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Proteolytically activated anti-bacterial hydrogel microspheres

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ABSTRACT

Hydrogels are finding increased clinical utility as advances continue to exploit their favorable material properties. Hydrogels can be adapted for many applications, including surface coatings and drug delivery. Anti-infectious surfaces and delivery systems that actively destroy invading organisms are alternative ways to exploit the favorable material properties offered by hydrogels. Sterilization techniques are commonly employed to ensure the materials are non-infectious upon placement, but sterilization is not absolute and infections are still expected. Natural, anti-bacterial proteins have been discovered which have the potential to act as anti-infectious agents; however, the proteins are toxic and need localized release to have therapeutic efficacy without toxicity. In these studies, we explore the use of the glutathione s-transferase (GST) to anchor the bactericidal peptide, melittin, to the surface of poly(ethylene glycol) diacrylate (PEGDA) hydrogel microspheres. We show that therapeutic levels of protein can be anchored to the surface of the microspheres using the GST anchor. We compared the therapeutic efficacy of recombinant melittin released from PEGDA microspheres to melittin. We found that, when released by an activating enzyme, thrombin, recombinant melittin efficiently inhibits growth of the pathogenic bacterium *Streptococcus pyogenes* as effectively as melittin created by solid phase peptide synthesis. We conclude that a GST protein anchor can be used to immobilize functional protein to PEGDA microspheres and the protein will remain immobilized under physiological conditions until the protein is enzymatically released.

1. Introduction

Most modern advances in medical technology put sterility as a first point in design principles. Conventionally, these systems are passively sterilized to prevent infection before they are used. Being an incomplete process, materials implanted or inserted into the body are expected to have an incidence of infection with even the best methods of sterilization. Nosocomial infections from passively sterilized materials, *e.g.* catheters, artificial joints, pacemakers and drug pumps, cause more than 1.5 million deaths each year [1–3]. Therefore, methods beyond passive sterilization are necessary. Active anti-infectious materials are thought to be superior to the currently used, passively sterilized, noninfectious materials in maintaining sterility in non-sterile environments.

Recent reports of actively anti-infectious systems are emerging. These systems include the use of charged polymers that interact with membranes of bacteria [4], nitric oxide coatings [5], antibiotic coatings [6], silver coatings [7], and antibody coatings [8] (reviewed in [9]). These systems have been shown to actively inhibit the growth of invasive bacteria and reduce the risk of infection. Local delivery of anti-bacterial therapy has been achieved by polymeric microspheres [10–12]. Further improvements are necessary to limit local infections.

Hydrogels are becoming more prevalent in medical use as delivery systems [13,14], implants [14], drug excipients [14,15], and coatings [16]. Hydrogels are highly adaptable and have the potential as a platform for modifying other materials. In this report, we describe a microsphere-based, active antibacterial hydrogel delivery system. The hydrogel microsphere system is, to our knowledge, the first activated by the local immune response to bacterial infection into releasing an anti-bacterial compound. The system utilizes the tripeptide glutathione (GSH), a highly versatile molecule readily attachable to most surfaces. GSH was immobilized onto poly(ethylene glycol) diacrylate (PEGDA) hydrogel microspheres to allow for a modular system of anchoring therapeutic glutathione s-transferase (GST) fusion proteins. The GSH–GST interaction has long found utility in recombinant protein technology [17] but not in drug delivery to date.

Here, we present data that GST fusion proteins may be bound to GSH-laden microspheres. To deliver specific proteins during infection, thrombin-cleavable sites were engineered in the junction between GST and the fused protein to serve as an activation site. Upon thrombin cleavage, the target protein dissociates from the immobilized GST. Microspheres with GST attached to green fluorescent protein (GFP) were made as a control to demonstrate proper formation of the complex and proper release by thrombin (Fig. 1A).

