

COMMUNICATIONS

Use of a Genetically Engineered Protein for the Design of a Multivalent MRI Contrast Agent

Lindsay S. Karfeld,^{†,‡} Steve R. Bull,^{†,§} Nicolynn E. Davis,[‡] Thomas J. Meade,^{§,||,#} and Annelise E. Barron^{*-†,‡,§}

Departments of Chemical and Biological Engineering, Chemistry, and Biochemistry and Molecular and Cell Biology, and Feinberg School of Medicine, Northwestern University, Evanston, Illinois 60208. Received April 25, 2007;

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The majority of clinically used contrast agents (CAs) for magnetic resonance imaging have low relaxivities and thus require high concentrations for signal enhancement. Research has turned to multivalent, macromolecular CAs to increase CA efficiency. However, previously developed macromolecular CAs do not provide high relaxivities, have limited biocompatibility, and/or do not have a structure that is readily modifiable to tailor to particular applications. We report a new family of multivalent, biomacromolecular, genetically engineered protein polymer-based CAs; the protein backbone contains evenly spaced lysines that are derivatized with gadolinium (Gd(III)) chelators. The protein's length and repeating amino acid sequence are genetically specified. We reproducibly obtained conjugates with an average of 8–9 Gd(III) chelators per protein. These multivalent CAs reproducibly provide a high relaxivity of $7.3 \text{ mM}^{-1} \text{ s}^{-1}$ per Gd(III) and $62.6 \text{ mM}^{-1} \text{ s}^{-1}$ per molecule. Furthermore, they can be incorporated into biomaterial hydrogels via chemical cross-linking of the remaining free lysines, and provide a dramatic contrast enhancement. Thus, these protein polymer CAs could be a useful tool for following the evolution of tissue engineering scaffolds.

One significant barrier to the development of new generations of biocompatible materials, particularly tissue engineering hydrogels, is the inability to noninvasively evaluate the properties and performance of the biomaterial over time (1–6).

* Corresponding author. Annelise E. Barron, Department of Bioengineering, Stanford University, W300B James H. Clark Center, 318 Campus Drive, Stanford, CA 94305-5440; Phone (650) 725-7665; Fax (650) 723-8544; Email aebarron@stanford.edu.

[†] Both authors contributed equally.

[‡] Department of Chemical and Biological Engineering.

[§] Department of Chemistry.

^{||} Department of Biochemistry and Molecular and Cell Biology.

[#] Feinberg School of Medicine.

[‡] Present address: Department of Bioengineering, Stanford University, W300B James H. Clark Center, 318 Campus Drive, Stanford, CA 94305-5440; Phone (650) 725-7665; Fax (650) 723-8544; E-mail aebarron@stanford.edu.

Magnetic resonance imaging (MRI) is capable of whole animal or human imaging at high spatial and temporal resolution and is an ideal modality for evaluating tissue engineering scaffolds *in vivo* (7–11). Exogenous contrast agents (CAs) increase the relaxation rate ($1/T_1$) of water protons and therefore improve image contrast. However, current clinically used CAs have low relaxivities ($3\text{--}7 \text{ mM}^{-1} \text{ s}^{-1}$) (12) and thus must be used at high concentrations for useful MRI signal enhancement (12).

T_1 CAs provide positive contrast by employing a paramagnetic ion (typically gadolinium, Gd(III)). The efficacy of a contrast agent is dominated by three parameters: q , the number of coordinated water molecules; τ_m , the residence lifetime; and τ_R , the rotational correlation time (13). Current clinically used small-molecule CAs are limited by their fast τ_R . More recently, these small-molecule CAs have been attached via covalent or noncovalent interactions to a number of macromolecules, including albumin (14), carbohydrates (15), linear polymers such

as polylysine (16), dendrimers (17), viral capsids (18), and liposomes (19). These multivalent CA designs are advantageous because they increase τ_R , Gd(III) concentration, and CA retention *in vivo*. Previously, researchers have tracked tissue engineering scaffolds *in vivo* by MRI without using any exogenous CAs (20, 21) or using soluble small-molecule CAs with a single Gd(III) chelator (22, 23). Recently, Bull et al. synthesized a self-assembled peptide amphiphile (PA)-based biomaterial scaffold, in which an MRI CA was incorporated, creating the first biomaterial designed for subsequent MRI-based *in vivo* fate mapping (11).

