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Formation and Characterization of Polyglutamate Core−Shell Microspheres

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Biocompatible vesicles have been and remain of intense interest for drug delivery and biomedical contrast imaging.[1] Of particular interest are protein microspheres, in part due to their stability and in part to their biocompatibility. We present here a new class of protein core−shell microsphere made with polyglutamic acid, whose size is sufficiently small to permit extravasation (i.e., escape) from the blood pool, particularly in regions with leaky vasculature, e.g., tumors. In contrast to previous core−shell protein microspheres (generally made from serum albumin), these sodium polyglutamate (SPG) microspheres are not held together by covalent cross-links, and yet they are extremely stable.

We have previously shown that albumin (e.g., bovine serum albumin, BSA), forms core−shell microspheres whose outer protein shell is stabilized by interprotein covalent disulfide cross-linking of cysteine residues.[2] They are easily formed by applying high-intensity ultrasound to a two-phase system of aqueous protein solution and a nonaqueous liquid (e.g., vegetable oil, an FDA permitted injectable); the cross-linking arises from redox reactions by hydroperoxyl radicals (i.e., protonated superoxide: $HO_2^{\bullet}$) formed during acoustic cavitation.[2] Such microspheres have been used as biomedical imaging contrast agents for magnetic resonance imaging (MRI),[3] for optical coherence tomography (OCT),[4] and, with gas-filled microbubbles, for sonographic imaging (e.g., Albunex, the first FDA approved echo contrast agent).[5] In all cases, these protein microspheres are comparable in size to an erythrocyte (i.e., several microns in diameter) and, thus, trapped in the blood pool. The ability to make smaller submicron protein microspheres that would be capable of escaping into poorly developed vasculature, such as found in tumors,[6] has not been previously achieved.

In this study, we use poly(α,L-glutamic acid) (PG), which is composed of L-glutamic acid molecules linked together through amide bonds. It is made synthetically and is degraded to glutamic acid molecules by cathepsin, an enzyme present in lysosomes.[7] This polymer has been conjugated to hydrophobic drugs such as Taxol to enhance uptake and retention; some of these conjugates are now in clinical trials.[8]

Recently, large poly(α,L-glutamic acid) microspheres (∼5 μm) were reported in passing using ultrasound under strongly acidic conditions.[9] From this prior report, it is probable that their microspheres are *not* core−shell (n.b., at low pH, PG is insoluble at MW > 6000). Contrary to our results here, Gedanken and co-workers[9] were unable to form microspheres by their methods above pH 4.5. *Solid* microspheres of polyglutamic acid have been synthesized using other methods.[10]

In our preparations, a 5 wt % solution of sodium poly-α,L-glutamate (SPG) was layered with vegetable oil and then sonicated using a 2-mm diameter ultrasonic tip (20 kHz, 50 W/cm^2, 3 min), which produces spheres that are less than one micron in diameter (Figure 1). These microspheres are core−shell, as shown in the cross-sectional TEM image (Figure 2A). To further demonstrate the core−shell nature of these spheres, toluene and oil were mixed prior to encapsulation and the resulting microspheres were imaged with SEM. As the toluene is pumped away, the microspheres partially collapse but retain their structural integrity (Figure 2B). The hydrophobic core and polymer shell nature of these spheres is important for their use in biomedical applications: the core can contain a variety of imaging agents (even simultaneously for multimodal imaging), and the protein shell can be easily modified to target specific organs or tumor types.[11]

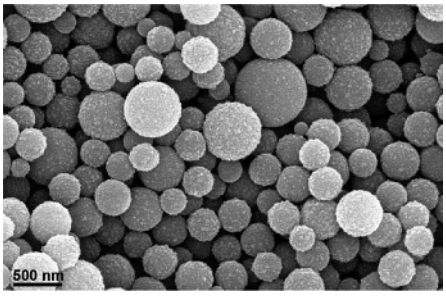

Figure 1. SEM image of 400 nm (±100 nm) (Supporting Information) sodium polyglutamate microspheres, formed using sonication of 5 wt % SPG with vegetable oil at pH 7.4. The microspheres were stained with OsO_4 and dehydrated prior to imaging. Other carboxylic acid polymers also produce similar microspheres under similar conditions, e.g., sodium polyaspartate (SPA).

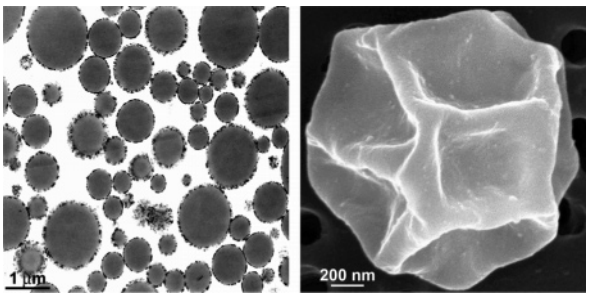

Figure 2. (A) Cross-sectional TEM image of SPG microspheres. (B) SEM image of PG sphere with 4:1 toluene/oil interior made from sonication of 2.5 wt % SPG. Spheres were stained with OsO_4 and dehydrated prior to imaging.

