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COMMUNICATIONS

Published on Web 02/23/2006

Tumor Targeting by Surface-Modified Protein Microspheres

Farah Jean-Jacques Toublan, Stephen Boppart, and Kenneth S. Suslick*

Department of Chemistry and Beckman Institute for Advanced Science and Technology, University of Illinois at Urbana−Champaign, 600 South Mathews Avenue, Urbana, Illinois 61801

Received July 5, 2005; E-mail: ksuslick@uiuc.edu

Core−shell protein microspheres are micron-sized, highly biocompatible structures, with an outer shell made from disulfide cross-linked protein roughly 50 nm thick and a core containing air or a nonaqueous liquid.[1] They are easily prepared during ultrasonic emulsification, particularly of serum albumin.[1,2] Such microspheres have been used in multiple biomedical imaging modalities, including magnetic resonance imaging (MRI), thermometric and oximetric imaging, sonography (e.g., Albunex, the first FDA approved echo contrast agent), and optical coherence tomography (OCT).[2−4] The ability of these vesicles to encapsulate hydrophobic material has also generated interest in drug delivery applications.[5,6] To date, however, selective targeting of protein microspheres to tumors has not been effectively achieved. Because of the availability of amine and carboxylic acid groups on the surface of the microspheres, most surface modification methods explored so far have been covalent in nature. Here we report a noncovalent, electrostatic layer-by-layer (LBL) modification that successfully targets protein microspheres to the integrin receptors that are overexpressed in several tumor types.

LBL self-assembly relies on interactions between a template and macromolecules for the deposition of material one layer at a time to the template's surface.[7] LBL self-assembly can be controlled by many different types of interactions: electrostatic, receptor−ligand, and metal−ligand binding interactions.[7,8] The LBL technique has recently found widespread applications in the coating of three-dimensional structures, such as polymer tubules, latex spheres, inorganic colloidal materials, and even a few types of biological cells.[9−13]

Our protein microspheres are core−shell vesicles with a vegetable oil core and a bovine serum albumin (BSA) shell that is made up of plates of cross-linked protein subunits which tile over the oil core like armadillo scales. The BSA molecules in the shell are not significantly denatured and are held together by the sonochemical formation of disulfide bonds via inter-protein cysteine oxidation.[1] These core−shell microspheres are highly charged due to the numerous ionizable groups present in the BSA shell. Under physiological conditions, BSA typically has 185 counterions resulting from both acidic and basic surface residues, with a net charge of −17 at pH 7.[14] For a typical 2 μm microsphere, there are $\sim 10^6$ BSA molecules per shell. Thus, the protein microspheres are sufficiently charged for electrostatic adhesion of polyelectrolytes onto their surface. We have found that these negatively charged vesicles are excellent templates for LBL electrostatic adhesion. In this paper, we show that protein microspheres can be selectively targeted to human tumor cells by using an LBL approach to modify their surface with integrin-receptor specific peptide ligands.

Integrin receptors are heterodimer, transmembrane receptors that have a wide range of functions: cell survival, migration, proliferation, differentiation, and death. Recently, these receptors have been shown to play a key role in cancer metastases and tumorigenesis.[15,16] There are over 25 known integrin receptors, and most of these recognize the small tripeptide turn sequence arginine−glycine−aspartic acid (RGD).[17,18] Integrin receptors are overexpressed in several tumor types, and the RGD tripeptide motif has been used as a label for these tumor cells and their vasculature.[19,20]

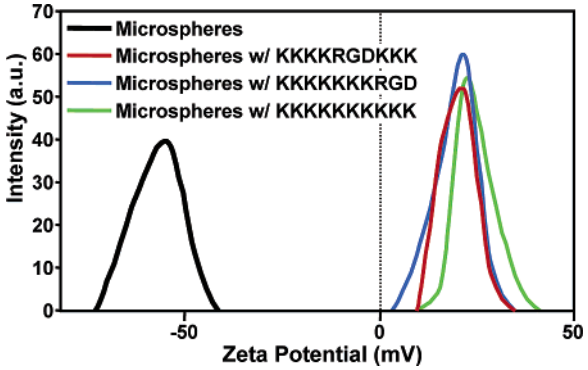

Figure 1. The ζ-potential measurements of native BSA microspheres (black) and BSA microspheres after LBL modification with polylysine (KKKKKKKKKK) (green) or RGD polylysine peptides (red and blue). A negative ζ-potential means that the microsphere has a net negative surface charge, and vice versa.

We synthesized three different peptides with an RGD motif embedded at the ends or in the middle of a highly positively charged, polylysine sequence: at the amino terminus, RGD-KKKKKK; in the middle, KKKKRGDKKK; and at the carboxy terminus, KKKKKKKRGD. The positively charged lysine residues electrostatically secure the RGD motif to the surface of the microspheres. An additional decapeptide polylysine, K_{10}, was prepared as a control. These peptides were synthesized using standard Fmoc peptide chemistry with a semiautomatic solid-phase peptide synthesizer, purified using HPLC, and characterized by MALDI-TOF-MS.

