ResearchGate

See discussions, stats, and author profiles for this publication at: https://www.researchgate.net/publication/43225819

Functionalizable and Ultrastable Zwitterionic Nanogels

ARTICLE *in* LANGMUIR · MAY 2010

Impact Factor: 4.46 · DOI: 10.1021/la100664g · Source: PubMed

CITATIONS
31

READS
23

7 AUTHORS, INCLUDING:

Luo Mi
University of Washington Seattle
16 PUBLICATIONS **272** CITATIONS
SEE PROFILE

Zhiqiang Cao
Wayne State University
34 PUBLICATIONS **1,613** CITATIONS
SEE PROFILE

Qiuming Yu
University of Washington Seattle
59 PUBLICATIONS **1,820** CITATIONS
SEE PROFILE

Available from: Luo Mi
Retrieved on: 17 January 2016

Langmuir
Letter
pubs.acs.org/Langmuir
© 2010 American Chemical Society

Functionalizable and Ultrastable Zwitterionic Nanogels

Gang Cheng,[†,‡] Luo Mi,[‡] Zhiqiang Cao,[‡] Hong Xue,[‡] Qiuming Yu,[‡] Louisa Carr,[‡] and Shaoyi Jiang*[,‡]

[†]*Department of Chemical and Biomolecular Engineering, University of Akron, Akron, Ohio 44325, and* [‡]*Department of Chemical Engineering, University of Washington, Seattle, Washington 98195*

Received February 12, 2010. Revised Manuscript Received April 4, 2010

Multifunctional biomimetic nanogels based on zwitterionic poly(carboxybetaine methacrylate) (pCBMA) were synthesized by inverse microemulsion free-radical polymerization. pCBMA nanogels exhibited excellent stability in 100% fetal bovine serum. Dextran labeled with fluorescein isothiocyanate (FITC-dextran) was encapsulated in nanogels as a model drug. Encapsulated FITC-dextran exhibited controlled release from the pCBMA nanogel. Additionally, pCBMA nanogels carry abundant carboxylate groups as functional groups used to conjugate ligands to the nanogels for targeted drug delivery. Flow cytometry results obtained showed that pCBMA nanogels conjugated with cyclo[Arg-Gly-Asp-D-Tyr-Lys] ligands dramatically improved the nanogel uptake by primary human umbilical vein endothelium cells. Functionalizable zwitterionic pCBMA nanogels hold great potential as targeted drug-delivery vectors for biomedical applications.

Introduction

Hydrogels have been broadly used as implantable tissue scaffolds,[1] surface coatings for implantable sensors,[2] wound dressings,[3] and drug-delivery vectors[4] because of their high water content, biocompatibility, and low cytotoxicity.[5] Recently, there has been increased interest in developing hydrogel particles on the nanometer scale (i.e., nanogels) as drug-delivery carriers because of their high drug-loading capacity, excellent biocompatibility, and responsiveness to environmental factors such as temperature and pH.

One of the major challenges of current nanoparticle drug-delivery carriers is limited blood circulation time after intravenous (IV) systemic administration and quick uptake by the liver and spleen because of nonspecific protein adsorption onto the particles from blood.[6] The stability of nanoparticles in blood is critical to the success of drug delivery and nanoparticle-based diagnostics. To achieve a prolonged blood circulation time, nanoparticles are modified with neutral and hydrophilic materials to reduce nonspecific protein adsorption from blood.[7] Although many materials have been developed to resist nonspecific protein adsorption, very few materials can achieve ultralow fouling, which is defined as less than 5 ng/cm^2 adsorbed fibrinogen.[8] Our previous studies demonstrate that zwitterionic poly(carboxybetaine methacrylate) (pCBMA) highly resists nonspecific protein adsorption even from undiluted blood plasma and serum.[9−14]

There is an increased interest in developing localized therapeutic methods to enhance drug-delivery efficiency and reduce the side effects of drugs. The most commonly used method is to covalently link targeting moieties to drug-delivery vectors. This method requires functional groups such as tetrazole[15] and carboxylate groups[16] to be incorporated into the drug-delivery vectors. Excessive unreacted functional groups, however, can cause nonspecific protein adsorption, thus reducing the blood circulation time of the nanoparticles. The pCBMA nanogel has abundant functional groups for the convenient and effective immobilization of biomolecules. Any unreacted activated groups will be converted back to nonfouling zwitterionic groups upon hydrolysis. Thus, pCBMA can both prevent nonspecific binding and immobilize ligands in the material.

