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Microspheres of Mixed Proteins

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Abstract: This paper describes the synthesis of mixed proteinaceous microspheres (MPMs) by the sonochemical method. The current fundamental research follows the research of Suslick and co-workers who have developed a method by which high-intensity ultrasound is used to make aqueous suspensions of proteinaceous microcapsules filled with water-insoluble liquids.[1] By using high-intensity ultrasound, we have synthesized microspheres made of a few different proteins. The three proteins used in the current experiments are bovine serum albumin (BSA), green fluorescent protein (GFP), and cyan fluorescent protein–glucose binding protein–yellow fluorescent fused protein (CFP-GBP-YFP). The two synthesized microspheres made of mixed proteins are BSA-GFP and BSA-(CFP-GBP-YFP). This paper presents the characterization of the sonochemically produced microspheres of mixed proteins. It also provides an estimate of the efficiency of the sonochemical process in converting the native proteins to microspheres.

Keywords: core–shell structures · micelles · proteins · sonochemistry · surface modification

Introduction

Ultrasonic emulsification is a well-known process and occurs in biphasic systems. Emulsification is necessary for microcapsule formation. Protein microspheres have a wide range of applications, including drug and oxygen delivery systems,[2–3,12] contrast agents for sonography,[4] and MRI.[5–7]

Micrometer-sized air-filled or liquid-filled proteinaceous microspheres (PMs) were synthesized from various kinds of proteins, such as bovine serum albumin (BSA),[8–10] human serum albumin (HAS),[11] and hemoglobin (Hb)[12] by the sonochemical method developed by Suslick and co-workers. In the late 1960s, a modified polymerization method for the preparation of proteinaceous microspheres was developed by Rhodes, Scheffel, Wagner, and Zolle et al.[13–15] The microsphere formation was accomplished by either heat denaturation at various temperatures or by cross-linking with carbonyl compounds in the ether phase. Other cross-linking agents, such as glutaraldehyde, were also used. Furthermore, air-filled human serum albumin microspheres were made by Dick and Feinstein[16,17] as contrast agents in echosonography. However, these methods yielded microspheres with a short storage life, low microbubble stability, or high toxicity. The first liquid-filled proteinaceous microspheres were prepared by Suslick. They were made of BSA and were filled with n-dodecane, n-decane, n-hexane, cyclohexane, or toluene. The synthesis was conducted under a high-intensity ultrasonic probe, and 1.5×10^9 microcapsules per mL were obtained upon sonicating the precursor solution under air or O_2. The average diameter of the PM was 2.5 μm with a narrow size distribution (Gaussian distribution $=\pm1.0$ μm). The mechanism of the sonochemical formation of PM has been discussed previously.[18] According to this mechanism, the microspheres are formed by chemically cross-linking cysteine residues of the protein with HO_2 radicals formed around a micron-sized gas bubble or a nonaqueous droplet. The chemical cross-linking is responsible for the formation of the microspheres, and is a direct result of the chemical effects of ultrasound radiation on an aqueous medium.

In the current fundamental studies, we have extended Suslick's method and applied it to more than one protein,

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and have succeeded in forming mixed protein microspheres (MPMs). For this purpose we have used GFP (recombinant green fluorescent protein),[19] CFP-GBP-YFP(cyan fluorescent protein, glucose binding protein, and yellow fluorescent fused protein),[20] and BSA proteins as candidates for building mixed protein microspheres. It is worth mentioning that all the studied proteins have cys (cysteine) residues[21] that are responsible for and capable of the formation of S–S bonds (cross-linking) and facilitate the formation of the MPMs. Two of the three different proteins used in the current experiments, GFP and CFP-GBP-YFP, are fluorescent.[22] The GFP protein has a major excitation peak at a wavelength of 395 nm and a minor one at 475 nm. Its emission peak is at 509 nm, which is in the lower green portion of the visible spectrum. CFP-GBP-YFP was excited at 436 nm, and the emission measured at 480 nm. Three possible arrangements can be foreseen as originating from the assembly of two different kinds of proteins in forming microsphere structures. First, the combined two proteins together form the microsphere's walls. Second, the first protein forms the microsphere's walls, while the second protein is encapsulated inside the liquid-filled proteinaceous microsphere bubble. Third, each kind of protein forms separated one-protein microspheres. To prove the arrangement of different proteins in a microsphere structure, we analyzed and characterized the products by light microscopy (Apo-Tome AxioImager.z1 microscope)[23] and by DLS (dynamic light scattering) measurements. Microscopic observation was carried out for the microspheres to determine the arrangement of fluorescent proteins in MPM structures. In addition, the microscopic images (the z-stack images) were analyzed by the 'Imaris' software image analysis program,[24–25] which provides a refined data set and, ultimately, a more precise evaluation of protein expression patterns. The 'Imaris' program enables one to flip and rotate the z-stack in real time, and also to employ the Clipping Plane command that allows a microcapsule to be cut in half for internal review. This is very useful for investigating protein localization. The 'Imaris' program enables the possibility of localizing the fluorescent proteins GFP and CFP-GBP-YFP in the MPM structure.

