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Biocompatible Protein Nanocontainers for Controlled Drugs Release

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ABSTRACT We designed a biocompatible carrier for controlled release of hydrophobic drugs. The designed carrier was prepared by sonicating oil in a protein aqueous solution forming a protein nanocontainer composed of an inner gel core and an outer protein shell. Two model drugs were loaded into the designed nanocontainers by dissolving drugs in the oil phase before sonication. The loading capacity was up to 0.9 mg/mL for the amphiphilic drug rifampicin, while it reached to 19 mg/mL for the hydrophobic drug indomethacin. The encapsulated drugs were released at different temperatures. At 37 °C, only less than 20% of the drug was released due to the protection by the gel core. Increasing temperature to 40 °C led to a completely release of the remaining drug. The drug release showed drastic temperature dependence. The biocompatibility of the protein nanocontainers was evaluated by incubating the nanocontainers in the 3T3 cell and B-LCL cell lines. Both experiments indicated an excellent biocompatibility of the designed nanocontainers.

KEYWORDS: biomedical application · colloids · core/shell nanoparticles · drug delivery · stimuli-responsive materials

Approximately 40% of the drugs in development and 60% of molecules obtained directly from synthesis are poorly soluble in water.[1] Direct administration of these drugs is usually associated with problems. For example, the aggregates of hydrophobic drugs in circulation can cause embolization of blood capillaries before the drug reaches the required sites.[2] Additionally, the low solubility of hydrophobic drugs in combination with excretion and metabolic degradation hinders the maintenance of therapeutically significant systemic concentrations.[3] One approach to circumvent these problems is to load the hydrophobic drugs into a biocarrier followed by delivery of the carrier to the site of desired action and release the encapsulated drugs *in situ* under control.[4] Encapsulating drugs into a biocarrier is also a potential solution for drug resistance by hiding cytotoxic drugs in the carrier.[5] The requirements for a biocarrier include small size, biocompatibility, biodegradability, high loading capacity, and prolonged circulation. Among the developed drug carriers which include liposomes,[6] polymeric micelles,[7] dendrimers,[8] polyelectrolyte capsules,[9] ceramic nanoparticles,[10] protein cage architectures,[11] virus-derived capsid nanoparticles,[12] and polyplexes,[13] liposomes are the first carrier to reach clinics.[14] Hydrophobic drugs can be loaded into the lipid bilayer of liposomes and delivered in circulation. The limitations for liposomes to encapsulate hydrophobic drugs are the efficacy of drug loading procedures currently available and the difficulty in controlled release. Therefore, efficient encapsulation of hydrophobic drugs into a biocarrier and release drugs under control is still a challenge.

In view of drug loading efficacy, emulsification is an effective method to load hydrophobic drugs into the oil droplets by simply dissolving drugs in the oil phase before emulsification.[15,16] The oil droplets can be coated in such a way that they do not interact with plasma proteins and are not trapped by the reticuloendothelial system (RES), instead remaining intact in the tissue or blood for hours, days, and even weeks. Suslick and colleagues have proposed a new method to prepare stable emulsions *via* sonicating oil in a protein solution forming a protein microsphere composed of an inner oil core and an outer protein shell.[17,18] Sonication not only leads to the emulsification of oil in an aqueous protein solution, but also induces the cross-linking of protein molecules adsorbed at the surface of oil droplets thus forming a stable protein shell. Because of the protection by the protein shell, the prepared microspheres are stable for months at room temperature. The protein microspheres have been prepared for nearly 20 years. However, their development as drug carriers is limited due to the

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Received for review February 15, 2010 and accepted April 13, 2010.

