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Targeting of Cancer Cells Using Quantum Dot-Polypeptide Hybrid Assemblies that Function as Molecular Imaging Agents and Carrier Systems

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Abstract

We report a highly tunable quantum dot (QD)-polypeptide hybrid assembly system with potential uses for both molecular imaging and delivery of biomolecular cargo to cancer cells. In this work, we demonstrate the tunability of the assembly system, its application for imaging cancer cells, and its ability to carry a biomolecule. The assemblies are formed through the self-assembly of carboxyl-functionalized QDs and poly(diethylene glycol-L-lysine)-poly(L-lysine) (PEGLL-PLL) diblock copolypeptide molecules, and they are modified with peptide ligands containing a cyclic arginine-glycine-aspartate [c(RGD)] motif that has affinity for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins overexpressed on the tumor vasculature. To illustrate the tunability of the QD-polypeptide assembly system, we show that binding to U87MG glioblastoma cells can be modulated and optimized by changing either the conditions under which the assemblies are formed or the relative lengths of the PEGLL and PLL blocks in the PEGLL-PLL molecules. The optimized c(RGD)-modified assemblies bind integrin receptors on U87MG cells and are endocytosed, as demonstrated by flow cytometry and live-cell imaging. Binding specificity is confirmed by competition with an excess of free c(RGD) peptide. Finally, we show that the QD-polypeptide assemblies can be loaded with fluorescently labeled ovalbumin, as a proof-of-concept for their potential use in biomolecule delivery.

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The table of contents entry

Application of quantum dot (QD)-polypeptide assemblies as potential dual imaging and targeted drug-delivery systems. The self-assembly between the QDs (red) and PEGLL-PLL diblock copolypeptide molecules (green) forms a nano-scale assembly that can take the shape of a shell-like structure. Receptor-mediated binding and endocytosis of assemblies modified with cancer-targeting ligands would allow molecular imaging and delivery of loaded drugs to be performed simultaneously.

Keywords

hybrid materials; quantum dots; supramolecular materials; self-assembly; nanostructures

1. Introduction

Two important aspects of cancer therapy are detection of malignant cells and targeted delivery of therapeutic agents. Quantum dots (QDs), modified with ligands that mediate binding to tumor receptors, have been used to image cancer cells due to their superior brightness, photostability, and tunable emission wavelength compared to conventional organic dyes.^[1-5] Carriers, such as polymersomes^[6,7] and liposomes,^[8-10] have been used for delivery of therapeutic agents to cancer cells to increase drug efficacy and decrease toxic side effects.^[8, 9] Currently, there are only a few systems that offer a “two-in-one” solution for simultaneous imaging and targeted delivery of drugs to cancer cells, and the available systems lack versatility and tunability.^[11-14] For example, most systems are suitable for the delivery of only certain types of therapeutic agents (e.g., hydrophobic drugs) and their physical (e.g., size, shape) and surface properties cannot be easily modified, ultimately limiting their scope of applications. To address these needs, we developed a highly tunable QD-polypeptide hybrid assembly system, which is formed through the supramolecular assembly (self-assembly) between carboxyl-functionalized QDs and poly(diethylene glycol-L-lysine)-poly(L-lysine) (PEGLL-PLL) diblock copolypeptide molecules.^[15] To target the QD-polypeptide hybrid assemblies to tumor cells, we functionalized them with ligands that bind to receptors overexpressed on the tumor neovasculature and mediate their endocytosis. We also showed that the QD-polypeptide assemblies could be loaded with biomolecules. Thus, this assembly system has potential for simultaneous imaging of cancer cells and targeted delivery of therapeutic cargo (**Fig. 1**).

We chose QDs as one component of the hybrid assembly system because their superior fluorescence properties will allow for noninvasive and high-resolution imaging of cancer cells.^[1-3] Block copolypeptides were chosen as the other component because of their material versatility as compared to phospholipids. This material versatility is attributed to the ability to synthesize block copolypeptides with a diverse range of chemical functionalities and to tailor their chemical properties, such as block lengths and polydispersity, using a wide range of polymer chemistries.^[6, 16] Combined with the ability to vary the self-assembly conditions, these features impart extensive tunability to the assembly properties. In particular, they allow the physical properties of the QD-polypeptide hybrid assembly system, including size, shape,^[15] surface potential, and targeting-ligand multivalency to be easily modulated. The modulation of these physical properties can in turn be exploited to tune the assembly's biochemical and biophysical attributes such as biocompatibility, colloidal stability, binding, and drug-loading characteristics. In addition, the components of the hybrid assembly system—PEGLL-PLL and QDs—can be replaced with other block copolymers and/or nanoparticles to further increase the diversity of the assembly's chemical functionality,^[17, 18] such that its properties can be tailored for a broad range of applications. For example, other positively charged polyelectrolytes may be used in

place of PEGLL-PLL to allow for the real-time observation of gene delivery into targeted cells.^[19-21]

