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# Hierarchical assembly of protein microspheres

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ABSTRACT

We demonstrate two techniques for the hierarchical assembly of protein microspheres into larger structures. In the first, static self-assembly driven by the buoyancy force of low-density protein microspheres creates a close-packed ensemble, which is freeze dried to form a protein scaffold. The internal pore size and surface area can be controlled through the protein microsphere diameter. In the second technique, directed-assembly of magnetic nanoparticle functionalized protein microspheres is demonstrated. An applied magnetic field directs the assembly into flexible fibers with lengths controlled by the microsphere number density. Taken together, these techniques provide a starting point to explore the design and assembly of complex functional materials.



## 1. Introduction

Hierarchical assembly of individual building blocks is an important route toward more complex materials [1–4]. The complex functionality of a macroscopic material emerges from interactions occurring at the nano and meso-scales. The power of hierarchical design is most evident in nature where biological cells are the fundamental building blocks that assemble into tissues, organs and systems. While the complexity of biological cells is beyond our current synthetic capabilities, the central design motif – flexible, spheroidal units that can be functionalized both internally and externally – represents an important step towards increasingly complex materials.

Protein microspheres were developed in 1990s and consist of a hollow protein shell encapsulating an interior oil phase [5]. These microspheres are easy to synthesize using a green, low-cost sonochemical method and are amenable to variation of the microsphere size and type of protein [6–9]. Both the interior and exterior of the microspheres can be modified to impart specific functionality to the microspheres. Up to now the focus has been on the individual properties of protein microspheres for applications such as multi-modal contrast agents [10]. However, they also have great potential as building blocks for the design and assembly of materials. It is therefore essential to develop techniques to controllably assemble functionalized microspheres into more complex hierarchical structures.

Here we demonstrate two techniques for the hierarchical assembly of protein microspheres. First, static self-assembly driven by the buoyancy force forms a close-packed ensemble that is freeze dried to create a porous protein scaffold. Control of the internal pore size and surface area suggests that these materials may be useful as tissue scaffolds and enzyme reactors. Second, directed-assembly of magnetized protein microspheres is driven with an applied magnetic field. The resulting flexible fibers are promising for artificial cilia for micro-propulsion and mixing.

## 2. Experimental

Synthesis of protein microspheres followed a standard routine [5]. The protein Bovine Serum Albumin (BSA, Sigma-Aldrich), and *n*-dodecane (Kanto Chemical) were used as received. The oil phase (6.7 mL *n*-dodecane) was carefully layered on 10 mL of aqueous BSA protein solution (0.1 – 5 w/v%). A high intensity ultrasonic horn (Sonics Vibra-Cell VC750) was placed at the water/oil interface and sonicated at 560 W for 3 min. The solution was maintained below 30 °C in an ice bath. After the synthesis, the protein microspheres are separated by 5 cycles of centrifugation (1000 × gravity, 10 min) and washing. Complete removal of excess protein was confirmed by UV–vis spectroscopy [11]. The resulting microspheres were analyzed by optical microscopy. To prepare the porous scaffold a 7 mL aliquot of purified microspheres is freeze dried in a small petri dish for 48 h. The microstructure of the scaffold was analyzed by SEM and the surface area was measured by BET.

Magnetic functionalization was accomplished by encapsulating magnetite nanoparticles inside the protein microspheres. Magnetite ($Fe_3O_4$ Sigma-Aldrich) was dispersed at 0.9 w/v % in corn oil and used as the oil phase in the standard synthesis and then

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purified as described above. The microspheres were analyzed by TEM-EDS for nanostructure and composition, TGA for composition and VSM (vibrating sample magnetometry) to determine the magnetization. Assembly of the magnetic microspheres was accomplished using a strong rare-earth magnet (3600 Gauss surface field). Optical microscopy and SEM were used to study the resulting assemblies.

## 3. Results and discussion

*Static assembly of protein scaffolds*: Protein microsphere diameter is a critical parameter for assembling hierarchical structures. In ultrasonic synthesis the emulsification of oil in water can be controlled through the concentration of surfactant [12,13]. For protein microspheres the protein itself acts as the surfactant allowing the microsphere size to be controlled through protein concentration [5]. Fig. 1 shows the diameter of the protein microspheres versus concentration of BSA protein. It is seen that increased concentration from 0.1 w/v% to 5 w/v% results in decreased mean diameter from 22.3 μm down to 2.7 μm. The inset of Fig. 1 shows optical microscope images for several concentrations. The microspheres have a uniform shape and relatively good size dispersion (Supplementary Fig. S1). The size dispersion increases with diameter from 39.6% (2.7 μm) to 58.6% (22.35 μm). Han et al. demonstrated that this polydispersity could be accounted for by the uneven ultrasonic energy distribution [14]. Nevertheless, polydispersity is beneficial for assembly into macro-sized materials because it promotes tighter packing as discussed below.

