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Engineering nanocages with polyglutamate domains for coupling to hydroxyapatite biomaterials and allograft bone

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ABSTRACT

Hydroxyapatite (HA) is the principal constituent of bone mineral, and synthetic HA is widely used as a biomaterial for bone repair. Previous work has shown that polyglutamate domains bind selectively to HA and that these domains can be utilized to couple bioactive peptides onto many different HA-containing materials. In the current study we have adapted this technology to engineer polyglutamate domains into cargo-loaded nanocage structures derived from the P22 bacteriophage. P22 nanocages have demonstrated significant potential as a drug delivery system due to their stability, large capacity for loading with a diversity of proteins and other types of cargo, and ability to resist degradation by proteases. Site-directed mutagenesis was used to modify the primary coding sequence of the P22 coat protein to incorporate glutamate-rich regions. Relative to wild-type P22, the polyglutamate-modified nanocages (E2-P22) exhibited increased binding to ceramic HA disks, particulate HA and allograft bone. Furthermore, E2-P22 binding was HA selective, as evidenced by negligible binding of the nanocages to non-HA materials including polystyrene, agarose, and polycaprolactone (PCL). Taken together these results establish a new mechanism for the directed coupling of nanocage drug delivery systems to a variety of HA-containing materials commonly used in diverse bone therapies.

1. Introduction

Each year more than 2 million bone grafting procedures are performed to stimulate bone repair or regeneration in orthopedic, neurological and dental applications [1]. Autogenous bone is the gold standard graft material as it contains the patient's own bone-forming cells and osteogenic proteins, and also provides a scaffold to support bone growth. However, there are limitations associated with autogenous grafts including the restricted amount of donor bone available and the considerable risk of postoperative pain and morbidity at the donor site [1]. Accordingly, allograft bone and synthetic bone-mimetic biomaterials are commonly used as alternatives to autograft [2]. Good clinical outcomes are achieved with allografted bone, but allograft has diminished osteoinductivity due to processing steps that eliminate cells and also denature or destroy osteoregenerative proteins [1,2]. Synthetic graft materials, such as those comprised of the calcium phosphate hydroxyapatite (HA), also lack osteoinductivity [3]. For these reasons, the development of methods that enable functionalization of allograft or synthetic biomaterials with osteoinductive molecules holds potential for creating graft substrates that have clinical efficacy comparable to that of autografted bone.

In this study we investigated a method for functionalization of graft materials including cadaveric-derived bone allograft, bulk HA, and HA-containing composite tissue engineering scaffolds. The strategy utilized is based on the mechanism by which native bone-binding proteins associate with the biologic HA present in bone mineral. Bone binding proteins such as bone sialoprotein and osteocalcin contain poly-acidic amino acid domains consisting of contiguous aspartate (D) or glutamate (E) residues that interact with the calcium within HA [4–6]. Prior studies from our group and others have shown that polyaspartate or polyglutamate domains can be used to anchor multiple bioactive peptides onto HA including the integrin-binding peptide, RGD [7–9], the proteoglycan-binding peptides, FHRRIKA and KRSR [10], and an osteoinductive collagen-derived peptide, DGEA [11]. For example, the addition of a heptaglutamate domain (E7) to the DGEA peptide markedly increased the amount of peptide loaded onto synthetic HA

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[11] as well as cortical bone allograft [12], and E7-DGEA peptides were retained on these substrates for at least 2 months *in vivo*. Improved coupling of DGEA to HA via the E7 domain was shown to have a significant biologic effect, in that E7-DGEA stimulated greater osteoblastic differentiation of mesenchymal stem cells (MSCs) and more robust *in vivo* bone formation than unmodified DGEA [11].

While osteoinductive peptides are being widely investigated as a tool to enhance graft integration, most of the peptides employed have a limited number of amino acids, encoding a finite amount of biologic information. The goal of the current study was to adapt the polyglutamate approach to achieve anchoring of other types of biomodifiers, specifically protein nanocage structures derived from the P22 bacteriophage. The benefit associated with nanocages is that these structures can be loaded with a variety of cargo including full-length proteins [13], imaging agents [14], and small therapeutic molecules [15]. The protein shell of bacteriophage P22 represents an attractive nano-scale drug delivery system due to the large cargo capacity, resistance to proteolytic cleavage, and stability under extreme pH and temperature [16–19]. Co-expression of the P22 scaffolding and coat proteins in a pET vector/BL21 (DE3) expression system results in the assembly of 60 nm diameter P22 virus-like particles made up of 420 identical subunits of coat protein surrounding approximately 300 molecules of scaffolding protein (Fig. 1) [20]. Fusion of cargo molecules such as green fluorescent protein (GFP) to the scaffolding protein results in their incorporation into the closed shell (Fig. 1) [13]. An insertion tolerant, externally exposed loop on the coat protein has been identified [17,18]. In the assembled protein shell, as a result of local pentameric and hexameric symmetry, the loops are clustered into twelve patches each containing five loops and sixty patches each containing six loops. To engineer nanocages with HA-selective binding sites, we used site directed mutagenesis to insert a diglutamate (E2) sequence into the loop region. As a result of local clustering, each P22 protein shell presented seventy-two high density negative charge patches to the exterior of each shell. These E2-P22 capsids were tested for binding to allograft and synthetic HA, with the expectation that the E2 modification would increase the binding of the nanocages to HA, providing a mechanism for enhanced delivery of cargo to sites of bone regeneration.

