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Diblock Copolymer Micellar Nanoparticles Decorated with Annexin-A5 Proteins

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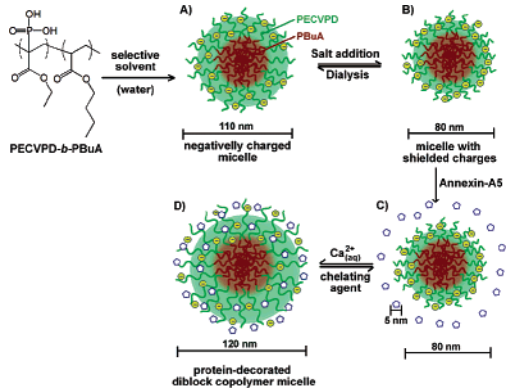
The ability of amphiphilic block copolymers to self-assemble into ordered structures (micelles, lamellae, vesicles, etc.) if dissolved in a selective solvent is well-known.[1] In recent years, the interest in these nano-organized objects as potential delivery vehicles for bioactive agents has grown enormously, due to the fact that they can not only stabilize hydrophobic molecules with otherwise limited water solubility but also decrease their eventual high toxicity to healthy cells.[1d,2] However, targeting tumor sites and associated cells still remains a significant challenge for the development of micelle-mediated drug delivery systems and in vivo imaging assays.[3] Several strategies have been developed to achieve these goals, which consist mainly in (i) the use of stimuli (pH, temperature, applied magnetic fields)-responsive macromolecules at near physiological conditions,[4] and (ii) the utilization of molecular markers recognizing specific sites in cancerous tissues.[3a] In both cases, landmark advances seem to be those linking the biological world, with its elaborate architectures, properties, and functions, to delivery nano-carriers.

Within this context, the interaction between nanosized lipid-based assemblies and annexins—a family of proteins that participate in a variety of membrane-related processes, such as blood coagulation and inflammation, and bind to biological membranes through *negatively charged phospholipids* in a calcium-dependent manner—has recently attracted increasing attention worldwide.[5] Such protein binding process is reversible, and removal of calcium ions by chelating agents leads to a liberation of annexins from the phospholipid matrix (Supporting Information (SI), Section 1). Depending on the environmental surroundings, protein-decorated assemblies may interact with specific types of receptors. For instance, detection assays for tumors with high apoptotic rates have been developed on the basis of the strong Annexin-A5 binding to phosphatidylserine (PS) moieties, whose exposition is considered as a hallmark of apoptosis.[6] Consequently, great potential for simultaneous in vivo monitoring[6] and cancer treatment is expected for Annexin-A5-guided assemblies (e.g., micelles and vesicles), which can be ultimately engineered to load either image contrast agents, such as hydrophobic iron nanoparticles, or active drugs.

In this perspective, our challenge is to build protein-decorated polymer assemblies in order to give (bio)functionality to more robust objects (micelles made from block copolymer or nano-organized bulk films) and offer another possibility as compared to liposomes with thin and deformable bilayers.[7]

Herein we report the (original) reversible binding of Annexin-A5 protein to diblock copolymer micellar nanoparticles having poly((1-ethoxycarbonyl)vinylphosphonic diacid) (PECVPD) and poly(*n*-butyl acrylate) (PBuA) as corona- and core-forming segments in water, respectively, as summarized in Scheme 1. PECVPD-*b*-PBuA block copolymer can be synthesized by Atom Transfer Radical Polymerization (ATRP).[8] In the present study, $PBuA_{35}-Br$ was used as macroinitiator to polymerize the DECVP monomer.[9] Subsequent two-step conversion of $-P(O)(OCH_3)_2$ groups into $-P(O)(OH)_2$ phosphonic diacid afforded the mentioned amphiphilic block copolymer (SI, Section 2).

Scheme 1. Successive Steps during the Formation of PECVPD-*b*-PBuA Micellar Aggregates in Water and Their Protein Decoration



When dissolved in water (a solvent thermodynamically good for PECVPD), the $PECVPD_{30}$-*b*-$PBuA_{35}$ diblock self-assembled, as expected, into spherical micellar aggregates. Therefore, these nano-objects are formed by a hydrophobic PBuA core and a *hydrophilic PECVPD corona bearing negatively charged phosphate moieties* (Scheme 1A), *which are potential binding sites for Annexin-A5*.

The polyelectrolyte behavior of $PECVPD_{30}$-*b*-$PBuA_{35}$ aggregates at near physiological pH (7.5) and in the presence of salts (conditions at which Annexin-A5 is stable) was first examined in detail by Static and Dynamic Light Scattering (SDLS) and Transmission Electron Microscopy (TEM) (SI, Section 3) in order to establish the micelle structure before protein decoration. As observed in DLS experiments summarized in Figure 1.I, the self-assembly of PECVPD-*b*-PBuA copolymer in pure water (no added salt) originated two distinct relaxation times of well-defined spherical nano-objects (also identified in cryo-TEM (Figure 1.II.A) and TEM micrographs (Figure S14)), whose apparent hydrodynamic diameters ($2R_H^{app}$) were 8 and 110 nm (curve A). Upon salt addition (either NaCl or $CaCl_2$) (curve B), one single relaxation time was observed corresponding to a size of $2R_H = 78$ nm. Such a decrease was also confirmed by cryo-TEM (Figures 1.II.B). The addition of 1.3 mg/mL of Annexin-A5 in a buffer solution (20 mM Tris-HCl, pH 8.0, 0.02% NaN_3, ~200 mM NaCl) to a 0.5 mg/mL $PECVPD_{30}$-*b*-$PBuA_{35}$ micellar solution in the presence of 50 mmol/L of NaCl did not provoke noticeable changes in both particle size and distribution profile (curve C). Under these experimental conditions,

