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COMMUNICATIONS

Published on Web 09/13/2002

Synthesis of Vesicles on Polymer Template

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Received April 8, 2002

A novel type of self-assembled materials was recently synthesized by reacting a block ionomer, poly(ethylene oxide)-*b*-poly(sodium methacrylate) (PEO-*b*-PMA), with single-tail cationic surfactants.[1,2] The surfactant ionic groups form ion pairs with PMA chain, while the surfactant tails segregate into hydrophobic clusters. PEO segments prevent macroscopic phase separation and stabilize complexes in aqueous dispersions. Resulting complexes spontaneously arrange into small vesicles (ca. 80−100 nm) composed of closed bilayers from PMA-bound surfactant, with corona from PEO chains.[2] Such complexes are in thermodynamic equilibrium with the surfactant ions present in solution. These materials can be used as pharmaceutical drug carriers. However, the complexes formed by single-tail surfactants are not stable and can disintegrate upon dilution that may occur in biological fluids. One way to stabilize them is to chemically link surfactant components with each other. A reactive single-tail surfactant, isothiuroniumethylhexadecyldimethylammonium bromide (C_{16}SU) that dimerize upon cleavage of isothiuronium group at elevated temperature or alkali pH was developed recently.[3] Herein, we report the reactive stabilization of vesicles from C_{16}SU cations assembled on anionic PEO-*b*-PMA template. The template-directed immobilization of C_{16}SU into the block ionomer complex results in formation of vesicles that are stable in a broad range of environmental conditions (pH, temperature, salt concentration).

The diblock copolymer PEO-*b*-PMA was reacted with C_{16}SU at various compositions of the mixture.[4] When the concentration of the surfactant was close to that of the carboxylate groups the particle net charge was close to zero (i.e., the complexes were stoichiometric). Despite charge neutralization, the resulting dispersions were optically transparent or slightly opalescent. Diameters of the complex particles remained rather small (80−90 nm) in the entire range of compositions; the polydispersity indexes were ca. 0.17.[5] Mixing of components followed by treatment with the oxidizing agent resulted in formation of complexes containing surfactant dimers (PEO-*b*-PMA/*d*C_{16}SU).[6] Preparation of the complexes in the presence of 10 mM of dithiotreitol (DTT), which prevents the formation of disulfide bonds, led to the complexes with C_{16}SU in the monomer form (PEO-*b*-PMA/*m*C_{16}SU). The formation of disulfide bonds between surfactant molecules was accompanied with the increase in the size of PEO-*b*-PMA/*d*C_{16}SU complexes as well as a slight decrease of the net charge of the particles, which is consistent with the removal of positively charged isothiouronium groups[3] (Table 1). Subsequent measurements with these samples showed no change in ζ-potential and size of the complexes for at least several weeks. The morphology of the complexes was examined by electron microscopy using negative staining and freeze fracture techniques.[7] Typical micrographs of PEO-*b*-PMA/*d*C_{16}SU complexes are presented in Figure 1. The images reveal small spheres similar to those observed previously with vesicles of block ionomer complexes of PEO-*b*-PMA and cationic single-tail surfactants.[2] The particle morphology practically did not change upon oxidation of C_{16}SU molecules as similar images were observed for PEO-*b*-PMA/*m*C_{16}SU complexes (data not shown).

Table 1. ζ-Potential and Effective Diameters of PEO-*b*-PMA/*m*C_{16}SU and PEO-*b*-PMA/*d*C_{16}SU Stoichiometric Complexes (10 mM borate buffer, pH 9.5)

complex	D_{eff}, nm	ζ-potential, mV
PEO-*b*-PMA/*m*C_{16}SU	86	−0.1
PEO-*b*-PMA/*d*C_{16}SU	99	−4.0

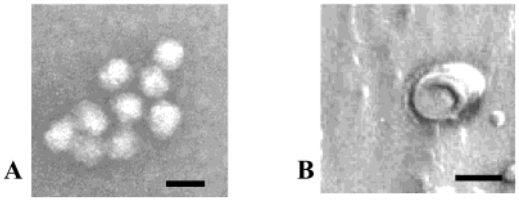

Figure 1. Microphotographs of PEO-*b*-PMA/*d*C_{16}SU complexes obtained using (A) negative staining and (B) freeze fracture techniques. Bar corresponds to 100 nm.

