High-capacity functional protein encapsulation in nanoengineered polypeptide microcapsules†

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Addition of polyethylene glycol to aqueous assembly solutions of oppositely charged polypeptides enables high-capacity "loading" of functional protein in biocompatible microcapsules by template-supported layer-by-layer nanoassembly.

Interest is rising in the discovery, development, and prescription of peptide and protein therapeutics. The pharmacodynamics of such drugs can be improved and controlled by encapsulation. Delivery systems based on liposomes, polymeric micelles, and polymeric hydrogel-based microencapsulation have been developed for this purpose.[1] Hurdles remain, however, in the engineering of micro- and nanosystems for drug delivery. These include materials biocompatibility, encapsulation efficiency, and stability of fabricated structures in a complex biological environment. Here, we report early findings on high-capacity functional protein encapsulation in polypeptide microcapsules.

Template-supported layer-by-layer assembly (LBL) of oppositely charged polyelectrolytes can be used to "nanoengineer" multilayer films and microcapsules of high stability and selective permeability.[2] Functional microcapsules are promising for controlled drug delivery. Encapsulation of protein by transfer into preformed multilayer shells has been reported. "Loading" is achieved by a change of the surrounding environment,[3] which can create "pores" in capsule walls and increased permeability.[4–7] The method is complicated, however, by deposition of encapsulant on the outer surface of the capsule, and achieving high-efficiency encapsulation at low cost remains a significant challenge.

An alternative approach involving LBL would be to adsorb a polyelectrolyte multilayer film onto a macromolecule-coated "template" which can later be dissolved (Fig. 1). Conceptually appealing, this approach suffers from the displacement of adsorbed macromolecules during deposition of highly charged polyions, promoting the return of macromolecules to the solvent.[8,9] To be practically useful, the approach should limit and permit control over dislodgement from the templates of the adsorbed macromolecules—*e.g.*, therapeutic proteins or nucleic acids prepared at considerable expense—during assembly of the encapsulating film. Later in the capsule engineering process, biological properties of the capsules could be "tuned" by design of polypeptide chains for film assembly,[10] and capsule stability could be controlled by formation of "natural," disulfide crosslinks under mild reaction conditions.[11,12]

Here, high-capacity, high-activity loading of a model protein therapeutic into polypeptide microcapsules has been achieved by addition of polyethylene glycol 300 (PEG300) to aqueous solutions of oppositely charged polypeptides used in capsule assembly. The model protein was glucose oxidase (GOx), chosen for its useful enzymatic properties. Poly(L-lysine) (PLL) (MW ~14.6 kDa) and poly(L-glutamic acid) (PLGA) (MW ~13.6 kDa) were selected for the pilot study because they are readily available from a commercial source; encapsulation involving designed peptides will be reported separately. All chemicals were from Sigma (USA) unless indicated otherwise.

Lyophilized PLL and PLGA were reconstituted in 10 mM Tris, 0.5 M NaCl, pH 7.4. Microcapsules were made by stepwise adsorption of a PLL/PLGA multilayer film[13] on GOx-coated spherical colloidal particles of $CaCO_3$ (PlasmaChem GmbH, Germany), followed by template dissolution (Fig. 1). Positively charged at neutral pH, the templates had a diameter of 4.4 ± 0.4 μm. The known nanoporosity and therefore high surface area of the templates allowed a large amount of GOx to be adsorbed and encapsulated. Maximum adsorption was about 75 μg mg^{-1} $CaCO_3$ microparticles in 10 mM Tris buffer, pH 7.4 (see ESI†). Shell assembly was followed by template dissolution in 0.2 M ethylenediaminetetraacetic acid. Template dissolution under mild conditions, *e.g.* neutral pH, is favorable for preserving protein function and capsule structure.[14–17]

Loss of GOx from template particles during film deposition was monitored by UV absorbance at 280 nm. GOx, but neither PLL nor PLGA, has aromatic groups and can absorb at this wavelength. 5 mg GOx-saturated $CaCO_3$ particles were added to 0.1 mL 1 mg mL^{-1} PLL or PLGA with or without 50% PEG300. Without PEG300, up to 1/2 of the adsorbed GOx molecules were released into solution in the first PLGA deposition step, despite the encapsulating PLL layer (Fig. 2). About 1/5 GOx was lost during the second PLGA adsorption step. Further GOx loss occurred during assembly of the third PLGA layer, though less than in the