MPMs have a potential application as a fluorescent detection of microspheres. In cell and molecular biology, the mixed protein microspheres could be introduced into organisms by endosythosis to follow the microspheres in the cell. In addition, since we have already demonstrated that drugs can be encapsulated in a PM in a 3 min sonication process,[26–27] the fluorescence of the MPM can help in tracing the release of the drug upon the disintegration of the sphere. The modified forms of MPM (e.g., MPM, which consists of two different biologically active proteins) could be used as a biosensor. The mixed proteins can serve for the microscopic monitoring of proper targeting. We propose using a mixture of fluorescent protein with a nonfluorescing protein that contains a mutation. Since these proteins are found in the same microshpere if one is properly targeted the second goes along. In this way, we can assure that we targeted the protein to the proper nuclear locale. If we use a nuclear protein, we anticipate localizing the protein to the nucleus. The advantages of MPMs over the single PMs are as follows: 1) To make one PM of a fluorescent protein would be very expensive because of its price. On the other hand, to synthesize fluorescent microspheres (MPMs) we need a small amount of the expensive fluorescent proteins and a large amount of the less expensive nonfluorescent protein. 2) The MPM enables two parts to be targeted in the organism at the same time.

Results and Discussion

The formation, characterization, and properties of the sonochemically made PM were recently reviewed.[28] The mechanism of the MPMs' formation is similar to the mechanism of PMs' formation. Aqueous sonochemistry caused by the implosive collapse of bubbles produces $^{\bullet}OH$ and $^{\bullet}H$. The radicals so produced form H_2, H_2O_2, and, in the presence of O_2, superoxide $^{\bullet}HO_2$. Hydroxyl, superoxide, and peroxide radicals are all potential protein cross-linking agents. The cysteine, which is present in BSA, CFP-GBP-YFP, and GFP proteins, is oxidized by the superoxide radical. The microcapsules are held together by protein cross-linking through disulfide linkages from cysteine oxidation.

Although we have previously shown that the microspherization of proteins can also happen in the absence of cysteines,[21] in the case in which cysteine is not present, we had to lower the pH to form the spheres. In the current case, in which cysteines exist in the protein, under neutral pHs, the S–S bonds are the dominant factor stabilizing the sphere.

After the sonication, we could identify three different kinds of microspheres that were synthesized sonochemically: pristine BSA microspheres (one-protein microspheres), pristine (CFP-GBP-YFP)/GFP microspheres (one-protein microspheres), and BSA-(CFP-GBP-YFP)/BSA-GFP MPM (mixed protein microspheres). A schematic diagram of the arrangement and assembly of proteins leading to microsphere formation is presented in Figure 1.

The morphology of the microspheres was determined by using light microscopy (Apo-Tome AxioImager.z1 microscope). Figure 2 shows a micrograph depicting three kinds of microspheres: a) pristine BSA, b) BSA-(CFP-GBP-YFP), and c) BSA-GFP. As expected, the BSA microspheres are colorless and their average size was calculated to be 2.34 microns.

