Evaluation of Multivalent, Functional Polymeric Nanoparticles for Imaging Applications

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A series of multivalent, functional polymer nanoparticles with diagnostic/imaging units and targeting ligands for molecular targeting were synthesized with the loading of the chain-end-functionalized GRGDS peptide targeting sequence (model system based on integrin αvβ3) ranging from 0 to 50%. Accurate structural and functional group control in these systems was achieved through a modular approach involving the use of multiple functionalized macromonomer/monomer units combined with living free radical polymerization. In cellulo results show an increase in uptake in αvβ3 integrin-positive U87MG glioblastoma cells with increasing RGD loading and a possible upper limit on the effectiveness of the number of RGD peptides for targeting αvβ3 integrin. Significantly, this increased targeting efficiency is coupled with in vivo biodistribution results, which show decreased blood circulation and increased liver uptake with increasing RGD loading. The results demonstrate the importance of controlling ligand loading in order to achieve optimal performance for therapeutic and imaging applications for multivalent nanoparticle-based systems.

KEYWORDS: polymeric nanoparticles · click chemistry · cellular uptake · pharmacokinetics

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targeting moieties and diagnostic units onto a polymeric nanoparticle scaffold and to exploit this high level of control to understand and evaluate the competing effects of ligand loading on nanoparticle binding affinity and uptake in cellulo compared to the effect on their pharmacokinetic properties. Here we demonstrate that the modular and tunable nature of the synthetic approach to these multifunctional comb nanoparticle (CNP) carriers allows for the design of systems with increased specific integrin binding and cellular uptake, optimal blood retention, and RES response based on an intermediate loading of targeting peptides. Of the many molecular targets available, \( \alpha_v\beta_3 \), a well-studied type of integrin upregulated in tumor angiogenesis, metastasis, inflammation, certain cardiovascular abnormalities, and bone resorption,20 was selected as a model system for evaluation. To synthesize agents capable of detecting \( \alpha_v\beta_3 \), small peptides containing the amino acid sequence Arg-Gly-Asp (RGD), which bind to \( \alpha_v\beta_3 \) with high affinity, were linked to the polymeric backbone of nanoparticles at various concentrations. Notably, the novel modular and tunable synthetic approach ensures accurate control over conjugation of RGD peptides to the backbone. Finally, this series of RGD-comb nanoparticles were radiolabeled with \(^{64}\text{Cu} \) \( (T_{1/2} = 12.7 \text{ h}, \beta^+ = 17.86\%) \), a positron emitter commonly used in positron emission tomography (PET), via the DOTA \((1,4,7,10\text{-tetraazacyclododecane-1,4,7,10-tetraacetic acid})\) chelator for evaluations in cellulo and in vivo.

RESULTS AND DISCUSSION

Synthesis of Comb Copolymers. Herein we describe the synthesis, in cellulo, and pharmacokinetic in vivo evaluation of a class of multifunctional nanoparticles as a model system for developing structure/bioperformance relationships. Using the binding of RGD to the integrin \( \alpha_v\beta_3 \) as a prototypical system, well-defined amphiphilic graft copolymers and associated comb nanoparticles (CNPs) having a controlled number of RGD peptide targeting moieties were prepared. The modular approach used in this study is based on four important building blocks: (a) poly(ethylene glycol) (PEG) as a hydrophilic, protein-resistant unit;\(^{21,22}\) (b) methyl methacrylate as a hydrophobic backbone which controls self-assembly; (c) \(1,4,7,10\text{-tetraazacyclododecane-N,N,N',N''-tetraacetic acid} \) (DOTA) as a chelator for imaging with the positron emitter \(^{64}\text{Cu} \) \( (T_{1/2} = 12.7 \text{ h}, \beta^+ = 17.86\%) \); and (d) GRGDS as a linear targeting peptide. The key to this strategy is the preparation of functional monomers and macromonomers, where incorporation of the desired targeting ligands and diagnostic units into these target structures allows for a more reproducible level of incorporation during living free radical polymerization. This degree of control also permits the spatial location of the building blocks along the polymer backbone to be manipulated, important for both nanoparticle self-assembly and activity of the various components (i.e., targeting moieties should be at the surface).\(^{18}\)