Published online April 15, 2010. 10.1021/nn100307j

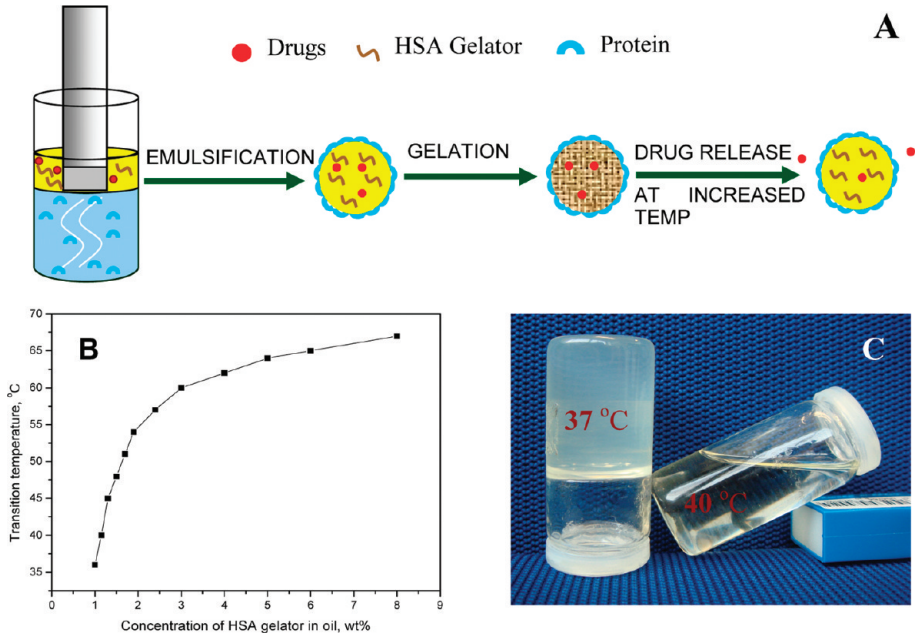

Figure 1. Schematic illustration of sonochemical preparation of a protein nanocontainer with an inner gel core and an outer protein shell (A); the drug encapsulated in the nanocontainer can be released at an increased temperature by transforming the gel core into an oil core. The transition temperature of the gel core is dependent on the concentration of HSA organic gelator (B); at the concentration of 1.3 wt % HSA, the oil mixture exhibits gel state at 37 °C while it changes to liquid at 40 °C (C). The left flask in panel C shows the gel above air.

scarcity of the core/shell structure, bio-properties, and drug loading and release features of the protein microspheres.

In this paper, we design a new biocarrier based on the protein microsphere by introducing gelation into the microsphere. The designed carrier has a gel core and protein shell structure. The gel core is responsible for drug loading and controlled release, while the protein shell plays important roles in biocompatibility and prolonged circulation. The protein shell is first confirmed in this paper. The biocompatibility and controlled release of the designed nanocontainers are evaluated in the following.

RESULTS AND DISCUSSIONS

The preparation of protein nanocontainers is illustrated in Figure 1A. Hydrophobic drugs and HSA (12-hydroxystearic acid) gelator are dissolved into the oil phase first. After sonication, the drug and the gelator are encapsulated spontaneously into the oil droplets. During sonication, the protein molecules adsorbed at the surface of the oil droplets are cross-linked forming the protein shell. When the resultant samples are cooled down from 45 °C to room temperature (about 25 °C), the liquid core of the protein container is transformed into a gel core due to the gelation of HSA. The gel core can reverse to liquid core at an increased temperature, resulting in the release of encapsulated drugs.

The HSA gelator, obtained from castor oil, is chosen as the gelator because it is commercially available and biocompatible.[19] Its molecules readily aggregate in solvents and form a fiber-like structure through hydrogen bonding between the hydroxyl groups at C12 and carboxyl groups that are aligned head-to-head.[20] The fibrous aggregates can entangle, forming a network at low temperature. The organic liquid can be immobilized in the network by surface tension.[21] The networks formed by fibrous aggregates are reminiscent of macromolecular gels and polymer. However, they are thermoreversible. When the temperature increases, the gel core reverses to a liquid core. The transition temperature is determined by the nature of the organic matrix and the concentration of the gelator (HSA). Figure 1B shows the change of the transition temperature with the concentration of HSA in a vegetable oil system. The transition temperature increases with the increase of HSA concentration, reaching 67 °C at 8 wt % HSA concentration. Since our containers are expected to be used in biological systems, we take 40 °C as the transition temperature, which corresponds to 1.3 wt % HSA concentration. The vegetable oil containing 1.3 wt % HSA is solid at 37 °C while it turns into liquid at 40 °C, as shown in Figure 1C.