Figure 2b shows the microspheres obtained upon the sonication of the BSA and CFP-GBP-YFP proteins. The figure does not represent the regular size of the blue microspheres. We have chosen to present microspheres that are much larger than the average blue microspheres obtained in the reaction, because these large microspheres exhibit a much stronger fluorescence signal than the BSA-(CFP-GBP-YFP) spheres. On the other hand, the color detected for the smaller spheres was much weaker. A strong blue sphere is observed at the center of each of the large two spheres. The

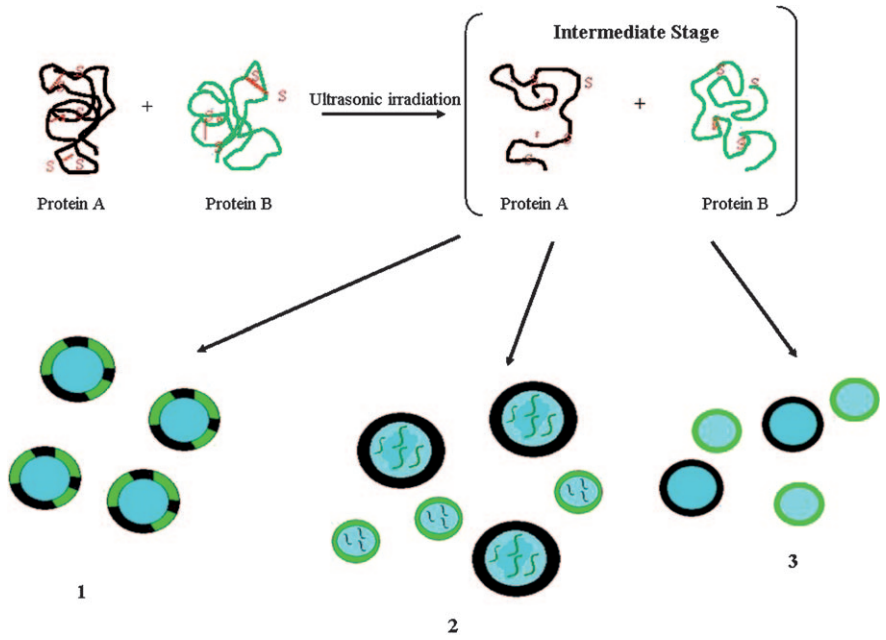

Figure 1. Schematic diagram of the arrangements and assembly of proteins leading to microsphere formation. A = any nonfluorescent protein; B = any fluorescent protein. 1) The combined two proteins together form the microsphere's walls. 2) The first protein forms the microsphere's walls, whereas the second protein is encapsulated inside the liquid-filled proteinaceous microsphere bubble. 3) Each kind of protein forms separated one-protein microspheres.

larger of the two blue spheres is about 25 microns in size and the smaller one is 12 microns. These MPMs were microtomed for further studies. The small microspheres behind and above the blue spheres are black and are surrounded by a white corona. Since the microspheres have different sizes, it is not possible to focus the illuminating beam on all the particles simultaneously. Thus, the corona is a result of focusing the illuminating beam on the blue spheres, and when the beam is focused on the black spheres the corona is not detected. At this stage, without further analysis, it seems that the blue microspheres are attributed to the CFP-GBP-YFP proteins or to the MPMs of BSA-(CFP-GBP-YFP), whereas the small black microspheres are due to BSA.

The microsphere size distribution of the three products (BSA, BSA-(CFP-GBP-YFP), and BSA-GFP) was examined by DLS measurements. When pristine BSA was sonicated, the DLS results yielded spheres with an average size of 2.34 microns (for BSA microspheres). When a mixture of BSA and GFP was sonicated, a bimodal size distribution was found in the DLS measurements. The average size for the major component of the BSA-GFP MPM was 1.406 μm (92.6%). The minor component of 7.4% showed an average size of 244 nm.

Only one DLS peak is obtained for the products of the sonicated mixture of BSA-(CFP-GBP-YFP). The average size of the spheres was found to be 3.525 μm. The results of the calculated size distribution of the microspheres are presented in Figure 3. The picture indicates that tBSA-(CFP-GBP-YFP) is the largest sphere, followed by the BSA spheres, and the BSA-GFP particles are the smallest. We attribute this order to the molecular weight of GFP and (CFP-GBP-YFP) proteins. The molecular weight and the size of the GFP is the smallest, which makes the spheres smaller. This number is increased in the (CFP-GBP-YFP) protein, making the spheres larger than in BSA-GFP. We assign the minor component (7.4%) in the BSA-GFP mixture with an average size of 244 nm to the GFP (one-protein) microspheres. In both mixtures, BSA-GFP and BSA-(CFP-GBP-YFP), a small amount (~5%) of pristine BSA-BSA (average size ≈2.3 microns) microspheres are detected. Since their contribution to the size distribution is negligible, the size order depicted in Figure 3 is a true presentation of the microspheres' sizes.