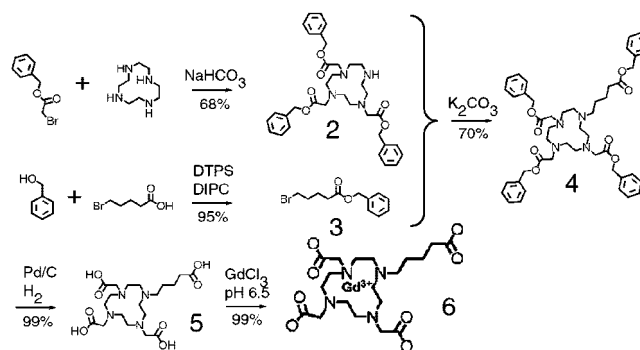
Here, we report the design, synthesis, and characterization of a novel family of multivalent, macromolecular CAs based on genetically engineered proteins with repetitive sequences that form the backbone for subsequent chemical modification with Gd(III) chelators. These "protein polymer" CAs have high relaxivities in aqueous solution. Moreover, they provide a dramatic contrast enhancement when covalently cross-linked into protein-based hydrogels. The artificial protein polymers were generated by controlled cloning and recombinant protein expression in *E. coli* and were designed to satisfy the specific criteria of water solubility, controlled reactivity, and ability to be cross-linked into hydrogels that could be useful for tissue engineering applications. In contrast to other biocompatible polymers such as dextran and other chemically synthesized and/or modified polymers, we can control the precise monomer composition, sequence, and chain length of these protein scaffolds. Affinity chromatography affords pure, monodisperse biopolymers with evenly spaced, primary amine reactive sites, which serve as an ideal scaffold for CA modification.

The protein polymer backbone we used comprises 30 repeats of the amino acid sequence GKAGTGSA, providing 30 reactive primary amines (ϵ -amino groups of lysines) that are available for derivatization (24). The 21 825 Da protein polymer (molar mass confirmed by MALDI; see Supporting Information (SI)) is water-soluble and can be produced in high yield. Circular dichroism spectroscopy shows that the protein adopts a random-coil (unfolded) configuration in aqueous solution (see SI) (25), thereby avoiding particular secondary structures that could potentially lead to decreased τ_R (such as a cylindrical α -helix). The macrocyclic chelate that coordinates the Gd(III) ion is a chemically synthesized derivative of 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid (DOTA) (26). The three acetate arms chelate the Gd(III), while the carboxylate group on the 5-carbon arm is used for covalent attachment to the primary amine of the lysine residues via amide bond formation.

A multistep controlled cloning method was used to create the 720 base pair gene that encodes the protein polymer (25), and standard transformation and expression protocols were used to produce the 264 amino acid–protein polymer, **1** (see SI) (27). The pET19b expression vector into which the gene was ligated contains an N-terminal histidine fusion tag, GH₁₀SSGHIDDDDKHM, enabling protein purification via Ni-NTA affinity chromatography under denaturing conditions (27). In a typical expression, up to ~60 mg/L of culture medium are obtained after purification. Further details are given in the SI.

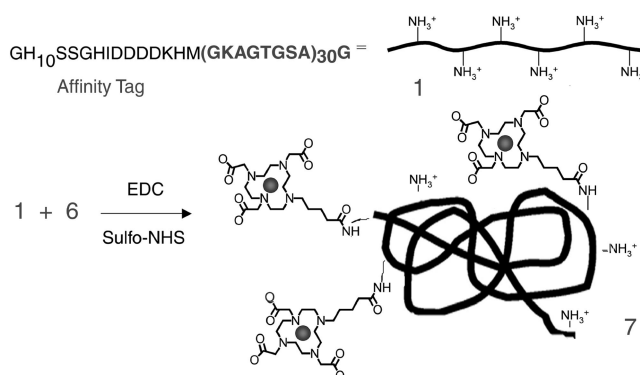
The Gd(III) chelator was synthesized using a hydrogenation-labile protection scheme with benzyl 2-bromoacetate and benzyl 5-bromopentanoate as the chelating arms of the macrocycle (Scheme 1). The addition of three benzyl 2-bromoacetate arms to cyclen was followed by the addition of benzyl 5-bromopentanoate. Global deprotection by hydrogenation of the chelator and subsequent metalation with GdCl₃ afforded **6**, which subsequently was conjugated to the protein polymer. The synthesis and characterization of the chelator is described in the SI.

Scheme 1. Synthesis of Gd(III) Chelator Using a Hydrogenation-Labile Global Deprotection Scheme^a



^a Gd(III) was chelated before conjugation to the protein to ensure that all chelators contained Gd(III).

Scheme 2. Conjugation of Gd(III) Chelator to Protein Polymer Backbone through Amide Bond Formation Using EDC and Sulfo-NHS, where ~8–9 Chelators Per Protein Molecule Are Available for Conjugation



