C, Larsson E, *et al*. Formation and growth of multicellular spheroids of human origin. *Int J Cancer*. 1983;31(5):523–33.
- Nederman T, Norling B, Glimelius B, Carlsson J, Brunk U. Demonstration of an extracellular matrix in multicellular tumor spheroids. *Cancer Res*. 1984;44(7):3090–7.
- Dufau I, Frongia C, Sicard F, Dedieu L, Cordelier P, Ausseil F, *et al*. Multicellular tumor spheroid model to evaluate spatio-temporal dynamics effect of chemotherapeutics: application to the gemcitabine/CHK1 inhibitor combination in pancreatic cancer. *BMC Cancer*. 2012;12:15.
- Monazzam A, Josephsson R, Blomqvist C, Carlsson J, Langstrom B, Bergstrom M. Application of the multicellular tumour spheroid model to screen PET tracers for analysis of early response of chemotherapy in breast cancer. *Breast Cancer Res*. 2007;9(4):R45.
- Frohlich E, Meindl C, Roblegg E, Ebner B, Absenger M, Pieber TR. Action of polystyrene nanoparticles of different sizes on lysosomal function and integrity. *Part Fibre Toxicol*. 2012;9:26.
- Hussain S, Pluckthun A, Allen TM, Zangemeister-Wittke U. Antitumor activity of an epithelial cell adhesion molecule targeted nanovesicular drug delivery system. *Mol Cancer Ther*. 2007;6(11):3019–27.
- Singh AK, Kilpatrick PK, Carbonell RG. Application of antibody and fluorophore-derivatized liposomes to heterogeneous immunoassays for D-dimer. *Biotechnol Prog*. 1996;12(2):272–80.
- Lonez C, Lensink MF, Kleiren E, Vanderwinden JM, Ruyschaert JM, Vandenbranden M. Fusogenic activity of cationic lipids and lipid shape distribution. *Cell Mol Life Sci*. 2010;67(3):483–94.
- Enmon Jr RM, O'Connor KC, Lacks DJ, Schwartz DK, Dotson RS. Dynamics of spheroid self-assembly in liquid-overlay culture of DU 145 human prostate cancer cells. *Biotechnol Bioeng*. 2001;72(6):579–91.
- Bozzola JJ, Russell LD. *Microscopy, principles and techniques for biologists*. Sudbury, MA, USA: Jones and Bartlett Publishers; 1999:32.
- Qaddoumi MG, Ueda H, Yang J, Davda J, Labhasetwar V, Lee VH. The characteristics and mechanisms of uptake of PLGA nanoparticles in rabbit conjunctival epithelial cell layers. *Pharm Res*. 2004;21(4):641–8.
- Duzgunes N, Nir S. Mechanisms and kinetics of liposome-cell interactions. *Adv Drug Deliv Rev*. 1999;40(1–2):3–18.
- Lee KD, Nir S, Papahadjopoulos D. Quantitative analysis of liposome-cell interactions *in vitro*: rate constants of binding and endocytosis with suspension and adherent J774 cells and human monocytes. *Biochemistry*. 1993;32(3):889–99.
- Wrobel I, Collins D. Fusion of cationic liposomes with mammalian cells occurs after endocytosis. *Biochim Biophys Acta*. 1995;1235(2):296–304.
- Blumenthal R, Weinstein JN, Sharrow SO, Henkart P. Liposome-lymphocyte interaction: saturable sites for transfer and intracellular release of liposome contents. *Proc Natl Acad Sci U S A*. 1977;74(12):5603–7.
- Rigotti A, Acton SL, Krieger M. The class B scavenger receptors SR-BI and CD36 are receptors for anionic phospholipids. *J Biol Chem*. 1995;270(27):16221–4.
- Lonez C, Lensink MF, Vandenbranden M, Ruyschaert JM. Cationic lipids activate cellular cascades. Which receptors are involved? *Biochim Biophys Acta*. 2009;1790(6):425–30.
- Rejman J, Oberle V, Zuhorn IS, Hoekstra D. Size-dependent internalization of particles *via* the pathways of clathrin- and caveolae-mediated endocytosis. *Biochem J*. 2004;377(Pt 1):159–69.
- Davies Cde L, Berk DA, Pluen A, Jain RK. Comparison of IgG diffusion and extracellular matrix composition in rhabdomyosarcomas grown in mice *versus in vitro* as spheroids reveals the role of host stromal cells. *Br J Cancer*. 2002;86(10):1639–44.