The different ways of controlling the size of proteinaceous microspheres were previously studied.[28]

To prove the arrangement of different proteins in the microsphere structure, we analyzed and characterized the products by light microscopy (Apo-Tome AxioImager.z1 microscope). We analyzed the Apo-Tome images by the 'Imaris' program to demonstrate the location of the fluorescent protein in the MPM structure. The 'Imaris' program enabled us to "cut" the microspheres into different planes (*xy*, *xz*, and *yz* planes), which allowed us to locate the different proteins in the microsphere structure (see the Supporting Information).

An example of a "reverse" BSA-(CFP-GBP-YFP) microsphere is shown in Figure 4a, in which the fluorescent CFP-GBP-YFP protein was encapsulated inside the liquid-filled BSA microsphere bubble. Figure 4b shows a BSA-(CFP-GBP-YFP) mixed protein microsphere, in which the walls are a mixture of the two proteins, BSA and CFP-GBP-YFP. The light emitted from the microsphere's walls does not spread homogeneously over the whole surface and only patches of blue are observed. We can clearly identify fragments of the blue color originating from the MPM wall that are assigned to the fluorescent CFP-GBP-YFP protein. Figure 4c and d show three kinds of microspheres in the same solution, PM, MPM, and "reverse" MPM. Unlike Figure 4a, in which homogeneous fluorescence originated throughout the content of the sphere, Figure 4d reveals many colored spheres in which the green color is located in only a small part of the volume. These kinds of spheres are clearly mixed spheres, in which the two proteins, BSA and GFP, construct the walls of the sphere. Figure 4c illustrates BSA-(CFP-

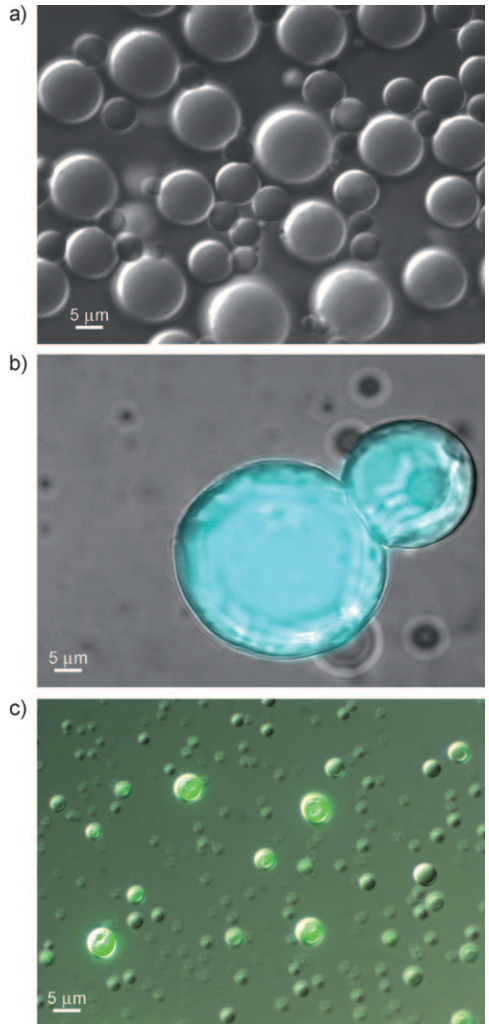

Figure 2. a) Apo-Tome image of BSA-BSA one-protein microspheres. b) Apo-Tome image of BSA-(CFP-GBP-YFP) MPM. c) Apo-Tome image of BSA-GFP MPM.

GBP-YFP) MPMs and pristine BSA spheres. As stated previously, these BSA spheres are only 5% of the total spheres observed in our studies.

