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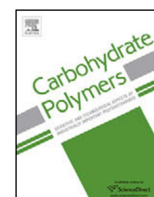

Macroporous chitosan hydrogels: Effects of sulfur on the loading and release behaviour of amino acid-based compounds

Lisa Elviri*, Maliheh Asadzadeh, Roberta Cucinelli, Annalisa Bianchera, Ruggero Bettini

Dipartimento di Farmacia, Università degli Studi di Parma, Parco Area delle Scienze 27/A, 43124 Parma, Italy

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Chitosan is a biodegradable, biocompatible polymer of natural origin widely applied to the preparation of functional hydrogels suitable for controlled release of drugs, peptides and proteins. Non-covalent interactions, expecially ionic interactions, are the main driver of the loading and release behaviour of amino acids or peptides from chitosan hydrogels. With the aim to improve the understanding of the mechanisms governing the behaviour of chitosan hydrogels on peptide uptake and delivery, in this paper the attention was focused on the role played by sulfur on the interactions of chitosan hydrogels with sulfur-containing amino acids (AA) and peptides. Hence, loading and release experiments on cysteine, cystine and glutathione (—SH containing amino acid, dipeptide and tripeptide, respectively) as well as on glycine and valine as apolar amino acids were carried out. For these puroses, chitosan hydrogels were prepared in an easy and reproducible manner by a freeze-gelation process on a poly-L-lysine coated support. The hydrogel surface pore size, uniformity and distribution were tested. Optimal results ($D_{50} = 26 \pm 4\,\mu m$) were obtained by using the poly-L-lysine positively-charged surface. The loading results gathered evidenced that the sulfur-containing molecules presented an increased absorption both in terms of rate and extent by chitosan hydrogels with respect to nonpolar amino acids, mainly due to ionic and hydrogen bond interactions. ATR-FTIR analysis carried out on chitosan hydrogels, with and without the AA related compounds to study putative interactions, supported these apparent sulfur-dependent results. Finally, chitosan hydrogels displayed excellent retention capabilities (AA release <5%) for all AA, strongly supporting the use of chitosan hydrogels as matrix for controlled drug release.

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1. Introduction

In the last years, the interest of the scientific community towards the development of innovative functional materials has grown exponentially due to their effective contribution to the progress in many clinical, physical and analytical fields (Lin, Huang, & Dufresne, 2012). These materials attracted a great deal of interest for applications in drug delivery, in soft tissue regeneration, as biosensors, enzyme immobilization or for sample preparation in food and environmental analyses as they present several interesting properties, i.e. tunable shape and porosity, biocompatibility, possible surface functionalization (Dey, Bera, & Raj, 2013; Kopeček & Yang, 2012; Weyers & Linhardt, 2013; Kuan, Yee-Fung, Yuen, & Liong, 2012).

For the preparation of functional materials several challenging issues have to be addressed, including chemical and biological stability, process repeatability, shape tuning and material selection. On this account, a variety of approaches and polymers have been promptly tested (Buwalda et al., 2014).

Among natural polymers, chitosan has been widely investigated for the preparation of functional hydrogels because of its biodegradability, biocompatibility and low toxicity (Shukla, Mishra, Arotiba, & Mamba, 2013; El Kadib, Bousmina, & Brunel, 2014; Dash, Chiellini, Ottenbrite, & Chiellini, 2011; Censi, Di Martino, Vermonden, & Hennink, 2012). Chitosan is a β-(1–4)-linked D-glucosamine and *N*-acetyl-D-glucosamine natural polysaccharide presenting a cationic nature imparting the capability to bind with a wide variety of polyanionic species through electrostatic-pH dependent interactions (Bianchera et al., 2014). Focusing on clinical applications, it is able to form bio- and mucoadhesive hydrogels with the anionic proteoglycans widely distributed within the organism and presents excellent applications in local controlled delivery of both low and high molecular weight drugs (Garg, Singh, Arora, & Murthy, 2012; Bernkop-Schnurch & Dunnhaupt, 2012). In addition, since chitosan hydrogels present mechanical and compositional similarities with native extracellular matrix, they can be used as support for cell growth

* Corresponding author. Tel.: +39 0521 905087; fax: +39 0521 905006.
E-mail address: lisa.elviri@unipr.it (L. Elviri).

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during tissue regeneration (Bettini, Romani, Morganti, & Borghetti, 2008; Romani et al., 2008; Dutta, Rinki, & Dutta, 2011).

In the last decade, different innovative strategies have been proposed to obtain systems for efficient, suitable and controlled drug delivery (Alvarez-Lorenzo & Concheiro, 2013; Kim et al., 2014; Mishra, Hubenak, & Mathur, 2013). Hydrogels, with their several applications for the oral, rectal, ocular, transdermal and subcutaneous drug release, represent an excellent choice as potential bioactive supports (Vemula et al., 2013; Overstreet, Dutta, Stabenfeldt, & Vernon, 2012; Bae et al., 2014). Their use as tools for the targeted delivery of active molecules results in a localized increase in the concentration of the drug, a preferential absorption of the drug at the site of release and a concurrent reduction of systemic exposure and side effects.

For instance, a time- and space-controlled delivery is highly desired for endogenous or synthetic peptides with antimicrobial activity (Schmidt & Wong, 2013; Lohan & Bisht, 2013; Romani et al., 2013a,b; Cabassi et al., 2013). As a matter of fact, as small molecules, they can (i) be easily modified in the host-pathogen interaction, (ii) cause cell damage or (iii) undergo premature degradation.

