Protein Microspheres

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Pure Protein Microspheres by Calcium Carbonate Templating**

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A progressive increase in the number of proteins used as therapeutic agents is driven by the high biological activity and specificity of proteins and also advances in biotechnology, which offers new proteins with tailored therapeutic properties.[1] The use of nano- and microcarriers with proteins is a main strategy for site-specific and prolonged drug delivery. A major challenge in protein drug delivery is the formation of protein particles with well-defined characteristics: size, morphology, composition, and density. These characteristics are critically important to achieve high bioavailability with a particular administration route. Conventional methods to produce protein nano- and microparticles include crystallization,[2] spray- and freeze-drying,[3] and incorporation in polymeric matrices or liposomes.[4] These methods, however, often present significant obstacles for control over particle morphology and size, protein stability due to utilization of organic solvents, and exposure to high temperatures or the gas–water interface. Unforeseen negative impacts of the additives/excipients that are generally used in these methods might also arise. Beyond that, monodispersity is often a key parameter to achieve high systemic bioavailability and well-defined release profiles. Thus, the development of new methods to formulate monodisperse additive-free protein particles is an important challenge.

Nanotechnology is making substantial inputs into the field of material development for drug delivery. Herein we present a new method to fabricate pure micrometer-sized insulin microspheres by templating onto porous pH-decomposable $CaCO_3$ microcores. Insulin is a glucose-regulating hormone that is used daily by patients suffering from diabetes; we use this important therapeutic protein as a model protein. Insulin particles are formulated by a one-step procedure in aqueous solution without additives or organic solvents. The microspheres are then characterized by optical and electron microscopy to reveal their structure and the mechanism of formation.

Templating by porous sacrificial microparticles composed of calcium carbonate has been introduced as a novel strategy to fabricate polymeric-matrix-type microcapsules at gentle template decomposition conditions (EDTA or acidic pH) using the layer-by-layer approach.[5] The nontoxic nature of these uniform and relatively monodisperse templates, high loading capacity, low price, easy preparation, and mild decomposition conditions stimulated utilization of the cores for template-assisted synthesis to produce biologically active polymeric capsules,[6] multicompartment[7] and stimuli-responsive capsules,[8] and capsules loaded with materials of a different nature, such as organic solvents, pharmaceuticals, enzymes, DNA, phospholipids, and polysaccharides.[6a,9] Decomposable cores from porous silica have been used as alternative templates to produce microparticles from protein–polymer complexes.[10]

Figure 1 shows microsphere fabrication without any additives and in one step. Regions of stability of $CaCO_3$ microcores (soluble at acidic pH) and insulin (insoluble in pH range 4.5–7.5; see the Supporting Information) have been identified. If the insulin solution is titrated with hydrochloric acid in the presence of $CaCO_3$ microcores starting from pH 9.5 and ending with pH 5.2, a few intermediate states are observed (Figure 1). In the vicinity of the isoelectric point (pI of insulin 5.3[11]), the solubility of insulin is dramatically decreased because it becomes more nonpolar in the polar solvent (water) woing to the decrease of the net charge of aminoacids. The decrease in pH below 8.0 induces a colloidal instability of insulin that promotes protein flocculation in the pores of $CaCO_3$ microcores (Figure 1 a,b). Adsorption of protein molecules on the surface of calcium carbonate promotes surface-mediated nucleation, which results in the growth of insoluble protein agglomerates in the cores but not in bulk solution. Non-ionic surfactants behave similarly, aggregating on glass surfaces.[12] The $CaCO_3$ core is slightly negatively charged (ζ potential of about -8 mV)[5a] under

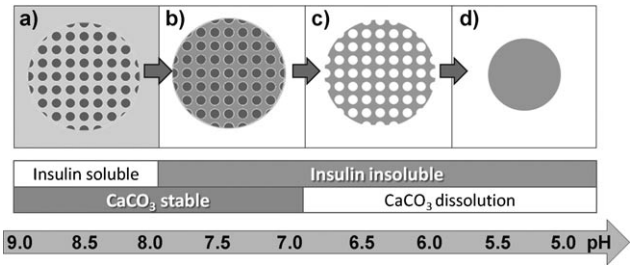

Figure 1. The fabrication of insulin microspheres by templating from $CaCO_3$ microcores. a) The $CaCO_3$ microcores with insulin solution; b) insulin loading by isoelectric precipitation; c) dissolution of the $CaCO_3$ template; d) shrinkage of the porous protein matrix to a compact sphere. Stability regions of insulin and $CaCO_3$ cores are presented for the pH range of 9.0 to 5.0 shown.

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these conditions (pH 9.0), which does not prevent protein adsorption (insulin is also negatively charged) on the microcores followed by exclusive precipitation in the pores of the microcores at pH values lower than 9.0. During titration at acidic pH, the $CaCO_3$ core is decomposed (Figure 1b,c) followed by shrinkage of the porous insulin matrix to more compact protein microspheres/beads (Figure 1c,d). The shrinkage is driven by water removal from the pores in the protein matrix; these pores were created after decomposition of the $CaCO_3$ template. At the final pH value, which coincides with the insulin pI (zero net charge of aminoacids), protein–protein interactions are established mostly by hydrophobic interactions, which promotes water removal from the pores and particle contraction. As core decomposition occurs under mild conditions and with strong interprotein interactions, it does not induce destruction of the protein matrix (Figure 1c), as found by analysis of the protein content in the supernatant after core decomposition.