The coupling reaction between the lysine residues of **1** and **6** was carried out in aqueous 0.1 M 4-morpholineethanesulfonic acid (MES), 0.5 M NaCl buffer with 1-ethyl-3-(3-dimethylammoniumpropyl) carbodiimide hydrochloride (EDC), and *N*-hydroxysulfosuccinimide (sulfo-NHS), which are water-soluble peptide bond-forming agents (Scheme 2) (28). We chose to conjugate premetallated chelators to the protein polymer in order to simplify the synthetic process, avoid solubility issues, and guarantee that each chelator contains a Gd(III) ion. MALDI-MS analysis of the conjugate confirmed that an average of ~8–9 out of a possible 31 lysines per molecule are typically derivatized using this procedure ($n = 4$), but we have achieved up to 15 Gd(III)-conjugated sites per protein molecule in some cases. The conjugation efficiency of this reaction is limited by a competing hydrolysis reaction involving water molecules. The derivatization with Gd(III) chelators introduces a small degree of polydispersity, since the positions of the chelators along the backbone are different in each individual CA, but these CAs can still be well-characterized by MALDI due to the monodispersity of the protein polymer backbone. As determined through relaxivity and inductively coupled plasma (ICP)-MS measurements, the CAs display relaxivities of $7.3 \pm 0.4 \text{ mM}^{-1} \text{ s}^{-1}$ per Gd(III) ion ($n = 4$) and $62.6 \pm 3.5 \text{ mM}^{-1} \text{ s}^{-1}$ per protein molecule complex, which is the product of the number of Gd(III) chelators per protein backbone times the relaxivity per Gd(III). Synthetic polymers such as dendrimers and metallofullerenes display molecular relaxivities of $\sim 35 \text{ mM}^{-1} \text{ s}^{-1}$ (29) and $\sim 60 \text{ mM}^{-1} \text{ s}^{-1}$ (30), respectively. There are a few examples of natural protein-based CAs that display greater molecular relaxivities (cowpea chlorotic mottle virus, $\sim 200\text{--}400 \text{ mM}^{-1} \text{ s}^{-1}$ per viral conjugate (31), apoferritin, $\sim 800 \text{ mM}^{-1} \text{ s}^{-1}$ per protein complex (32), MS2

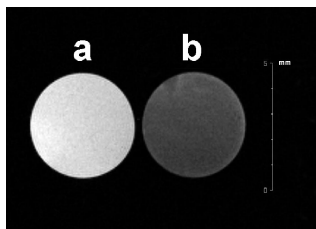


Figure 1. MR images acquired at 14.1 T with TR 250.0 ms, TE 10.2 ms. (a) Glutaraldehyde-cross-linked hydrogel doped with **7** with an added Gd(III) concentration of 0.8 mmol. (b) Control hydrogel without contrast agent.

viral capsid, $\sim 7200 \text{ mM}^{-1} \text{ s}^{-1}$ (18)). Even though some of these CAs have shown higher relaxivities per molecule, our new protein polymer CAs avoid some disadvantages associated with these other CAs. As compared with synthetic polymers, protein polymers consist of amino acids, so they are biodegradable and should not have any toxic byproducts (chelated Gd(III) is nontoxic). The above-mentioned natural protein CAs are less easily tunable and modifiable, so the relaxivity, size of the CAs, toxicity, *in vivo* circulation time, and mode of excretion cannot be as easily modulated. On the other hand, protein polymers can be precisely designed for the particular application and point of use; they can be customized to vary protein lengths and the spacing and number of lysines for conjugation to Gd(III) chelators. Additionally, these protein polymer CAs can be derivatized with other biological moieties for additional functionality, such as targeting and intracellular translocation.

To demonstrate one useful application of these new protein polymer-based CAs, tracking the evolution of tissue engineering hydrogels over time, we prepared 5.8% (w/v) protein-based hydrogels in aqueous solution in 5 mm NMR tubes for MR imaging experiments. The protein-CA conjugate, **7**, was doped into a solution of unconjugated protein polymer, **1**, and the free amines were cross-linked with 45 μL of 25% glutaraldehyde to form a gel (see SI). One advantage of these protein polymer-based CAs is that at least 50% of the primary amines on the backbone remain underivatized and are available for covalent cross-linking to form the hydrogel, as well as assisting in water solubility. Thus, once cross-linked to other proteins within a hydrogel, the multivalent CAs can only be removed from the hydrogel as a result of hydrolysis of the scaffold and not as a result of diffusion. The MR images show that the hydrogel incorporating **7** is homogenous and exhibits dramatically higher MRI contrast than the hydrogel formed from a protein polymer control (Figure 1).

In conclusion, a combination of biosynthetic and chemical methods has been used to create high-contrast, multivalent, protein polymer-based MRI CAs. We have demonstrated a reliable synthetic protocol that allows conjugates to be characterized by MALDI and provides reproducible relaxivity results, both per molecule and per Gd(III). As shown in MR images, this protein polymer-based CA can be incorporated into a hydrogel and yields dramatic contrast enhancement, which could be a useful tool for following the evolution of tissue engineering scaffolds *in vivo*. We plan to employ other cross-linking mechanisms and hydrogel media to create a family of biocompatible tissue engineering scaffolds that can be fate-mapped *in vivo* by MRI, enabling tracking of both the cellular ingrowth and the scaffold degradation over time.

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Supporting Information Available: Procedures on protein polymer, chelator, and CA synthesis and characterization including SDS-PAGE, MALDI, circular dichroism, and NMR data. This material will be available free of charge via the Internet at <http://pubs.acs.org>.

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