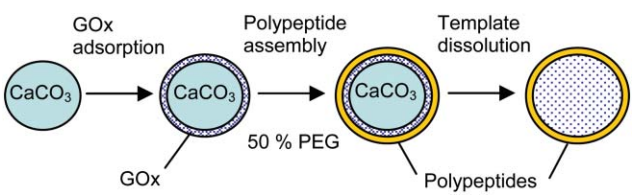

Fig. 1 Nanoassembly of polypeptide microcapsules. GOx was loaded by adsorption onto colloidal $CaCO_3$ particles and encapsulation with polypeptides by LBL. PEG increases GOx loading efficiency.

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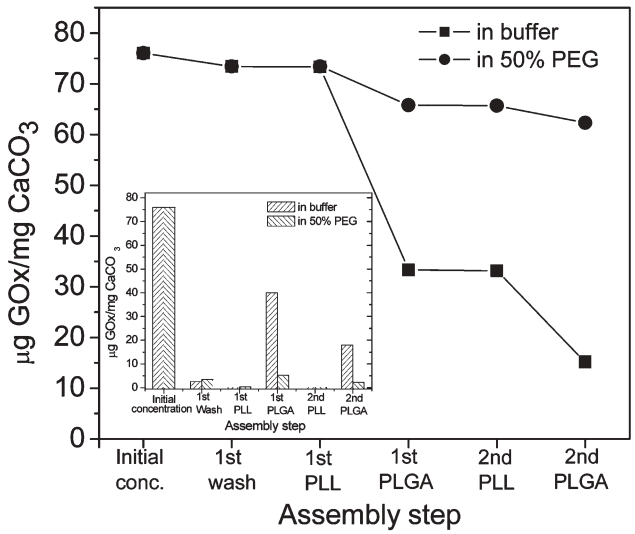

Fig. 2 Retention of GOx on $CaCO_3$ templates during deposition of the encapsulating PLL/PLGA film. The first points represent initial GOx "loading" on templates. Succeeding points show remaining GOx on the templates in subsequent peptide assembly and particle rinsing steps. Only two deposition cycles are shown. Inset, complementary data showing the release of GOx from GOx-loaded template particles as measured in the supernatant of washing and assembling solutions.

previous steps (not shown). By contrast, addition of 50% PEG300 greatly increased retention of GOx on the templates during the first two PLGA deposition steps, and total loss was only ~10% (Fig. 2).

The loading of GOx into polypeptide microcapsules (PLL/PLGA, 12 layers) has been confirmed by fluorescence microscopy. Strong fluorescence emission of 12-layer capsules indicates that a substantial amount of GOx labeled with Cy3 (Amersham Biosciences, UK) was present (Fig. 3). Probably, some of the labeled GOx is associated with the capsule wall, but not all of it. There are three possible fates of a GOx molecule on dissolution of the template: remain associated with the capsule wall, dissociate from the capsule wall and diffuse throughout the capsule interior, or penetrate into the capsule wall. The last possibility is improbable, because the encapsulating polypeptides, which have a high linear density of charge, will bind to themselves more tightly than they will bind to GOx; electrostatic interactions are stronger

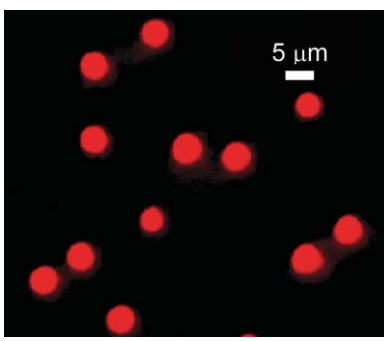

Fig. 3 Fluorescence micrograph of Cy3-labeled GOx after loading into polypeptide microcapsules. PLL and PLGA were not labeled. Capsule diameter is similar to the dimensions of human capillaries.

than possible hydrophobic interactions between GOx and the capsule wall. Some GOx molecules will remain associated with the capsule wall, but many of them will be released into the space created by dissolution of the core particle. Electrostatic interactions between GOx particles and the capsule wall will be relatively weak, and there will be an energetically favorable increase in entropy on release of the protein from the capsule wall, because there are more ways of placing a GOx molecule in the capsule interior than on the capsule surface. Further study by confocal microscopy will seek to determine the proportion of GOx free to diffuse inside the capsules.