In this work, we demonstrate that the QD-polypeptide assemblies can be modified with cyclic peptide ligands containing an arginine-glycine-aspartate [c(RGD)] motif that has affinity for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins overexpressed on the tumor vasculature.^[3, 22-24] We assess the binding and uptake of these c(RGD)-modified assemblies (termed c(RGD)-assemblies) in U87MG glioblastoma cells that overexpress these integrin receptors.^[3, 24, 25] In addition, we show that the tumor cell binding characteristics of the QD-polypeptide assemblies can be modulated and optimized, thus, highlighting their tunability. Finally, we load ovalbumin, a model biomolecule, into the c(RGD)-assemblies as proof of concept that they can be used to carry biomolecular cargo. Collectively, our data demonstrate that these QD-polypeptide assemblies have promise as highly tunable, dual imaging and biomolecule carrier agents for cancer diagnostics and therapy applications.

2. Results and Discussion

2.1. Synthesis and Characterization of c(RGD)-PEGLL-PLL Conjugates

To create the modified assemblies, we first covalently conjugated c(RGD) ligands to PEGLL-PLL diblock copolypeptide molecules. Subsequently, QDs were added to the conjugate solution to allow the spontaneous formation of c(RGD)-assemblies. The c(RGD)-PEGLL-PLL conjugates were synthesized using a heterobifunctional crosslinker, sulfo-succinimidyl 4-[N-maleimidomethyl]cyclohexane-1-carboxylate (sulfo-SMCC) which contains an amine-reactive N-hydroxysuccinimide (NHS) ester and a sulfhydryl-reactive maleimide group. The NHS ester reacted with either the PLL side chain or the N-terminus of the PEGLL-PLL to form a maleimide-activated PEGLL-PLL molecule. Covalent conjugation of cyclo(Arg-Gly-Asp-D-Phe-Cys) (c(RGDfC)) to PEGLL-PLL occurred through the reaction of the maleimide group of the activated PEGLL-PLL with the free –SH group of the cysteine residue. Further details on the synthesis and characterization of the c(RGD)-PEGLL-PLL conjugates are presented in SI 1.

Because there is $\gg 1$ primary amine present per PEGLL-PLL molecule, it is possible to conjugate more than one c(RGD) molecule to each PEGLL-PLL. We showed that the conjugation ratio, which is defined as the number of c(RGD) molecules conjugated per PEGLL-PLL molecule, could be increased by increasing either the molar excess of the crosslinker or c(RGD) (SI 1). Our ability to tune the conjugation ratio indicates that the conjugation chemistry is efficient and provides a way to vary the number of c(RGD) ligands per assembly. This is beneficial since the multivalency of RGD-containing carrier constructs has been shown to have significant impact on their binding affinity and receptor-mediated internalization.^[22, 26]

2.2. Formation and Characterization of c(RGD)-Assemblies

Next, we formed the c(RGD)-assemblies and characterized their physical properties such as size, shape, zeta-potential, and stability. Spontaneous formation of c(RGD)-assemblies occurred with the addition of QDs to the c(RGD)-PEGLL-PLL conjugate solution (**Fig. 2**). The proposed mechanism of c(RGD)-assembly formation can be found in SI 2. Due to the

resolution limit of optical microscopy, the morphology of the assembly interior could not be resolved in Fig. 2. However, based on our previous studies^[15] we expect the c(RGD)-assemblies to adopt the shape of a shell-like structure, in which the shell corresponds to the cluster of QDs that are interacting closely with the PLL residues, and the interior is hollow and filled with the solvent medium, e.g., water (Fig. 1 and SI 2). DLS measurements showed that the assemblies were quite polydisperse in size (variance of particle-size distribution = 0.6) with an average hydrodynamic diameter of 477 ± 19 nm; these observations were consistent with fluorescence microscopy imaging data (Fig. 2). The zeta-potential of the c(RGD)-assemblies was measured to be 27.1 ± 0.6 mV; the positive value is expected due to the presence of lysine residues of the PEGLL-PLL molecules.

