Journal of Controlled Release 171 (2013) 57–62

Contents lists available at SciVerse ScienceDirect

Journal of Controlled Release

journal homepage: www.elsevier.com/locate/jconrel

A biohybrid hydrogel for the urate-responsive release of urate oxidase

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ARTICLE INFO

Article history:
Received 21 March 2013
Accepted 29 June 2013
Available online 6 July 2013

Keywords:
Biohybrid material
Closed-loop control
DNA-protein interaction
Stimulus-sensing material
Gouty arthritis

ABSTRACT

Functional biomaterials that detect and correct pathological parameters hold high promises for biomedical application. In this study we describe a biohybrid hydrogel that detects elevated concentrations of uric acid and responds by dissolution and the release of uric acid-degrading urate oxidase. This material was synthesized by incorporating PEG-stabilized urate oxidase into a polyacrylamide hydrogel that was crosslinked by the uric acid-sensitive interaction between the uric acid transcription factor HucR and its operator *hucO*. We characterize the uric acid responsiveness of the material and demonstrate that it can effectively be applied to counteract flares of uric acid in a mouse model. This approach might be a first step towards a biomedical device autonomously managing uric acid burst associated to gouty arthritis and the tumor lysis syndrome.

1. Introduction

Functional biomaterials that autonomously detect and correct a disease state hold high promises for biomedical application [1–3]. The key to the synthesis of such autonomous materials is the availability of sensor molecules that translate pathological signals into a change in material characteristics. Prototypes of such functional materials were engineered to be responsive to elevated glucose levels to autonomously trigger the release of insulin in a self-sufficient therapeutic approach against diabetes [4]. These materials were synthesized by crosslinking polymers *via* glucose-binding proteins [5,6] or by doping pH-responsive materials with glucose oxidase that translates the glucose concentration into a lowered pH [7]. However, these approaches were rather specific to the target molecule glucose and are unlikely generically applicable to different disease-associated metabolites. This limitation was recently addressed by introducing prokaryotic metabolite-responsive transcription factors as sensors and effectors for the synthesis of metabolite-responsive biohybrid hydrogels [1,2,8,9]. Prokaryotes have evolved a large array of transcription factors that control gene expression by directly binding or unbinding target promoter DNA in response to the concentration of specific metabolites like sugars, amino acids or other primary and secondary metabolites [10]. Applying such transcription factor-DNA pairs to crosslink polyacrylamide resulted in the formation of hydrogels that could dose-dependently be dissolved upon addition of the specific metabolite triggering the dissociation of both partners. This setup was applied to synthesize tetracycline-responsive hydrogels based on the tetracycline transcription factor TetR and its operator *tetO* [8,11]. In a similar configuration, a hydrogel was recently described that dissolved in the presence of elevated uric acid concentrations that are typically associated with gouty arthritis or the tumor lysis syndrome [9,12].

In this study we capitalize on this uric acid-responsive biohybrid hydrogel to develop a device that detects elevated levels of uric acid and responds by the release of urate oxidase to degrade excess uric acid concentrations. For this aim we first increase the stability of clinically approved *Aspergillus flavus* urate oxidase (rasburicase [13]) by pegylation and subsequently incorporate this protein into the uric acid-responsive hydrogel. We demonstrate that this material dissolves in response to a pulse in uric acid concentration and that it effectively counteracts uric acid surges in mice. These results may represent the first step towards a biomedical device autonomously managing surges in serum uric acid as associated with gouty arthritis [14] or the tumor lysis syndrome [15].

2. Materials and methods

2.1. Vector construction, protein production, purification and characterization

The expression vector pCG62 encoding Uox-H_6 was constructed by amplifying a synthetic urate oxidase gene from plasmid pUC57-smUox [12] using oligos OCG124 (5′-ccatccatatgtctgctgtgaaggccgcaagatatggc-3′) and OCG125 (5′-cctgaaagcttttaatggtgatggtgatggtgcagcttgctcttcagag-3′, annealing sequences underlined) and cloning the PCR product *Nde*I/*Hind*III into plasmid pRSetmod [16] under the control of the phage T7 promoter. The expression vector pCG50 encoding H_6-HucR_2-C has been described earlier [9]. The vector phucO_{24} (pCG66) was synthesized

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http://dx.doi.org/10.1016/j.jconrel.2013.06.037

(Geneart, Regensburg, Germany) harboring 24 repeats of the *hucO* operator motif each separated by a random 6 nucleotide spacer.

