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Brief/Technical Note

Improved Enzyme Activity and Stability in Polymer Microspheres by Encapsulation of Protein Nanospheres

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INTRODUCTION

The sustained delivery of proteins from bio-compatible polymers has attracted remarkable interest (1–4). However, the necessary encapsulation of proteins in hydrophobic polymer microspheres remains challenging when using proteins in aqueous solutions (4). For example, when proteins are encapsulated in biocompatible poly(lactic-co-glycolic) acid (PLGA) microspheres using standard water-in-oil-in-water (w/o/w) methods, substantial protein instability is observed. This is primarily due to protein adsorption and subsequent unfolding at the water–oil interfaces (4). Solid-in-oil-in-water (s/o/w) encapsulation procedures avoid the instability problems associated with the first emulsification step in w/o/w methods by employing dry solids (4). However, a common problem with encapsulation methods that use suspensions of dry protein powders is the potentially low encapsulation efficiency which is due to the size and shape of the protein powder particles (5). Companies have gone through quite some investment in method development to overcome this by producing small particles by, *e.g.*, spray- or spray-freeze drying (6,7). Recently, an interesting article showed that several enzymes were formulated as solid nanoparticles by solvent-precipitation and encapsulated in PLGA microspheres by a s/o/w protocol (8). With the use of lysozyme as the model enzyme, good stability after encapsulation and release was obtained.

In this study, we propose an alternative and simple method to encapsulate proteins as solid nanoparticles in PLGA microspheres. We have shown previously that nano-sized dry protein-spheres can be obtained by co-lyophilization of various proteins with methyl-β-cyclodextrin (MβCD) followed by dispersion in ethyl acetate (9). The formulation is scalable because the protein particle size solely depends on the ratio of protein-to-additive during lyophilization (9). In addition to potentially improving the encapsulation efficiency in s/o/w encapsulation methods, the drug particle size is highly relevant because it can influence bioavailability, release, and stability of the drug (10).

Herein, we encapsulated spherical horseradish peroxidase (HRP) nanoparticles in PLGA microspheres by a s/o/w method. Our method of nanoparticle formation typically leads to 100% of recovered enzyme activity (9). Horseradish peroxidase was chosen as the model enzyme because we have accomplished protein nanoparticles using it (9); it is very susceptible to denaturation during *in vitro* release (11) and has been used as a model to study the crossing of proteins through the hemato-encephalic barrier into the brain (12). Ethyl acetate was used during encapsulation instead of dichloromethane (the conventional solvent) because it is less toxic (13). Furthermore, ethyl acetate is the solvent most conveniently used to suspend the protein-MβCD co-lyophilizate to obtain protein nanoparticles (9).

MATERIALS AND METHODS

Materials

Peroxidase (type II) from horseradish (essentially salt-free, lyophilized powder; 150–250 units/mg solid), hydrogen peroxide, 2,2'-azino-bis(3-ethyl-benz-thiazoline-6-sulfonic acid) (ABTS), poly(vinyl alcohol) (PVA, 87-89% hydrolyzed, M_W 31,000–50,000), potassium phosphate, ethyl acetate (anhydrous, 99.8%), and MβCD were purchased from Sigma-Aldrich (St. Louis, MO). PLGA with a copolymer ratio of 50:50 and a molecular weight of 61.6 kDa was obtained from Lakeshore Biomaterials (Birmingham, AL).

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Preparation of HRP Nanoparticles

HRP nanoparticles were prepared as we described in detail by (9). Briefly, HRP (40 mg) and MβCD (160 mg) were co-dissolved in 10 ml of nanopure water, and this solution was frozen in liquid N_2 and lyophilized for 48 h using a Labconco FreeZone 6 L freeze drier at a condenser temperature of −45°C and a pressure of <60 μm of Hg. Protein nanoparticles were formed by suspending the HRP/MβCD co-lyophilizate in 40 mL of ethyl acetate. This suspension was sonicated for 30 s with a model 3510 ultrasonic cleaner from Branson Cleaning Equipment Co. (Shelton, CT) followed by centrifugation for 10 min at 5,500 rpm and 4°C in a Hermle Z 323 K with a Hermle Rotor 220.80 V02 from Labnet Int. (Woodbridge, NJ). Finally, the supernatant was removed with a Pasteur pipette and the HRP nanoparticles allowed to air dry.

Protein Lyophilization

Forty milligrams of horseradish peroxidase (as supplied by the vendor) was dissolved in 10 mL of deionized water at pH 6.5. The excipient was co-dissolved at the desired ratio. The samples were rapidly frozen in liquid N_2 and lyophilized for 48 h using a Labconco FreeZone 6 L freeze drier at a condenser temperature of −45°C and a pressure of <60 μm of Hg.

