Hybrid Materials

Micrometer-Sized Spherical Assemblies of Polypeptides and Small Molecules by Acid–Base Chemistry**

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Microspheres have long been recognized for their potential uses in drug delivery and isolation of chemical reactions. Many such systems require sacrificial templates and/or surfactants for their self-assembly, or otherwise use organic solvents.[1] We have shown that microspheres can be obtained directly by self-assembly of Cys_nLys_m block copolypeptides with either citrate-coated silver and gold nanoparticles[2a] or CdSe/CdS nanocrystal quantum dots,[2b] or of poly-L-lysine (PLL) with citrate-coated CdSe quantum dots.[3] These assemblies can be mechanically stabilized by adding an outer layer of negatively charged colloidal silica. Herein we report that large spherical assemblies can be obtained without nanoparticles but simply by reaction of one of several polyelectrolytes and certain small, functionalized molecular counterions.[4] These assemblies can then be further functionalized, and we show how those based on polyamines can be protected by silica either in the form of colloidal silica or by condensation of silicic acid. Many earlier efforts have concentrated on the ability of multivalent ions to aggregate oppositely charged polymers, and such systems have been described by theory[5] and studied experimentally,[6] particularly in the case of DNA.[7] However, in none of these studies was sphere formation observed. In other studies, spherical assembly using polyelectrolytes was observed, but these approaches have required either amphiphilic block copolymers,[8] proteins,[9] two different polyelectrolytes,[10] hydrophobic molecules,[11] or the presence of both components of an insoluble salt (calcium carbonate).[12] However, to our knowledge, the work reported herein is the first demonstration of spherical assembly using only a single polyelectrolyte with one small counterion without stabilization by an inorganic species.

Directed biomineralization can provide novel methods for the assembly of highly ordered structural materials, as demonstrated by the recent in vitro utilization of some biological or biomimetic peptides.[13–16] We have shown that synthetic poly(amino acid)s can mimic silicateins and direct the formation of silica structures, such as spheres.[13] Kröger, Sumper and co-workers have reported that silaffins and polyamines from diatoms can form 0.3–1-μm silica spheres in the presence of inorganic phosphates.[14a–d] In an extension of this work, Brunner, Lutz, and Sumper have very recently shown that sulfate and phosphate induce microscopic phase separation of polyallylamine (PAA) and that this phase-separated state in turn has high silica-precipitation activity and yields silica spheres.[14e] Clarson and co-workers have shown that silica microspheres can be obtained from PLL using silicic acid in phosphate or citrate buffers.[15] We now show that our preformed assemblies also condense silicic acid, and propose that the formation of microspheres in the work of the groups of Sumper, Brunner, and Clarson may be understood by initial formation of spherical templates, like ours, prior to silica condensation. This model is similar to the microscopic phase-separation picture put forth by Brunner, Lutz, and Sumper.[14e] The silica spheres from these systems are in direct contrast to the disordered precipitates that result when multivalent anions are not used.[16]

In extension of our work based on citrate-stabilized nanoparticles,[2,3] we obtained assemblies by the reaction of citrate (final concentration 0.5 wt.%) and PLL[17] (final concentration 0.6 wt.%) at pH 7. After mixing the two components, the solution immediately turned from clear to cloudy. Within a few minutes of mixing, spheres were observed by light microscopy, as illustrated in Figure 1a. Thus, we have found a route to assembly that eliminates the need for nanoparticle reactants. After drying, these assem-

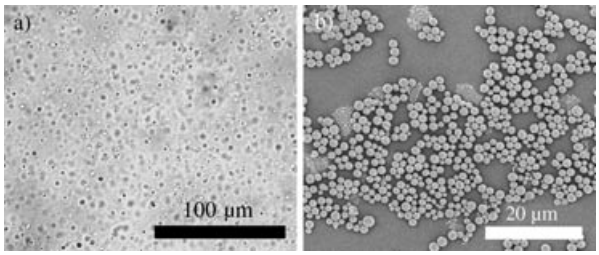

Figure 1. PLL/citrate assemblies: a) optical image, without colloidal silica, b) SEM image after modification by colloidal silica.

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blies cling to the glass slide and lose their shape. However, they can be stabilized by a protective silica shell through the addition of colloidal silica that condenses on the preformed assemblies (Figure 1b).

To investigate how generalizable the sphere formation process is, we explored a host of multivalent organic acids other than citrate (Table 1). PLL-containing spheres were obtained in the presence of two other triacids (isocitrate and trimesate), but with neither diacids nor ethylenediaminetetracacetic acid (EDTA). Assuming that the role of the counterion is to bridge polycations, the diacids may not offer the required kinetic or thermodynamic cooperativity. The failure of EDTA to form spheres with PLL at any pH value is puzzling, but may be related to the better hydration of its carboxylate groups.