As aggregation can significantly impair the biophysical or biological properties of carrier systems,^[5, 8, 27] we characterized the colloidal stability of the QD-polypeptide assemblies. The assemblies formed using PEGLL₁₁-PLL₁₂₉ and PEGLL₁₁-PLL₉₀ diblock copolypeptide systems were found to be stable in serum-containing culture medium for > 3 hours at room temperature (SI 3).

2.3. Tunability of the QD-Polypeptide Assembly System

The QD-polypeptide assembly system is highly tunable because its physical properties such as size, shape,^[15] surface potential, and ligand multivalency can be modulated by varying several parameters. These parameters include the conjugation ratio, PEGLL_x-PLL_y architecture, and charge ratio (R') (see Experimental). Here, the PEGLL_x-PLL_y architecture refers to a diblock copolypeptide molecule that has a particular set of PEGLL and PLL block lengths. The ability to modulate the physical properties of the assembly system in turn provides potential paths of tuning attributes such as colloidal stability, immunogenicity, and binding.^[6, 8, 22, 26, 28, 29] For example, we previously showed that the QD-polypeptide assemblies can be varied from sizes between 30 nm and 2 μ m by altering either the PEGLL_x-PLL_y architecture or R' value.^[15] Variations in the assembly size could in turn affect *in vivo* clearance rate,^[8, 27] receptor-mediated binding affinity,^[30, 31] and extravasation into tumors.^[9, 21] Here, to highlight the tunability of our hybrid assembly system, we showed that its cell binding characteristics can be modulated by varying the PEGLL_x-PLL_y architecture and R' value. These findings led to the generation of an optimal c(RGD)-assembly system that bound to integrin-expressing cancer cells with low levels of non-specific binding (see Section 2.4 below).

2.3. (A) Minimization of Non-specific Binding of Assemblies by Changing the PEGLL_x-PLL_y Architecture—To illustrate an aspect of the tunability of the QD-polypeptide assembly system, we show how the level of non-specific cell binding can be modulated by changing the PEGLL_x-PLL_y architecture, i.e., the number of PEGLL residues (x) versus PLL residues (y). Non-specific binding is known to result from electrostatic interactions between positively charged moieties and the negatively charged proteoglycans on cell membranes.^[10, 22, 32] In the case of the QD-polypeptide assemblies, the positively charged moieties correspond to the PLL residues. Therefore, when a PEGLL_x-PLL_y molecule having a higher ratio of x:y was used, the resulting QD-polypeptide assembly exhibited a lower zeta potential. Specifically, unmodified assemblies formed using

PEGLL₁₁-PLL₁₂₉ and PEGLL₂₀-PLL₇₂ diblock copolypeptide systems with $R' = 5.8 \times 10^{-3}$, were measured to have zeta-potentials of 45.9 ± 1.8 and 18.5 ± 0.6 mV, respectively. In addition, flow cytometry analyses using U87MG cells showed that the level of non-specific binding exhibited by the PEGLL₂₀-PLL₇₂ assemblies was consistently lower than that exhibited by the PEGLL₁₁-PLL₁₂₉ assemblies at all concentrations tested (**Fig. 3A**). These findings not only illustrated the tunability of the QD-polypeptide assembly system, but suggested that the PEGLL₂₀-PLL₇₂ diblock copolypeptide system would lead to reduced non-specific binding compared to PEGLL₁₁-PLL₁₂₉. Thus, the former system was used to synthesize c(RGD)-assemblies that were used for the cell-binding and imaging experiments below (Section 2.4).

2.3. (B) Optimization of c(RGD)-Assembly Binding by Varying Charge Ratio

(R')—To further demonstrate the tunability of the system, we showed that the binding characteristics of c(RGD)-assemblies could be modulated by changing their charge ratio, R' . R' is defined as the molar ratio of QD carboxyl functional groups present in the QD suspension to the lysine (PLL) residues present in the polypeptide solution (see Experimental). Previously, we showed that the size of QD-polypeptide assemblies could be increased by increasing the R' value.^[15] Since nanostructure size has been shown to affect receptor-mediated binding affinity and cellular uptake,^[30, 31] we hypothesized that integrin-binding levels could be modulated by varying the R' value of the c(RGD)-assemblies.