Addition of simple salts to polymer−surfactant complexes leads to destabilization of the system of the salt bonds. This is usually accompanied by initial aggregation of the particles followed by complete disappearance of the complex species at the higher salt concentration.[8] For example, complexes of PEO-*b*-PMA anions and cetyltrimethylammonium cations completely disintegrate in the presence of ca. 0.5 M NaCl. In contrast, the size of PEO-*b*-PMA/*d*C_{16}SU particles increased from ca. 100 to 160 nm upon elevating NaCl concentrations from 0.3 to 0.5 M and then remained practically constant at the higher salt concentrations (at least up to 0.8 M used in this study). Conversely, at NaCl concentrations higher than 0.4 M large aggregates (>250 nm) were formed in systems containing PEO-*b*-PMA/*m*C_{16}SU complex. Similar aggregation was also observed in the solutions of C_{16}SU in the absence of the block ionomer upon addition of the salt. Therefore, formation of the disulfide bonds between C_{16}SU molecules stabilized the surfactant species assembled on the block ionomer templates. It appeared that these species remained stable in the dispersion even at the high ionic strengths when the salt bonds between PMA and surfactant cations cannot exist. Using pyrene-labeled PEO-*b*-PMA[9] we

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10.1021/ja020509p CCC: $22.00

demonstrated that binding of $C_{16}SU$ cations to the polyanion chains results in quenching of the pyrene fluorescence. The fluorescence remained quenched in the presence of the oxidizing agent when the PEO-*b*-PMA/*d*$C_{16}SU$ complexes were formed. Upon addition of salt a sharp increase of fluorescence intensity was observed at 0.3−0.5 M NaCl, which indicates removal of the block ionomer chains. However, the fluorescence restoration was incomplete as an additional increase of fluorescence intensity (20%) was observed after vesicle disintegration in Triton X-100. It is possible that the incomplete fluorescence restoration is due to entrapment of a part of PEO-*b*-PMA chains in the aqueous pool of the *d*$C_{16}SU$ vesicles even though the chains at the external surface were dissociated upon addition of salt.

Stability of the vesicles was further examined upon changing environmental pH. The ionization degree of PMA decreases at lower pH, resulting in the decrease of the number of salt bonds between the block ionomer and surfactant. At pH 3 there is less than 10% of the salt bonds left as was shown for mixtures of PEO-*b*-PMA with various single- and double-tail surfactants.[10] No particles were detected in the PEO-*b*-PMA/*m*$C_{16}SU$ system at pH 3. In contrast, there was practically no change in the size of PEO-*b*-PMA/*d*C_{16}-SU particles upon variation of pH in the range from 3 to 9. This reinforces the conclusion that *d*$C_{16}SU$ species preformed on the PEO-*b*-PMA template are preserved after depleting the salt bonds.

The size of PEO-*b*-PMA/*d*$C_{16}SU$ particles did not change upon variation of the temperature from 23 to 60 °C. The size of PEO-*b*-PMA/*m*$C_{16}SU$ particles initially increased after elevation of the temperature followed by restoration of the initial size within several minutes. Such behavior was not noticed in the case of PEO-*b*-PMA/*d*$C_{16}SU$ complexes. This observation provides additional evidence of higher mobility of the surfactant molecules included into the block ionomer complexes in the monomer form compared to those in the dimer form.

The entrapment experiments using fluorescein isothiocyanate-dextrans (FITC-Dex) of various molecular weights suggested that the particles of PEO-*b*-PMA/*d*$C_{16}SU$ complex containing the dimer form of surfactant efficiently encapsulate and retain hydrophilic macromolecules.[11] The fraction of FITC-Dex entrapped in the PEO-*b*-PMA/*d*$C_{16}SU$ complexes varied from 0.13 to 0.19% for dextrans with various molecular weights. In contrast, the particles of PEO-*b*-PMA/*m*$C_{16}SU$ complexes eluted from the column did not practically retain FITC-Dex. It is possible that PEO-*b*-PMA/*m*C_{16}-SU complexes undergo some structural rearrangements accompanied with the release of FITC-Dex in the conditions of elution (10 mM Tris buffer, pH 8.8, containing 0.2 M NaCl). The size of such particles after the elution was larger than their initial size (130 vs 75 nm.). Conversely, such rearrangement might be hindered in the case of PEO-*b*-PMA/*d*$C_{16}SU$ complexes, which did not change practically in size after the elution (90 nm vs 80 nm). As a result, FITC-Dex molecules remained encapsulated into the PEO-*b*-PMA/*d*$C_{16}SU$ vesicles after the elution. Overall, these experiments provide an additional evidence of formation of PEO-*b*-PMA/*d*$C_{16}SU$ vesicles with internal aqueous volume. We assume that such vesicles are composed of bilayers from polymethacrylate anion-bound surfactant, with a corona from PEO chains stabilizing these complexes in aqueous media. It has to be noted, however, that both PEO-*b*-PMA/*d*$C_{16}SU$ and PEO-*b*-PMA/*m*$C_{16}SU$ vesicles appeared to be quite permeable to small molecules, such as carboxyfluorescein, which cannot be entrapped.