Table 1: Microsphere synthesis at room temperature.[a]

Acid	n_{COOH}	p$K_a(n)$	PPL	PLO	PLH	PLR
citric	3	6.43	5.5–9.0	5.5–9.5	4.5–6.0	precipitate
isocitric	3	6.40	5.5–9.0	5.0–9.5	5.0–6.0	precipitate
trimesic	3	4.7	4.5–8.0	4.5–9.0	4.0–6.0	precipitate
EDTA	4	10.26 (6.16)	NO	NO	NO	6–10
carbonate	2	10.33	NO	NO	NO	NO
alkanedicarboxylic acids, $n(CH_2) = 0$–6	2	3.85–5.69	NO	NO	NO	NO (precipitate with oxalate)
tartaric	2	4.34	NO	NO	NO	NO
malic	2	5.2	NO	NO	NO	NO
fumaric	2	4.54	NO	NO	NO	NO

[a] Entries indicate which combination of small organic acid and polycation yields assemblies or precipitates, and the approximate pH ranges over which assemblies are visible in the optical microscope. NO = no assembly or precipitate.

Having found some variability in one assembly component, we varied the cationic poly(amino acid). The triacids were seen to create spheres with poly-L-ornithine (PLO) and with poly-L-histidine (PLH). PLH provides a particularly interesting case in that spheres were not obtained at pH 7 but only below the pK_a value of the imidazole side chain, 6.0. This result reflects the fact that assembly requires charged groups. Indeed, in all cases the lower pH boundary for assembly was defined by the highest pK_a value of the acid; in other words, all the carboxyl groups must be deprotonated, and the spheres disassemble at pH values roughly one unit below the acid pK_a. This behavior is reversible: the spheres reassemble upon increasing the pH value. Similarly, spheres will not form if the polymer chain is under protonated, and assembly is only seen up to a pH value similar to the formal pK_a of the polypeptide side chains. This situation suggests that the spheres form primarily by electrostatic attraction, which is most likely accompanied by COO^-/NH_3^+ acid–base hydrogen bonding that provides further stabilization. Their stability is also sensitive to the ionic strength of the solution; the spheres shrink and finally become unobservable under the optical microscope at salt concentrations of roughly double the final value of $[COO^-]$ and $[NH_3^+]$. The size of the assemblies depends on the ratio of $[COO^-]$ and $[NH_3^+]$. In agreement with the observations of Brunner, Lutz, and Sumper in the PAA/phosphate system,[14e] we found that the size of the PLL/citrate assemblies reach a maximum as the $[COO^-]$:$[NH_3^+]$ ratio is increased.

Poly-L-arginine (PLR), with its pK_a 12.5 side chain, showed remarkably different behavior with the triacids, by forming a precipitate. The PLR-triacid systems were coaxed into assembling spheres by heating the solution, which indicates there is an important entropic contribution to both the stability of the spheres and their kinetics of formation. We are currently exploring the nature of these assemblies as well as the temperature dependence of their formation. Also, unlike the other cationic poly(amino acid)s, PLR formed spheres with EDTA. This may be because the highest pK_a of EDTA is below that of the arginine side chain, so a proton transfers from EDTA to the side chain and creates an additional acid–base bridge.

The microsphere assembly was further extended by combining anionic poly(amino acid)s with cationic molecules containing multiple amine groups. Poly-L-aspartate (PLD) and poly-L-glutamate (PLE) did not form spheres with any of the divalent or trivalent cations explored,[18] but spherical assembly for both polymers did occur with pentalysine and tetraethylenepentamine. Tetravalent tris(ethyleneamine)amine also gave spheres with PLD; however, with PLE, assembly required cooling and produced a combination of spheres and precipitate.

The surfaces of the assemblies are chemically active, and a shell of colloidal silica can be deposited onto the surface of assemblies of polycations. The addition of colloidal silica to a solution of these assemblies gives them a protective shell (Figure 1b). Addition of colloidal silica leads to a redistribution of the polymer: confocal microscopy of spheres made from FITC-labeled PLL (FITC = fluorescein isothiocyanate) showed that the polymer is evenly distributed.[19] However, upon reaction with colloidal silica, the polymer is pulled, presumably because of coulombic attraction to the negatively charged silica nanoparticles, to the inside rim of the silica coat (Figure 2).[4,20]

Inspired earlier work[14e,15] we also obtained silica spheres by adding prehydrolyzed tetramethylorthosilicate (TMOS) to a solution of preformed polycation/citrate spheres.[21] Condensation of the silicic acid was evident under the optical microscope, which showed a dark, thin shell, and from the stability of the resulting spheres after drying (Figure 3). We performed confocal microscopy on these spheres, as we had done with those coated with colloidal silica, to verify the templating action of the assemblies. Cross-sectional images of spheres after silicic acid condensation also show that their interiors are indeed full of polymer. Moreover, there is no central cavity as was observed for the colloidal silica-coated spheres (Figure 4).