Numerous biomedical applications of amino acid-grafted chitosans were reviewed by Casettari, Vllasaliu, Lam, Soliman, and Illum (2012).

The delivery of amino acids or peptides using chitosan or its derivatives as matrix forming systems relies on different strategies that share the common goal of improving chitosan loading efficiency towards the amino acid or peptide. The easiest and most frequent approach is driven by the ionic characteristics of the protein to be adsorbed and leads to the covalent association of chitosan with positively charged groups that can form polyelectrolytes complexes with oppositely charged proteins such as those deriving from plasma; the modification with negatively charged groups is less frequent. In a work by Zubareva et al. (2013) chitosan was derivatized in order to obtain nanogels having positive (hexanoyl-chitosan) or negative (succinoyl-chitosan) surface charge and their ability to form complexes with differently charged peptides was evaluated: significant passive sorption was found only between nanogels and peptides with opposite charges, with a binding efficiency increasing with the intensity of the charge. Such behaviour was confirmed in the case of differently charged proteins, with a more pronounced effect for low molecular weight proteins. Electrostatic interactions played the main role but hydrophobic, van der Waals forces and hydrogen bonds were also operative. Hoven, Tangapasuthadol, Angkitapaiboon, Vallapa, and Kiatkamjornwong (2007) prepared quaternary ammonium functionalized positively-charged or sulfonate-functionalized negatively-charged chitosan films and particles, tailoring the extent of surface modification. This had a remarkable impact on their ability to interact with proteins if compared to non-treated chitosan films. In negatively-charged films, electrostatic forces were the main drivers of the interaction and adsorption was promoted as a function of charge density, while surface hydrophobicity had no influence. Unexpectedly, positively-charged films adsorbed proteins independently of their charge: the adsorption trend paralleled a decrease of water contact angle but a role in protein adsorption by swelling ability or state of water in the gel was excluded. On the other hand, Romani et al. (2013a,b) demonstrated that chitosan films, produced with a method able to increase the amount of water in the matrix, afforded a negligible interaction with blood cells due to the rearrangement of polymer chains that masked the cationic nature of chitosan surface, being this rearrangement ascribed to the large amount of water in chitosan films.

Thus, the study of non-covalent interactions of amino acids or short peptides with chitosan hydrogels represents a chief topic in drug delivery and may offer crucial basic information on the mechanism of peptide or protein adsorption onto chitosan structures as well as on the design of efficient delivery systems.

In such a context, with the aim to improve the understanding of the mechanisms governing the properties of chitosan hydrogel as possible peptide delivery system, in this paper we focused on the role played by sulfur on the interaction of chitosan hydrogels with sulfur containing amino acid compounds. For this purpose, a simple and reproducible freeze-gelation procedure was adapted to prepare hydrogels with uniform pore size and distribution. Hence, loading and release experiments on cysteine, cystine and glutathione (—SH containing amino acid, dipeptide and tripeptide, respectively) and glycine and valine as nonpolar amino acids at neutral pH were carried out.

The affinity of the polymer for the studied amino acids or peptides, both in the loading and in the release phase, was investigated with a soaking approach (Bianchera et al., 2014) and quantified by using an in-house validated reversed phase liquid chromatography-electrospray-mass spectrometry method. Furthermore, the small molecule-polymer structural features were investigated by attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy in order to characterize possible non-covalent interactions.

2. Materials and methods

2.1. Materials

Chitosan fine powder (deacetylation degree min = 90%, molecular weight distribution 60–80 kDa) was purchased from A.C.E.F. (Piacenza, Italy). Formic acid was from J.T. Baker (Deventer, Netherland). Water was obtained with a MilliQ element A10 System (Millipore, S. Francisco, CA, USA). Cystine (Di-CYS), cysteine (CYS), reduced glutathione (GSH), glycine (GLY), valine (VAL), raffinose penta-hydrate and potassium hydroxide were from Sigma-Aldrich (St. Louis, MO, USA). All other reagents of analytical grade were obtained from Sigma-Aldrich.

2.2. Chitosan purification

Chitosan was purified by alkaline precipitation. Briefly, a 2% chitosan solution was prepared in 1% acetic acid aqueous solution and stirred until complete dissolution. A 3% KOH aqueous solution was prepared in order to have half volume of the chitosan solution and added drop wise at a rate of 60 drops min^{-1} to induce chitosan precipitation. The obtained dispersion was filtered through filter paper on a Buckner funnel then rinsed three times with 96% ethanol. The resulting slurry was then transferred on a 40 °C oven and dried for 1 h. The resulting yellowish powder was sieved to collect the fraction with particle size <60 μm.

2.3. Hydrogel preparation

Hydrogels were prepared by modifying the method described elsewhere (Bettini et al., 2008). Briefly, a 4.5% w/v chitosan solution was prepared by dissolving purified chitosan in a 1% v/v acetic acid aqueous solution, then raffinose penta-hydrate was added at a final concentration of 290 mM as thickening agent. After complete dissolution the solution was cast into 10 mm diameter rubber rings between two solid surfaces and frozen at −60 °C overnight. Different surfaces were tested: silanized glass, silica, polytetrafluoroethylene (PTFE), polyvinylidene fluoride (PVDF) and poly-L-lysine. Frozen hydrogels were then transferred in a cold mixture of KOH 5% w/v aqueous solution and 96% v/v ethanol (4:6, v/v) and left to gelify at −20 °C for 24 h. Hydrogels were then rinsed three times in deionized water and kept in water until use.

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