Figure 2A shows the optical transmission image of the prepared protein nanocontainers. They have a spherical shape with average diameter of 250 nm and a narrow size distribution, which is confirmed by the DLS (dynamic light scattering) measurement, as shown in the inset of Figure 2A. The originally prepared protein containers are nano- to micrometer sized with a broad size distribution due to the uneven power distribution in the sonic cell.[22] Two at-

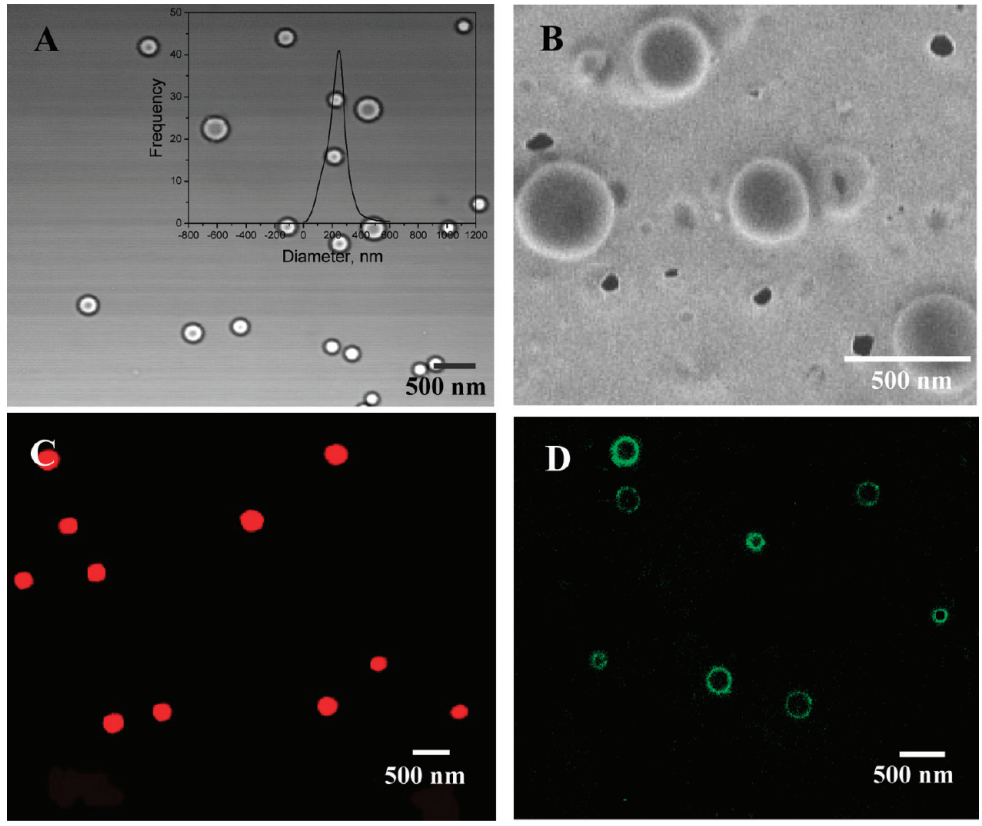

Figure 2. Micrographs of the prepared protein nanocontainers; (A) an image by transmission optical microscopy and the size distribution of the protein nanocontainers (inset of panel A); (B) a scanning electron microscopy image; (C) a confocal fluorescence microscopy image of the protein nanocontainers loaded with red dye (rhodamine B); (D) a confocal fluorescence microscope image of the protein shell labeled with a green dye (FITC).