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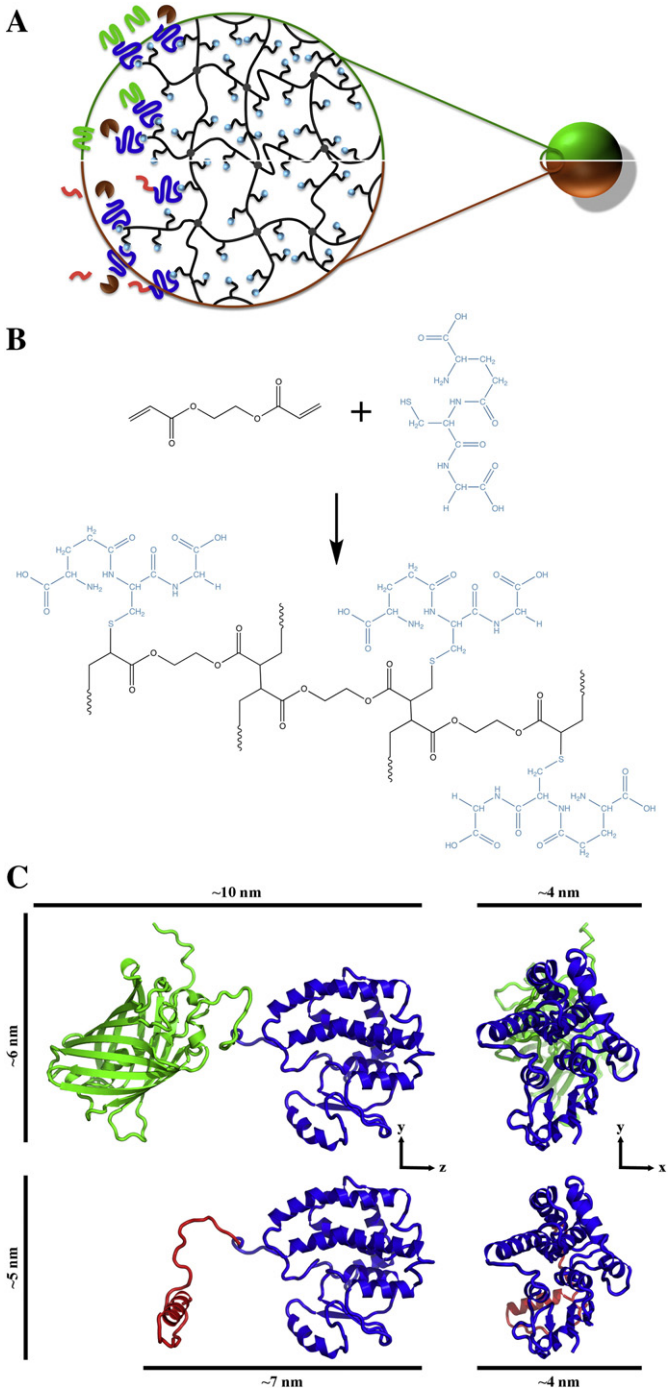

Fig. 1. Schematic of PEGDA–GSH microspheres and predicted structure of GST–GFP and GST–melittin. (A) Schematic showing half of a GST–GFP (green/top) and half of a GST–melittin (orange/bottom) microsphere with a magnified region of the microspheres suggesting the surface and internal structure. In the magnified region, black entanglements indicate the crosslinked (gray circles) PEGDA mesh with pendant glutathione (GSH; gold spheres). GST (blue)–GFP (green) and GST–melittin (pink) fusion proteins are shown binding to GSH. Thrombin (brown) is shown cleaving melittin or GFP from GST fusion partners by acting on a thrombin cleavage site in linker fragment. (B) Schematic of the chemical structure of the PEGDA–GSH hydrogel. (C) Predicted three-dimensional structures of GST (blue)–GFP (green) fusion protein and GST (blue)–melittin (red) fusion protein monomers [18] used to estimate the size of the fusion proteins. Distances are approximations of the longest three dimensions of the fusion proteins.

As a model anti-bacterial therapeutic, melittin was attached to microspheres to act as a lytic peptide against bacterial pathogens [19–21] following cleavage. Thrombin cleavage was chosen due to the fact that thrombin is activated by the host immune system at sites of inflammation caused by bacterial pathogens, such as the Gram-positive bacterium *Streptococcus pyogenes*, among others [22]. Both the therapeutic molecule and protease are models and can be readily altered in this platform. GSH-laden PEGDA hydrogel microspheres, thus, can interact with GST-fused proteins and act as a delivery system for antibacterial proteins or peptides, such as melittin. It is reasoned that proteolysis by host-derived thrombin at sites of infection will facilitate release of melittin and subsequently, the killing of pathogenic bacteria.