The objective of this study is to investigate the nonfouling and functionalization properties of pCBMA nanogels for targeted drug delivery. pCBMA nanogels were synthesized using an inverse microemulsion method.[17] Dextran labeled with fluorescein isothiocyanate (FITC-dextran) was encapsulated into nanogels as a model drug. The release rate of FITC-dextran was determined. Human umbilical vein endothelial cells (HUVECs) were used as a model cell system to test the targeting capability of pCBMA nanogels conjugated with cyclo[Arg-Gly-Asp-D-Tyr-Lys] (cRGD), which can selectively bind cells such as HUVECs expressing $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins.[18]

*Corresponding author. E-mail: sjiang@u.washington.edu.

(1) Langer, R.; Vacanti, J. P. *Science* **1993**, *260*, 920–926.
(2) Galeska, I.; Hickey, T.; Moussy, F.; Kreutzer, D.; Papadimitrakopoulos, F. *Biomacromolecules* **2001**, *2*, 1249–1255.
(3) Corkhill, P. H.; Hamilton, C. J.; Tighe, B. J. *Biomaterials* **1989**, *10*, 3–10.
(4) Peppas, N. A.; Bures, P.; Leobandung, W.; Ichikawa, H. *Eur. J. Pharm. Biopharm.* **2000**, *50*, 27–46.
(5) Peppas, N. A.; Hilt, J. Z.; Khademhosseini, A.; Langer, R. *Adv. Mater.* **2006**, *18*, 1345–1360.
(6) Raemdonck, K.; Demeester, J.; De Smedt, S. *Soft Matter* **2009**, *5*, 707–715.
(7) Vinogradov, S.; Batrakova, E.; Kabanov, A. *Colloids Surf., B* **1999**, *16*, 291–304.
(8) Tsai, W. B.; Grunkemeier, J. M.; Horbett, T. A. *J. Biomed. Mater. Res.* **1999**, *44*, 130–139.
(9) Zhang, Z.; Chen, S. F.; Chang, Y.; Jiang, S. Y. *J. Phys. Chem. B* **2006**, *110*, 10799–10804.
(10) Chang, Y.; Chen, S. F.; Zhang, Z.; Jiang, S. Y. *Langmuir* **2006**, *22*, 2222–2226.
(11) Chang, Y.; Chen, S. F.; Yu, Q. M.; Zhang, Z.; Bernards, M.; Jiang, S. Y. *Biomacromolecules* **2007**, *8*, 122–127.
(12) Zhang, Z.; Chen, S. F.; Jiang, S. Y. *Biomacromolecules* **2006**, *7*, 3311–3315.
(13) Ladd, J.; Zhang, Z.; Chen, S.; Hower, J. C.; Jiang, S. Y. *Biomacromolecules* **2008**, *9*, 1357–1361.
(14) Yang, W.; Xue, H.; Li, W.; Zhang, J.; Jiang, S. Y. *Langmuir* **2009**, *25*, 11911–11916.
(15) Tominey, A.; Andrew, D.; Oliphant, L.; Rosair, G. M.; Dupre, J.; Kraft, A. *Chem. Commun.* **2006**, *23*, 2492–2494.
(16) Nayak, S.; Lee, H.; Chmielewski, J.; Lyon, L. A. *J. Am. Chem. Soc.* **2004**, *126*, 10258–10259.
(17) Shi, L. J.; Khondee, S.; Linz, T. H.; Berkland, C. *Macromolecules* **2008**, *41*, 6546–6554.
(18) Ogawara, K. I.; Rots, M. G.; Kok, R. J.; Moorlag, H. E.; van Loenen, A. M.; Meijer, D. K. F.; Haisma, H. J.; Molema, G. *Hum. Gene Ther.* **2004**, *15*, 433–443.