U87MG cells, which have a high level of $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin expression,^[3, 24, 25] were incubated with c(RGD)-modified and unmodified assemblies (negative control) formed with varying R' values. Flow cytometry analysis showed that the mean fluorescence intensity associated with the c(RGD)-assemblies increased dramatically as R' increased from 1.8 to 8.5×10^{-3} ; this increase became less pronounced as $R' > 8.5 \times 10^{-3}$ (**Fig. 3B**). Furthermore, at each R' value, the level of non-specific binding exhibited by the unmodified assemblies was consistently lower than the binding of c(RGD)-assemblies, indicating integrin-mediated cell binding. The level of integrin-mediated binding was also observed to increase steadily up to $R' = 8.5 \times 10^{-3}$. This finding might be attributable to an increase in the assembly size^[15] (resulting in a reduced curvature) and the number of amphiphilic molecular units (see SI 2), as R' increases. As a consequence, more c(RGD) moieties per assembly can potentially interact with the integrin receptors resulting in binding avidity effects.^[30] The decrease in the level of integrin-specific binding as $R' > 8.5 \times 10^{-3}$ could be due to c(RGD) moieties being “buried” by the substantial amount of QDs present at high R' values and/or the saturation of the integrin receptors (**Fig. 3B**).

2.4. c(RGD)-Assemblies as Imaging and Carrier Systems that Bind to Cancer Cells

Collectively, the experiments above led to a c(RGD)-assembly system, synthesized with c(RGD)-PEGLL₂₀-PLL₇₂ conjugates and an R' value of 8.5×10^{-3} , that has optimal binding characteristics, including a high level of integrin-mediated binding and relatively low non-specific binding. We next provide proof-of-concept that this assembly system has: 1) the capability to target receptors present on the surface of cancer cells and 2) the potential to function as a biomolecule carrier agent.

2.4. (A) Binding and Imaging of Cancer Cells Using c(RGD)-Assemblies—To determine the integrin binding capability of the c(RGD)-assemblies, cells were incubated with either c(RGD)-modified or unmodified assemblies at 4°C for 3 h and analyzed by flow cytometry. The c(RGD)-assemblies bound cells with a fluorescence intensity that was 195% greater than the unmodified assemblies, demonstrating affinity for integrins (**Fig. 4A**). The specificity of the c(RGD)-assemblies for integrin receptors was illustrated by a substantial decrease in binding upon addition of excess free c(RGDfC) molecules.

We next performed live-cell imaging experiments to show that the c(RGD)-assemblies were able to image U87MG cells through specific targeting of integrin receptors. Confocal microscopy images of U87MG cells showed binding and endocytosis of c(RGD)-assemblies incubated at 37°C for 1 h (**Fig. 4B**). In contrast, negligible QD fluorescence was observed when cells were co-incubated with a 100-fold molar excess of c(RGDfC) to competitively block integrin receptor binding. These results clearly show that the binding and uptake of the c(RGD)-assemblies occurred through specific integrin-mediated interactions.

2.4. (B) Loading of c(RGD)-Assemblies with Fluorescently Labeled Ovalbumin—As proof of principle that the c(RGD)-assemblies could potentially be used as carriers for biomolecular cargo, we loaded them with fluorescently labeled ovalbumin, which is used here as a model biomolecule. Ovalbumin is a hydrophilic protein that has a molecular weight of 45 kD and pI ~ 5, which makes it negatively charged at neutral pH. The co-localization of the ovalbumin fluorescence with that of the c(RGD)-assemblies suggests that successful loading has occurred (**Fig. 4C**). It is worth noting that other types of nanoparticle-polymer hybrid assemblies have also been loaded successfully with water-soluble compounds; in these cases, the loading occurred by means of encapsulation.^[18, 33] At present, however, we are unable to unambiguously determine whether the negatively charged ovalbumin is electrostatically adsorbed on the positively charged surface of the assemblies, or is encapsulated within the hollow interior of the assemblies. We expect the relative contributions of encapsulation and surface adsorption to cargo loading to depend on the physical properties (e.g., surface potential, size) of the particular QD-polypeptide assembly system used and the chemical/physical nature of the cargo (see below). Nevertheless, as previously demonstrated, effective intracellular drug delivery can be achieved regardless of if the mode of cargo loading is encapsulation^[12, 13] or surface adsorption.^[14, 34]

In future studies, molecules other than ovalbumin could be loaded into the QD- polypeptide assemblies. For example, we have previously shown the shape of the assemblies can be changed from a shell-like structure to a dense aggregate,^[15] which should allow for the encapsulation of hydrophilic and hydrophobic molecules, respectively. Depending on the chemical/physical nature of the cargo, loading could also be carried out by other modes such as surface adsorption^[14, 34] and conjugation to the assembly using hydrolysable linkers.^[35, 36] Furthermore, the desired mode of loading can potentially be tailored by tuning the physical properties of the QD-polypeptide assembly system. For instance, if encapsulation of negatively charged cargo is desired, the positive surface potential of the assemblies can be reduced (see Section 2.3) such that surface adsorption of the cargo molecules is inhibited.