The DOTA methacrylate, 3, was synthesized as shown in Scheme 1, from the bromomethacrylacyl methacrylate derivative, 1, and the tris-functionalized cyclen derivative, 2, allowing direct incorporation of the diagnostic \(^{64}\text{Cu} \)-DOTA units in the interior of the nanoparticle after deprotection and \(^{64}\text{Cu} \) insertion. The
RGD–PEG macromonomer, 6, was synthesized in two steps from a heterobifunctional PEG containing a hydroxyl and an azide chain end. The initial step involved introduction of the methacrylate functionality at the hydroxyl end of the heterobifunctional PEG through reaction with methacryloyl chloride to give 4. Following this, the acetylene-derivatized GRGDS peptide, 5, was attached using Cu(I) click chemistry, which proved to be orthogonal to the functional groups displayed on peptides as well as the polymerizable methacrylate unit, giving the desired macromonomer, 6, in excellent yield and purity (Scheme 2).23–25 Although a previous report by Dechantsreiter et al.26 points to improved affinity exhibited by cyclic peptides, in vitro experiments performed in our laboratory demonstrated that, although the lactam-cyclized peptide, c(RGDyK), had increased binding affinity for αvβ3 (3.7 nM) over the linear peptide (GRGDS, 15.9 nM), GRGDS had improved specificity for αvβ3 compared to other integrins (c(RGDyK), αvβ5: 171 nM, αllbβ3: 0.11 nM; GRGDS, αvβ5: >5000 nM, αllbβ3: 873 nM).27 Additionally, the cellular uptake was comparable for the cyclized and linear peptides.27

Copolymerization of these well-defined monomers with varying amounts of PEG methacrylate and methyl methacrylate comonomers allows the preparation of a library of functionalized comb copolymers. The polymerizations were performed to ca. 50% conversion, which gives low polydispersity and controlled molecular weight.
materials where the level of incorporation of the functionalized monomers agrees with the feed ratios.28–30 By controlling the relative hydrophobic/hydrophilic balance of the copolymers through the ratio of methyl methacrylate and PEG units, assembly into CNPs was observed and nanoparticles with approximately the same size (∼22 nm), irrespective of the level of incorporation of the RGD units obtained (Table 1). This allows variations with respect to CNP size to be eliminated and direct comparison between the samples with 0% RGD, 5% RGD, 10% RGD, 20% RGD and 50% RGD—CNPs having approximately 0, 7, 14, 28, and 70 RGD’s per CNP to be made. As has been previously shown, the stability of the $^{64}$Cu-labeled-CNPs were tested in serum and cell culture conditions up to 48 h and no dissociation of $^{64}$Cu or degradation of the CNPs was observed.28 In our previous work, we showed the effects of PEO length on particle biodistribution as well as polymer assembly into core—shell nanoparticles by DLS, SANS and CryoTEM.29 The nanoparticle nature of our system provides for longer circulation times than similar random copolymer systems developed by Kopecek et al.31

**In Vitro Evaluation of $\alpha_v\beta_3$ Targeted CNPs by Isolated Integrin Binding Assays.** In evaluating the biological activity of RGD-functionalized CNPs toward $\alpha_v\beta_3$, the effect of multivalency on integrin binding affinity was assessed by performing isolated integrin binding assays. CNPs with variable levels of RGD attachment were evaluated for their binding affinity and specificity toward the integrins $\alpha_v\beta_3$, $\alpha_5\beta_1$, and $\alpha_4\beta_1$ in heterologous competitive binding experiments with biotinylated vitronectin ($\alpha_v\beta_3$, $\alpha_5\beta_1$) and fibronectin ($\alpha_4\beta_1$) as the competitive natural ligand, respectively. Nonlinear regression was used to fit the binding curves and calculate inhibitory concentration values of 50% (IC$_{50}$), with the $\alpha_v\beta_3$ binding kinetics following a classic sigmoid path that could be fit with a nonlinear regression curve (Figure 1). The results demonstrate a ca. 75-fold increase in $\alpha_v\beta_3$ binding between the 5% RGD—CNPs (IC$_{50}$ = 78.46 nM) and 50% RGD—CNPs (IC$_{50}$ = 1.08 nM), which is in contrast to the number of RGD targeting ligands where the increase is only a factor of 10.