- Lieleg O, Baumgartel RM, Bausch AR. Selective filtering of particles by the extracellular matrix: an electrostatic bandpass. *Biophys J*. 2009;97(6):1569–77.
- Stylianopoulos T, Poh MZ, Insin N, Bawendi MG, Fukumura D, Munn LL, *et al*. Diffusion of particles in the extracellular matrix: the effect of repulsive electrostatic interactions. *Biophys J*. 2010;99(5):1342–9.
- O'Neill CH, Riddle PN, Jordan PW. The relation between surface area and anchorage dependence of growth in hamster and mouse fibroblasts. *Cell*. 1979;16(4):909–18.
- Kuh HJ, Jang SH, Wientjes MG, Au JL. Computational model of intracellular pharmacokinetics of paclitaxel. *J Pharmacol Exp Ther*. 2000;293(3):761–70.
- Wiley HS, Cunningham DD. The endocytotic rate constant. A cellular parameter for quantitating receptor-mediated endocytosis. *J Biol Chem*. 1982;257(8):4222–9.
- Harwood Jr HJ, Pellarin LD. Kinetics of low-density lipoprotein receptor activity in Hep-G2 cells: derivation and validation of a Briggs-Haldane-based kinetic model for evaluating receptor-mediated endocytotic processes in which receptors recycle. *Biochem J*. 1997;323(Pt 3):649–59.
- Pluen A, Netti PA, Jain RK, Berk DA. Diffusion of macromolecules in agarose gels: comparison of linear and globular configurations. *Biophys J*. 1999;77(1):542–52.
- Kakkad SM, Solaiyappan M, O'Rourke B, Stasinopoulos I, Ackerstaff E, Raman V, *et al*. Hypoxic tumor microenvironments reduce collagen I fiber density. *Neoplasia*. 2010;12(8):608–17.
- Levick JR. Flow through interstitium and other fibrous matrices. *Q J Exp Physiol*. 1987;72(4):409–37.
- Westrin BA, Axelsson A. Diffusion in gels containing immobilized cells: a critical review. *Biotechnol Bioeng*. 1991;38(5):439–46.
- Mota M, Teixeira JA, Yelshin A. Immobilized particles in gel matrix-type porous media. Homogeneous porous media model. *Biotechnol Prog*. 2001;17(5):860–5.
- Rakesh V, Schweitzer AD, Zaragoza O, Bryan R, Wong K, Datta A, *et al*. Finite-element model of interaction between

- fungus polysaccharide and monoclonal antibody in the capsule of *Cryptococcus neoformans*. *J Phys Chem B*. 2008;112(29):8514–22.
41. Sparano JA, Malik U, Rajdev L, Sarta C, Hopkins U, Wolff AC. Phase I trial of pegylated liposomal doxorubicin and docetaxel in advanced breast cancer. *J Clin Oncol*. 2001;19(12):3117–25.
42. Stinchcombe TE. Nanoparticle albumin-bound paclitaxel: a novel Cremphor-EL-free formulation of paclitaxel. *Nanomedicine (London)*. 2007;2(4):415–23.
43. Gorodetsky R, Levitsky L, Vexler A, Shimeliovich I, Kassis I, Ben-Moshe M, *et al*. Liposome transduction into cells enhanced by haptotactic peptides (Haptides) homologous to fibrinogen C-termini. *J Control Release*. 2004;95(3):477–88.
44. de Lima MC, Simoes S, Pires P, Gaspar R, Slepishkin V, Duzgunes N. Gene delivery mediated by cationic liposomes: from biophysical aspects to enhancement of transfection. *Mol Membr Biol*. 1999;16(1):103–9.
45. Mok KW, Cullis PR. Structural and fusogenic properties of cationic liposomes in the presence of plasmid DNA. *Biophys J*. 1997;73(5):2534–45.
46. Juweid M, Neumann R, Paik C, Perez-Bacete MJ, Sato J, van Osdol W, *et al*. Micropharmacology of monoclonal antibodies in solid tumors: direct experimental evidence for a binding site barrier. *Cancer Res*. 1992;52(19):5144–53.
47. Goodman TT, Chen J, Matveev K, Pun SH. Spatio-temporal modeling of nanoparticle delivery to multicellular tumor spheroids. *Biotechnol Bioeng*. 2008;101(2):388–99.