3. Conclusions

We show that ligand-modified QD-polypeptide assemblies can bind to and illuminate cancer cells through specific receptor mediated interactions and can potentially be used as carriers to deliver biomolecular cargo. Specifically, we demonstrate the ability of c(RGD)- modified QD-polypeptide assemblies to bind to integrin receptors on U87MG glioblastoma cells, as determined by both flow cytometry and fluorescence microscopy. The successful loading of ovalbumin, a model molecule, into the assemblies also demonstrates their potential for drug delivery applications. To mediate intracellular cargo release, we expect the pH change that occurs within the endocytic pathway^[37, 38] to disrupt the electrostatic interaction that causes the self-assembly.^[17] Furthermore, we highlight the tunability of the QD-polypeptide assembly system by showing that its binding characteristics can be modulated, and hence optimized, by varying the R' value and the PEGLL_x-PLL_y architecture.

The tunability and versatility of this QD-polypeptide assembly system will allow it to be used for a broad range of applications. For example, the assemblies could be modified with a myriad of ligands against a variety of cancer biomarkers, thus endowing them with heterofunctional binding properties. These modification are possible because there are many reactive functional groups present on both the PEGLL-PLL (e.g., primary amines) and the QDs.^[39] In addition, the tunability of the QD-polypeptide assembly system could be exploited to change the assembly size to < 200 nm, which would allow for more optimal extravasation for *in vivo* applications.^[9, 40] Although we used c(RGD)-assemblies that have an average size of ~500 nm in this work, we previously showed that the size of the QD-polypeptide assemblies can be reduced to as small as 30 nm by changing the PEGLL_x-PLL_y architecture and/or R' value.^[15]

4. Experimental

Formation of the QD-Polypeptide Assemblies

Before use, the QD suspension (Qdot 605 ITK carboxyl quantum dots, Invitrogen) was diluted 1000-fold with Milli-Q water to a final QD concentration of 8 nM. Unmodified or c(RGD)-assemblies were spontaneously formed by mixing the desired amount of PEGLL-PLL or c(RGD)-PEGLL-PLL conjugate solution (0.25 – 0.5 mg/mL in water) with the QD suspension by repeated manual pipetting. The amount of QD suspension to be mixed with the polypeptide solution was determined based on the desired charge ratio, R' , which is defined as the molar ratio of QD carboxyl functional groups present in the QD suspension to the lysine (PLL) residues present in the polypeptide solution. According to Invitrogen, there are approximately 100 carboxyl functional groups per QD. Based on the definition of R' , R' can be taken to be proportional to the fraction of lysine residues of the PEGLL-PLL molecules that are charge neutralized by the QD carboxyl functional groups.

Zeta-Potential

QD-polypeptide assembly samples were prepared and diluted with Milli-Q water to the volume of the sample cell (250 μ L), while ensuring that the measured count rates ranged between 450 and 800 kcps. The samples were measured immediately after preparation at 25 °C using a ZetaPlus system (Brookhaven Instruments). Data were collected using the high

precision mode that is built into the *BIC Zeta Potential Analyzer* software. *Dynamic Light Scattering (DLS)*. Measurements were made using a 90Plus Particle Size Analyzer (Brookhaven Instruments) with a laser source emitting at 633 nm. Each QD- polypeptide assembly sample was measured immediately after preparation. All measurements were done at a fixed angle of 90°, temperature of 25 °C, and with the samples registering count rates that ranged between 80 and 200 kcps. Data were collected over an analysis time of 3 min, with first delay time of 5 µs and last delay time of 100 ms, and with a baseline difference kept within 1%. Data analyses of particle-size distributions were carried out using the *BIC Dynamic Research Software*. The CONTIN algorithm was used to analyze the acquired autocorrelation functions, and the reported particle-size distribution was intensity-weighted. Polydispersity of the assembly size is reported in terms of the variance of the particle-size distribution.

Fluorescence Optical Microscopy

Imaging of the QD-polypeptide assemblies was done using a Zeiss Axioplan 2 microscope. A small amount (~ 1 µL) of the QD-polypeptide assembly mixture was sandwiched between a glass slide and a cover slip, and the imaging was performed immediately. To capture the fluorescence of the QDs, which have an emission peak near 605 nm, a TRITC filter set was used.