The 10 and 20% RGD—CNPs had intermediate IC$_{50}$ values of 12.48 and 5.21 nM, respectively, with the significant finding being the nonlinear nature of the relationship between the IC$_{50}$ and the number of peptides (Table 2). The results indicate that increasing the number of targeting peptides per CNP translates to increased binding affinity to the integrin, with the nontargeted control CNP showing no appreciable binding to $\alpha_v\beta_3$ (Table 2). For example, doubling the number of peptides from the 5% sample to the 10% sample leads to a 6-fold improvement in binding, while a 10-fold increase in peptide attachment corresponds to a 75-fold improvement in the IC$_{50}$ value. This enhancement in the binding affinity can be attributed to both the multivalent nature of the nanoparticle design as well as the increased availability of the targeting peptides at the surface of the nanoparticle.

The RGD—CNPs were also screened against the integrins $\alpha_5\beta_1$ and $\alpha_4\beta_1$, found on macrophages and blood platelets, respectively. The binding to these two integrins was tested to evaluate the specificity of the multivalent CNPs toward the integrin $\alpha_v\beta_3$. Significantly, all four CNP constructs demonstrated no specific binding toward the integrins $\alpha_5\beta_1$ and $\alpha_4\beta_1$, with

![Figure 1. IC$_{50}$ binding curves for RGD—CNPs for the human integrin $\alpha_v\beta_3$, obtained by competition with biotinylated vitronectin.](image)

![Figure 2. Cell-associated $^{64}$Cu—RGD—CNPs at 37 °C at 30 min time point in U87MG glioblastoma cells as a function of RGD content. Blocking was carried out by coincubation with 1 μM nonradioactive nanoparticle solution (500 μL total volume).](image)

<table>
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<tr>
<th>CNPs</th>
<th>number of peptides per CNP</th>
<th>$K_{50}$ $\alpha_v\beta_3$ (nM)</th>
<th>$K_{50}$ $\alpha_5\beta_1$ (nM)</th>
<th>$K_{50}$ $\alpha_4\beta_1$ (nM)</th>
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<td>5%</td>
<td>7</td>
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<td>10%</td>
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<td>20%</td>
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<td>50%</td>
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IC50 values of >1000 nM. It should be noted that at higher concentrations weak binding was observed toward Rllbβ3. This is not surprising as modest binding would be expected to the β3 part of the integrin; however, the near thousand-fold enhancement of binding toward Rvβ3 when compared with Rvβ5 and Rllbβ3 in all four RGD-CNP constructs suggests a high selectivity for Rvβ3. It should also be noted that the small molecule analogue, GRGDS, was shown to have an IC50 of 15.9, >5000, and 873 nM for Rvβ3, Rvβ5, and Rllbβ3, respectively, displaying decreased selectivity when compared to the corresponding multivalent CNP. These results are promising for further application of these CNPs in clinical imaging and therapeutic settings.

Cell Uptake Assays. Cellular uptake assays were performed to evaluate the cellular uptake kinetics of the RGD-CNPs using αvβ3-positive U87MG human glioblastoma cells. The cellular associated radioactivity, which is the sum of the cell-internalized CNP fraction and cell surface-bound CNP fraction measured at a 30 min incubation time point and normalized to protein content, is presented in Figure 2. The 5, 10, and 20% RGD-CNPs demonstrated increasing internalization at 30 min post-incubation as the level of RGD functionalization increases, with average values of 10, 20, and 35% cell-associated fraction/mg protein, respectively. The 50% RGD-CNP demonstrated a slight decrease in cell uptake as compared to the 20% comb, with a value of about 30% cell-associated fraction/mg protein, suggesting an upper limit on the effect of increased RGD loading on cellular uptake efficiency, possibly due to the 50% RGD-CNP exceeding the optimal RGD peptide/integrin binding site ratio. Montet et al. have reasoned that this upper limit is reached when the density of RGD peptides on the CNP exceeds the density of RGD/integrin binding sites displayed by the cell membrane.32 Because there is only one RGD binding site per integrin, the number of RGD peptides per CNP that participate in binding is limited by the number of integrins displayed by the membrane. Thus, any “extra” RGD peptides present on the multivalent nanoparticle cannot participate in integrin binding. Decuzzi et al. have argued that other key factors, such as the threshold particle radius, the optimal particle radius, and the characteristic wrapping time, may also play a role in the specific and nonspecific interactions in the receptor-mediated endocytic performances of nanometer particles.33 The cellular uptake of the control peptide or the functionalized nanoparticles could be successfully blocked by the addition of an excess of the
corresponding nonradiolabeled peptide or CNP, respectively (Figure 2), consistent with the mechanism of receptor-mediated endocytosis of the RGD—CNPs.