A highly developed internal structure of $CaCO_3$ microcores can be seen in Figure 2a. In contrast, the insulin microspheres are compact beads (Figure 2c). Due to shrinkage of the pores in the protein matrix, insulin is homogeneously distributed in the microspheres (Figure 2b,d), at least on the scale of around 30 nm that corresponds to the pore size of $CaCO_3$ cores.[5b]

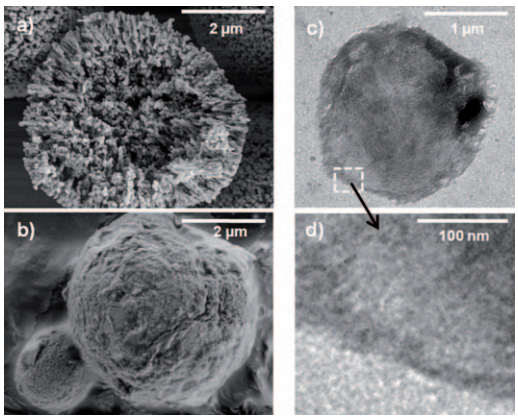

Figure 2. a,b) Scanning electron microscopy (SEM) images of a) $CaCO_3$ microcores (broken particle), b) insulin microspheres (a mixture of microspheres prepared at initial protein/$CaCO_3$ weight ratio 2% and 8% was used). c,d) Transmission electron microscopy (TEM) images of c) an insulin microsphere prepared at initial protein/$CaCO_3$ weight ratio 2%, d) magnified part of image (c).

Insulin loading in the microspheres has an upper and a lower limit. Below the initial protein/$CaCO_3$ weight ratio of 2%, the microspheres are not formed, probably because the stability of the protein matrix in the cores is not high enough to compensate the high osmotic pressure created during $CaCO_3$ core dissolution. A maximum of the loading capacity is reached at a ratio of 8–10% (Figure 3a, depicted by a broken line) that induces an appearance of the protein precipitates in solution together with the microspheres (Figure 3b,c; bulk precipitates depicted by arrows). Taking into account the low content of FITC-labeled insulin molecules (10%) mixed with unlabeled insulin and the low protein density in the microspheres with relatively homogeneous protein distribution, a distance between fluorescein molecules of longer than 10–15 nm can be estimated. Self-quenching, which takes place at interdye distance comparable to the Förster distance (4.2 nm for fluorescein[13]), is thus excluded and the fluorescence intensity is therefore proportional to the dye (that is, protein labeled with the dye) concentration.

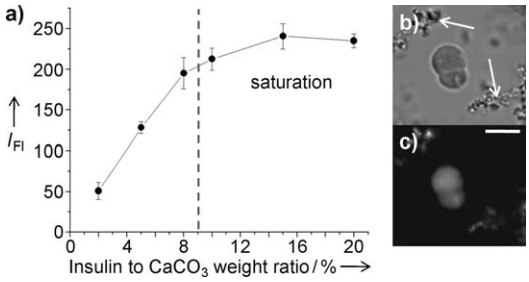

Figure 3. a) Adsorption isotherm for insulin microspheres. Integral fluorescence I_{Fl} is plotted versus the initial protein/$CaCO_3$ weight ratio used for insulin loading. b,c) CLSM images of insulin microspheres prepared at an initial protein/$CaCO_3$ weight ratio of 15%: a) transmission and b) fluorescent mode. Scale bar: 2 μm.

Shrinkage of insulin microspheres takes place after core removal, when there is no barrier to prevent collapsing of the porous protein matrix. The contraction extent is considerably increased with a decrease of protein loading into the $CaCO_3$ microcores (Figure 4a). This effect can be related to a release of larger amounts of water from the more porous and hydrated protein matrix formed at lower protein loadings. The collapsed protein matrix, however, contains a significant amount of water that is independent on the initial protein loading into the $CaCO_3$ cores. The protein density in insulin microspheres was found to be around 0.3 $g\,cm^{-3}$ for cores loaded with protein at protein/$CaCO_3$ ratios of from 2 to 15% (Figure 4b). The high water content is not surprising, because insulin molecules are not crystalline and rather amorphous, as shown by small-angle X-ray scattering (SAXS; see Supporting Information). Amorphous insulin could have some advantages compared to a crystalline phase. Bailey et al. reported that isoelectrical precipitation does not affect the secondary structure of insulin;[14] in general, changes in secondary structure are expected to be less pronounced for the more hydrated amorphous form than for a compact crystalline form. The stability of amorphous insulin towards chemical degradation has been reported to be higher than that of crystalline form.[15] The calculated protein density corroborates well with findings of Bailey et al., who has demonstrated that insulin precipitated in solution at a pH value of about 5 has a density of slightly below 0.3 $g\,cm^{-3}$ and the content of crystalline insulin is around 5%.[14]

