Protein Nanoparticles for Intracellular Delivery of Therapeutic Enzymes

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ABSTRACT: The use of enzymes as therapeutics is very promising because of their catalytic activity and specificity. However, intracellular delivery of active enzymes is challenging due to their low stability and large size. The production of protein-enzyme nanoparticles was investigated with the goal of developing a protein carrier for active enzyme delivery. β-Galactosidase (β-gal), an enzyme whose deficiency is the cause of some lysosomal storage disorders, was incorporated into enhanced green fluorescent protein nanoparticles prepared via desolvation. Particle size was found to be sensitive to the type of cross-linker, cross-linking time, and the presence of imidazole. The results indicate that β-gal activity is highly retained (>70%) after particle fabrication and >85% of protein is incorporated in the particles. Protein-enzyme nanoparticles exhibited higher internalization in multiple cell lines *in vitro,* compared with the soluble enzyme. Importantly, β-gal retained its activity following intracellular delivery. These data demonstrate that protein nanoparticles are a biocompatible, high-efficiency alternative for intracellular delivery of active enzyme therapeutics. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: therapeutic enzymes; protein delivery; nanoparticles; intracellular delivery; enzyme activity; cross-linking; desolvation; macromolecular drug delivery; particle size

INTRODUCTION

Enzyme therapeutics offer multiple advantages over small molecule drugs. Enzymes can bind their targets with high affinity and act with high specificity.[1] Most importantly, an enzyme's catalytic activity allows it to rapidly convert multiple target molecules into products, which significantly amplifies its effect compared with that of small molecules.[2,3] These unique properties make enzymes promising therapeutic agents, and an important and growing segment of pharmaceuticals.

Although most targets of United States Food and Drug Administration-approved enzymatic drugs are extracellular, there are many diseases for which treatment requires intracellular delivery.[4] Effective enzyme replacement for genetic diseases such as lysosomal storage diseases requires intracellular delivery to lysosomal compartments.[2,5] More therapeutic applications of intracellular enzymes are expected to emerge, including inhibition or reversal of ubiquitination for cancer[6] and phosphorylation of Tau protein for Alzheimer's disease.[7] Enzymes can effectively perform these functions,[8–11] but require modification, encapsulation, or immobilization on biocompatible matrices to improve their stability and limited distribution.[1,2,12] Hence, there is a critical need to develop delivery systems that will improve accessibility of enzymatic drugs to intracellular compartments.

There are many challenges associated with the delivery and retention of activity, given the large size of enzymes and their complex tertiary or quaternary structure that can be highly sensitive to the environment.[2] A delivery vehicle must be produced in conditions gentle enough for activity retention and be small enough to penetrate tissues and be internalized by cells.[13,14] The size of delivery vehicles should be in the nanometer range, but the exact size depends on the application. Enzymes packaged in a delivery vehicle possess several advantages over soluble formulations, such as higher stability, lower immune response and targeting capabilities.[12,13,15–19] Most delivery systems immobilize enzymes on the surface of another material or encapsulate them in polymeric, lipid, or mesoporous materials.[2,4,20–25] Other methods involve fusion of an enzyme to other proteins or peptides.[26] However, challenges remain, such as low encapsulation efficiencies, reduction in activity from fabrication conditions, or undesirable degradation products.[27]

A biodegradable, alternative method is to use protein particles for delivery of enzymes. Protein particles offer multiple advantages over their polymeric, inorganic, and liposomal counterparts including their high loading capacity, ease of production, surrounding protein environment, and amino-acid degradation products.[28,29] Most protein particles are studied for intracellular delivery of small molecule drugs,[29–35] only a few protein drugs have been investigated.[36,37] Little is known about the production or function of enzyme nanoparticles. Some proteins and enzymes have been encapsulated in albumin microspheres[38,39] or crystallized and cross-linked[40,41] but the large size of these particles hinders efficient delivery and intracellular uptake. Recently, particles made directly with glucose oxidase have been shown to be more efficient as biosensors than immobilized enzyme on the surface of gold nanoparticles.[42]

In this study, we report the production of protein particles as enzyme carriers that maintain enzymatic activity and increase cellular uptake. For this purpose, we studied

Abbreviations used: eGFP, enhanced green fluorescent protein; β-gal, β-galactosidase; GTA, glutaraldehyde; DTSSP, 3,3′-dithiobis(sulfosuccinimidylpropionate); BS^3, bis(sulfosuccinimidyl)suberate; ONPG, ortho-nitrophenyl-D-galactopyranoside.

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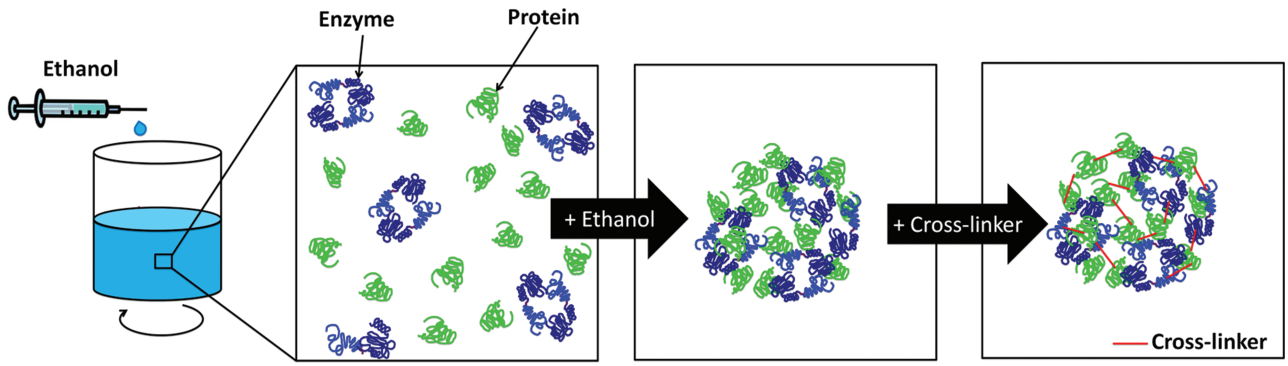