**Internalization Studies.** The cellular uptake trend of the 64Cu-labeled multivalent CNPs was clearly demonstrated by assays performed with the αvβ3-positive U87MG cells. Initial experiments to quantify the level of surface-bound CNPs versus internalized nanoparticles were complicated by strong adherence of the CNPs to the cell surface, preventing full removal by washing. In order to validate the internalization of the RGD—CNPs, confocal microscopy was therefore performed on the 20% RGD—CNP with αvβ3-positive U87MG cells. Since there was no fluorescent marker on the comb nanoparticle, the confocal imaging strategy involved targeting of the PEG units on the RGD—CNPs by a monoclonal anti-PEG antibody, which could be specifically detected by a fluorescent secondary marker. Significantly, the confocal images showed internalization of the 20% RGD—CNPs within 1 h of incubation with the cells (Figure 3), and the CNPs favored localization close to the perinuclear region inside the cell. The confocal images combined with the cellular studies using radioactive assays illustrate that high cellular uptake and internalization by receptor-mediated endocytosis can be achieved and manipulated through tuning of the nanoparticle structure.

**Biodistribution Studies.** Previous in vivo studies with nanoparticles bearing a targeting ligand (such as RGD, DNA aptamers, etc.) have shown that these moieties can lower the blood circulation lifetime and increase the immune response in comparison to nanoparticles that only have a PEG surface layer.11,18 The structural control over nanoparticle size and peptide loading coupled with the ability to introduce diagnostic units permits the ex vivo results described above to be compared with the in vivo pharmacokinetic behavior of the multivalent CNPs. Toward this aim, 64Cu-labeled CNPs with varying loadings of RGD were administered intravenously to normal rats, with blood retention, liver, and spleen accumulation studied in detail.

The blood retention profiles for the RGD—CNP series are shown in Figure 4. The 10, 20, and 50% RGD—CNP demonstrate relatively fast blood clearance to 1 h followed by stabilization of blood activity to 48 h. In contrast, the 5% RGD—CNPs exhibit a similar blood retention profile as the control particle without RGD, with the blood retention remaining high out to 48 h. These results show that, for the GRGDS ligand, low loadings have minimal effect on blood retention while higher loadings drastically decrease blood retention. There was no significant accumulation in the lungs, as expected from non-aggregating nanoparticles.

We monitored the liver and spleen uptake of the CNPs as the liver contains some of the principal phagocytic cells (e.g., Kupffer cells, hepatocytes, hepatic stellate cells) and both organs eliminate foreign particulate, macromolecules, and senescent cells.34 The liver and spleen uptake for our series of nanoparticles is shown in Figure 4 (middle and bottom panel). Both the 20 and 50% RGD—CNPs are quickly uptaken by the liver and spleen, and their concentrations slowly decrease presumably via urinary and fecal excretion, as increased excretion is seen for the RGD—CNPs (data not shown), whereas the 0, 5, and 10% RGD—CNPs show only moderate uptake in the liver and spleen. These results reveal that increases in the amount of RGD on a CNP surface can lead to a greater response from the RES of an organism. Future studies will examine different peptide sequences for targeting integrins in order to understand the effect of the nature of the peptide (hydrophilic/hydrophobic, charge, etc.) combined with its loading level and structural position on the biodistribution.