tempts were conducted in our group to prepare the nanosized protein containers. One is to optimize the preparation conditions in terms of sonic parameters, sonic modes, and oil and solution properties. It is found that pulse sonication conducted at the mode of sonication 2 s and pause 1 s can produce protein containers less than 1 μm in diameter. The pulse sonication can improve the mobility of droplets in the emulsion, which increases the chance of big droplets being close to the sound-emitting surface of the ultrasonic probe, where the big droplets are easily broken to small droplets. Hence, the particles prepared by pulse sonication have a smaller size. Another attempt we performed is to select the desired nanocontainers in the emulsion. Because of their lower density oil droplets are pushed up, and the force is higher for bigger particles which results in a higher creaming speed for the bigger particles in the emulsion. By controlling the viscosity of the emulsion it is therefore possible to obtain a gradient of droplet size in the creaming layer. After diluting the samples to double volume we selected protein containers less than 500 nm in diameter from the down gradient layer after 48 h aging. A systematic study on the relationship of viscosity and creaming velocity as well as dependence of size on creaming time is necessary to develop this novel concept for selection of the desired particles from solution. Figure 2B shows a SEM image of the prepared samples. The containers present a smooth and crack free surface. They attach to the glass slide while keeping a spherical shape, which indicates that the prepared containers are robust and can suffer the vacuum and coating process in the preparation of SEM samples. Figure 2C shows a confocal laser scanning micrograph (CLSM) of the prepared containers loaded with red dye (rhodamine B). The dye is distributed evenly in the container. At room temperature and 37 °C, the encapsulated dyes can be stably carried for weeks as long as the stability of protein nanocontainers is preserved. The presence of the protein shell is for the first time proven by using the labeled BSA protein coupled with a green fluorophor (FITC). The green proteins are seen to assemble at the surface of droplets forming a dense protein layer, as shown in Figure 2D. The high stability of the protein layer is attributed to the formation of disulfide bonds among the protein molecules, which is induced by sonication.[18]

We tested the biocompatibility of the designed protein nanocontainers. Different amounts of nanocontainers are added into the wells containing 3T3 cells. After 24 h incubation, the cell viability was determined by the MTT assay, as shown in the Figure

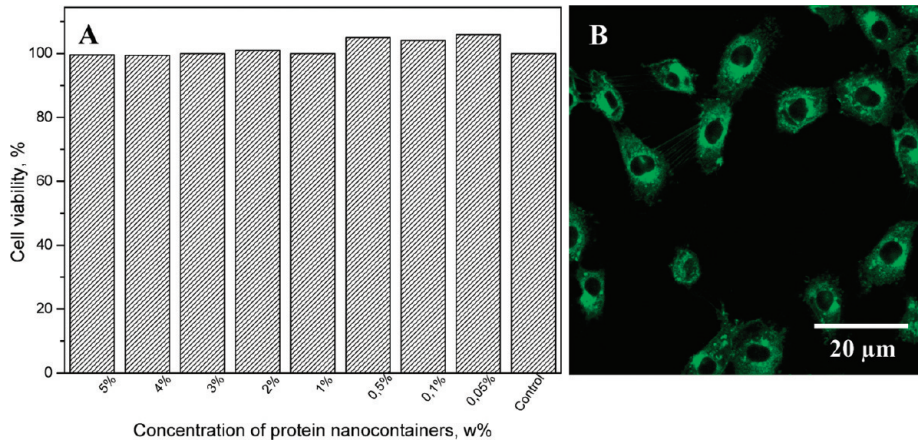

Figure 3. Biocompatibility and distribution of the prepared protein nanocontainers loaded with green coumarine 6 dye in 3T3 cells.

3A. The cells can survive in the containers solution even at a concentration as high as 5% in weight, which indicates a good biocompatibility of the prepared protein nanocontainers. This is attributed to the protection by the protein shell. We examined the distribution of protein nanocontainers in the cell solution. After being washed, the treated cells were observed by means of a confocal fluorescence microscopy. It is found that most containers are internalized into the cells, making the cells present green color, as shown in Figure 3B. The protein nanocontainers are mainly distributed in the cytoplasm while they seldom go into the nucleolus. Since the prepared protein nanocontainer has a positively charged surface (zeta potential of +8 mV), while the cell membrane has a negatively charged surface, it is not difficult to understand their internalization. We also test the biocompatibility of the protein nanocontainers in B-LCL cells. The results are similar to those obtained in the 3T3 cells. The easy internalization and high biocompatibility promises that the protein nanocontainers are excellent carriers for drug delivery.