Experimental Section

Chemicals. 2-(Dimethyl amino)ethyl methacrylate (DMAEMA), *N*,*N*′-methylene-bis-acrylamide (MBAA), phosphate-buffered saline (PBS), sodium salicylate, acetonitrile, ethyl ether, Span 80, Tween 80, and fluorescein isothiocyanate-dextran (FITC-dextran) (MW 10 000) were purchased from Sigma-Aldrich (St. Louis, MO). 1-Ethyl-3-[3-dimethylaminopropyl]-carbodiimide hydrochloride (EDC) was purchased from Pierce Biotechnology, Inc. (Rockford, IL). *N*-Hydroxysulfosuccinimide (sulfo-NHS) was purchased from Acros Organics (Geel, Belgium). Cyclo[Arg-Gly-Asp-D-Tyr-Lys] (cRGD) was purchased from Peptides International (Louisville, KY). Tetrahydrofuran (THF) and hexane were purchased from J. T. Baker (Phillipsburg, NJ). 2,2′-Azobis(4-methoxy-2.4-dimethyl valeronitrile) (V-70) was purchased from Wako Pure Chemical Industries (Osaka, Japan). 2-Carboxy-*N*,*N*-dimethyl-*N*-(2′-(methacryloyloxy)ethyl)ethanaminium (carboxybetaine methacrylate, CBMA) was synthesized by the reaction of DMAEMA and β-propiolactone using a method published previously.[9,12]

Inverse Microemulsion Polymerization of pCBMA Nanogels. In a typical reaction, pCBMA nanogels were prepared via inverse microemulsion polymerization, following a method reported previously.[17] The continuous-phase solution contains 40 mL of hexane, 1.4 g of Tween 80, 1.6 g of Span 80, and 8 mg of V-70. The solution was kept on ice. Aqueous monomer stock solutions were prepared by dissolving 229 mg (1 mmol) of CBMA and 4.6 mg (0.03 mmol) of MBAA in 0.5 mL of DI water. Then an aqueous stock solution was added to a 100 mL flask containing 40 mL of continuous-phase solution followed by vigorous shaking and 2 min of sonication. The flasks were purged with nitrogen at 4 °C for 30 min to remove dissolved oxygen. During polymerization, the reaction was kept at 40 °C with stirring and was protected under nitrogen for 4 h. For the synthesis of pCBMA nanogels containing FITC-dextran, the conditions are the same as those for pCBMA nanogels without dextran except that 10 mg of FITC-dextran was added to the aqueous stock solution.

Purification of Nanogels. Ten milliliters of the reaction solution was mixed with 30 mL of THF and stirred for 5 h to remove surfactants. The mixture was centrifuged for 40 min at 4400 rpm. The supernatant was discarded, and the precipitate was washed twice with 30 mL of THF. The final precipitate was resuspended in 4 mL of DI water for 4 h, and the aqueous solution was placed into a 100 kD molecular weight cutoff Amicon Ultra centrifugal filter device (Millipore, Burlington, MA) to remove the liquid. pCBMA nanogels were resuspended in 4 mL of DI water. The wash was repeated 10 times at room temperature. Then, the aqueous solution containing nanogels was filtered through a sterile 0.45 μm PTFE syringe filter and stored at 4 °C for further characterization. The concentration of nanogels was measured by weighing the material before and after lyophilization. The yields for nanogels containing no dextran with 1.5, 3, and 5% cross linker (molar concentration) are 36.2, 51.3, and 40%, respectively.

The hydrodynamic diameter and polydispersity index of pCBMA nanogels were analyzed by a Zetasizer Nano ZS dynamic light scattering (DLS) instrument (Malvern, U.K.) at a wavelength of 633 nm. A scattering angle of 173° was used, and the temperature was 25 °C. The dispersant refractive index and the viscosity of water were taken to be 1.330 and 0.8872 cP, respectively.

Release of Encapsulated Dextran from pCBMA Nanogels. The release of encapsulated FITC-dextran from pCBMA nanogels with 1.5% cross linker was determined. Forty milligrams of the purified pCBMA nanogels with FITC-dextran was resuspended in 20 mL of DI water. At time zero, 1 mL of solution was taken from the pCBMA nanogel solution, and its total fluorescence density was measured. At different time points, 1 mL of solution was placed into a 100 kD molecular-weight-cutoff Amicon Ultra centrifugal filter device (Millipore, Burlington, MA) and centrifuged at 4400 rpm for 90 min to collect flow-through for FITC fluorescence detection. The fluorescence density of the filtrate at 515 nm was measured at 25 °C with a fluorescence spectrophotometer (F-4500 fluorescence spectrophotometer, Hitachi, Japan) with an excitation wavelength of 495 nm and a cutoff wavelength of 500 nm. The percentage of released FITC-dextran was defined as the ratio of the fluorescence density of flow-through at different time points to the total fluorescence density at time zero.