Confocal Imaging of c(RGD)-Modified Assemblies Loaded with Fluorescently Labeled Ovalbumin

A solution of c(RGDyC)-PEGLL₁₁-PLL₁₂₉ (1 µL of 0.5 mg/mL) was mixed with fluorescently labeled ovalbumin (0.5 µL of 25 µg/mL) by vortexing. Alexa Fluor 647 labeled ovalbumin (Invitrogen) has absorbance and emission peaks of 650 nm and 665 nm, respectively. The appropriate amount of the QD suspension (80 nM) was then added such that $R' = 7.5 \times 10^{-3}$, and was mixed by repeated manual pipetting. Free ovalbumin that was not loaded into the assembly was not removed. The mixture was then imaged directly using a Leica SP2 microscope with a 40X oil-immersion objective having a numerical aperture (NA) = 1.25. To capture the fluorescence of the QDs and fluorescently labeled ovalbumin, the following settings were used: excitation/emission of 405 nm/580 – 620 nm (setting 1), 633 nm/640 – 670 nm (setting 2), respectively. Sequential scans were taken: one scan was acquired using setting 1 and the second scan was acquired immediately using setting 2. The images were then overlayed to determine the co-localization of the QD and ovalbumin fluorescence. To improve the signal- to-noise ratio, averaging (= 4) was used during the image acquisition. The absence of crossfluorescence was confirmed by imaging QD-polypeptide assemblies that had not been incubated with fluorescently labeled ovalbumin under setting 2.

Cell Culture

The human glioblastoma cell line U87MG (ATCC) was cultured in DMEM medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere containing 5% CO₂. All reagents were obtained from Invitrogen. *Live-Cell Imaging*. U87MG cell suspensions (50 µL of 10⁵ cells/mL) were seeded for 18 h

in each well of tissue-culture treated μ -Slide Angiogenesis (Ibidi). The cells were washed with phosphate buffered saline (PBS) pH 7.4, and incubated with the appropriate amount of the QD-polypeptide assembly mixture in integrin-binding buffer (IBB) for 1 h at 37 °C with 5% CO₂. The IBB has the following composition: 20 mM Tris (pH 7.5) with 1 mM MgCl₂, 1 mM MnCl₂, 2 mM CaCl₂, 100 mM NaCl, and 1 mg/mL bovine serum albumin (BSA) [41]. Subsequently, the cells were washed with ice-cold PBS three times and imaged using laser scanning confocal microscopy (Leica SP2 microscope). A 405 nm diode laser was used to excite the QDs, and QD emissions in the 580-620 nm range were detected using a photomultiplier tube. The cells were detected using the differential interference contrast (DIC) technique. Images were acquired using a 40X oil-immersion objective having NA 1.25. The DIC and fluorescence images were overlayed to determine the location of the QD fluorescence relative to the cell.

Flow Cytometry

U87MG cells grown to about 70 - 80% confluence were detached using 0.05% trypsin-EDTA. Approximately 350,000 cells were incubated with each QD- polypeptide assembly sample in IBB for 3 h at 4 °C with rotation. In the competitive-binding experiments, cells were incubated with a 2,500-fold molar excess of c(RGDfC) for 30 min on ice prior to the addition of the QD-polypeptide assembly mixture. Cells were then washed twice with ice-cold FACS buffer (PBS + 0.1% BSA) and analyzed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Treestar, Inc). The negative control for each c(RGD)-assembly sample corresponds to the unmodified assembly formed using the same PEGLL_x- PLL_y diblock copolypeptide system and R' value. In addition, the negative control contained the same concentration of PEGLL-PLL molecules as the c(RGD)-assembly mixture. The level of integrin-mediated binding was estimated by calculating the % difference between the geometric mean fluorescence intensities (GMFI) associated with the c(RGD)-assemblies and unmodified assemblies (negative control):

$$\frac{GMFI(c(RGD) - \text{assembly}) - GMFI(\text{unmodified assembly})}{GMFI(\text{unmodified assembly})} \times 100\%$$