Particle Size Determination

The size of HRP protein nanoparticles was determined by dynamic light scattering, and the size of the lyophilized HRP powder particles was determined by laser diffraction particle size analysis using a Hydro SM Mastersizer 2000 from Malvern Instruments. Both formulations were suspended in ethyl acetate. Background measurements were performed using ethyl acetate as the carrier solvent as described (9).

PLGA Microsphere Preparation

PLGA microspheres were prepared by a s/o/w encapsulation procedure developed in our laboratory (14). Briefly, 40 mg of lyophilized HRP powder or HRP nanoparticles were suspended in a solution of PLGA in 2 ml of ethyl acetate. The resulting solid-in-oil suspension was homogenized with a VirTis Tempest homogenizer using a 10-mm shaft at 40,000 rpm for 30 s. This suspension was added to a PVA solution and was homogenized at 40,000 rpm to produce the oil-in-water emulsion. The microspheres were allowed to harden for 3 h at 21°C under stirring. After 3 h, the microspheres were collected by vacuum filtration through a 0.45 μm cellulose acetate filter, washed twice with 50 ml of nanopure water, and vacuum-dried for 24 h at <60 μm Hg. The following conditions were varied for optimization purposes: PLGA concentration (50, 100, 200, and 360 mg/mL), PVA concentration (1%, 5%, 10%, and 20% *w/v*), homogenization time (2, 4, 6, and 10 min), and volume of the aqueous phase (40, 60, 80, and 100 mL).

Determination of the Actual Protein Loading and Encapsulation Efficiency

To determine the actual protein loading in PLGA microspheres, 10 mg of PLGA microspheres was dissolved in 1 ml of ethyl acetate and stirred for 30 min, followed by centrifugation at 5,000 rpm for 10 min. The supernatant was discarded and the pellet vacuum-dried for 30 min. This pellet (mostly protein nanoparticles) was dissolved in 1 ml of potassium phosphate buffer at pH 6.5. To separate the soluble and insoluble protein fractions, the samples were subjected to centrifugation at 5,000 rpm for 10 min, and the soluble fraction was removed with a Pasteur pipette. Protein in the buffer insoluble fraction was completely dissolved by adding 1 ml of 6 M urea. The protein concentration was determined by measuring UV absorbance at 280 nm. The encapsulation efficiency was determined as described (14).

Scanning Electron Microscopy (SEM)

A JEOL 5800LV Scanning Electron Microscope was used to obtain scanning electron microscopy (SEM) micrographs. A Denton Vacuum DV-502A was used to coat the protein nanoparticles and PLGA microspheres with gold. The size of the PLGA microspheres was determined from images using the Scandium software.

In Vitro Protein Release

In vitro release tests were carried out by placing 30 mg of PLGA microspheres in 1 ml of 10 mM sodium phosphate buffer at pH 7.4 and incubation at 37°C. Every 24 h, the samples were subjected to centrifugation (5,000 rpm for 5 min in a Beckman J-2B centrifuge) to pellet the insoluble components. One milliliter of the supernatant was removed and the concentration of released protein determined by absorbance measurement at 280 nm or by bicinchoninic acid assay (following manufacturer's instructions). Fresh release buffer was added every 24 h to maintain sink conditions. The concentration of released protein was used to construct cumulative release profiles. All release experiments were performed in triplicate, the data for each time point averaged, and the standard deviation calculated.

Determination of Enzyme Activity

HRP activity was determined by UV–vis spectroscopy using a Shimadzu 160 UV/Vis spectrophotometer at 22°C as described (11). The enzymatic reaction of HRP was performed in a 1-mL cuvette using 25 mM potassium phosphate buffer at pH 7.3, 10 mM of ABTS, 0.001 mM of HRP, and 20 mM of H_2O_2. The time-dependent absorbance changes at 414 nm were measured, and the slope was used to determine the initial velocities for each HRP sample. The residual activity was calculated with respect to the specific activity obtained from freshly prepared HRP solution. All samples were performed in triplicate.

Statistics

The data was statistically analyzed via one-way analysis of variance using the GraphPad Prism 4 Software (La Jolla, CA). A value of $p<0.05$ was considered significant.