Functionalization of pCBMA Nanogels. Fifty milligrams of pCBMA nanogels with 5% MBAA was resuspended in 2 mL of DI water. One hundred fifty-three milligrams of EDC and 23 mg of sulfo-NHS were added to the pCBMA nanogel solution, and the solution was incubated at 25 °C for 30 min to activate the carboxylate group of pCBMA nanogels. Then, 1.3 mg of cRGD was added to the activated pCBMA nanogel solution and the pH value of the solution was adjusted to 8.5−9.0. The reaction was incubated at 25 °C for 3 h. The reaction solution was placed into a 100 kD molecular-weight-cutoff Amicon Ultra centrifugal filter device (Millipore, Burlington, MA) to remove reactants. The nanogels were resuspended in 10 mL of DI water and again passed through a 100 kD molecular-weight-cutoff Amicon Ultra centrifugal filter device. The wash was repeated 10 times at room temperature.

Nanogel Cytotoxicity Assay. Cell viability was assessed using a Vybrant MTT cell proliferation assay kit (Invitrogen, Carlsbad, CA). Human umbilical cord vascular endothelial cells (HUVEC) were seeded in 96-well tissue culture plates at a density of 7000 cells/well and cultured in 100 μL of phenol red-free medium 200 supplemented with low serum growth supplement (Invitrogen, Carlsbad, CA). Cells were incubated in 100 μL of phenol red-free medium 200 with nanogels at various concentrations at 37 °C for 4 h, and then the metabolic activity was determined by using a MTT assay. After the medium was removed, 100 μL of phenol red-free medium 200 and 10 μL of 12 mM MTT stock solution in PBS were added to each well. The samples were incubated at 37 °C for 4 h. Then, the medium was removed and 50 μL of DMSO was added and incubated for 10 min. The absorbance at 570 nm was read with a 96-well plate reader (SpectraMax M5, Molecular Devices, Sunnyvale, CA). Cell viability was expressed as the percentage of absorbance of treated cells relative to the absorbance of cells that were not incubated with pCBMA nanogels. Each measurement had five replicate wells.

Flow Cytometry. HUVEC cells were seeded into 24-well tissue culture plates at a density of 10 000 cells/well and cultured in 500 μL of medium 200 supplemented with low serum growth supplement (Invitrogen, Carlsbad, CA) at 37 °C for 24 h. Then, cells were incubated with 500 μL of serum-free medium 200 containing 1.0 or 0.2 mg/mL FITC-dextran loaded pCBMA nanogels (5% MBAA) conjugated with or without cRGD at 37 °C for 4 h. After the medium was replaced with 500 μL of free medium 200 supplemented with low serum growth supplement, HUVEC cells were incubated at 37 °C for 12 h. Then, the medium was removed and the cells were washed three times with PBS. After detachment by trypsin, HUVEC cells were resuspended in PBS with 1% fetal bovine serum. The cellular uptake of pCBMA nanogels was analyzed by flow cytometry (FACScan, BD).

Results and Discussion

pCBMA nanogels were synthesized by inverse microemulsion free-radical polymerization.[17] Because the potential application of pCBMA nanogels as carriers involves temperature-sensitive biologically active compounds such as protein, DNA, or RNA, polymerization was initiated by using low-temperature free-radical imitator V-70 at 40 °C. The size of the nanoparticles can greatly influence their blood circulation time. It is reported that nanoparticles smaller than 200 nm have less chance to be cleared by Kupffer cells and splenic filtration.[19] Furthermore, it is also

(19) Moghimi, S. M.; Hunter, A. C.; Murray, J. C. *FASEB J.* **2005**, *19*, 311–330.