We loaded model drugs into the designed protein nanocontainers by dissolving drugs in the oil phase before sonication. We chose rifampicin as the first model drug. Rifampicn is a bacterial antibiotic drug. It is typically used to treat mycobacterium infections, including tuberculosis and leprosy. Rifampicin has to be administered daily for several months at a high dose, which could induce serious drug resistant tuberculosis. Microencapsulation was proposed for sustained or controlled release of this drug to improve patient compliance and reduce the emergence of drug resistance.[23] However, the selection of suitable carriers for rifampicin is still an open issue. Here we attempt to load rifampicin into the designed protein nanocontainers. Figure 4A shows the loaded content of the rifampicin in the protein nanocontainers. The loaded content is calculated as follows:

$$\text{loaded content} = (W_0 - W_s)/V$$

where W_0 is the total weight of the added drug, W_s is the weight of unencapsulated drug in the solution after filtration, and V is the volume of the oil. Figure

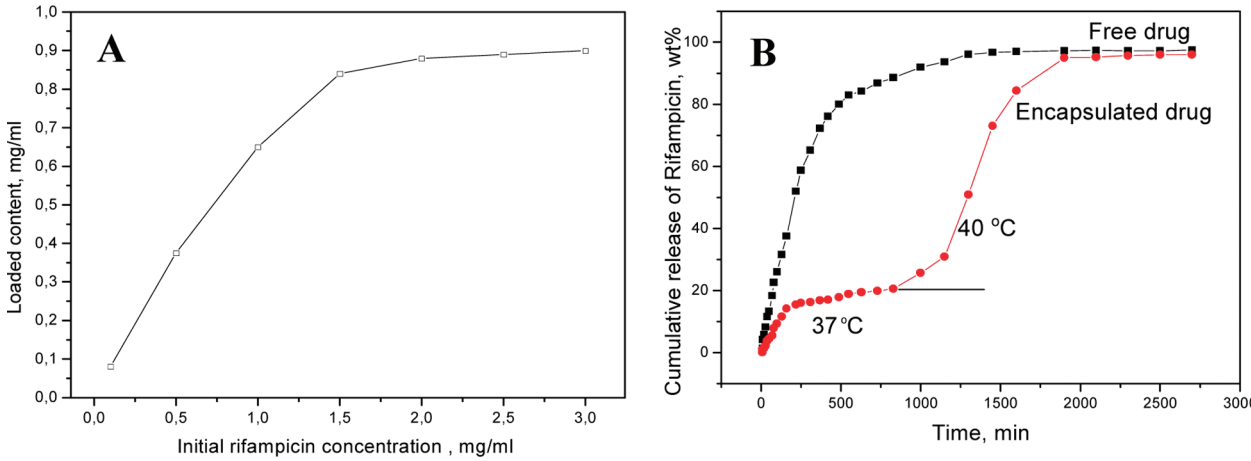

Figure 4. Loaded content (A) and release profile in a dialysis bag (B) of rifampicin by the protein nanocontainers. In B (red curve) the suspension was kept at 37 °C for 14 h then heated to 40 °C, that is, above the transition temperature. The black curve indicates the release of the free drug from a dialysis bag.

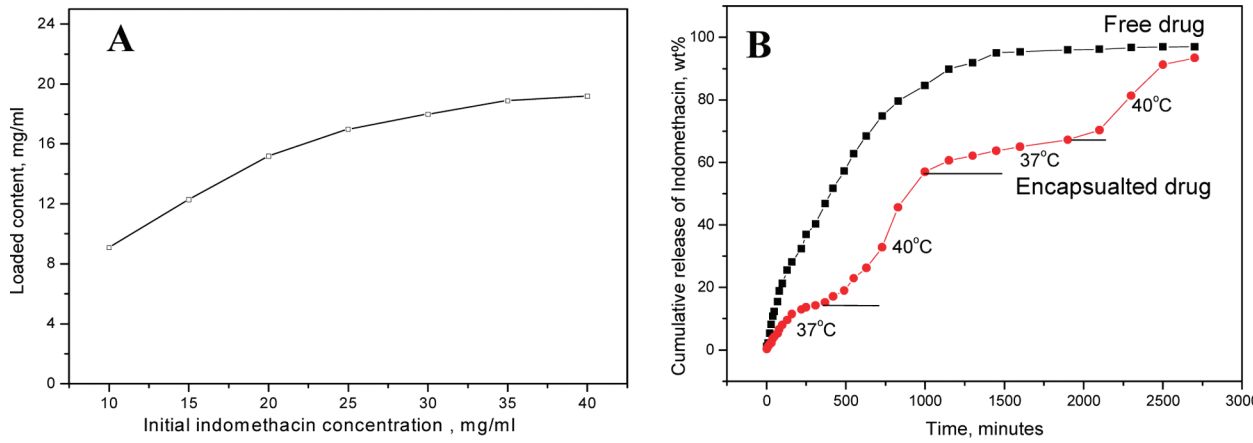

Figure 5. Loaded content (A) and release profile in a dialysis bag (B) of indomethacin by the protein nanocontainers; the red curve in B demonstrates the multistage controlled release triggered by temperature, and the black curve shows the release of the free drug from a dialysis bag.