**CONCLUSIONS**

The tunable and modular synthesis of functionalized comb copolymers has enabled facile development of a library of multivalent nanoparticles incorporating diagnostic 64Cu—DOTA units with varying amounts of surface-accessible RGD peptides for αvβ3 targeting. By controlling the level of targeting moieties, while maintaining similar nanoparticle sizes, fundamental structure/bioperformance relationships were developed and the effects of functional group density on pharmacological profiles and biological behavior explored. The in cellulo results indicate an upper limit of RGD loading for effective cellular uptake in αvβ3-positive U87MG glioblastoma cells, while the in vivo results show a balance between the level of targeting ligand and biodistribution. This ability to screen a library of well-defined nanoparticles is critical in designing the optimal material parameters for therapeutic and imaging applications while at the same time developing a fundamental understanding of the interaction of functionalized nanoparticles with cellular and in vivo systems. The lessons learned from model αvβ3-targeted CNPs can thus be applied for a variety of molecular targets and targeting ligands using the CNP nanoparticle scaffold.

**EXPERIMENTAL SECTION**

**Materials.** Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification unless otherwise stated, and functionalized poly(ethylene glycol) (PEG) derivatives were obtained from Intezyne Technologies, (Tampa, FL). 64Cu was prepared on the Washington University Medical
School CS-15 Cyclotron by the 14(Nil;p)39Cu nuclear reaction at a specific activity of 50–200 mCi/μg (end of bombardment), as previously described.28 The buffers used for 39Cu labeling were treated with Chelex-100 resin (Bio-Rad Laboratories, Hercules, CA) before use. Tris-4-butylerter-DOTA and 1,4,7,10-tetraazacyclododecane were purchased from GE Healthcare (Piscataway, NJ). 2-(Bromoacetoxy) ethyl methacrylate,37 dihedral radical addition—fragmentation transfer (RAFT) agent,37 1,4,7,10-tetraazacyclododecane-1,4,7,10-tris-(butyl acetate) (DOTA)38, and 4-pentynoic anhydride39 were prepared as previously reported. Vitronecin, fibronectin, RGD-PEGMA (40 mg, 0.0072 mmol) were dissolved in DMSO and 1.27 g of Milli-Q water followed by the additions of DMSO and 10 mL of Milli-Q water and freeze-dried (yield 132 mg, 78%).

**Synthesis of DOTA Methacrylate (DOTA-MA) (3).** DOTA (1.92 g, 3.74 mmol) and 2-(bromoacetoxy)ethyl methacrylate (1.02 g, 4.06 mmol) were dissolved in acetonitrile (50 mL) followed by the addition of K2CO3 (0.62 g, 4.48 mmol), and the reaction mixture was then stirred overnight at room temperature. Dichloromethane (50 mL) was added to the reaction which was extracted with water (25 mL), concentrated by rotary evaporation, and purified by flash chromatography (DCM/ MeOH, 95:5) to obtain a viscous clear oil (yield 1.81 g, 71%).

**Synthesis of Poly(ethylene glycol)(methyl ether methacrylate) (PEGA).** Poly(ethylene glycol) monomethyl ether (5.0 kDa, mPEG, 5.0 g, 1.00 mmol) was dissolved in dichloromethane (25 mL) and triethylamine (5 mL). Freshly distilled methacryloyl chloride (0.70 mL, 0.75 g, 7.1 mmol) was added dropwise at 0 °C, and the reaction mixture was allowed to stir overnight under argon. The reaction was quenched with water, filtered, and the organic phase was washed with 10% NaHSO4 (w/v), dried over anhydrous MgSO4, and concentrated in vacuum to ca. 10 mL. The product was precipitated by adding cold diethyl ether (100 mL) and dried in vacuum (yield 1.11 g, 74%).

**Synthesis of Acidoploy(ethylene glycol)methacrylate (Nz-PEGMA) (4).** Polyethylene glycol)(monomethyl, (50 kDa, 1.50 g, 0.30 mmol) was dissolved in dichloromethane (15 mL) and triethylamine (3 mL). Freshly distilled methacryloyl chloride (0.70 mL, 0.75 g, 7.1 mmol) was added dropwise at 0 °C, and the reaction mixture was allowed to stir overnight under argon. The reaction was quenched with water, filtered, and the organic phase was washed with 10% NaHSO4 (w/v), dried over anhydrous MgSO4, and concentrated in vacuum to ca. 3 mL. The product was precipitated by adding cold diethyl ether (100 mL) and dried in vacuum (yield 1.11 g, 74%).