2. Materials and methods

2.1. Expression and purification of recombinant proteins

Proteins were expressed and purified by methods previously reported [23]. In brief, GFP was cloned from the pWiz-GFP plasmid (Aldevron, Madison, WI) into the pGex-6p-1 plasmid (GE Healthcare, Waukesha, WI) by primer extension PCR. A hexa-histidine tag and thrombin cleavage site (LVPRGS) were added to the N-terminal of GFP prior to insertion, generating pJB-HTS-GFP [24]. Melittin (GIGAVLKVLTTGLPALISWIKRKRQ) [19,20] was codon optimized using JCAT [25]: (AGC *GGA TCC* GGT ATC GGT GCT GTT CTG AAA GTT CTG ACC ACC GGT CTG CCG GCT CTG ATC TCT TGG ATC AAA CGT AAA CGT CAG TAG *GAA TTC* TCA CG) and cloned into the same vector replacing GFP after removal by BamHI and EcoRI (NEB, Cambridge, MA) generating pJB-HTS-melittin [23].

Proteins were expressed in BL21 and Rosetta (Novagen/EMD Millipore, Billerica, MA) *Escherichia coli* cells for GST–GFP and GST–melittin, respectively. In both cases, bacterial cells were grown to an absorbance at 600 nm (A_{600}) of 0.4 at 37 °C before addition of 0.1 mM IPTG [24] to induce protein production. Cells were removed to 25 °C and incubated for 16 h to produce the fusion proteins. Bacterial cells were centrifuged before re-suspension in lysis buffer (50 mM $NaHPO_4$, 300 mM NaCl, buffered to pH 8.0). Cells were lysed by three freeze–thaw cycles on dry ice and 4 °C before three 15-second sonication (Misonix model XL2015) rounds at 40% intensity with 15-second incubation on ice between sonication rounds. The lysate was centrifuged at 12,000 RPM and 4 °C for 30 min to pellet the insoluble material. GST–GFP was predominantly in the soluble fraction and was purified by Ni-NTA chromatography (Qiagen, Germantown, MA) according to the manufacturer's recommendation. GST–melittin was predominantly in the insoluble fraction and was extracted by 1% Tween-20. The extract was then purified by Ni-NTA chromatography yielding a column elution product determined to be approximately 50% GST and 50% GST–melittin.

2.2. PEGDA microsphere formation, protein loading, and characterization

All chemicals are from Thermo Fisher Scientific (Waltham, MA) unless otherwise noted. PEGDA microspheres were created through a modified process of reverse phase emulsion polymerization [24,26]. PEGDA (300 μL; MW = 575 g/mol, Sigma-Aldrich, St. Louis, MO) was diluted in 300 μL PBS containing 30 mg reduced glutathione (GSH) or 12 mg (equimolar with GSH) reduced cysteine (Sigma-Aldrich, St. Louis, MO). The microspheres made in the presence of glutathione and cysteine are referred to as PEGDA–GSH and PEGDA–cys, respectively. Mineral oil (2 mL) was added to a borosilicate culture tube (1.5 mm diameter, 10 mm length). Monomer solution (100 μL) was added to the culture tube while vortexing for 10 s at full speed. While still vortexing, 100 μL of 20% ammonium persulfate (in PBS) was added followed by 50 μL N,N,N′,N′-tetramethylethylenediamine. The mixture was vortexed for an additional minute. Deionized water (2 mL) was added prior to centrifugation at 4 °C, 4000 RPM for 1 min to recover the microspheres. Microspheres were washed over the course of a week at 4 °C with greater than a 10-fold volume of PBS and with 5 to 10 changes of PBS per day. Microspheres were loaded over the course of 3 h with 50 to 100 μg of protein in PBS. Loading occurred on a rotator wheel, with gentle rotation