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Tunable drug-loading capability of chitosan hydrogels with varied network architectures

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ABSTRACT

Advanced bioactive systems with defined macroscopic properties and spatio-temporal sequestration of extracellular biomacromolecules are highly desirable for next generation therapeutics. Here, chitosan (CT) hydrogels were prepared with neutral or negatively charged cross-linkers in order to promote selective electrostatic complexation with charged drugs. CT was functionalized with varied dicarboxylic acids, such as tartaric acid, poly(ethylene glycol) bis(carboxymethyl) ether, 1,4-phenylenediacetic acid and 5-sulfoisophthalic acid monosodium salt (PhS), whereby PhS was hypothesized to act as a simple mimetic of heparin. Attenuated total reflectance Fourier transform infrared spectroscopy showed the presence of C=O amide I, N–H amide II and C=O ester bands, providing evidence of covalent network formation. The cross-linker content was reversely quantified by proton nuclear magnetic resonance on partially degraded network oligomers, so that 18 mol.% PhS was exemplarily determined. Swellability (*SR*: 299 ± 65–1054 ± 121 wt.%), compressibility (*E*: 2.1 ± 0.9–9.2 ± 2.3 kPa), material morphology and drug-loading capability were successfully adjusted based on the selected network architecture. Here, hydrogel incubation with model drugs of varied electrostatic charge, i.e. allura red (AR, doubly negatively charged), methyl orange (MO, negatively charged) or methylene blue (MB, positively charged), resulted in direct hydrogel–dye electrostatic complexation. Importantly, the cationic compound, MB, showed different incorporation behaviours, depending on the electrostatic character of the selected cross-linker. In light of this tunable drug-loading capability, these CT hydrogels would be highly attractive as drug reservoirs towards e.g. the fabrication of tissue models in vitro.

1. Introduction

In in vivo tissue engineering, multifunctional material systems should temporally mimic natural tissues, exhibiting controlled macroscopic properties and inducing specific biological signals to local stem cells [1]. In vivo, these functions are provided by the extracellular matrix (ECM), a highly organized supramolecular hydrogel structure binding and stabilizing growth factors in a spatio-temporal, controlled manner [2]. ECM sulfated glycosaminoglycans, such as heparin, protect bound growth factors from proteolytic degradation, potentiating their bioactivity by facilitating cell receptor interactions [3]. Consequently, the design of advanced bioactive systems mimicking ECM growth factor features in a defined, application-dependent, temporal fashion is currently considered one of the greatest challenges in regenerative medicine [4,5].

Functional biomaterials, including hydrogels [5–11], sponges [12], scaffolds [13], electrospun meshes [14] and capsules [15], have been extensively investigated for the localized loading and controlled release of growth factors. Among the different carrier systems, hydrogels are attractive material candidates since they are generally biocompatible, biodegradable and can mimic ECM architecture over different length scales [16,17]. At the same time, their high swelling and hard-to-control elasticity and degradability must be carefully addressed in order to ensure a timely and sustained material performance without compromising loading and release profiles [9]. Using functionalized dextran hydrogels, Schillemans et al. applied reversible electrostatic interactions for post-loading and release of proteins [18]. Here, protein incorporation was not homogeneous and nearly complete release was observed following 50 h. Aiming at sustained and localized delivery, Jeon et al. synthesized growth-factor-encapsulating hydrogels [19]. Here, covalently linked heparin segments were expected to mimic ECM growth-factor-binding sites, although release was found to be mainly driven by diffusion rather than network degradation. In an effort to control loading and release capability independently of network characteristics, Freudenberg et al. described a modular system of biohybrid

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hydrogels based on covalently cross-linked heparin and star-shaped poly(ethylene glycols) [5]. Although mechanical properties and biofunctionality could be ruled independently, up to only ~5 wt.% loading was observed,[1] while nearly 20 wt.% of loaded growth factor was released within 7 days. Besides biopolymer-based hydrogels, surface-modified synthetic hydrogel networks have also been established, whereby pH-triggered, hour-scale release of model drugs was demonstrated [11]. From all the aforementioned examples, it appears rather clear that successful clinical translation of current hydrogel systems as controlled drug delivery systems has been deterred by setbacks such as (i) poor loading efficiency, (ii) large initial burst release and (iii) limited control in material properties. An interesting approach to address these challenges is based on the design of functional hydrogel reservoirs allowing for the controlled loading of a wide range of biomolecules, whereby stimulus-triggered drug release can be obtained. To reach this goal, the molecular network architecture must be thoroughly investigated, so that defined structure–property–function relationships can be established [20].