**Dynamic Light Scattering (DLS).** DLS measurements were carried out on a Brookhaven BI-9000AT Digital Autocorrelator (Holtville, NY) equipped with an Avalanche photodiode detector and a MG vertically polarized 35 mmV helium–neon 633 nm laser and operated by the 9KDLSW control program. All samples were filtered through 0.45 μm and run in triplicate for 10 min at 25 °C, at 1 mg/mL, and at a fixed 90° angle. Hydrodynamic diameter and distribution of particles were determined by
fitting the correlation functions with the ISDA analysis software.

Radio-labeling of DOTA-CNPs with $^{64}$Cu. $^{64}$Cu chloride (5–10 μL in 0.5 M HCl) was diluted with 0.1 M ammonium acetate buffer (pH 5.5, 50–100 μL). The CNP solutions (3 mg/mL, 30–200 μL) were two-fold diluted with acetate buffer, $^{64}$Cu-acetate (2–5 μCi) was added, and the mixture was incubated at 80 °C for 1 h. Following radiolabeling of the CNPs, 5–10 μL of 10 mM aqueous ethylenediamine tetraacetic acid (EDTA) solution was added and incubation continued for another 10 min at room temperature (RT) with the labeling yield determined by radio-ITLC. The resulting solution was incu- bated at 80 °C, a 10 mM aqueous ethylenediamine tetraacetic acid (EDTA) was added and the resulting solution was incubated for 10 min at RT. Aliquots were then analyzed by radio-ITLC: the $^{64}$Cu-labeled nanoparticles remained at the origin while the $^{64}$Cu-EDTA complex migrated with an Rf of 0.9. The number of DOTA chelates attached to the comb copolymers was determined from the counts per minute (CPM) in the radiochromatogram, with the following equation:

$$n_{\text{chelates}} = n_{\text{copolymer}} \times \frac{\text{CPM}}{\text{Rf} < 0.3}$$

**FPLC Analysis.** An aliquot (100 μL, ca. 15 μCi) of the $^{64}$Cu-labeled CNPs was injected into a Superose 12 gel filtration column (GE Healthcare Biosciences) and eluted with 20 mM HEPES and 150 mM NaCl (pH 7.3) at 0.8 mL/min. The UV wavelength was preset to 280 nm, and the radioactivity was monitored by an in-line radio detector. Under these conditions, the retention times of the native nanoparticles were 8–10 μL while the retention times of free $^{64}$Cu and $^{64}$Cu-EDTA were 20–22 μL. Samples with RCP > 95% were used for cells or animal studies.

**Biodistribution Studies.** All animal studies were performed in compliance with guidelines set by the Washington University Animal Studies Committee. Normal female Sprague–Dawley rats (180–200 g, n = 4 per time point) anesthetized with 1–2% vaporized isoflurane and injected via the tail vein with approxi- mately 15 μCi in 200 μL of PBS (80–100 μg/kg rat body weight). At each time point, the rats were anesthetized prior to sacrifice. Organs of interest were removed and blotted dry. The activity was measured in a gamma counter. The total activity in the blood was calculated assuming 6% of the rat body weight.41 Diluted standard doses (1:100) were prepared and counted along with the samples. All of the data were corrected for $^{64}$Cu decay. The percent injected dose per gram of tissue (%ID/g) values were calculated using the following equation:

$$\%\text{ID/g} = \left(\frac{\text{cpm in sample} - \text{background}}{\text{cpm total}}\right) \times 10^2$$

**Cell Culture.** All cell handling was aseptically performed in a laminar flow hood. U87/MG human glioblastoma cell lines were purchased from the American Type Culture Collection and were grown until 60–75% confluence in T75 tissue culture flasks. The cells were maintained at a concentration of 1 $\times$ 10^6 cells/mL in Iscove's modified Dulbecco's medium (GIBCO-BRL) at 37 °C in a humidified atmosphere with 5% CO2 in a Revco Elite II incubator. To determine cell density, equal amounts of cell suspension and trypan blue exclusion were added to a hemo- cytometer to calculate a cells/mL concentration and ensure cell viability.