A low protein density is advantageous for pulmonary delivery in deep lungs.[16] Particles prepared in this study have a geometric diameter (d_g) from 2 to 4 μm (Figure 4a) that corresponds to an aerodynamic diameter (d_a) from 1.1 to 2.2 μm (respirable range[17]), because for spherical particles in water, d_a is equal to d_g multiplied by the square root of the

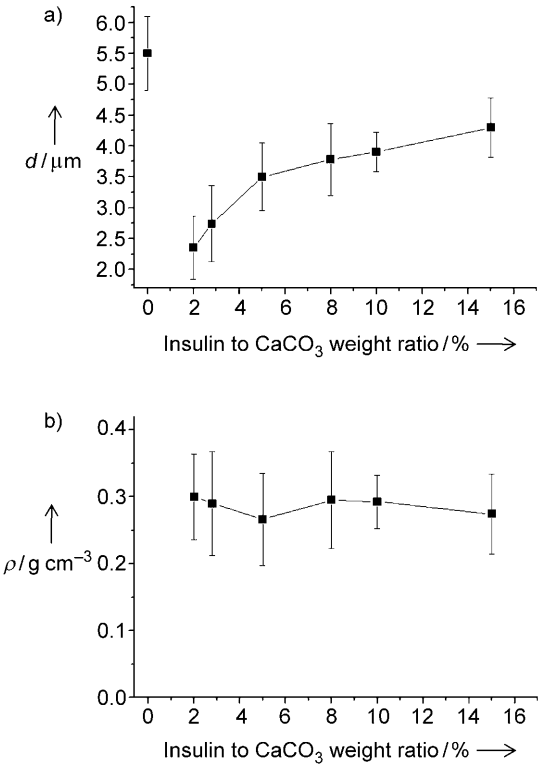

Figure 4. a) Diameter *d* of insulin microspheres and b) protein density ρ in the microspheres as a function of initial protein/$CaCO_3$ weight ratio used for insulin loading. The diameter of bare $CaCO_3$ cores (5.5 μm) is presented at zero protein/$CaCO_3$ weight ratio.

particle density.[14] The finding that the microspheres studied herein have the same protein density as precipitates formed in bulk[14] (Figure 3b,c) indicates that the shrinking insulin matrix (Figure 1c,d) is relatively dynamic but not a frozen structure at the insulin pI. A study on protein mobility (interprotein interactions) in amorphous protein assemblies is of high fundamental and applied interest and is a topic of our future studies. Another direction of the current research with the insulin microspheres includes a study on protein release and cell biology experiments.

In conclusion, we show that pure insulin microspheres can be fabricated by protein templating at isoelectric points on decomposable porous microcores from $CaCO_3$. The main features of the microspheres include uniform size, spherical shape, monodispersity, and no additives or harsh preparation conditions with minimal processing steps. We should stress that the effective method of preparing organic nanoparticles of defined size is not confined to insulin but is of more general applicability. Inspecting Figure 1, it can be seen that the crucial requirement is an overlap of the template stability and drug solubility along with solubility for a certain parameter (here pH) and otherwise insolubility upon template destruction. $CaCO_3$ is a suitable decomposable template for many reasons, but also many other proteins or even small drugs fulfill the conditions cited above.

The features of the protein microspheres make the microspheres valuable for protein delivery and show potential to achieve high systemic bioavailability and avoid potential complications owing to the presence of additives. The approach developed herein can be generalized for many other proteins that can be precipitated at conditions under which $CaCO_3$ microcores are decomposed (that is, acidic pH or the presence of EDTA).

Experimental Section

FITC-labeled and unlabeled insulin from bovine pancreas with 0.5% zinc content of was purchased from Sigma (Germany). $CaCO_3$ microtemplates were prepared according to the procedure described previously,[5b] average particle diameter (5.5 ± 0.6) μm. $CaCO_3$ particles (10 mg) were dispersed in insulin solution (15 mL) with the pH value adjusted to 9.5. The insulin content was chosen to obtain a protein/$CaCO_3$ mass ratio from 2 to 20%. Stock insulin contains 10% (w/w) of insulin-FITC. The suspension was slowly titrated with 0.1M HCl until pH 5.2, followed by dialysis for one day (Float-A-Lyser G2 dialysis tubes, cut-off 0.5–1 kDa, Spectra/Por, USA) against water (2 L) with the pH value adjusted to 5.2. The microspheres were stored at 4°C as a suspension or lyophilized. All experiments were carried out at room temperature.

The relative content of insulin in the microspheres was calculated using the integral fluorescence from insulin microspheres as a function of initial protein/$CaCO_3$ weight ratio. The protein density was calculated taking into account an average size, mass, and porosity of $CaCO_3$ particles[5b] and also the adsorption isotherm (Figure 3a). 30–40 particles were treated to determine the average cumulative fluorescence (Figure 3a) and the microsphere diameter and protein density (Figure 4). For details of CLSM, TEM, SAXS, and insulin titration experiments, see the Supporting Information.

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