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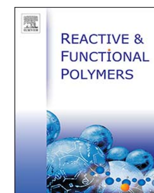

Thermoresponsive hydrogels with covalently incorporated trehalose as protein carriers

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ABSTRACT

A series of thermoresponsive hydrogels containing covalently incorporated trehalose, the well-known protein stabilizing disaccharide, was synthesized with the aim to obtain bioprotective carriers for protein release. Smart materials with trehalose present only in cross-links or both in cross-links and as pending moieties, were fabricated by redox-initiated radical copolymerization, with *N*-isopropylacrylamide used as the main monomer. In order to modify thermoresponsive properties, hydrogels containing more hydrophilic comonomers, acrylamide or *N*-(2-hydroxyethyl)acrylamide, were also obtained. The susceptibility of the cross-linker to undergo acid-catalyzed hydrolysis was used to disintegrate the polymer network and estimate the composition of the synthesized materials. The properties of hydrogels, which include: swelling capacity, thermoresponsive behavior, rheological characteristic, internal microstructure and the rate of acid-catalyzed degradation, were found to be dependent on both trehalose as well as hydrophilic comonomers. BSA and β-Galactosidase were chosen as model proteins for the release study from obtained hydrogels. The release profiles were shown to vary significantly depending on hydrogel form, the polymer network composition and temperature.

1. Introduction

Protein and peptide therapeutics have become mass-scale products and have achieved a significant role in almost every field of medicine, such as the treatment of cancer, inflammatory diseases, in vaccines and in diagnostics [1]. However, the fragile three-dimensional structure of proteins makes them very susceptible to proteolytic and chemical degradation as well as physical unfolding and aggregation in body fluids, which leads to the fast loss of their bioactivity [2–4]. The half-lives of many commonly used biomacromolecules like insulin, oxytocin hormone, parathyroid hormone or vasopressin are less than half an hour [5,6]. Providing a bioprotective microenvironment is therefore of crucial importance for the delivery of therapeutic proteins. One of the strategies used to increase exogenous protein stability *in vivo* is to employ matrices within which they are entrapped. Many studies describe the potential role of smart hydrogels in the delivery of bioactive compounds. Their porous structure and high water content makes them suitable for holding high amounts of water-soluble compounds like proteins. Based on the polymer origin, hydrogels can be classified as natural, synthetic or hybrid materials with each possessing its own advantages and limitations. Compared to biopolymer-derived hydrogels, which potentially could induce immunogenic reactions and introduce pathogen contamination, synthetic hydrogels are bioinert [7]. Moreover, they have a well-defined chemical composition and allow for precise control over material properties and offer the possibility of providing a desired biofunctionality. Considerable emphasis is placed on hydrogels bearing reactive functional groups enabling the matrix to interact with the components of biological systems, thus ensuring drug stabilization and targeted delivery of the drug to specified sites. Functionalization of the polymer may be performed by incorporating a large variety of cell-specific ligands to polymer chains. There is a still growing interest in the development of functional hydrogels with a therapeutic or diagnostic potential, capable of targeting carbohydrate-binding receptors.

Glycosylated polymers containing carbohydrates as pendant groups are promising candidates for drug delivery to specific cells and tissues because certain carbohydrates present on the polymer surface could mimic specific interactions that naturally occur between glycoproteins

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found on the cell surface and carbohydrate-binding proteins. Such interactions are involved in a variety of biological processes, *e.g.*: cell growth, cancer metastasis, bacterial and viral infection [8]. Although single carbohydrate-protein interaction is usually weak, the interaction of multiple protein subunits with a multivalent display of carbohydrates could increase the binding strength and specificity significantly. It was found that carbohydrate-decorated polymers can mimic these natural multivalent carbohydrate–receptor interactions and promote high-affinity binding to specific cells [9]. When properly recognized, a drug may be released from the carrier to selectively target appropriate cellular organelle. Recently, many studies on the delivery of bioactive compounds focus on hydrogels containing pending carbohydrates. The typical example includes synthetic hydrogels containing a terminal galactose moiety that provide hepatocyte anchorage *via* asialoglycoprotein receptors (ASGP-R) expressed on the hepatocyte cell surface [10–19]. There is also interest in developing mannosylated hydrogels due to the fact that mannose receptors are highly expressed in immune system cells, *i.e.* macrophages and dendritic cells [20,21]. Another carbohydrate ligand commonly incorporated into drug delivery systems is glucose [22–26]. Great interest in this ligand may arise from the fact that in human cancers the insulin-independent GLUT-1 is overexpressed and induces the consumption of large amounts of D-glucose compared to healthy cells.

Various synthetic glycohydrogel carriers have been studied for the delivery of different classes of therapeutic agents, ranging from small molecules, *e.g.* olansazine [12,24,25], propranolol [14,27], doxorubicin [11,13], iodoazomycinarabinofuranoside [15], oridonin [18], vancomycin [20]; medium sized molecules, *e.g.* insulin [26], siRNA [23], DNA [22], genes [19] or macromolecules, like BSA [10,16,28]. One smart hydrogel type includes materials, which show environmentally stimulated phase transition. Some well-known examples include hydrogels based on poly(*N*-isopropylacrylamide) (PNIPAM), which is a thermoresponsive polymer exhibiting lower critical solution temperature (LCST) and undergoes reversible conformational change around the physiological temperature [29–31]. Among these are some examples of PNIPAM-based hydrogels containing sugar components, which were tested *in vitro* for encapsulation and release of macromolecules [10,22,23,26,28]. Quan and Zhu [10] have described galactose-decorated, injectable, thermoresponsive microgels for BSA release. Narain et al. [28] also studied BSA release from glucose-based hydrogels containing an acid labile cross-linker. Dual temperature and pH sensitivity was used in cationic nanogels containing pending glucose as gene delivery carriers to hepatocytes [22] or in glucosamine-carrying microgels for insulin release [26].