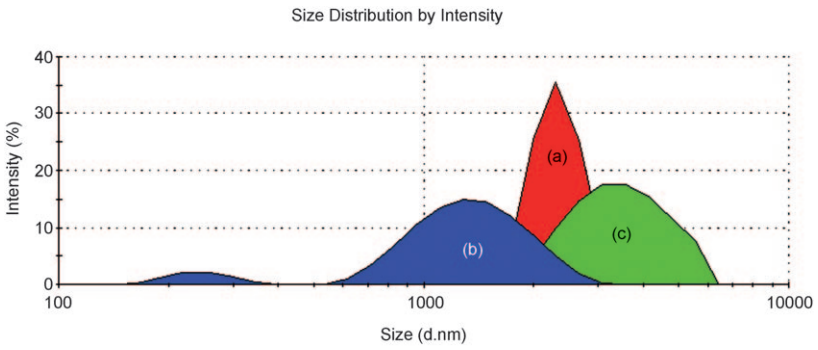

Figure 3. DLS measurement of MPM size distribution. The average sizes of three examples are: a) 2.34 microns (for BSA microspheres), b) average size for BSA-GFP MPM is 1.406 μm (92.6%) and 244.0 nm (7.4%), c) average size for BSA-(CFP-GBP-YFP) spheres is 3.525 μm.

The calculated abundance of each kind of microsphere ("reverse" microspheres, BSA-(CFP-GBP-YFP)/BSA-GFP MPMs, pristine BSA, and pristine GFP/(CFP-GBP-YFP) spheres) was studied by using the 'Scion' image analysis program. These calculations have revealed differences in the particle size of the four kinds of the above-mentioned microspheres. To make this kind of analysis, we first need to identify each kind of microsphere. In "reverse" microspheres, in which the fluorescent (GFP/CFP-GBP-YFP) protein was encapsulated inside the liquid-filled BSA PM, the light (green for GFP or blue for CFP-GBP-YFP protein) was emitted from the inner part of the spheres. The BSA-(CFP-GBP-YFP)/BSA-GFP MPMs emitted the light, which is not homogeneously spread, from the microspheres' walls. The pristine BSA spheres have no fluorescent signal. Pristine GFP or CFP-GBP-YFP PMs emitted the homogeneously spread green or blue light, respectively, from the PM walls.

Figures 5 and 6 present the percentages and average sizes of the different structures for MPMs of BSA-GFP and BSA-(CFP-GBP-YFP), respectively. Figure 5 is divided into a and b, each emphasizing different microspheres. In Figure 5a, in which the green color dominates, it is easier to detect the microspheres with green patches or with a complete green wall. On the other hand, Figure 5b emphasizes the more blackish colors and, therefore, helps to detect the pristine BSA microspheres. The results show that the major components of the BSA-GFP and BSA-(CFP-GBP-YFP) solutions after the sonication are mixed protein microspheres, namely, microspheres the skeleton of which is composed of the two proteins. Figure 5c shows the abundance of the different kinds of microspheres for the BSA-GFP solution. For this solution, four types of microspheres were created: a) pristine BSA microspheres (3.6%) with an average size of 2.3 μm, b) pristine GFP microspheres (3.7%) with an average size of 435 nm, c) reverse GFP-BSA MPMs (8.4%) with an average size of 1.657 μm, and d) GFP-BSA MPM (84.3%) with an average size of 1.25 μm. For the BSA-GFP solution we got a total 92.7% of GFP-BSA MPMs with an average size of 1.453 μm. These results confirm the previous calculation of the Gaussian distribution of particle size (Figure 3), in which the average size for the major component (BSA-GFP MPM) was 1.406 μm (92.6%). The same calculations were made for the BSA-(CFP-GBP-YFP) solution (Figure 6), in which the following results were observed: a) pristine BSA microspheres (4.3%) with an average size of 2.1 μm, b) pristine CFP-GBP-YFP microspheres (4.3%) with an average size of 5.66 μm, c) reverse (CFP-GBP-YFP)-BSA MPMs (7.1%) with an average size of 1.5 μm, and