Earlier studies described stabilization of preformed lipid vesicles including polymerization of the surfactant monomers within the lipid bilayers, polymerization of the monomeric counterions at the external and internal leaflets of lipid membranes and grafting the vesicle surface with nonionic polymers.[12] This work for the first time describes the template-assisted self-assembly of vesicles from single-tail surfactants, followed by dimerization of the surfactant molecules. The vesicles are stabilized in dispersion due to the presence of PEO chains, can effectively encapsulate hydrophilic macromolecules, and are resistant to the changes in environmental characteristics. They may be useful as drug and gene delivery systems.

Acknowledgment. This work was supported by NSF DMR-0071682 (A.V.K.), NIH DK46052 (F.C.S.), and NSERC STR-0181003 (A.E.). We thank R. Vaughn (UNMC) and Randy Nessler (University of Iowa) for carrying out TEM experiments.

Supporting Information Available: Structure of $C_{16}SU$ and dimerization reaction scheme (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(4) PEO-*b*-PMA was synthesized by anionic polymerization as described before [Kabanov, A. V.; Bronich, T. K.; Kabanov, V. A.; Yu, K.; Eisenberg, A. *Macromolecules* **1996**, *29*, 6797−6802]. The block lengths were 176 and 188 repeating units for PEO and PMA, respectively. The concentration of carboxylate groups in the copolymer samples was estimated by potentiometric titration. The complexes were prepared by mixing PEO-*b*-PMA aqueous solution and $C_{16}SU$ solution in water/methanol mixture (75/25 v/v %) at room temperature. The composition of the mixture is expressed as a ratio of the concentration of $C_{16}SU$ to the base-molar concentration of the methacrylic acid units in the system and varied from 0.1 to 1.0. The final concentration of the PEO-*b*-PMA carboxylate groups was 0.4 mM, pH 8.2 to 9.5 (10 mM Tris buffers or 10 mM borate buffers saturated with N_2 were used in this study). No additional mechanical agitation commonly used for vesicle preparation was applied.

(5) Particle effective diameter and ζ-potential were determined by photon correlation spectroscopy using a "ZetaPlus" ζ-potential analyzer (Brookhaven Instrument Co.) equipped with the multiangle option. The sizing measurements were performed at 23 °C at detection angle 90°.

(6) At 25 °C and pH 9.5 the hydrolysis of isothiuronium group has a halftime of several minutes, and is therefore much slower than the electrostatic binding of the surfactant cations to the block ionomer chains. To ensure that all $C_{16}SU$ molecules incorporated into the complexes form dimers, the PEO-*b*-PMA/$C_{16}SU$ systems were treated with 10% excess of H_2O_2 or by DMSO (1 vol %) to form disulfide bonds. Under these conditions the formation of the surfactant dimers is practically complete, as was demonstrated by colorimetric assay using 5,5′-dithiobis(2-nitrobenzoic acid) [G. S. Tarnowsky et al. *Arch. Biochem. Biophys.* **1965**, *110*, 210−216].

(7) Preparation of samples for TEM experiments was described in detail in our previous publication [ref 2].

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(9) PEO-*b*-PMA was labeled with pyrene using previously described method [Krakovyak, M. G., et al. *Vysokomol. Soedin.* **1969**, *11*, 2499−2504]. Pyrene fluorescence was recorded at $\lambda_{ex} = 342$ nm and $\lambda_{em} = 376$ nm.

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(11) Stoichiometric PEO-*b*-PMA/$C_{16}SU$ complexes were prepared in 10 mM Tris buffer (pH 8.8) in the presence of 5 mg/ml FITC-Dex with various molecular weights (4000, 9500, and 19500) followed by treatment with oxidizing agent or DTT, respectively. The resulted mixtures were stirred for 1 h, and then the salt concentration was adjusted to 0.2 M with NaCl. Nonentrapped FITC-Dex was separated by gel penetration chromatography on Sepharose Cl-6B column with 10 mM Tris buffer (pH 8.8) containing 0.2 M NaCl as eluent. Complex particles emerged in the void volume as was confirmed by photon correlation spectroscopy, whereas the nonentrapped FITC-Dex was retained. The concentration of FITC-Dex retained in the vesicles was determined by fluorescence intensity measurements ($\lambda_{ex} = 490$ nm and $\lambda_{em} = 516$ nm).

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JA020509P