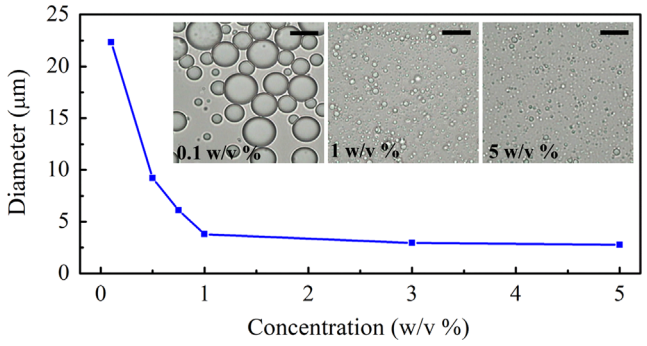


**Fig. 1.** Mean size distribution of BSA microspheres prepared from various BSA concentrations. (Inset) Optical microscopic images showing the resulting protein microspheres. All scale bars are 20 μm.

The as synthesized microspheres are buoyant in solution due to their low-density interior oil phase. The microspheres close-pack at the surface much like bubbles at the surface of a glass of beer. Freeze drying the close-packed protein microspheres produces a porous protein scaffold as shown in Fig. 2(a). The scaffold is lightweight, self-supporting, flexible and easily handled with the look and feel of cotton. Fig. 2(b) shows that the microstructure consists of a network of protein with interconnected spherical pores. The network is formed during freeze drying by first dehydration of water surrounding the spheres pulling them together tightly then the dodecane oil phase slowly escapes through small openings in the 30 nm thick protein shell leaving a protein scaffold with nearly spherical pores.

For specific applications it will be necessary to optimize the pore size. Fig. 2(c and d) shows the resulting microstructures of scaffolds prepared from spheres with mean diameters of 9.2 μm and 2.7 μm. The larger diameter microspheres create a more open structure than the small diameters. BET analysis shows that larger spheres result in a higher surface area (24.4 $m^2/g$) compared to the smaller spheres (15.1 $m^2/g$). The larger spheres are more polydisperse, which allows them to pack more tightly. Since the microspheres are hollow the close packing results in more included pore volume, a lower mass density and therefore a higher surface area per unit gram. The ability to control the microstructure in addition to the type of protein opens up many

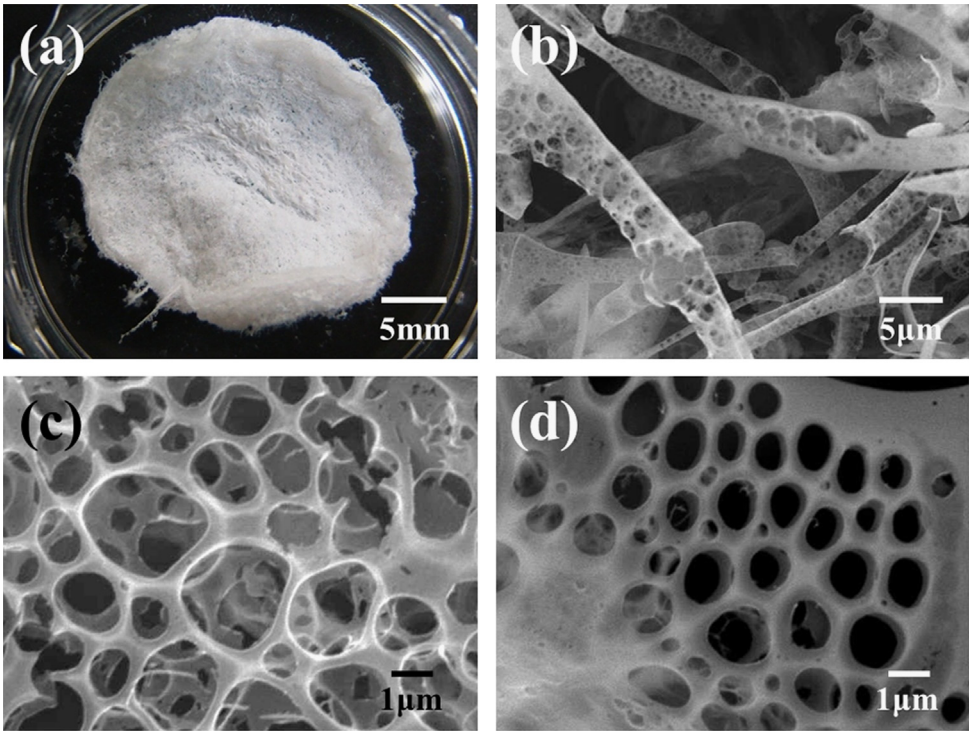


**Fig. 2.** (a) Photograph of a freeze-dried protein scaffold. (b) SEM image of the fibrous microstructure. Higher magnification SEM images of individual fibers for (c) 0.5 w/v% and (d) 5 w/v% BSA concentrations.