The activity of encapsulated GOx without a label was measured by a colorimetric assay involving Amplex Red (Molecular Probes, USA).[18] When capsules were assembled without PEG300, measured GOx activity tended to be about 1/10 that prior to film deposition, independent of film thickness in the 6–12 layer range (Fig. 4). Addition of "inert" PEG300 to the PLL and PLGA assembly solutions substantially enhanced efficiency of capsule loading according to measurement of the enzyme activity. About 4×10^{-12} g GOx was loaded per capsule with PEG; *i.e.*, over 80 mg mL^{-1}, assuming a density of $CaCO_3$ of 2.7 g cm^{-3}, no change in size of capsules during core dissolution, and a template volume of 4.6×10^{-11} cm^3. This concentration is about half of the calculated maximum based on measured adsorption of GOx onto $CaCO_3$ templates. By contrast, without PEG300, about 5-fold less GOx was loaded by assay with Amplex Red, roughly 1/10 the calculated maximum. The data are consistent with a reduction in protein solubility in an aqueous environment by addition of PEG[19] and consequent reduction of loss of GOx from the template surface during polyelectrolyte film assembly. PEG300 might limit interaction between GOx and the polyelectrolyte layers. Leaching of GOx from the capsules was tested, by washing the capsules three times with Tris buffer and centrifuging, and found to be insignificant.

This work has outlined a straightforward means of high-efficiency loading of functional protein in nanoengineered polypeptide microcapsules. Evidently, PLL/PLGA shells prevent leakage of the biological model without precluding the permeability of small molecules. Glucose and oxygen (dissolved in the

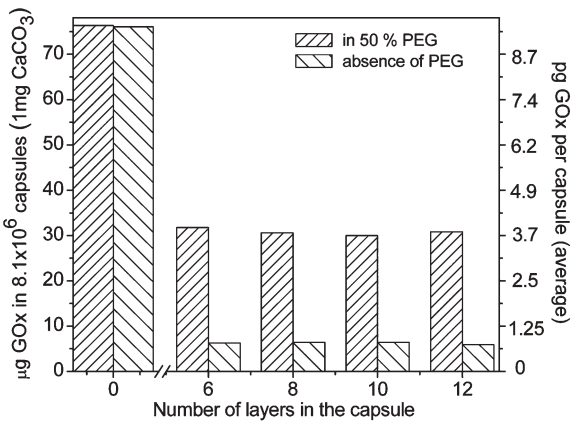

Fig. 4 Measured amount of encapsulated GOx as a function of number of layers of polypeptide. The amount of material retained in the capsule after template dissolution is shown, with and without addition of PEG in the polypeptide assembly solutions. Capsules of fewer than 6 layers were unstable in solution.

buffer) must pass through the capsule wall for the enzymatic reaction to occur. The reaction product, H_2O_2, must then diffuse out from the capsule and react with Amplex Red to generate resorufin in a reaction catalyzed by peroxidase. The assay thus indirectly indicates that the polypeptide microcapsule is semi-permeable; a quantitative study of permeability will be reported elsewhere. Addition of PEG300 to the polypeptide adsorption solutions significantly increased the efficiency of protein loading. Other bio-friendly polymers could presumably substitute for PEG300, for example, poly(vinyl alcohol), poly(propylene glycol), and poly(acrylic acid). The small size of PEG300 will limit its being trapped in the film during polypeptide assembly. The use of a template core dissoluble under relatively mild conditions is favorable for preservation of polypeptide shell integrity and biological functionality. The inherent biocompatibility of the encapsulating polypeptides presents advantages for biomedical applications over the more common non-biodegradable synthetic polyelectrolytes[4–7] used in LBL. This work thus advances LBL for development of bioreactors, biosensors, artificial cells and delivery devices for medically interesting biologicals.

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