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Stability and cell uptake of calcium carbonate templated insulin microparticles

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ABSTRACT

Therapeutic proteins are an integral part of today's pharmaceutical practice, but they still present challenges from the drug delivery point of view. In this work, a new approach is studied based on hard templating for fabrication of microparticles composed of pure insulin, which may enable effective delivery, for instance pulmonary delivery. The approach is both simple and versatile: the protein particles are prepared by selective precipitation into porous $CaCO_3$ microtemplates, followed by full decomposition of the template at the isoelectric point of the protein (pH 5.2). Control over the main material parameters (mechanical properties, porosity, morphology and stability at physiological conditions) are critical for the envisioned application in drug delivery. It is demonstrated that these critical parameters can be significantly tuned by a slight final pH variation around the isoelectric point (pH range 4–6) and by the denaturation degree of insulin. Electrostatic interactions and inter-protein crosslinking in the protein particles as well as their internal structure are considered, to explain the variation in the particle properties. The particle property parameters are explored using atomic force microscopy, optical microscopy and circular dichroism spectra. Finally, phagocytic clearance of the protein particles in vitro was studied to explore possible enhancements in particle fabrication to improve the efficiency of insulin delivery by inhalation.

1. Introduction

Biopharmaceuticals, i.e. molecules that are based on sugars, nucleic acids or proteins, hold great promise for advanced medical therapies. To date, virtually all existing biopharmaceuticals are based on proteins or peptides [1,2]. The significance of protein-based drugs is, to a large extent, due to techniques such as genetic engineering, which allow virtually any class of pharmaceutically active proteins to be produced on a large scale, including hormones, vaccines, cytokines, growth factors, antibodies or antibiotics [3]. However, administration and handling of protein-based drugs pose strict limits to the development of new therapies because of the inherent side effects, e.g. inflammatory response, fast degradation, low retention time, limited control over the release profile and drug bioactivity. Therefore, future developments in biopharmaceuticals are directed towards microscale engineering of protein-based therapeutic compounds to reduce the above-mentioned limitations [4–7]. One obvious approach would be formulating protein-based drugs into particulate form [8–10]. By controlling parameters such as size, density and mechanical properties, one can modulate the release profile and increase protein mechanical stability and stability against degradation. Advanced particulate systems may also include stealth properties and specific release in response to controlled stimuli in vivo [5]. Classic techniques toward microparticulate proteins are lyophilization [11], spray drying [12] or fabrication of pure protein crystals [13]. Protein aggregates can be stabilized by polymers to form microparticles as well [14–16]. However, although these techniques yield microparticulate proteins, the prepared particles are often polydisperse, e.g. in size and density. This hampers control over the release profile of the active component and other relevant parameters such as mechanical properties, colloidal stability and retention time. As an alternative, templating techniques are employed to generate materials with tailored properties and improved uniformity of the particles' material parameters: size, morphology, composition and porosity [17,18].

This work focuses on so-called "hard templating", and the general fabrication procedure can be summarized by two steps: (1) a "hard" but porous inorganic or polymeric template particle is infiltrated with the desired protein species; (2) the inorganic particle is subsequently removed, yielding precise replicates of the inverse template morphology initially. Using this approach, the material parameters of interest can be controlled simply by selection of the template type. For example, the mechanical properties or therapeutic payload can be conveniently tuned when varying the

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template porosity. Templating via porous $CaCO_3$ vaterite particles as hard templates has been identified as a suitable approach for the preparation of fragile biomolecular particles, owing to the template removal in mild conditions [19]. Other template systems often involve hash conditions, e.g. pyrolysis or hydrofluoric acid etching for the removal of polymer [17] or silica templates [20]. Moreover, by controlling the salt concentration and/or stirring speed and time during synthesis, the morphology of $CaCO_3$ microtemplates and thus the size of the resulting templated protein particles can be controlled in a wide range [21]. Furthermore, $CaCO_3$ particles can be obtained with a narrow size distribution and well-defined internal morphology, making them useful as templates for biomolecular species, in particular [22–24]. Most importantly, the $CaCO_3$ templates can be removed in slightly acetic conditions (pH < 6) or by mild treatment with complexing agents such as EDTA. Because of these advantages, templating by porous sacrificial microparticles composed of $CaCO_3$ is now a well-established strategy also for the fabrication of various microparticulate systems, including capsules made of synthetic polymers [25], biopolymers [26,27] and single-component capsules [28,29].

Recently, it was shown that pure, monodisperse protein particles can also be obtained by $CaCO_3$ templating without any additional stabilizers [30,31]. Such unmodified pure protein systems are not stabilized by chemical crosslinkers or other components, but the protein particles are stable as a result of physical inter-protein interaction at the isoelectric point. For many biomedical applications, such additive-free proteins are favored over heterogeneous systems, because additional components, e.g. crosslinkers, may hamper the activity of the protein or lead to undesired side effects, not to mention drug approval issues. The preparation of these pure protein particles makes use of the differential solubility of the protein payload and the $CaCO_3$ matrix, depending on the pH of the solution (Fig. 1).