Figure 1. Schematic diagram of the desolvation process. Ethanol is added drop by drop to a solution of carrier protein and enzyme. The protein and enzyme precipitate into nanoparticles that are stabilized by cross-linking.

desolvation and size tuning of enhanced green fluorescent protein (eGFP) as an enzyme carrier. eGFP has the added advantage of fluorescence for intracellular delivery tracking and imaging. We incorporated β-galactosidase (β-gal), a hydrolase homo-tetramer, whose deficiency is the cause of lysosomal storage disorders GM1 gangliosidosis and Morquio B disease[43] into eGFP nanoparticles and show retention of enzymatic activity. In multiple cell types, we found that uptake of particles was greater than uptake of soluble protein. Finally, we successfully demonstrate intracellular delivery of active enzyme *in vitro* and the potential of protein nanoparticles as therapeutic enzyme drug carriers.

MATERIALS AND METHODS

Materials and Cell Lines

β-Galactosidase (from *Escherichia coli*, lyophilized powder >500 U/g) and glutaraldehyde (GTA, 8% solution) were purchased from Sigma–Aldrich (Saint Louis, Missouri). 3,3´-Dithiobis(sulfosuccinimidylpropionate) (DTSSP) and bis(sulfosuccinimidyl)suberate (BS^3) were obtained from Pierce (Rockford, Illinois). eGFP was expressed in *E. coli* and purified with Ni-NTA agarose (Qiagen, Valencia, California) (Fig. S1, Supporting Information). HeLa, SK-BR-3, and NIH/3T3 cells were purchased from American Type Culture Collection (Manassas, Virginia). HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% (v/v) fetal bovine serum (FBS). SK-BR-3 cells were grown in McCoy 5A Media and supplemented with 10% FBS. 3T3 cells were cultured in DMEM and supplemented with10% calf serum. All media were supplemented with 1% (v/v) penicillin/streptomycin and cells were incubated at 37°C in a 5% CO_2 humidified atmosphere.

Preparation of Particles

Protein particles were prepared by desolvation as described by Weber et al.[44] Briefly, 6 mg/mL of protein (pure eGFP or 1:24 enzyme:eGFP) was dissolved in water, NaCl solution or imidazole solution (0–250 mM in a 300 mM NaCl, 50 mM NaH_2PO_4 buffer; pH 8). Hundred microliters of protein solution was desolvated by continuous, drop-by-drop addition of 400 μL ethanol or acetone at a rate of 1 mL/min (Fig. 1). After desolvation, cross-linker was added at a ratio of cross-linker to lysines of 1:2.2. After 2 h stirring, unless otherwise stated, the cross-linking reaction was stopped by centrifugation at 1000*g* for 1 min and removal of supernatant. Particles were resuspended in phosphate buffered saline (PBS, 10 mM NaH_2PO_4, 137 mM NaCl, 2.7 mM KCl, 2 mM KH_2PO_4; pH 7.4) and sonicated on ice for 1 s every 15 s at 30% amplitude, for 5 min. To determine the yield, the amount of protein not precipitated during desolvation was measured by BCA protein assay (Pierce). Following centrifugation, the concentration of protein in the supernatant was measured and the yield was estimated to be 100% minus the percentage of protein in the supernatant. Data presented are the arithmetic mean of the yield of three samples.

Determination of Particle Size and Zeta Potential

Particle size distribution was measured by dynamic light scattering using a Zetasizer Nano ZS90 (Malvern Instruments Ltd., Westborough, Massachusetts). The samples were measured in PBS at 25°C and a scattering angle of 90°. Average particle size was calculated as the arithmetic mean of the distribution of at least three batches of particles and the SD was calculated as the variance between average diameters of batches. Zeta potential was determined by measuring the electrophoretic mobility of nanoparticles in PBS and 10 mM HEPES buffer using the same instrument.

Determination of β-Gal Activity

The activity of β-gal was measured by quantification of hydrolysis of ortho-nitrophenyl-D-galactopyranoside (ONPG) using a colorimetric β-Gal Assay Kit (Invitrogen, Carlsbad, California). Activity in particles was measured by diluting the particles to a final concentration of 0.5 ng/mL of β-gal and analyzing according to kit instructions. For quantification of activity of β-gal in cells, HeLa and 3T3 cells were seeded at a density of 1×10^4 per well in a 96-well plate in their growth medium. After 16 h, the cells were incubated with particles (87.5 μg/mL) in growth medium for 6 h. The cells were washed five times with PBS, then lysed and assayed for hydrolysis of ONPG. The number of moles of ONPG that are hydrolyzed are calculated using the following equation:

$$\text{nmoles of ONPG hydrolyzed} = \frac{(OD_{420})(\text{final volume})}{\left(4500 \frac{\text{nL}}{\text{nmoles cm}}\right)(1\ \text{cm})}$$