Table 1. Sizes of pCBMA Nanogels with or without Encapsulated FITC-dextran in DI Water

sample	diameter (nm) in DI water (polydispersity index)
1.5% MBAA	104 (0.12)
1.5% MBAA + dextran	100 (0.16)
3% MBAA	117 (0.25)
3% MBAA + dextran	109 (0.15)
5% MBAA	99 (0.20)
5% MBAA + dextran	94 (0.21)

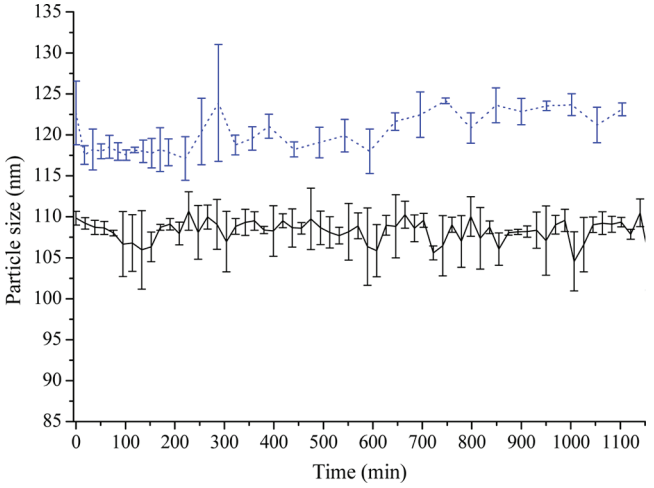

Figure 1. Stability of pCBMA nanogels with 3% MBAA (blue dotted line) and 1.5% MBAA (black solid line) in 100% fetal bovine serum at 37 °C as a function of time.

reported that small particles (< 200 nm) can more effectively extravasate into tumors.[20] In this study, the size of the nanogels was maintained below 200 nm by adjusting the ratio and the concentration of the two surfactants (Tween 80 and Span 80). The hydrodynamic size and polydispersity of pCBMA nanogels (Table 1) were analyzed by dynamic light scattering in DI water. The sizes of pCBMA nanogels with 1.5, 3, and 5% MBAA (molar concentration) were 104, 117, and 99 nm, respectively. The size distribution of nanogels with 1.5% MBAA is the narrowest among these nanogels.

The stability of zwitterionic nanogels in the complex medium was investigated (Figure 1) by measuring the size change of nanogels as a function of time in 100% fetal bovine serum (FBS). Both nanogels with 1.5 and 3% MBAA retained their original sizes after an 18 h incubation in 100% FBS. The major challenge for the intravenous administration of nanogels is that the adsorption of blood proteins on the nanogels can destabilize the nanogel and lead to fast clearance by liver, spleen, and macrophage cells before the nanogel can reach its intended target.[6] Neutral and hydrophilic materials such as poly(ethylene glycol) (PEG)[21] and zwitterionic carboxybetaine polymers[22] have been coated onto nanoparticles to reduce nonspecific protein adsorption. Recently, zwitterionic materials have been systemically studied for their ability to resist nonspecific protein adsorption on various surfaces, and it was reported that protein adsorption from 100% human blood plasma and serum on

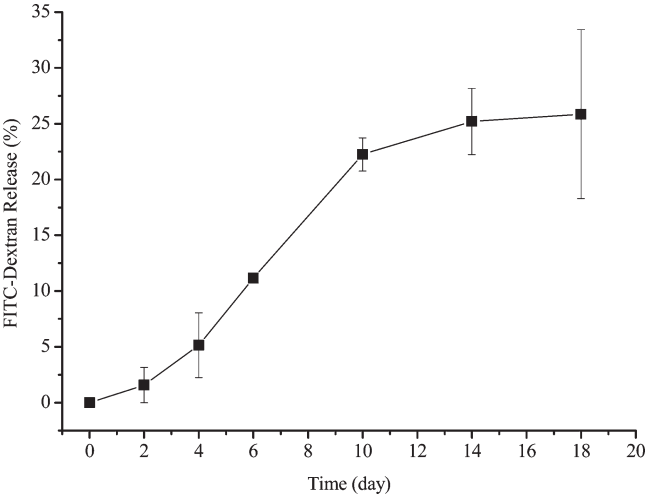

Figure 2. *In vitro* FITC-dextran release from pCBMA nanogels with 1.5% MBAA. FITC-dextran release was measured by a fluorescence spectrophotometer. The results are averaged from three replicates.