RESULTS AND DISCUSSION

Accomplishing encapsulation and sustained release of proteins from biocompatible polymers is still challenging due to protein susceptibility to unfolding, inactivation, and aggregation. Several approaches have been used to improve protein stability in polymer microspheres, *e.g.*, the addition of stabilizers such as sugars, polymers (*i.e.*, PEG), and salts (5,15). In this context, we explored employing solid protein nanoparticles in the encapsulation procedure because we recently found excellent stability of various proteins when forming the particles using MβCD in conjunction with lyophilization and suspension in ethyl acetate (9). The model protein HRP was formulated as solid nanoparticles by co-dissolving HRP and MβCD at a 1:4 mass ratio followed by lyophilization for 24 h. The specific activity of lyophilized HRP was 87±1% and that of HRP co-lyophilized with MβCD 85±2% after reconstitution in buffer. This demonstrates that the excipient was not an efficient lyoprotectant in the case of HRP. The HRP/MβCD powder was suspended in ethyl acetate in order to dissolve the MβCD followed by sonication for 30 s. The ethyl acetate containing MβCD was removed, and the solid protein nanoparticles were collected by centrifugation as described (9). The recovered specific activity for the nanoparticles was 100±4% after reconstitution in buffer. The size of these HRP nanoparticles was determined to be 148±16 nm by dynamic light scattering (9). Figure 1 shows the spherical shape and size of the protein nanoparticles obtained by this method.

We proceed to explore the effect of the protein nanoparticle formulation in protein stability during encapsulation and release from PLGA microspheres. The encapsulation parameters chosen were the optimum parameters identified for the incorporation of proteins in PLGA microspheres by a s/o/w technique (see Supplemental material for details) (14). SEM micrographs of the HRP protein nanoparticles of the PLGA microsphere loaded with HRP nanoparticles and lyophilized HRP (for comparison) are shown in Fig. 2. The two formulations generated spherical PLGA particles in the micrometer range with quite some variation in size. We then studied the effect of protein nanoparticles on protein activity, encapsulation efficiency, specific activity, insoluble aggregates, microsphere size, initial release, and residual activity. In general, the optimum encapsulation procedure should lead to minimum protein instability, maximum encapsulation efficiency, and low burst release. In addition, a small PLGA particle size (<50 μm) may be more desirable because of its reduced initial protein release and its tendency to display constant release rates (zero-order protein release kinetics) (15). Maintaining protein integrity is crucial because protein aggregates administered to the body can cause severe immune reactions (16). Table I shows the results obtained for the encapsulation of HRP in PLGA microspheres. As expected, we obtained an increase in encapsulation efficiency (EE) by incorporation of the HRP nanoparticle formulation. The EE was with 55±1% for the HRP nanoparticles significantly higher ($p<0.0002$) than the 39±4% for the lyophilized powder (Table I). The enhanced EE can mainly be attributed to the smaller size of HRP nanoparticles (148 nm) compared with about 1,000 nm for lyophilized HRP.

After encapsulation, the specific activity of HRP could be completely recovered (100±4%) for HRP nanoparticles but dropped to 87±2% for lyophilized HRP (Table I). Similarly, significantly ($p<0.001$) less encapsulation-induced aggregate formation was observed for HRP nanoparticles (3±2%) compared with lyophilized HRP (12±1%). The residual HRP activity after 24 h of *in vitro* release for the encapsulated nanoparticles was with 100±1% significantly ($p<0.00001$)

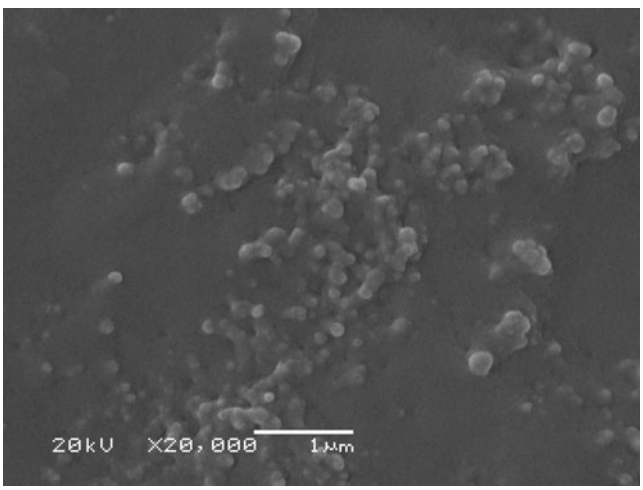

Fig. 1. Scanning electron micrographs of protein nanoparticles formed after co-lyophilization with MβCD in a 1:4 mass ratio, suspension in ethyl acetate, and sonication