2. Materials and methods

2.1. Capsid Expression System

Co-expression of wild-type P22 scaffolding and coat proteins using the P22 assembler plasmid generates P22 virus-like particles (VLPs) in a BL21 DE3 expression system (Fig. 1). The P22 assembler plasmid has been previously manipulated to express a fusion of GFP and truncated P22 scaffolding (GFP/141-303 gp8) that fully encapsulates and retains the GFP molecule at the same packaging efficiency as wildtype P22 scaffolding (gp8) during self-assembly [13]. Site-directed mutagenesis was performed on the GFP expressing P22 assembler plasmid in order to replace codon 183 (T) of P22 coat protein with the desired coding sequences. Plasmids from successful colonies were purified and the presence of the intended mutagenesis was confirmed by sequencing.

2.2. Purification of capsids

BL21 DE3 containing the P22 assembler plasmid was grown to ~0.6 OD, induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and incubated for an additional 4 h. Cells were then pelleted, lysed by french press, and the lysate debris cleared by centrifugation. Capsids were purified from the clarified lysate by pelleting through 20% (w/v) sucrose at 40K (Beckman type 42.1) for 2 h followed by sedimentation through a 5–20% (w/v) sucrose gradient at 38K (Beckman SW41) for 30 min. Sucrose gradient fractions were isolated and analyzed by SDS-PAGE to identify the VLP fractions. Pooled VLP fractions were then characterized by electrospray ionization mass spectrometry (ESI-MS) and transmission electron microscopy (TEM) to confirm proper coat protein expression, capsid morphology, and packaged GFP/141-303 gp8. Molecular graphics were performed with the UCSF Chimera package. Chimera was developed by the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (supported by NIGMS 9P41GM103311).

2.3. Material substrates

HA powder (MP Biomedicals, Solon, Ohio) was measured (5–50 mg) into eppendorf tubes or pressed into disks using a 15.875 mm die under 3000 psi. All disks were sintered at 1000 °C and allowed to cool in the furnace at decreasing intervals before being stored under sterile conditions. Electrospun scaffolds ± nano-HA were fabricated as described previously [21]. Briefly, two types of scaffolds were produced by electrospinning: (1) 100% polycaprolactone (PCL) and (2) 80wt% PCL +20wt% HA (PCL/HA). 15 kV voltages were applied using a high-voltage power supply (Gamma High Voltage Research, Ormond Beach, FL). A syringe pump was used to feed polymer solution into the needle at a feed rate of 2 mL/h and scaffolds were collected onto an aluminum collecting plate. The allograft used for these studies was commercial-grade, mineralized cortical particulate bone graft (OraGRAFT®), obtained from LifeNet Health (Virginia Beach, Virginia). For the assays of binding selectivity, polystyrene dishes or tissue culture dishes coated with poly-D-lysine were purchased from Corning (Corning, New York).

2.4. Capsid coating onto materials

GFP loaded P22 or E2-P22 nanocages were used at equimolar concentrations based on protein quantification values. Working concentrations of 0.1–1.0 μM were used for coating materials, and for every experiment, solutions were checked to ensure that wild-type P22 and E2-P22 had equivalent starting fluorescence values.

2.5. Imaging of nanocage binding to substrates

P22 or E2-P22 capsid solutions at 1 μM were coated onto bulk HA disks, electrospun scaffolds (100%PCL and PCL/HA), and agarose for 2 h, or particulate HA for 2 min. Following coating, substrates were washed three times for 1 min with

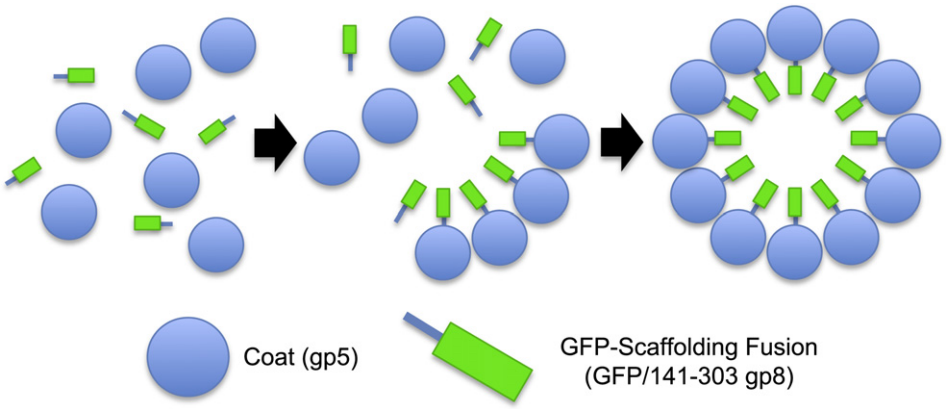

Fig. 1. *P22 capsid expression system.* Co-expression of P22 coat (gp5) and GFP-scaffolding fusion (GFP/141-303 gp8) in BL21(DE3) results in the assembly of 60 nm diameter VLPs. Assembly is mediated by the C-terminus of the scaffolding protein and results in the incorporation of GFP into the closed VLP shell at an approximate ratio of 420 coat subunits to 300 scaffolding subunits. As previously described, the GFP-scaffolding fusion remains incorporated in the closed shell and demonstrates a mechanism of packaging non-native proteins into the P22 VLPs [13].