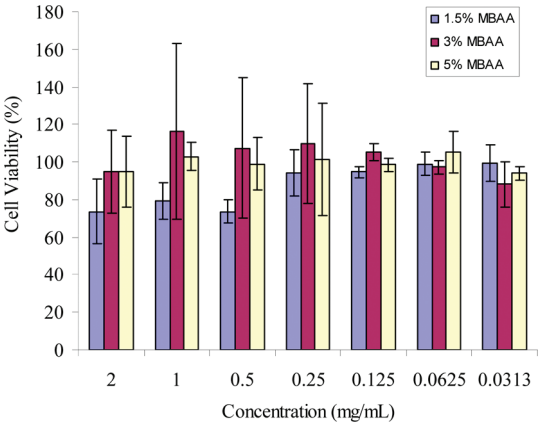

Figure 3. Cytotoxicity of pCBMA nanogels against HUVECs as determined by MTT assay.

pCBMA-coated gold surface is less than 0.3 ng/cm^2.[14,23] These nanogels based on ultralow fouling zwitterionic carboxybetaine are very promising as ultrastable drug-delivery carriers.

FITC-dextran was encapsulated in pCBMA nanogels as a model drug. Figure 2 shows the controlled release of FITC-dextran from pCBMA nanogel as a function of time. Twenty-five percent of the FITC-dextran initially encapsulated in the pCBMA-2 hydrogel was released after 18 days. Because of their strong hydration and excellent biocompatibility, hydrogels have been extensively studied as controlled-release drug-delivery vectors. Macromolecules can be released from the matrix through diffusion or environmental stimuli such as changes in pH and temperature. Environmental stimuli can lead to faster release because of the decomposition of the matrix or the increased pore size of the matrix.[5,24] To investigate the intrinsic capacity and properties of novel zwitterionic pCBMA as a drug-delivery carrier, in this study the release rate of encapsulated FITC-dextran is simply controlled by diffusion, which is determined by the molecular weight of the encapsulated drug and the pore size of the nanogels. FITC-dextran with a molecular weight of 10 kD and 1.5% cross linker was used. The release rate can be adjusted by controlling the ratio between MBAA and CBMA monomers

(20) Couvreur, P.; Vauthier, C. *Pharm. Res.* **2006**, *23*, 1417–1450.
(21) Gref, R.; Luck, M.; Quellec, P.; Marchand, M.; Dellacherie, E.; Harnisch, S.; Blunk, T.; Muller, R. H. *Colloids Surf., B* **2000**, *18*, 301–313.
(22) Matsuura, K.; Ohno, K.; Kagaya, S.; Kitano, H. *Macromol. Chem. Phys.* **2007**, *208*, 862–873.
(23) Cheng, G.; Li, G.; Xue, H.; Chen, S.; Bryers, J. D.; Jiang, S. Y. *Biomaterials* **2009**, *30*, 5234–5240.
(24) Lin, C. C.; Metters, A. T. *Adv. Drug Delivery Rev.* **2006**, *58*, 1379–1408.