In conclusion we have shown the existence of a new chemical entity that self-assembles from low concentrations

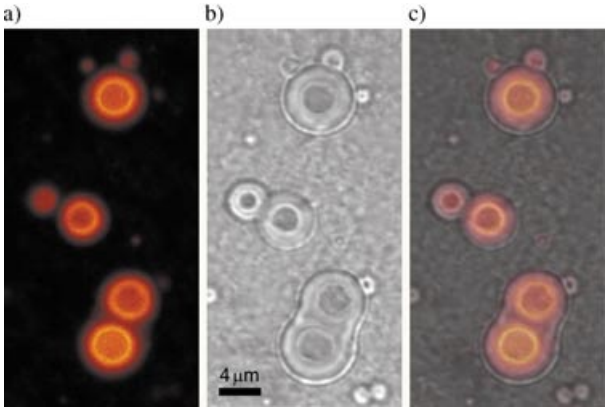

Figure 2. Cross-sectional images of a colloidal-silica-coated sphere made from FITC-labeled PLL and trimesate, a) the polymer fluorescence in a plane through the center of the sphere, b) the optical transmission image at that focal plane, c) an overlay of (a) and (b).

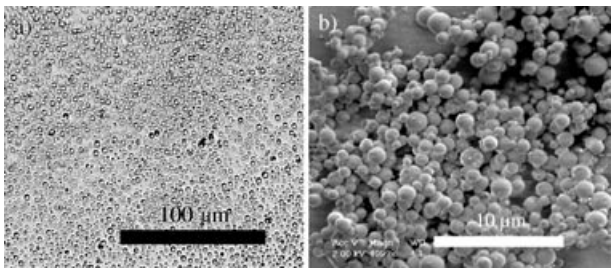

Figure 3. PLO/isocitrate spheres functionalized by condensed silicic acid: a) an optical image, the spheres have a thick, dark outline, in contrast to those in Figure 1a, b) SEM image of a centrifuged sample.

of polyelectrolyte and an oppositely charged, multivalent ion. We have shown that all naturally occurring charged poly(amino acid)s can assemble into microspheres when combined with a proper counterion. We have also shown assembly with PLO, and believe that spheres may be obtained with other synthetic polyelectrolytes as shown for PAA by Brunner, Lutz, and Sumper.[14e] The strength of the interaction between the two components is predictably influenced by pH value, and is also affected by salt concentration and temperature. Importantly, the surface of the spheres is chemically active, as shown by the silica condensation reactions, and could be further functionalized for applications in delivery or detection.

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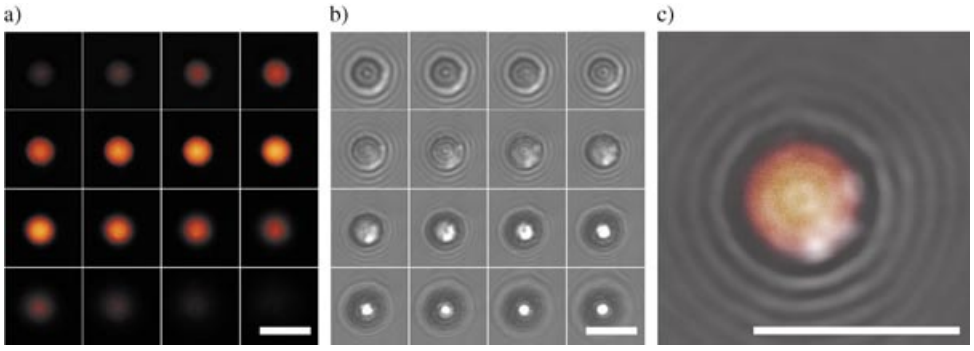

Figure 4. Cross-sectional images of FITC-labeled PLL/isocitrate spheres functionalized by condensed silicic acid. Galleries of the fluorescence (a), and transmission (b) responses at different sections through a sphere, c) an overlay of the two images at the central focal plane. The scale bars represent 5 μm.

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[17] Poly-L-lysine hydrochloride (30 kDa), FITC-labeled poly-L-lysine (70 kDa), poly-L-histidine (10 kDa), poly-L-arginine (30 kDa), poly-L-ornithine (50 kDa), poly-L-glutamate (15 kDa) and poly-L-aspartate (35 kDa) were obtained from Sigma and used as received.

[18] The amines used that were unsuccessful in sphere formation are: 1,4-bis(3-aminopropyl)piperazine, 3,3′-diamino-*N*-methyldipropylamine, melamine, diethylenetriamine, 2,6-diaminopyridine, *N*,*N*,*N*′,*N*′,*N*″-pentamethyldiethylenetriamine, 1-(2-aminoethyl)-piperazine, 1,3-diaminopropane, 1,6-diaminohexane, 1,8-diaminooctane, and 1,12-diaminododecane.

[19] The uncoated spheres are mobile in solution before adhering to the glass slide and losing shape, and were thus difficult to observe with this technique. However, all independent scans show them to be full of polymer.

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[21] TMOS was prehydrolyzed at a final concentration of 0.1M, in HCl (pH 2–3), for 5 min, before adding a small amount of this precursor (≈5% v/v) to a solution of polycation/citrate spheres. The prehydrolyzed TMOS could also be quickly neutralized before addition to the spheres.