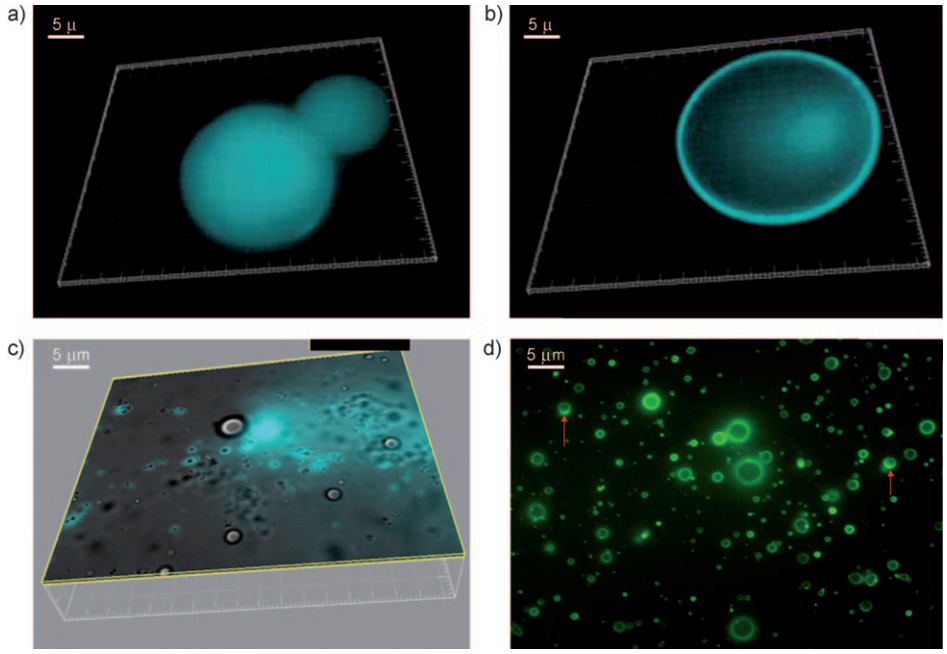

Figure 4. a) The Imaris image for BSA-(CFP-GBP-YFP) MPM. The BSA protein forms the microsphere's walls and the CFP-GBP-YFP protein is encapsulated inside the liquid-filled proteinaceous microsphere bubble. b) The CFP-GBP-YFP protein forms the microsphere's wall. c) The Imaris image with BSA-(CFP-GBP-YFP) MPM and one-protein microspheres: BSA-BSA (without a fluorescent signal) and (CFP-GBP-YFP)-(CFP-GBP-YFP) (with a strong fluorescent signal in the blue portion of the visible spectrum). d) Apo-Tome (z-stack) image of BSA-GFP MPM.

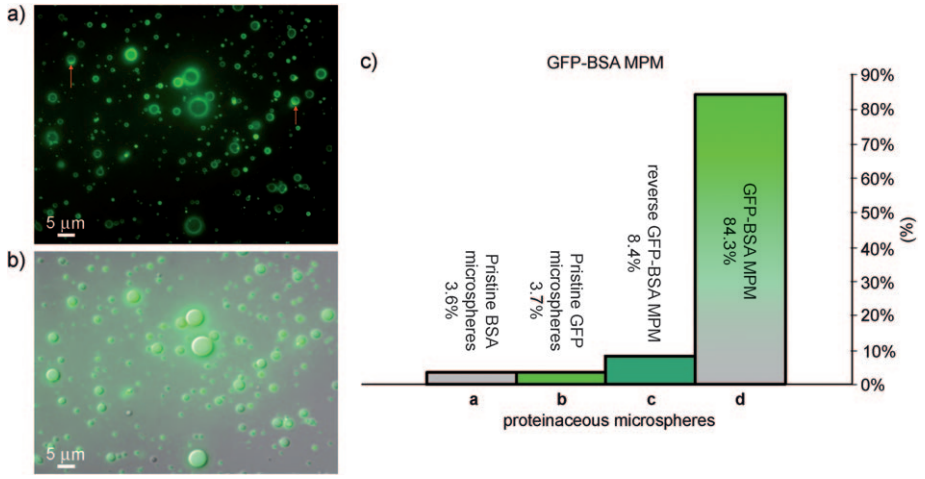

Figure 5. The percentage and size distribution of the various types of the BSA-GFP microspheres. a) Apo-Tome (z-stack one-color channel) image of BSA-GFP MPM. b) Apo-Tome image of BSA-GFP MPM (a and b are the same images). This figure was used for monitoring the percentage and average size of the pristine BSA microspheres. c) Histogram for abundance of each of the MPM structures. The calculations of the percentage of each of the MPM structures and particle size distribution employed the 'Sicon' image software program: a = the abundance of pristine BSA microspheres (3.6%) with an average size of 2.3 µm, b = pristine GFP microspheres (3.7%) with an average size of 435 nm, c = reverse GFP-BSA MPMs (8.4%) with an average size of 1.657 µm, and d = GFP-BSA MPM (84.3%) with an average size of 1.25 µm.