In the sonochemical formation of BSA microspheres, disulfide cross-linking gives the spheres their long-lived stability. BSA oligomers had been detected in sonicated BSA solutions, and gel electrophoresis showed that the oligomers were returned to the monomer by mild disulfide reducing agents. With no disulfide bonds possible to stabilize these new SPG microspheres, the mechanism of stabilization must be different. To see if covalent bonds were formed during the formation of SPG spheres, sonicated and nonsonicated (i.e., control) solutions of 1500−6000 MW polymer were examined by MALDI-MS. No dimers, oligomers, or other changes were observed in the mass spectrum after the solution was sonicated (Supporting Information).

Radical scavengers were also added to the reaction mixture prior to sonication to test their effect on sphere formation. In contrast to BSA microspheres (which are strongly inhibited by radical traps),

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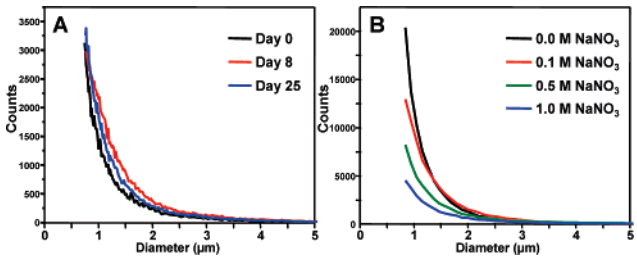

Figure 3. (A) Microsphere stability vs time. Particle counting data (Coulter Multisizer IIE) for a single sample of SPG microspheres. (B) Microsphere stability vs added ionic strength after 1 h exposure of SPG microspheres to solutions containing $NaNO_3$. Without addition of $NaNO_3$, the Na^+ concentration was 0.4 M.

when hydroquinone or glutathione were added to the SPG solution, no decrease in the number of spheres was observed. Again, we find no evidence that SPG spheres are covalently cross-linked by radicals.

The robustness of the SPG spheres is striking, given the lack of covalent cross-linking. By particle counting (Coulter Multisizer IIE), the polyglutamate spheres have a half-life of more than 1 month when stored at 2 °C (Figure 3A). Thermal stability was also tested: the SPG microspheres were stable for more than an hour even at 60 °C (Supporting Information).

The intermolecular interactions that might be responsible for holding the SPG microspheres together include hydrogen bonding, van der Waals, hydrophobic, and electrostatic interactions, all of which can be effected by changes in pH and ionic strength.[12] More specifically, we believe that the dominant interaction between the polymer chains are a network of hydrogen bonds or ion pairs: $[RCO_2^-...M^+...^-O_2CR]^-$ where $M^+ = H^+$ or Na^+. Convincingly, the asymmetric CO_2 vibration in the IR (spectra in Supporting Information) of SPG microspheres (1608 cm^{-1}) is 54 cm^{-1} higher in energy than that of a simple SPG solution (1554 cm^{-1}), which is consistent with an ion-paired or hydrogen bonded carboxylate compared to a free carboxylate.[13]

A proton bridging between two carboxylates is the strongest type of hydrogen bond[14] and is consistent with the pH profile of these microspheres' stability. The effective pK_a of SPG is ~6, with deprotonation occurring over the broad range of pH 4.5−9 (Supporting Information). The microspheres are stable when stored in solutions ranging from pH 4.8 to 12 but lose stability at lower pH: at pH 3, more than half of the microspheres are destroyed in 1 h (Supporting Information). The formation of microspheres is also affected by pH: the optimal pH is about 7, and decreasing the pH to 6 increased sphere size and heterogeneity. The instability of the microspheres at lower pH, where most of the carboxylates will be protonated, indicates the importance of neutral carboxylic acid−carboxylate anion interactions in holding the microspheres together.

As expected for our proposed network of hydrogen bonding or ion pairing, increasing ionic strength also has a dramatic effect on microsphere stability, both during formation and upon addition to preformed microspheres. The concentration of sodium ions in a 5 wt % SPG solution is ~0.4 M, but increasing the ionic strength (even by relatively small amounts, e.g., 0.1 M added $NaNO_3$) significantly decreases microsphere formation (Figure 3B). In addition, preformed microspheres are nearly completely destroyed when subjected for 1 h to additional ionic strength (added 0.1 M $NaNO_3$). If the microspheres are first washed to remove excess counterion, however, and then placed in physiological condition buffers, the SPG microspheres were unchanged after 24 h (Supporting Information).

Changing the surfactancy of the solution can affect sphere size by changing surface tension of the solution. Decreasing the concentration of the polymer solution from 5 to 2 wt % increased the surface tension and, consequently, increased the average size of the spheres and their heterogeneity. Further increasing the polymer concentration to 7 wt % did not change sphere size and neither did addition of surfactants such as Tween or Triton-X.

In summary, a new and simple method for the sonochemical formation of biocompatible core−shell microspheres has been demonstrated. These polyglutamate microspheres are highly stable throughout the pH, ionic strength, and temperature ranges encountered in vivo. The stability of polyglutamate microspheres appears to be due to hydrogen bonding networks and *not* covalent cross-linking.

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Supporting Information Available: Further characterization data. This material is available free of charge via the Internet at http://pubs.acs.org.

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