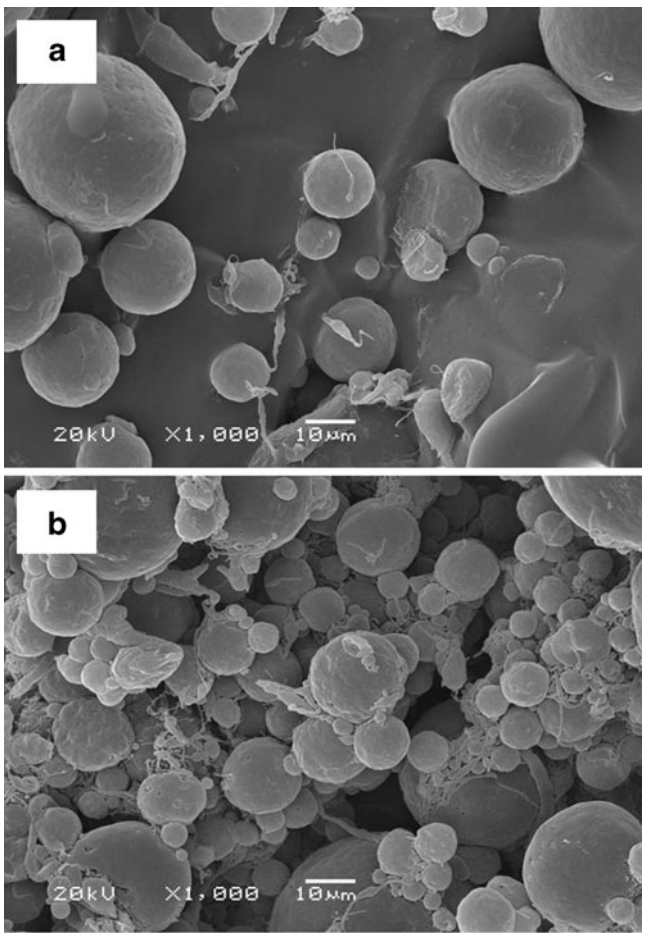

Fig. 2. Scanning electron micrographs of PLGA microspheres loaded with lyophilized HRP powder (**a**) and HRP nanoparticles (**b**)

Table I. Characteristics of HRP-Loaded PLGA Microspheres Obtained by the Encapsulation of HRP Formulated as Nanoparticles and Lyophilized Powder

HRP formulation[a]	Encapsulation efficiency (%)[b]	Specific activity (%)[c]	Insoluble aggregates (%)[d]	Microsphere size (μm)[e]	Initial release (%)[f]	Residual activity (%)[g]
Nanoparticle	55±1*	100±4*	3±2**	12±4	20±1**	100±1*
Lyophilized powder	39±4	87±2	12±1	20±11	58±1	65±9

HRP horseradish peroxidase, *PLGA* poly(lactic-co-glycolic) acid

[a] The encapsulation conditions used for both protein formulations were as follows—360 mg/mL PLGA, 2 mL of ethyl acetate; homogenization time of 2 min at 40,000 rpm, and 80 mL of 10% PVA in water (See "Materials and Methods" section for details)

[b] The encapsulation efficiency (EE) was calculated using the actual and theoretical loading of the PLGA microspheres

[c] The specific activity is the percentage with respect to that of the freshly dissolved HRP. The specific activity values were calculated for the soluble protein fraction

[d] Percentage amount of HRP that formed buffer-insoluble aggregates upon encapsulation in PLGA microspheres

[e] Size of PLGA microspheres obtained upon encapsulation of HRP (see "Materials and Methods" section for details)

[f] The initial release was determined based on the amount of protein in the release buffer after 24-h release

[g] Protein activity that remains after encapsulation in PLGA microspheres. The residual activity was calculated with respect to the specific activity obtained from freshly prepared HRP solution

*$p<0.0001$ level of significance *versus* lyophilized powder value

**$p<0.001$ level of significance *versus* lyophilized powder value

higher than that of the lyophilized formulation (65±9%). The increase in protein activity and decrease in aggregate formation observed after encapsulation of HRP nanoparticles is likely due to their faster encapsulation in the polymer microspheres, thus decreasing the chance for the protein interaction with the o/w interface (the most detrimental step in the encapsulation process).