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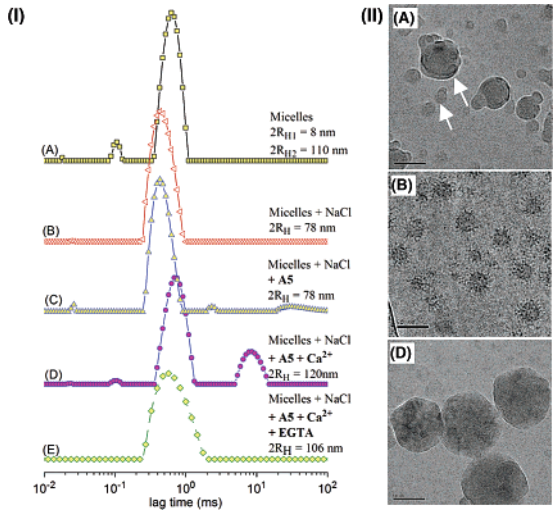

Figure 1. (I) Distribution of relaxation times A(t) obtained using CONTIN analysis of autocorrelation functions C(q,t) (DLS) recorded during preparation steps of protein (Annexin-A5)-decorated diblock copolymer micellar aggregates. In all cases, the relaxation frequency ($\Gamma = \tau^{-1}$) is q^2-dependent (q is the wavevector). (II) Cryo-TEM images corresponding to solutions A, B, and D in Scheme 1 and Figure 1.I (scale bar = 50 nm).

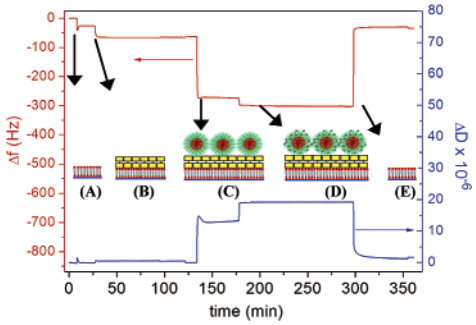

Figure 2. QCM-D data showing the formation of Annexin-A5/PECVPD-*b*-PBuA assemblies, as follows: (A) deposition of a lipid bilayer; (B) deposition of A5 dimers; (C) after addition of $PECVPD_{30}$-*b*-$PBuA_{35}$ micellar solution; (D) further addition of Annexin-A5; (E) after addition of EGTA. Solutions B−D contained 2.0 mmol/L of $CaCl_2$.

negatively charged phosphate groups are exposed at the corona/solvent (water) interface, with no detectable interaction with the protein.

Binding of Annexin-A5 onto PECVPD-*b*-PBuA micellar aggregates was achieved by adding 2.0 mmol/L of $CaCl_2$ (close to physiological content) (curve D), thus originating *(bio)functionalized polymer-based assemblies*, as demonstrated by DLS (Figure 1.I), cryo-TEM (Figure 1.II), and Quartz Crystal Microbalance with Dissipation monitoring (QCM-D) (Figure 2) experiments. According to DLS results, this phenomenon caused a 40 nm increase in the mean diameter value (from 78 nm, curve B/C, to 120 nm, curve D). Such a large size increase was further confirmed by cryo-TEM images (Figures 1.II.D) and suggested the migration of Annexin-A5 into the micelle corona, in a process favored by the existence of phosphate groups along the micelle corona. The protein binding efficiency determined by SLS after separation of unbound protein by ultracentrifugation was higher than 85% (SI, Section 4). The accessibility of pendant phosphonic diacid moieties in the polymer backbone is almost quantitative, as judged by potentiometric acid−base titration curves (Figure S15). This behavior was quite different when compared to that of Annexin-A5/liposome systems, for which the size shift due to protein surface binding was virtually equivalent to a protein monolayer thickness (2 × 4 nm) because liposomes exhibit phosphate moieties exposed at their periphery (Scheme S1). The slow relaxation mode in curve D was attributed to large aggregates in solution, possibly induced by protein interaction connecting different particles since Annexin-A5 has multiple potential Ca^{2+} binding sites.[5a]

The addition of EGTA (a Ca^{2+}-selective chelating agent) to solutions containing Annexin-A5/micelle assemblies resulted in a clear decrease in the size (curve E in Figures 1.I and S16) as a result of Annexin-A5 release from the micelle.

Similarly, QCM-D measurements revealed variations in the resonance frequency and dissipation values upon addition of PECVPD-*b*-PBuA micellar solution to a monolayer of chemically engineered "double" Annexin-A5 molecules (Figure 2C). The same behavior could be evidenced when Annexin-A5 was added to a layer of deposited micelles (Figure 2D). These assembled objects were stable and not affected by rinses. As for DLS, disassembly occurred when a chelating agent was added (Figure 2E).

In summary, polymeric micellar assemblies were decorated with Annexin-A5 protein, originating nanosized structures capable of participating in molecular recognition processes, while having a cargo space for hydrophobic molecules. The latter can be easily tailored through rather simple polymer chemistry. These new objects, whose decoration is reversible, find applications, for example, in micelle-mediated target imaging and drug delivery and controlled fabrication of biochips.

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Supporting Information Available: Diblock copolymer synthesis, DLS and SLS data on PECVPD-*b*-PiBuA micelles, and protein/liposome assemblies, experimental details. This material is available free of charge via Internet at http://pubs.acs.org.

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