Previous work highlighted the synthesis of pure insulin microparticles [30] and the mechanism of precipitation in the porous $CaCO_3$ matrix [31]. The present work also focuses on insulin-based microparticles because of the growing demand for improved insulin therapies due to an increasing numbers of patients suffering from diabetes. The aim of this work is to fabricate microparticulate insulin, employing well-controlled material properties (size, structure, stability and mechanics) that are important for the application as a drug. For example, it is known that the size and stiffness of drug carriers may strongly affect the release characteristics (e.g. circulation time) [32] and also their immune response [33].

These parameters may also affect particle adhesion on surfaces and thus colloidal stability, shelf life and the ability to target specific tissues. Consequently, the mechanical properties, porosity, degree of swelling, size, stability and release profile are controlled by adjusting the pH and degree of insulin denaturation. For example, circular dichroism (CD) spectra indicate large differences in activity and solution stability when insulin is stored at high pH (>10) greatly affecting the mechanics and stability of the particles. On a change in pH, the particles exhibit different surface charge, and the swelling behavior is also changed, thus indicating changes in stability in physiological conditions. The results demonstrate new design strategies for protein particle synthesis with tunable material properties. Via atomic force microscopy (AFM) and optical microscopy, these properties are next tested in different media. After characterization of the material properties, the uptake and degradation of the particles by macrophages is tested. The idea is to test the rate of particle uptake as a function of the particle size in order to identify morphologies with the smallest uptake propensity to improve delivery and activity of the drug in vivo.

2. Experimental section

2.1. Particle preparation

FITC-labeled (I2383) and unlabeled insulin (I5500) from bovine pancreas with a content of 0.5% zinc were purchased from Sigma–Aldrich (Germany). $CaCO_3$ particles were prepared as described previously [31]. Controlled denaturation of insulin was conducted in solution at pH 11 via addition of NaOH and incubation at 4 °C for 48 h. Non-denatured particle samples were prepared by immediate processing of fresh insulin solutions at pH 9.5. Insulin particle preparation was based on hard templating, as described in detail previously [31]. Briefly, first, at pH > 9 (adjusted by NaOH/HCl), the $CaCO_3$ templates (5 μm in diameter) were dispersed in protein (insulin) solution. The initial protein/$CaCO_3$ mass ratio was 15%. Then the pH was decreased by controlled addition of 50 mM acetic acid buffer (pH 5.2) via a peristaltic pump at a slow rate, resulting in gradual insulin precipitation and dissolution of the templates (Fig. 1). The pH titration was finished after 45 min, after reaching a pH of 5.2, if not stated otherwise. After synthesis, the particles were purified by 1 day dialysis (Float-A-Lyser G2 dialysis tubes, cut-off 3.5 kDa, SpectraPor, USA) in 2 liters of 10 mM acetic acid pH 5.2.

2.2. Characterization of material parameters

Optical microscopy was conducted on an Olympus IX 71 equipped with a Zeiss HRm camera. Phase contrast and fluorescence images were collected with a 20× Objective (PLN 20XPH, N.A. 0.4, Olympus).

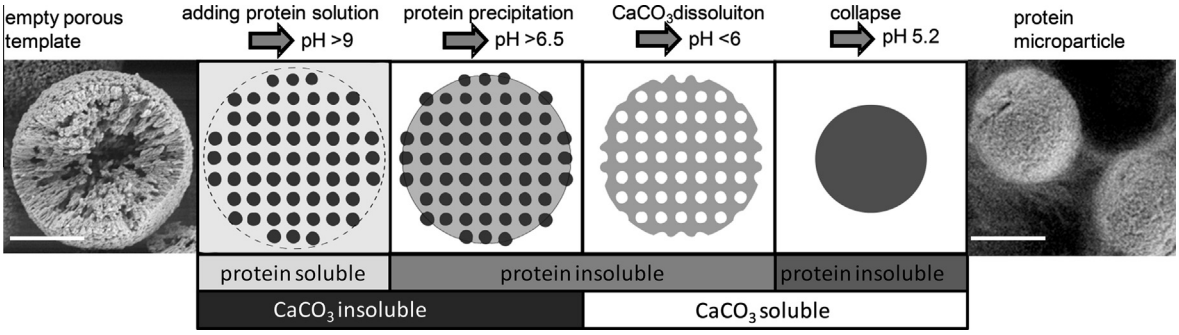

Fig. 1. Fabrication of insulin microspheres by templating via $CaCO_3$ particles. Starting with the empty templates, the insulin solution is added at pH 9.5. Then, the pH is gradually decreased by addition of acetic acid buffer, resulting in selective precipitation that is completed at pH 6.5. Significant dissolution of the template particles starts at the same pH and is typically continued up to the isoelectric point of insulin at pH 5.2 to dissolve $CaCO_3$ completely. Under these conditions, the particle hydrophobicity is maximum and the particle collapses. Scale bar: 2 μm.