Chitosan (CT) is the second most abundant natural polymer on earth and serves as a structural polysaccharide for many phyla of lower plants and animals. CT is derived from the partial deacetylation of chitin, thereby resulting in the only linear cationic polysaccharide. It contains glucosamine and *N*-acetyl glucosamine units along its polymer backbone, so that it mimics the chemical composition of ECM glycosaminoglycans. In light of its suitable biodegradability, biocompatibility, immunological, antibacterial and wound-healing properties as well as good mechanical and film-forming properties [21,22], CT has been widely applied in the biomedical field as a wound dressing [23], haemostat [24] and scaffold for tissue engineering [25,26], and as controlled drug [27] and gene [28] delivery vehicles. At the same time, CT's polycationic nature should be carefully considered for the successful formulation of bespoke drug delivery systems, due to the potential electrostatic repulsion of the polymer backbone with positively charged growth factors, such as BMP-2 or FGF-2 [29]. Furthermore, a non-controllable electrostatic complexation of the material surface with cells may be observed following protonation of CT amino groups in vitro or in vivo, ultimately leading to cytotoxic effects. To circumvent these issues, chemical functionalization of CT can be carried out, whereby reaction of amino and hydroxyl terminations leads to the establishment of covalent, neutralized net points, so that the polycationic character of CT is controlled [30–32]. CT has been carboxymethylated [33,34], grafted with phenylalanine [35] and cross-linked with low-molecular-weight segments [36], leading to a broad range of polymers. However, despite such enormous polymer variability, the fact that CT solubility is restricted to acidic conditions imposes several constraints in terms of accomplishing selective and tunable functionalization, so that systematic changes in molecular organization are highly challenging. Indeed, reacted products might be unstable at acidic pH, potentially resulting in the occurrence of side reactions and reduced reaction yield, so that material properties and drug loading/release functionalities cannot be systematically varied.

This work aimed at establishing a novel synthetic approach for the formation of bioactive CT-based systems displaying tunable network architecture and superior drug-loading functionality. It was hypothesized that selective CT functionalization could be accomplished in aqueous systems with bifunctional segments of varied molecular weight, backbone rigidity, wettability and electrostatic charge, so that bespoke hydrogels with defined macroscopic properties could be formed. Based on the selected network architecture, it was hypothesized that hydrogel drug-loading functionality could result from the electrostatic complexation of the network chains with systematically varied model drugs. To reach this goal, L(+)-tartaric acid (TA), poly(ethylene glycol) bis(carboxymethyl) ether (PEG), 1,4-phenylenediacetic acid (4Ph) and 5-sulfoisophthalic acid monosodium salt (PhS) were selected as dicarboxylic acids for CT functionalization. TA and PEG have been previously applied to CT for the design of pH-responsive nanoparticles [32] and drug delivery hydrogels [36]. Here, both compounds were employed as flexible, aliphatic, neutral cross-linkers of varied molecular weight. 4Ph and PhS were selected as rigid, aromatic segments, whereby the only difference between the two molecules was the presence of a sulfonic acid group in the PhS benzene ring, aimed at mimicking the growth factor binding sites of heparin in vivo. Consequently, incubation in solutions containing either allura red (AR) as doubly negatively charged, methyl orange (MO) as negatively charged and methylene blue (MB) as positively charged, model drugs was carried out in order to explore hydrogel loading functionality [37]. Thus, CT systems with systematically adjusted material properties and loading efficiencies via selective changes in network architecture were expected to be established. Ongoing research is focusing on the evaluation of the controlled drug release functionality in the presented chitosan systems.

2. Materials and methods

2.1. Materials

Low-molecular-weight (50,000–190,000 Da) CT, *N*-(3-dimethylaminopropyl)-*N*-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), PEG (average $M_n \sim 600$ Da) and hydrochloric acid solution (37%) were purchased from Sigma Aldrich (Japan). 1-hydroxybenzotriazole (HOBt) was purchased from Kishida Chemical Co., Ltd (Japan). PhS, 4Ph and TA were purchased from Tokyo Chemical Industry Co., Ltd (Japan). Allura red, methylene blue and methylene orange were purchased from Tokyo Chemical Industry Co. Ltd (Japan), Kishida Chemical Co., Ltd (Japan) and Nacalai Tesque, Inc. (Japan), respectively. Deuterium oxide was purchased from Wako Pure Chemical Industries, Ltd (Japan).

2.2. Formation of CT-based hydrogels

CT-based hydrogels were prepared by dissolving CT (0.15 g) in a HOBt (0.12 g)–water (4.23 g) solution [38]. Once the CT solution was obtained, an equimolar content of dicarboxylic acid (either TA, PEG, 4Ph or PhS) with respect to the CT amino functions was activated in water (0 °C, pH 4, 30 min) with EDC (1 or 2.3 M) in the presence of NHS. A three-fold excess of EDC/NHS with respect to the dicarboxylic acid molar content was introduced. NHS-activated dicarboxylic acid mixture was mixed with previously obtained CT solution and incubated at room temperature under gentle shaking, in order to allow for the nucleophilic addition reaction of CT amino groups to the cross-linker carboxylic functions to occur. Complete gel formation was observed following 1 h reaction of CT with selected NHS-activated dicarboxylic acids. Resulting hydrogels were thoroughly washed with distilled water, followed by vacuum-drying at room temperature.

2.3. Chemical characterization of hydrogel networks

Attenuated total reflectance Fourier transform infrared (ATR FT-IR) spectroscopy was carried out on dry samples using a Perkin-Elmer Spectrum 100 FT-IR spectrophotometer with diamond ATR attachment. Scans were conducted from 4000 to 400 cm^{-1} with 16 repetitions averaged for each spectrum at 4 cm^{-1}

[1] Hydrogel incubation was carried out with 5 μg ml^{-1} FGF-2 solution (9 .ng of FGF-2 loaded per μl of hydrogel; 200 μl of FGF-2 solution applied per cm^2 hydrogel; hydrogel thickness: 50 μm).