Next, we determined the release properties of the PLGA microspheres by *in vitro* measurements (Fig. 3a). The initial burst release was significantly ($p<0.0002$) lower for the HRP nanoparticle formulation (20±1%) than for lyophilized HRP (58±1%). This was not likely caused by microsphere size differences. Even though the PLGA microspheres obtained seemed with 12±4 μm to be somewhat smaller for encapsulated HRP nanoparticles compared with those obtained with lyophilized HRP powder (20±11 μm), there was no statistical significance ($p<0.1$). Given that the microsphere size was not very different, drug particle size should be of major importance (17). Larger drug particles will statistically be closer to the surface of the microspheres and therefore release should be faster. The reduction in burst release obtained with the HRP nanoparticles is likely due to the decrease in HRP particle size. Due to their smaller size, protein nanoparticles are encapsulated inside the microsphere rather than attached to or close to the surface of the polymer (18). In addition, the spherical shape of protein nanoparticles may have contributed to the decrease in initial release. It has been shown that the shape of the particle also influences the initial release (7). Spherical particles have a more uniform distribution in the polymer matrix than irregularly shaped particles and therefore have a lower burst release (7).

The sustained release profile for lyophilized HRP was dominated by a fast release lasting only a few days (Fig. 3a). In contrast, microspheres loaded with protein nanoparticles showed a sustained release for 25 days. After this time, the release slowed down due to depletion of the microspheres. Such linear release profiles are important for many sustained release applications in which a constant daily dosage is aimed for (*e.g.*, hormone replacement treatment). It is evident that HRP nanoparticles produced much more desirable release properties than the lyophilized HRP powder. The incomplete

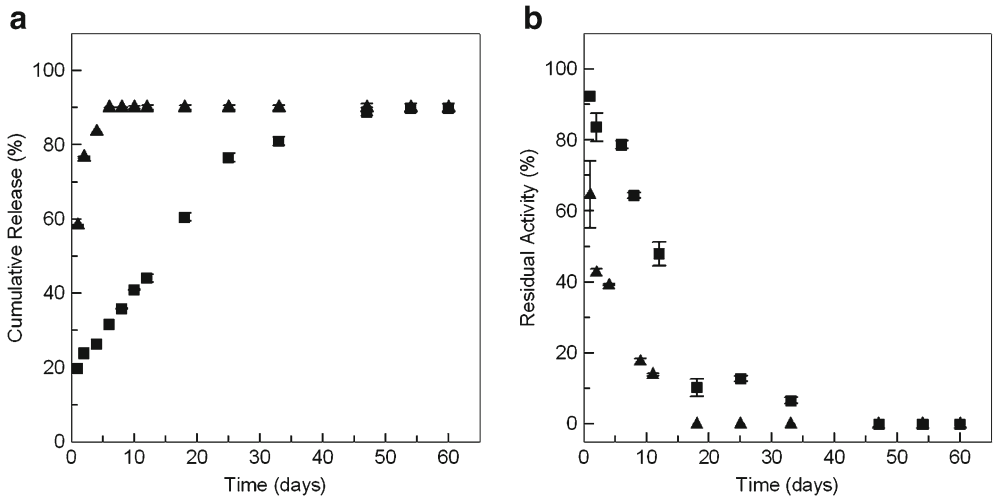

Fig. 3. Cumulative release profiles (**a**) and residual activity during *in vitro* release (**b**) of microspheres containing HRP nanoparticles (*squares*) and lyophilized HRP powder (*triangles*)

release can be attributed to the formation of buffer-insoluble protein aggregates and to protein adsorption to the degraded microsphere surface (19). (Note that we have not investigated release of soluble protein aggregates in this investigation.)

Figure 3b shows the residual activity of HRP nanoparticles and lyophilized HRP at different time points of *in vitro* release from PLGA microspheres. The activity of encapsulated lyophilized HRP dropped to only 10% during the first 10 days of release. In contrast, HRP nanoparticles maintained ca. 50% of activity during the same period of time. Moreover, protein nanoparticles retained some activity (~15%) for a prolonged time (25 days) of *in vitro* release. Still, additional stabilization (*e.g.*, prevention of a drop in the pH value upon release) must be employed to overcome the activity loss (19).

CONCLUSIONS

In this work, we demonstrate a simple and effective method to encapsulate protein nanoparticles in PLGA microspheres. The use of protein nanoparticles revealed substantial advantages over a lyophilized protein formulation by not only increasing the EE, reducing burst release, and improving release profiles for prolonged times, but also by enhancing protein activity during release from PLGA microspheres. The most likely explanation for this is a more complete and rapid encapsulation of nanoparticles in the PLGA matrix minimizing deleterious protein–water interactions in the process. This technology based on the use of protein nanoparticles could be very useful for the improvement of the stability of large and fragile therapeutic enzymes in sustained release applications.

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