Uox-H_6 was produced in *Escherichia coli* BL21 Star™ (DE3) (Invitrogen, Cat. no. C6010-03). Cultures were inoculated at OD_{600} = 0.1 and cultivated at 37 °C in shaking flasks. Protein production was induced by 1 mM IPTG at OD_{600} = 1.2 for 5 h. Production conditions for H_6-HucR_2-C have been described elsewhere [9]. After protein production the cells were harvested by centrifugation (6500 ×*g*, 8 min, 4 °C), re-suspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, 10 mM β-mercaptoethanol (β-me), 10% (w/v) glycerol, pH 8.0) and disrupted using a French press (APV Systems, Albertslund, Denmark, model APV-2000) (3 cycles at 1000 bar, 4 °C). The lysates were cleared by centrifugation (30,000 ×*g*, 30 min, 4 °C) and purified by Ni^{2+}-NTA-affinity chromatography (2.5 ml column volume /l production culture, Qiagen, Hilden, Germany, Cat. no. 30210) according to the manufacturer's protocol except that all buffers were supplemented with 10% (w/v) glycerol and 10 mM β-mercaptoethanol. After elution, the buffer of the proteins was exchanged to binding buffer (60 mM Tris/HCl, 200 mM NaCl, 10 mM β-me, 4% (w/v) glycerol, 0.075% (w/v) Brji58, pH 8.0) *via* three dialysis cycles (3500 kDa MWCO, Thermo-Scientific, Rockford, IL, Cat. no. 68035). Proteins were quantified using the Bradford method (Bio-Rad, Hercules, CA, Cat. no. 500-0006) with BSA (Fluka, Rotkreuz, Switzerland, Cat. no. 05479) as standard. For assessing protein purity and size, samples were resolved on 12% SDS-PAGE with subsequent Coomassie staining. Urate oxidase activity was quantified using the Amplex Red Uric Acid/Uricase Assay Kit (Invitrogen, Carlsbad, CA, Cat no. A22181).

2.1.1. Pegylation and characterization of urate oxidase

For pegylation of urate oxidase, 1 mg/ml Uox-H_6 was mixed with 4 mg/ml (800-fold molar excess) 8-azaxanthine (Sigma-Aldrich, Munich, Germany, Cat. no. 11460) in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 8.0) for protecting the active Uox site from pegylation. Subsequently, the mix was added to a 5–40-fold molar excess of succinimide-functionalized polyethylene glycol (750 Da, Sigma-Aldrich, Munich, Germany, Cat. no. 712531) and incubated for 2 h at room temperature. The enzyme was subsequently transferred to binding buffer by ultracentrifugation (10 kDa MWCO, Corning, Lowell, UK, Cat. no. 431483) and pegylation was confirmed by SDS-PAGE with subsequent Coomassie staining.

2.2. Production and characterization of plasmid phucO_{24}

The plasmid phucO_{24} was transformed in *E. coli* Top10 cells (Life Technologies, Darmstadt, Germany, Cat. no. C4040-10) that were subsequently cultivated in TB medium (12 g/l casein, 24 g/l yeast extract, 12.5 g/l KH_2PO_4, 2.3 g/l K_2HPO_4, 4 g/l glycerol, pH 7.2) for 20 h at 30 °C and 170 rpm in 2 l baffled flasks. Cells were harvested by centrifugation (6500 ×*g*, 8 min, 4 °C) and plasmids were isolated and purified using Jetstar 2.0 Gigaprep columns (Genomed, Löhne, Germany, Cat. no. 240006). Purified plasmids were resuspended in binding buffer, adjusted to 2 μg/μl and stored at −80 °C.

2.2.1. DNAse I sensitivity of phucO_{24}

The effect of H_6-HucR_2-C on the DNAse I sensitivity of phucO_{24} was evaluated. To this aim, 10 μg (corresponding to 125 pmol *hucO* motifs) phucO_{24} was pre-incubated for 30 min at room temperature with 21 μg (500 pmol) H_6-HucR_2-C in binding buffer supplemented with 2.5 mM $MgCl_2$ and 0.5 mM $CaCl_2$. Subsequently, 2 units of DNAse I (NEB, Ipswich, MA, Cat. no M0303S) were added followed by a 1 h incubation at 37 °C. As control, samples were prepared without H_6-HucR_2-C. The samples were resolved by electrophoresis on a 1% (w/v) agarose gel in 0.5 × TAE buffer (20 mM Tris, 20 mM acetic acid, 0.5 mM EDTA, pH 8) and DNA was visualized by staining with ethidium bromide (0.5 μg/ml in 0.5 × TAE) and illumination at 254 nm.