d) (CFP-GBP-YFP)-BSA MPM (84.3%) with an average size of 3.3 µm.

We have also studied the influence of different ratios of BSA: GFP/(CFP-GBP-YFP) on the composition of the MPM. Increasing the BSA fluorescent protein ratio in the precursor solution from 2.2:1 to 6.6:1 caused an increase in the percentage of pristine BSA microspheres in the resulting solution (from ~3–5 to ~12%). This result is explained by the larger amount of BSA, which will favor the formation of pristine BSA microspheres.

Conclusions

In this work, we have synthesized mixed proteinaceous microspheres by the sonochemical method. We have proved that there are three possible arrangements that can be obtained from the assembly of two different kinds of proteins in microsphere structures. First, the combined two proteins form the microsphere's walls. Second, the first protein forms the microsphere's walls and the second one is encapsulated inside the liquid-filled proteinaceous microsphere bubble. Third, each kind of protein forms one-protein microspheres. The 'Imaris' images indicated the location of the different proteins in the microsphere structures. We have found that there are differences in the particle's size in the case of one-protein microspheres against mixed protein microspheres. These differences result from the molecular weight of proteins.

We are currently trying to verify our results by using FPLC measurements. We are also planning to bind antibodies that are specific to CFP-GBP-YFP or GFP to the MPM surface to find out the protein percentage in the microsphere walls.

Experimental Section

Sonochemical preparation of BSA microspheres: Bovine serum albumin (BSA, molecular weight 66 kDa), 96–99% albumin (Sigma) was used

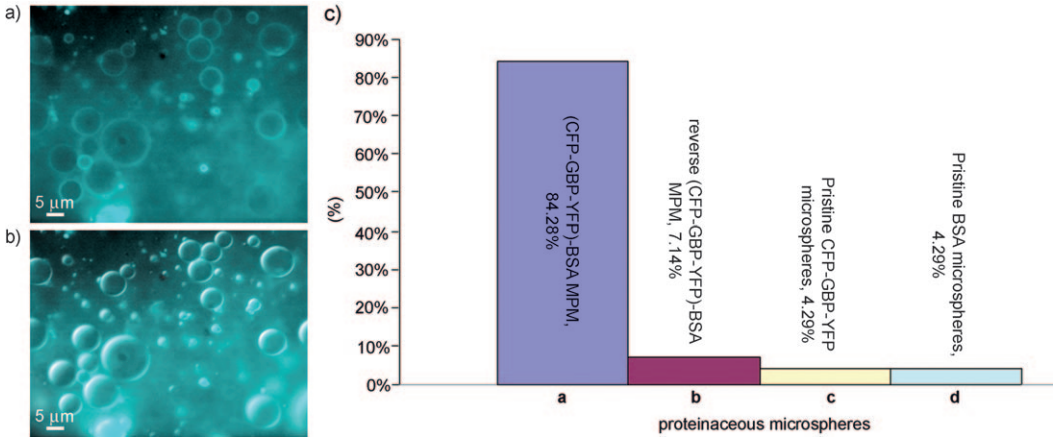

Figure 6. The percentage and size distribution of the various types of the (CFP-GBP-YFP)-BSA microspheres. a) Apo-Tome (z-stack one-color channel) image of BSA-(CFP-GBP-YFP) MPM. b) Apo-Tome image of BSA-(CFP-GBP-YFP) MPM. (Figure 5a and b) are the same images. Figure 5b was used for monitoring the percentage and average size of the pristine BSA microspheres. c) Histogram for abundance of each of the MPM structures. The calculations of the percentage of each of the MPM structures and particle size distribution employed the 'Sicon' image software program. a = the abundance of pristine BSA microspheres (4.3 %) with an average size of 2.1 μm, b = pristine CFP-GBP-YFP microspheres (4.3 %) with an average size of 5.66 μm, c = reverse (CFP-GBP-YFP)-BSA MPMs (7.1 %) with an average size of 1.5 μm, and d = (CFP-GBP-YFP)-BSA MPM (84.3 %) with an average size of 3.3 μm.