The purified peptides are then used in the LBL electrostatic adhesion to decorate the surface of the protein microspheres. The success of the adhesion was determined by measuring the ζ-potential (i.e., net particle charge) of the microspheres before and after adhesion of peptides. As shown in Figure 1, the ζ-potential of the microspheres from −54 mV before modification to +22 mV after peptide adhesion occurred. In addition, we are able to visualize the effects of peptide adhesion by adhesion of a second layer of negatively charged silica nanoparticles. In this process, the microspheres were exposed first to the polycationic RGD-containing peptides to reverse their surface charge, and then to silica colloids (∼100 nm), which are negatively charged at pH 7.4 and do not adhere to native (i.e., negatively charged) BSA microspheres. The SEM images of the microspheres in Figure 2 confirm the adhesion of the positive peptides since a uniform layer of silica nanoparticle is produced. These polylysine sequences, even though relatively small (i.e., ∼1000 amu), allow for uniform and complete coverage of the microspheres.

10.1021/ja0544455 CCC: $33.50

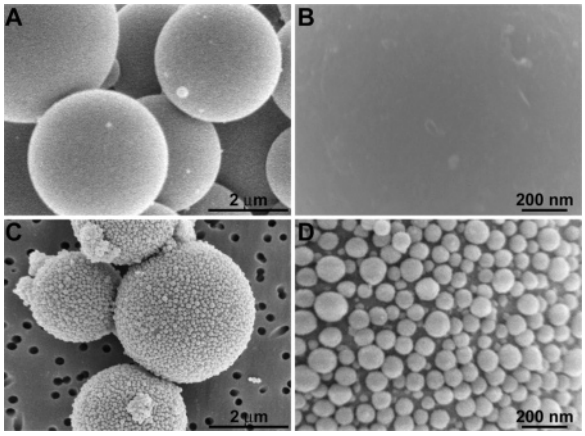

Figure 2. SEM visualization of LBL adhesion: native BSA microspheres (A) and close-up (B) versus silica-coated microspheres using a RGD polylysine peptide to reverse surface charge (C) and close-up (D).

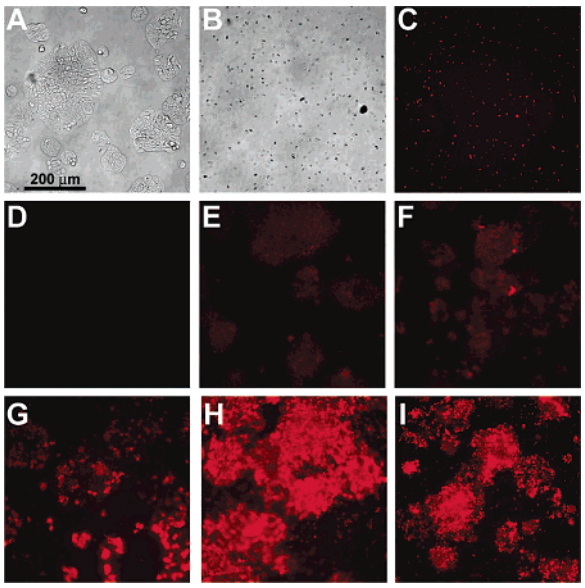

Figure 3. Uptake of fluorescent microspheres by HT29 tumor cells. Bright field micrographs of (A) cells and (B) fluorescent microspheres containing Nile red. Fluorescence micrographs of (C) fluorescent microspheres, (D) cells, (E) cells exposed to unlabeled microspheres, (F) cells exposed to K_{10}-coated microspheres, (G) cells exposed to KKKKRGDKKK-labeled microspheres, (H) cells exposed to RGDKKKKKK-labeled microspheres, and (I) cells exposed to KKKKKKKRGD-labeled microspheres.

We determined the efficacy of the RGD-modified microspheres in tumor targeting by using HT29 tumor cells in vitro. HT29 cells are human colon tumor cells which are known to overexpress integrin receptors.[21−23] For the targeting experiments, microspheres were synthesized with 250 μg/mL Nile red fluorescent dye in vegetable oil in their core. These microspheres were modified with K_{10} and silica colloids (microspheres in Figure 3, micrograph C). The resulting fluorescent microspheres are then used as a fluorescent tag to identify microsphere adhesion to and uptake by HT29 tumor cells. The silica-modified fluorescent-core microspheres are decorated with the RGD-containing peptides. Then the adherent cells are incubated with these red fluorescent RGD-modified microspheres in serum free media at 37 °C under 5% CO_2. The silica layer gives the microsphere sufficient density to be heavier than water and therefore come in contact with the HT29 cells. Following incubation, excess microspheres are removed by exhaustive washings of the cells with PBS. Finally, the cells are imaged using a Leica-IRE2 inverted fluorescence microscope. The fluorescence micrographs in Figure 3 demonstrate that binding of RGD-modified microspheres is increased relative the unmodified ones. The microspheres modified with the RGDKKKKKK peptide exhibit the best binding to the tumor cells, with the KKKKRGDKKK peptide binding the least.

In summary, we have prepared serum albumin microspheres whose surface has been modified by layer-by-layer electrostatic adhesion of peptides containing an integrin-receptor specific sequence. These modified microspheres can be utilized as an effective target for integrin expressing cells and more specifically colon tumor cells in vitro. This should be an easily generalized method for the labeling of microspheres with peptide ligands to other important cell membrane receptors, which opens a new tool for both targeted imaging and targeted drug delivery.

Acknowledgment. This work was supported by the NSF and in part the NIH (HL25934). We also acknowledge DOE support (DEFG02-91ER45439) to the Center for Microanalysis of Materials, UIUC Seitz Materials Research Laboratory. We thank Professors J. Lewis for access to instrumentation in her laboratory, and Mrs. Wei Luo for valuable discussions.

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JA0544455