2.3. Hydrogel synthesis

For hydrogel synthesis, 600 μg (14.4 nmol) H_6-HucR_2-C (2.5 μg/μl in binding buffer) was mixed with 286 μg phucO_{24} (corresponding to 3.6 nmol *hucO* monomer) and incubated at room temperature for 30 min. Subsequently, 500 ng pegylated (PEG:Uox-H_6 = 40:1, mol:mol) Uox-H_6 (1 μg/μl stock solution) was added prior to concentrating the mix to 80 μg/μl (expressed as H_6-HucR_2-C concentration) by ultrafiltration (5000 Da MWCO, Corning, Cat. no. 431482). Gels were synthesized by mixing 7.5 μl of the HucR/phucO_{24} mix with 5 μl of 6% (w/v) poly(AAm-co-Ni^{2+}-NTA-AAm) in binding buffer [17,18]. The mix was transferred to a dialysis membrane (3500 Da MWCO, Thermo-Scientific, Rockford, IL, Cat. no. 68035) and dialyzed against binding buffer without β-me for 16 h at 4 °C. Subsequently, hydrogels were removed from the dialysis membrane by centrifugation (17,000 ×*g*, room temperature, 1 min) into an Eppendorf tube. Subsequently, hydrogels were swollen in 1.5 ml binding buffer for 2 h at room temperature.

2.4. Hydrogel characterization

2.4.1. Mechanical hydrogel characterization

For mechanical characterization, hydrogels with a total volume of 50 μl were prepared as disks with 0.5 mm height. Storage and loss moduli (G′ and G″) were obtained in small-strain oscillatory shear experiments using a modular advanced rheometry system II (Thermo Scientific, Waltham, MA) with a 20 mm parallel steel plate geometry at 20 °C. The gap was adjusted starting from the original sample height and compressing the sample to 0.15 mm in order to prevent slippage. Frequency-sweep experiments were conducted in a constant strain (20%) mode as a function of frequency (0.01 to 2.0 Hz).

2.4.2. Urate-inducible hydrogel dissolution

For release studies hydrogels (12.5 μl volume) were transferred to 1.5 ml binding buffer without β-me supplementation or to 1.5 ml synthetic body fluid representing the ion concentrations in human plasma (142 mM Na^+, 5 mM K^+, 2.5 mM Ca^{2+}, 1.5 mM Mg^{2+}, 125 mM Cl^-, 27 mM HCO_3^-, 1 mM HPO_4^{2-}, 0.5 mM SO_4^{2-}, 50 mM Tris, pH 7.4). The samples were supplemented with increasing amounts of uric acid sodium salt and hydrogel dissolution was monitored by quantifying the release of hydrogel building blocks into the supernatant by Bradford assay or by a spiked fluorophore (mCherry) as described earlier [19]. The values were normalized to the total protein content in the hydrogel as determined after complete hydrogel dissolution induced by the addition of 25 mM EDTA sodium salt pH 8.0 and 10 mM β-me.

2.4.3. Cell culture compatibility assay

Human embryonic kidney (HEK-239T) cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml gentamicin at 37 °C in a humidified atmosphere with 5% CO_2. For cell culture compatibility assays, 60,000 cells/well were grown in a 24-well plate in 0.5 ml medium in the presence of a hydrogel (50 μl volume) containing 10 μg Uox-H_6 for 24 h prior to quantifying cell proliferation using the WST-1 assay (Roche Diagnostic, Mannheim, Germany, Cat. no. 1644807). The conversion of WST-1 to the colored formazan was followed at 440 nm at 37 °C for 2 h (data acquisition every 2 min).

2.4.4. DNAse I sensitivity of hydrogels

Hydrogel sensitivity to DNAse I was evaluated by incubating gels (12.5 μl) in 750 μl binding buffer supplemented with 2.5 mM $MgCl_2$,