without any further purification. The preparation of BSA microspheres followed the typical synthesis of proteinaceous microspheres, as described previously.[29] In brief, dodecane (20 mL, 98.0 % Fluka) was layered over a water solution (30 mL) of 5 % w/v protein. The volume ratio between the 5 % (w/v) BSA aqueous solution and dodecane was kept constant at 3:2, respectively.

General conditions for the preparation of MPM: Herein we outline the common conditions used in the preparation of MPMs. MPMs have been synthesized with a high-intensity ultrasonic probe (Sonic and Materials, VC-600, 20 kHz, 0.5 in. Ti horn, at 78 % amplitude). The volume of the acoustic chamber was 25 mL and the total volume of all the ingredients was 17.7 mL. The bottom of the high-intensity ultrasonic horn was positioned at the aqueous–organic interface, employing an acoustic power of about 150 $W cm^{-2}$ with an initial temperature of 22 °C in the reaction cell. The sonication lasted for 3 min at 22 °C by using an ice-cooling bath to maintain the low temperature. At the end of the reaction, the temperature in the reaction cell reached 28 °C. The rise in temperature from 22 to 28 °C (measured by a thermocouple) did not change the conformation of the proteins. The temperature in the reaction cell should not rise above the denaturation temperature of the proteins (the recommended temperature is 5 °C below the denaturation temperature of the proteins). A separation flask was used to separate the product from the mother solution. The separation was accomplished within a few minutes due to the lower density of the microspheres, relative to the density of water. To obtain a more complete separation of the proteinaceous microspheres from the mother solution, the separation flasks were placed in a refrigerator (4 °C) for 24 h. After the separation, the residual aqueous phase and the organic solvent (dodecane) were removed and the product was resuspended in water.

While the GFP was purchased from Alpha-Diagnostic, the CFP-GBP-YFP was not purchased from outside sources and was purified according to the following description.

CFP-GBP-YFP purification: A BL21 bacterial *E. coli* strain[30] transformed with a plasmid expressing the desired protein was induced for protein synthesis. The protein was purified by using a Talon metal affinity resin (Clontech). The CFP-GBP-YFP protein was identified by Western Blot analysis[31] and reveals the presence of the CFP-GBP-YFP protein at 90 kDa.[32]

Preparation of BSA-(CFP-GBP-YFP) microspheres: The three starting materials used for the preparation of mixed proteinaceous BSA-(CFP-GBP-YFP) microspheres were: 1) a 5 % w/v aqueous solution of BSA (bovine serum albumin, 96–99 % Sigma–Aldrich), 2) a CFP-GBP-YFP protein aqueous solution (0.15 $mg mL^{-1}$), and 3) dodecane (98.0 % Fluka). The amount of water-soluble CFP-GBP-YFP protein in all the reactions varied from 0.5 to 1.5 mL. The ratio of BSA:(CFP-GBP-YFP) varied from 2.2:1 to 6.6:1. The CFP-GBP-YFP protein was added to the aqueous solution of the BSA. The volume ratio of the 5 % (w/v) BSA aqueous solution and dodecane was kept constant, 3:2, respectively.

Preparation of BSA-GFP microspheres: For the preparation of mixed proteinaceous GFP-BSA microspheres, the following reactants were used: 1) a 5 % w/v aqueous solution of BSA (bovine serum albumin, 96–99 % Sigma–Aldrich), 2) GFP (recombinant green fluorescent protein; Alpha-Diagnostic), 3) dodecane (98.0 % Fluka). The volume ratio of the 5 % (w/v) BSA aqueous solution and dodecane was kept constant, 3:2, respectively. The GFP protein was added to the aqueous solution of the BSA (ratio BSA: GFP varied from 2.2:1 to 6.6:1).

Characterization methods: See the Supporting Information.

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