**Competitive Binding Assay.** The 96-well plates (Nunc Immuno Plate with MaxiSorp) were coated with 100 μL of human integrin αvβ3 or αvβ5 (Chemicon Intl, Inc.) or αvβ3 (EMD Chemicals) (1 μg/mL) in coating buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM CaCl2, 1 mM MgCl2, 0.1% [v/v] NaN3) for 1 h at 4 °C. The plates were then blocked with blocking buffer (3% BSA in coating buffer, 200 μL per well) for 1 h at 4 °C. For the radioactive labeling, aliquots of the 64Cu-labeled nanoparticle solution were added along with 14 nM biotinylated vitronectin solution for integrins αvβ3 and αvβ5, and 14 nM biotinylated fibronectin for integrin αvβ3. Biotinylated vitronectin or fibronectin (chemicon) was prepared by using sulfo-biotin-NHS (Pierce Biotechnology). The reagents were allowed to bind to the respective integrins for 2 h at 37 °C. Following incubation, the plates were washed three times with the binding buffer to remove unbound reagents. Bound biotinylated vitronectin/fibronectin was detected by adding ExtrA- vidin alkaline phosphatase conjugate (Sigma) (1/5 000 dilution, 1 h, RT) using the p-nitrophenyl phosphate substrate solution as the chromogen (30 min, RT, in dark). Absorbance was read on the spectrophotometer at 405 nm. Each concentra- tion data point was evaluated in triplicate, and nonspecific binding was subtracted from each data point. Nonlinear regression was used to fit binding curves and calculate IC50 values (Prism, version 4.0, GraphPad). A control assay with a small peptide whose IC50 was previously known (e.g., cRGDyK, IC50 3.7 nM) was always run concurrently with the test assay to validate the accuracy of procedure and reagents.

**Cellular Uptake/Internalization Assay.** U87/MG human glioblastoma cells were grown to 60–75% confluence. The cells were harvested by mechanical dissociation and re- suspended in the binding medium (Iscoves MDM, 1% gluta- mine, 1% BSA, 0.1 mM Mg2+ and 0.1 mM Mn2+) in microfuge tubes. The $^{64}$Cu-labeled nanoparticle solution (1 nM, 20–50 μL) was added to the cell suspension. The samples were incubated for 0, 30, and 60 min in a cell incubator (37 °C, 5% CO2). For blocking experiments, the samples were co-incubated with 1 μM nonradioactive nanoparticle solution (500 μL total volume). After incubation, the samples were centrifuged at 1400 rpm for 1 min, and the radioactive medium was removed. Cell pellets were rinsed with ice cold binding buffer (500 μL) and centrifuged at 1400 rpm for 1 min. The surface-bound fraction was collected by suspending the cells in 500 μL strip buffer (5% Trypsin EDTA, pH 7.4), followed by incubation for 10 min at 37 °C. The cells were separated via centrifugation followed by one more washing with 500 μL strip buffer. The combined strip- buffer wash fractions constituted the surface-bound fraction. The radioactivity in each fraction was measured in a well counter (Packard II gamma counter). The protein content of each cell lysate sample was determined (BCA Protein Assay Kit, Pierce) to normalize the data.

**Microscopy.** The U87/MG cells were plated on tissue culture treated glass slides and incubated with 20% comb nanoparti- cles for 1.5 h at 37 °C. Following washing of the unbound nanoparticles were washed off, and the cells were fixed with 4% paraformaldehyde (PFA) solution and permeabilized with 0.2% Triton X-100 in PBS. Following fixation and permeabilization steps, PEG monoclonal antibody (20 μg/mL; Biodiag, Catalog #G01237M, Clone B141M; 30 min; 37 °C) was added to react with PEG units present on the internalized CNPs. After removal of the unbound antibody and washings with PBS (4 ×), secondary reagent for detecting the PEG antibody was added (Inovitro; SKU# A-21237; Alexa Fluor 647 Fab’; fragment of goat anti-mouse IgG (H+L); 2 mg/mL; 1/100 dilution; 20 min; RT). Final step involved washing off of any unbound reagent (PBS, 4 ×) and placing of mounting media before covering the slide. Confocal images were collected 24 h later with an Olympus FV1000 microscope using a 60 ×/1.20 M.O, 0.13–0.21 NA water immersion objective. Fluorescence emission was detected.
REFERENCES AND NOTES