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Gao XH, Cui YY, Levenson RM, Chung LWK, Nie SM. *Nat. Biotechnol.* 2004; 22:969. [PubMed: 15258594]
2. Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S. *Science.* 2005; 307:538. [PubMed: 15681376]
3. Cai WB, Shin DW, Chen K, Gheysens O, Cao QZ, Wang SX, Gambhir SS, Chen XY. *Nano Lett.* 2006; 6:669. [PubMed: 16608262]
4. Ballou B, Lagerholm BC, Ernst LA, Bruchez MP, Waggoner AS. *Bioconjug. Chem.* 2004; 15:79. [PubMed: 14733586]
5. Dubertret B, Skourides P, Norris DJ, Noireaux V, Brivanlou AH, Libchaber A. *Science.* 2002; 298:1759. [PubMed: 12459582]
6. Antonietti M, Forster S. *Adv. Mater.* 2003; 15:1323.
7. Discher DE, Ahmed F. *Annu. Rev. Biomed. Eng.* 2006; 8:323. [PubMed: 16834559]
8. Lian T, Ho RJY. *J. Pharm. Sci.* 2001; 90:667. [PubMed: 11357170]
9. Alexis F, Rhee J, Richie JP, Radovic-Moreno AF, Langer R, Farokhzad OC. *Urol. Oncol. -Semin. Orig. Investig.* 2008; 26:74.
10. Campbell RB, Ying B, Kuesters GM, Hemphill R. *J. Pharm. Sci.* 2009; 98:411. [PubMed: 18563780]
11. Park J, von Maltzahn G, Ruoslahti E, Bhatia SN, Sailor MJ. *Angew. Chem. -Int. Edit.* 2008; 47:7284.
12. Nasongkla N, Bey E, Ren J, Ai H, Khemtong C, Guthi JS, Chin S, Sherry AD, Boothman DA, Gao J. *Nano Lett.* 2006; 6:2427. [PubMed: 17090068]
13. Kim J, Lee JE, Lee SH, Yu JH, Lee JH, Park TG, Hyeon T. *Adv. Mater.* 2008; 20:478.
14. Yu MK, Jeong YY, Park J, Park S, Kim JW, Min JJ, Kim K, Jon S. *Angew. Chem. -Int. Edit.* 2008; 47:5362.
15. Atmaja B, Cha JN, Marshall A, Frank CW. *Langmuir.* 2009; 25:707. [PubMed: 19072205]
16. Discher DE, Eisenberg A. *Science.* 2002; 297:967. [PubMed: 12169723]
17. Cha JN, Birkedal H, Euliss LE, Bartl MH, Wong MS, Deming TJ, Stucky GD. *J. Am. Chem. Soc.* 2003; 125:8285. [PubMed: 12837100]
18. Wong MS, Cha JN, Choi KS, Deming TJ, Stucky GD. *Nano Lett.* 2002; 2:583.
19. Morille M, Passirani C, Vonarbourg A, Clavreul A, Benoit J. *Biomaterials.* 2008; 29:3477. [PubMed: 18499247]
20. Luo D, Saltzman WM. *Nat. Biotechnol.* 2000; 18:33. [PubMed: 10625387]
21. Schatzlein AG. *J. Biomed. Biotechnol.* 2003; 2003:149. [PubMed: 12721518]
22. Temming K, Schiffelers RM, Molema G, Kok RJ. *Drug Resist. Update.* 2005; 8:381.
23. Wayner EA, Orlando RA, Cheresh DA. *Cell Biol J.* 1991; 113:919.
24. Kimura RH, Cheng Z, Gambhir SS, Cochran JR. *Cancer Res.* 2009; 69:2435. [PubMed: 19276378]
25. Taga T, Suzuki A, Gonzalez-Gomez I, Gilles FH, Stins M, Shimada H, Barsky L, Weinberg KI, Laug WE. *Int. J. Cancer.* 2002; 98:690. [PubMed: 11920637]
26. Janssen M, Oyen WJG, Massuger LFAG, Frielink C, Dijkgraaf I, Edwards DS, Radjopadhye M, Corstens FHM, Boerman OC. *Cancer Biother. Radiopharm.* 2002; 17:641. [PubMed: 12537667]
27. Ahl PL, Bhatia SK, Meers P, Roberts P, Stevens R, Dause R, Perkins WR, Janoff AS. *Biochim. Biophys. Acta-Biomembr.* 1997; 1329:370.
28. Gregoriadis, G. *Liposome technology.* 3rd edn.. Vol. II. Informa Healthcare; New York: 2007.
29. Khemtong C, Kessinger CW, Gao J. *Chem. Commun.* 2009; 24:3497.
30. Jiang W, Kim BYS, Rutka JT, Chan WCW. *Nat. Nanotechnol.* 2008; 3:145. [PubMed: 18654486]
31. Chithrani BD, Chan WCW. *Nano Lett.* 2007; 7:1542. [PubMed: 17465586]
32. Mislick KA, Baldeschwieler JD. *Proc. Natl. Acad. Sci. U. S. A.* 1996; 93:12349. [PubMed: 8901584]
33. Rana RK, Murthy VS, Yu J, Wong MS. *Adv. Mater.* 2005; 17:1145.