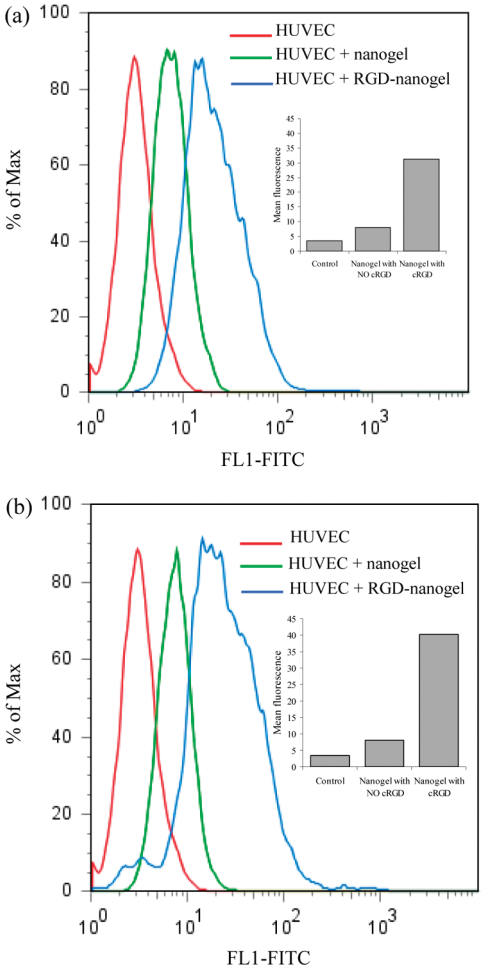

Figure 4. Flow cytometric analysis of the uptake of pCBMA nanogels [(5% MBAA), red (no pCBMA nanogels); green (FITC-dextran loaded pCBMA nanogels); and blue (FITC-dextran loaded pCBMA nanogels conjugated with cRGD)] at concentrations of (a) 0.2 and (b) 1 mg/mL.

in aqueous stock solutions, depending on the size and hydrophilicity of a drug used for a specific application.

The cytotoxicity of the nanogels was assessed by measuring the viability of primary HUVECs as a function of the concentration of pCBMA nanogels. The effect of the concentration of the cross-linker, MBAA, was also investigated. Results in Figure 3 show that pCBMA nanogels with 1.5% MBAA as the cross linker have minimal cytotoxicity even at high concentrations (2 mg/mL).

cRGD as a targeting ligand was conjugated to FITC-dextran-loaded pCBMA nanogels using EDC/sulfo-NHS chemistry in water. The cellular uptake of FITC-labeled nanogels was quantified with a flow cytometer. pCBMA nanogels conjugated with cRGD showed a higher uptake by HUVECs than by pCBMA nanogels without cRGD (Figure 4). pCBMA nanogels at a concentration of 1 mg/mL lead to a higher uptake of pCBMA nanogels than at a concentration of 0.2 mg/mL. The ratios of mean fluorescence intensities between cells containing cRGD-conjugated nanogels and cells containing bare pCBMA nanogels are 4.99 and 3.87 for nanogels at concentrations of 1 and 0.2 mg/mL, respectively. For conventional coatings on nanoparticles, mixed functional and nonfouling groups such as the hydroxyl-terminated poly(ethylene glycol) (PEG) and carboxylate-terminated PEG are commonly used.[25] Because of the limitation of conjugation efficiency, unreacted functional groups such as carboxylate and amine groups will cause nonspecific protein adsorption onto nanoparticle surfaces[26] in these traditional systems. However, pCBMA does not have such a problem because pCBMA can do both jobs of nonfouling and functionalization in one material. Any unreacted functional groups in pCBMA can be hydrolyzed back into nonfouling zwitterionic groups. Thus, the density of targeting agents on pCBMA nanogels can be controlled simply by adjusting the concentration of the targeting agent during ligand conjugation.

Conclusions

In this work, multifunctional nanogels based on pCBMA were synthesized by an inverse microemulsion free-radical polymerization method. pCBMA nanogels exhibited excellent biophysical stability in 100% fetal bovine serum and had minimal cytotoxicity. The controlled release of FITC-dextran encapsulated within pCBMA nanogels was demonstrated; FITC-dextran initially encapsulated in pCBMA-2 hydrogels was gradually released. The release rate depends on the particular hydrogels and drugs used and can be readily controlled. Furthermore, each side chain of the pCBMA nanogels contains a carboxylate group for ligand immobilization. The results obtained from flow cytometry indicated that cRGD ligands dramatically increased the uptake of nanogels by human umbilical vein endothelium cells. These functionalizable zwitterionic nanogels hold great potential as targeted drug-delivery vectors because of their ultrastability and multiple functionality all in one chemically uniform particle.

Acknowledgment. This work was supported by the Office of Naval Research (N000140910137).

(25) Farokhzad, O. C.; Cheng, J. J.; Teply, B. A.; Sherifi, I.; Jon, S.; Kantoff, P. W.; Richie, J. P.; Langer, R. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 6315–6320.

(26) Gessner, A.; Lieske, A.; Paulke, B. R.; Muller, R. H. *Eur. J. Pharm. Biopharm.* **2002**, *54*, 165–170.