Herein we report on the incorporation of another carbohydrate, trehalose, into the PNIPAM network. Trehalose is mainly known for its ability to protect proteins from inactivation or denaturation caused by a variety of stress conditions, including desiccation, dehydration or osmotic stress [32]. A few earlier reports have shown the covalent incorporation of trehalose into hydrogel networks [33–37]. The effectiveness of trehalose-based hydrogel to stabilize proteins against heat was demonstrated using phytase as an example [34]. Controlled release of ovalbumin and HRP from thiol-ene ethoxylated polyol formed with diacrylate trehalose monomers was demonstrated by Langer et al. [35]. In order to combine the bioprotective properties of trehalose and the characteristics of smart hydrogels, we synthesized PNIPAM-based thermoresponsive materials containing trehalose as novel protein carriers. We synthesized and characterized a series of PNIPAM hydrogels containing trehalose both in cross-links and as pending moieties in order to induce the bioprotective properties of matrices.

2. Experimental

2.1. Materials and general methods

Acrolein diethyl acetal, acrylamide (AM), ammonium persulphate (APS), anhydrous D-trehalose, Coomassie Brilliant Blue G-250, hydrochloric acid, *N*-isopropylacrylamide (NIPAM), phosphate buffer saline (PBS) tablets, *p*-toluenesulfonic acid and triethylamine were purchased from Sigma Aldrich, Acros Organics or Fluka and used directly without any purification. *O*-nitrophenyl-β-D-galactopyranoside (ONPG) (N1127), Bovine Serum Albumin (BSA) (A7030) and β-Galactosidase (β-Gal) from *Aspergillus oryzae* (G5160, 13 U·mg^{-1}) were purchased from Sigma Aldrich. *N*-(hydroxyethyl)acrylamide (HEAM) (Sigma Aldrich) were purified by passing through a column filled with basic aluminum oxide to remove the inhibitor. 4,6-*O*-acrylidene-α,α-D-trehalose (MAT) and 4,6:4′,6′-di-*O*-acrylidene-α,α-D-trehalose (DAT) were synthesized as we previously described [37]. Extra dry *N*,*N*-dimethylformamide (DMF) were purchased from Acros Organics and stored over molecular sieves under an inert atmosphere. Methanol and chloroform for column chromatography, tetrahydrofuran (THF) of HPLC grade, ethanol (96%), and phosphoric acid (85 wt% in H_2O) were purchased from Avantor Performance Materials Poland S.A.

Reactions were monitored by TLC on precoated plates of silica gel 60 F_{254} (Merck) and visualized by charring with 10% sulphuric acid in ethanol. Crude products were purified using column chromatography performed on silica gel 60 (70–230 mesh, Fluka). All evaporation were performed under diminished pressure at 50 °C. Lyophilization was carried out using freeze-dryer ALPHA 1-2 LD_{plus}, (CHRIST). NMR spectra were recorded using Varian spectrometer operating at 600 MHz with deuterium oxide used as a solvent and 3-(trimethylsilyl)propionic-2,2,3,3-d_4 acid sodium salt as an internal standard.

2.2. Synthesis of hydrogels

Hydrogels were synthesized at 25 °C by free-radical copolymerization of NIPAM, MAT and AM or HEAM with 2 mol% of DAT (0.024 mmol) used as a cross-linker. The detailed feed compositions and sample codes of the hydrogels are given in Table 1. The total

Table 1
Monomer feed compositions and physicochemical characteristics of hydrogels.

Sample code	Monomer feed composition[a] [mol%]					Yield [%]	Content of trehalose [wt%] actual (theor.)	VPTT [°C] in PBS		ESR	t_{deg} [h]	M_n [g/mol] (Đ)
	NIPAM	DAT	MAT	AM	HEAM			T_{Peak}	ΔT			
M0	98	2	–	–	–	87	2.5 (5.6)	31.8	1.8	32	147	16400 (2.1)
M5	93	2	5	–	–	73	5.4 (17.7)	32.4	2.6	45	91	15200 (1.8)
M10	88	2	10	–	–	64	6.9 (27.7)	33.1	3.2	61	48	12500 (1.4)
M5-A5	88	2	5	5	–	77	5.7 (18.1)	36.0	4.4	54	71	> 300000
M10-A5	83	2	10	5	–	64	7.0 (28.0)	36.7	5.1	66	50	> 300000
M5-H5	88	2	5	–	5	76	5.0 (17.7)	37.9	3.7	54	53	> 300000
M10-H5	83	2	10	–	5	63	6.6 (27.6)	38.7	4.8	68	43	> 300000

VPTT – volume phase transition temperature, ΔT = T_{Endset} − T_{Onset}, ESR – equilibrium swelling ratio, t_{deg} – degradation time, 25 °C, 1 M HCl.
[a] Monomers concentration: 1 M, APS/TMEDA concentration: 0.010/0.015 M.

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