34. Yang Y, Jiang J, Du B, Gan Z, Qian M, Zhang P. J. Mater. Sci. -Mater. Med. 2009; 20:301. [PubMed: 18791664]
35. Modi S, Jain JP, Domb AJ, Kumar N. Curr. Pharm. Des. 2006; 12:4785. [PubMed: 17168778]
36. Moon C, Kwon YM, Lee WK, Park YJ, Yang VC. J. Controlled Release. 2007; 124:43.
37. Simoes S, Moreira JN, Fonseca C, Duzgunes N, de Lima MCP. Adv. Drug Deliv. Rev. 2004; 56:947. [PubMed: 15066754]
38. Slepishkin VA, Simoes S, Dazin P, Newman MS, Guo LS, deLima MCP, Duzgunes N. J. Biol. Chem. 1997; 272:2382. [PubMed: 8999949]
39. Hermanson, GT. Bioconjugate Techniques. 1st edition edn.. Academic Press; 1996.
40. Cho K, Wang X, Nie S, Chen Z, Shin DM. Clin. Cancer Res. 2008; 14:1310. [PubMed: 18316549]
41. Silverman AP, Levin AM, Lahti JL, Cochran JR. J. Mol. Biol. 2009; 385:1064. [PubMed: 19038268]

Figure 1. Schematic of the application of quantum dot (QD)-polypeptide assemblies as dual imaging and targeted drug-delivery agents. The self-assembly between the QDs (red) and PEGLL-PLL diblock copolypeptide molecules (green), in which the PEGLL block adopts an α -helical conformation, forms an assembly that can take the shape of a shell-like structure. Receptor-mediated binding and endocytosis of assemblies modified with cancer-targeting ligands would allow molecular imaging and drug delivery to be performed simultaneously.

Figure 2. Fluorescence microscopy image of c(RGD)-modified assemblies, which appear as fluorescent “dots”, formed using c(RGDfC)-PEGLL₂₀-PLL₇₂ with a conjugation ratio of 4.6 and a charge ratio (R') = 8.5×10^{-3} (see Experimental). The negative control (inset), which corresponds to a QD suspension having the same QD concentration as that in the QD-polypeptide assembly mixture, did not exhibit any fluorescence. Scale bar: 3 μ m.

Figure 3.

Flow cytometry data performed using U87MG cells to illustrate the tunability of the QD-polypeptide assembly system. (A) Comparison of the level of non-specific binding exhibited by unmodified assemblies formed using two different architectures—PEGLL₂₀-PLL₇₂ and PEGLL₁₁-PLL₁₂₉—with $R' = 5.8 \times 10^{-3}$. Cells were incubated with varying concentrations of the unmodified assemblies; the concentration is reported as the concentration of the PEGLL-PLL molecules that constitute the assemblies. (B) Binding of QD-polypeptide assemblies as a function of the charge ratio (R'). c(RGD)-assemblies were formed using c(RGDfC)-PEGLL₂₀-PLL₇₂ conjugates having a conjugation ratio = 4.6; the Submitted to assembly concentration was ~ 0.7 pM. The level of integrin-mediated binding was estimated as the % difference between the mean fluorescence intensities associated with c(RGD)-modified and unmodified assemblies, and is depicted above the bars. See SI 4 for the calculation of the assembly concentration.

Figure 4.

Binding and microscopy data to demonstrate the feasibility of the c(RGD)-assembly system as a targeted imaging and biomolecule carrier system. (A) Binding of c(RGD)- assemblies to U87MG cells as evaluated by flow cytometry. To demonstrate integrin-binding specificity, cells were incubated with a 2500-fold molar excess of free c(RGDfC) prior to addition of the c(RGD)-assemblies. The assembly concentration was ~ 0.7 pM. (B) Binding and uptake of c(RGD)-assemblies by U87MG cells as evaluated by confocal microscopy. U87MG cells were incubated with ~ 0.1 pM of c(RGD)-assemblies in the absence (left) and presence (right) of a 100-fold molar excess of c(RGDfC). Scale bar: 20 μ m. In (A) and (B),

assemblies were formed using c(RGDfC)-PEG_{LL20}-PLL₇₂ conjugates having a conjugation ratio of 4.6 with $R' = 8.5 \times 10^{-3}$. (C) Confocal microscopy image showing the loading of Alexa Fluor 647 labeled ovalbumin into c(RGD)-assemblies. Co-localization of the ovalbumin fluorescence (red) with that of the QD-polypeptide assembly (blue) results in a pink color. A blow-up of the confocal microscopy image depicting the co-localization is shown (inset). Excess ovalbumin that was not loaded into the assemblies was not removed, and it formed aggregates (red) that were detected by confocal microscopy. Scale bar: 5 μm .