4A shows that the loaded content increases with the initially added drug concentration, but the value of loaded content is less than the added concentration because of the transfer of the drug from oil phase to aqueous phase during sonication. The transfer ratio is dependent on the hydrophobic property of the drug. Drugs with high hydrophobicity have low transfer ratio, presenting a high loading capacity in the protein nanocontainers, as shown in the second model drug. For the amphiphilic rifampicin, the loading capacity is up to 0.9 mg/mL. The release profile of the encapsulated rifampicin is shown in Figure 4B. Compared with the control experiment, the drugs encapsulated in the gel containers are released slowly at 37 °C by a factor of 2.5 slower, presenting a sustained release in the beginning. This release is attributed to a diffusion process of the drug loaded in the outer layer of the gel core where the drug is not stably fixed by the gel structure. This diffusion release can be reduced when the gel density is increased. The release stops after 3 h with more than 80 wt % drugs remaining inside containers. The remaining drugs could not be released at 37 °C even though we prolong the release time. When the temperature is increased to 40 °C, the remaining drug is released. We believe that the temperature inducing drug release is a result of gel core liquification. At the same time, we cannot exclude the heat-shrinking effect which has been well studied on the polyelectrolyte multilayer capsules prepared by the layer-by-layer techniques.[24,25] The detailed mechanism behind the temperature inducing controlled release is under study and not discussed in this paper. So far we can conclude that the designed nanocontainers can be used for controlled release of encapsulated materials. When the nanocontainers are incorporated with gold nanoshells or gold nanorods, a remotely controlled release can be achieved by infrared light irradiation because gold nanoshells and nanorods can adsorb infrared light and heat the gel core.[26,27]

The second model drug we chose is indomethacin which is a nonsteroidal anti-inflammatory drug commonly used to reduce fever, pain, stiffness, and swelling. Owing to its low solubility, a high dose is usually administered to achieve a therapeutic effect, which in turn causes serious side effects, such as headache, vertigo, dizziness, hearing loss. It is therefore important to develop a vehicle for the controlled release of indomethacin in order to achieve sustained medication clinically, especially for an alleviating pain during sleep at night. The indomethacin being loaded into the protein nanocontainers has a loading capacity of 19 mg/mL which is much higher that that of rifampicin (0.9 mg/mL). This high loading capacity is ascribed to the high hydrophobicity of the indomethacin. The loading efficacy of the rifampicin is up to 70% which is impressive for hydrophobic drugs encapsulation.[28] The release profile of the encapsulated indomethacin is shown in Figure 5B. At 37 °C, 17% of the drugs are released after 3.5 h reaching saturation. When the temperature is increased to 40 °C, the release starts again, which is ascribed to the gel core transformation to liquid core. When the temperature is decreased to 37 °C, the release is slowed down to form a stable state. The increase of temperature to 40 °C restores the normal release again. Therefore, the designed protein nanocontainers can be used for multistage controlled release to release drugs in a desired time and a required dose.

CONCLUSIONS

A novel controlled release platform was designed and prepared by introducing gelation into the protein microspheres forming a protein shell and gel core nanocontainer. The protein shell contributed to a good biocompatibility of the designed nanocontainers, while the gel core endowed the nanocontainers with a con-

trolled release triggered by temperature change. The designed nanocontainers present attractive features in easy drug loading, high loading capacity, and multistage controlled release. They are excellent carriers for hydrophobic drugs, especially for hydrophobic anticancer agents which are difficult to administer directly in circulation. Loading the hydrophobic anticancer agents in the protein nanocontainers can prolong the circulation of drugs and also prevent their destruction to healthy cells. When the containers are coated with targeting ligands[29] or magnetic nanoparticles,[30] the containers can be delivered to the site of interest. Under the trigger of remotely controlled vectors,[26,27] the drug can be released into the cancer cells under control resulting in an improved treatment of cancer with fewer side effects.

METHODS

Materials. Bovine serum albumin (BSA), 12-hydroxystearic acid (HSA) gelator, phosphate buffer solution (PBS), vegetable oil, coumarin 6, rhodamine B, rifampicin, and indomethacin were purchased from Sigma-Aldrich (Germany). All chemicals were used without further purification. The water used in all experiments was prepared in a three stage Millipore Milli-Q plus 185 purification system and had a resistance higher than 18.2MΩ · cm.

Preparation and Characterization of Protein Containers with Gel Inside. A 1.3 wt % portion of HSA gelator was dissolved in a vegetable oil forming a mixture which was kept in the form of liquid by maintaining the temperature above its gelation temperature (40 °C) until completion of sonication. A 1 mL portion of the oil mixture was layered on top of 5 mL of 5 wt % BSA solution in a cylindrical vessel which was attached to a sonicator (GEX 600, Sonics & Materials, Newtown, CT). The tip of the probe was placed at the interface of oil and solution, and the assembly was put in a circulating water bath to maintain the solution temperature at 40 °C during sonication; sonication was programmed to stop automatically above 45 °C. The sonication was carried out at 20 kHz frequency at a power of 40 W/cm^2 for 5 min in a mode of pulse sonication. After sonication, a white milky suspension was obtained. The dyes (coumarin 6 or rhodamine B) and hydrophobic drugs (rifampicin and indomethacin), as required, were loaded into the containers by dissolution in the oil phase before sonication. The size of the microspheres was measured by dynamic light scattering (Malvern Instruments, England). The morphology of samples was measured by a Gemini Leo 1550 scanning electron microscopy (SEM). Confocal fluorescence images were taken by a confocal laser-scanning system from Leica (Wetzla, Germany) equipped with a 100× oil immersion objective with a numerical aperture of 1.4. UV−vis measurement was performed in a CARY 50 UV−vis spectrophotometer (Varian, Germany) to quantify the released drug.

***In vitro* Biocompatibility Study.** The biocompatibility of the designed protein nanocontainers was evaluated by measuring the viability of 3T3 cells (mouse embryonic cells) and B-LCL cells (human B cells infected with Epstein−Barr virus) in the presence of different concentrations of the protein nanocontainers. The viability of the cells was determined by the MTT assay. The cells were seeded in 24 well plates at a density of 30000 cells per well in 500 μL cell medium. After plating twenty four hours, different amounts of the prepared nanocontainers were added in the wells. In case of the control sample, no nanocontainer was added in the well. After 24 h of incubation at 37 °C, 50 μL of MTT solution (5 mg/mL in PBS pH 7.4) were added into each well and the plates were incubated at 37 °C for 2 h. The solution was transferred to 96 well plates and immediately read on a microplate reader (Bio-rad, Hercules, CA, USA), at a wavelength of 490 nm. The experiments were performed in triplicate. Biocompatibility of the prepared nanocontainers was expressed as percentage of cell viability, which was calculated from the ratio between the number of cells treated with the nanocontainers and that of nontreated cells (control).

***In vitro* Drug Release Tests.** The release of drugs from protein nanocontainers was performed in a phosphate buffer solution (pH = 7.4). Protein nanocontainers loaded with drugs were loaded into dialysis tubing (13500 MW) and immersed into drug-free 600 mL PBS buffer which was kept in a temperature controlled water bath. The release experiments were conducted at a controlled temperature (37 and 40 °C). During the release, 1 mL sample of medium was taken out at a desired period and subjected to UV−vis measurement. The removed volume was replaced each time with 1 mL of fresh medium. A control experiment was conducted by putting the same amount of free drug into the dialysis tubing and measuring its release at the same condition. The quantitative analysis of drugs was based on UV−vis data since the intensity of the peaks (rifampicin at 333 nm and indomethacin at 320 nm) is dependent on the concentration of each drug. By referring to a calibration curve prepared separately, we determined the amount of released drugs after different time periods.

Acknowledgment. Y. Han thanks the Alexander von Humboldt Foundation. The work was supported by Nano Future Program of the German Ministry for Science and Education (BMBF) and PPP-China 2009/2010 project. We thank Z. Mao for his help with cell cultures.

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