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Collagen tissue treated with chitosan solution in H_2O/CO_2 mixtures: Influence of clathrates hydrates on the structure and mechanical properties

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ABSTRACT

A mixture of water/carbon dioxide is a "green" perspective solvent from the viewpoint of biomedical applications. Clathrate hydrates are formed this solvent under certain conditions and a very interesting question is the impact of clathrates hydrates on the structure and properties of bovine pericardium, which is used in biomedicine, in particular as a main part of biological heart valve prostheses. The aim of the present work is to investigate the influence of clathrates on the structure and mechanical properties of the collagen tissue treated with chitosan in H_2O/CO_2 mixtures under pressure 3.0–3.5 MPa and temperatures 2–4 °C. It was first found that the clathrate hydrates in this media due to the strong fluctuations "bomb" collagen tissue of bovine pericardium, which is manifested in the appearance of numerous small gaps (pores) with mean size of 225 ± 25 nm and large pores with size of 1–3 μ on the surface and within collagen matrices. High porosity leads to averaging characteristics of the organization structure in tissues with different orientation of the collagen fibers. As a result, the mechanical properties of the collagen tissue with a different orientation of the collagen fibrils become similar, which is quite different from their original properties. The structural changes caused by the influence of the environment clathrate hydrates led to a significant decrease of the tensile strength (30–47% in total, $p < 0.05$) and initial elastic moduli (74–83%, $p < 0.05$). However, the final elastic moduli and the maximum tensile virtually unchanged compared to the control. Nevertheless, it was found that the direct deposition of chitosan from the H_2O/CO_2 mixtures with clathrate improve the mechanical-strength properties of the porous matrices. We believe that these improved mechanical properties are achieved due to particularly deep and uniform impregnation of the collagen matrix with chitosan from its pressurized solutions in H_2O/CO_2 mixtures.

1. Introduction

Collagen is the most abundant protein in the extracellular matrix of animals and has been considered to be a group of proteins with a characteristic molecular structure – fibrillar structure (Ricard-Blum, 2011). Collagen offers low immunogenicity, a porous structure, permeability, good biocompatibility and biodegradability and has functions to regulate the morphology, adhesion, migration and differentiation of cells (Chevallay et al., 2000). All of these good performances make this natural polymer seem to be a promising biomaterial for scaffolds in tissue engineering. Collagen based materials in different physical forms (fibers, films, sponges, hydrogels, powder) are widely used for tissue engineering applications.

Despite the excellent biological properties of the pure collagen scaffold, it has poor mechanical properties and structural stability. Physical treatment or chemical agents can be used to achieve intermolecular cross-linking of collagen, thus modifying the properties of the collagen scaffold. Chemical modification is accomplished mainly by means of covalent of amine/imine linkage (Suchý et al., 2015). Glutaraldehyde (GA) is a synthetic cross-linking agent that has been widely used in the manufacturing of bioprosthesis. It produces collagen with a high degree of cross-linking, but with potential toxicity due to possible residue in the scaffold (Yoshioka and Goissis, 2008). In particular, this material is a general functional part of the bioprosthetic heart valves. The tissue is to be crosslinked mainly by glutaraldehyde (GA) to provide mechanical stability and absence of any foreign-body immune response of a patient (Cannegieter et al., 1994; Stein et al., 2001; Rahimtoola et al., 2003; Bach, 2003; Siddiqui et al., 2009). Such

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a leaflet of the valve is akin to its structure and properties to the native one being replaced. As far as bioprosthetic valves better emulate hemodynamic properties of native human valves as compared with mechanical substitutes, this leads to the much less damage of red blood cells and a lower risk of blood clot formation (Cannegieter et al., 1994; Stein et al., 2001; Rahimtoola et al., 2003; Bach, 2003; Siddiqui et al., 2009).

However, there is a very significant disadvantage associated with the use of glutaraldehyde as cross-linking agent of the collagen tissue in terms of biomedical applications. The main reason for valve failure is calcification of collagen tissue. It is known that one of the possible ways of the calcification of collagen tissue is interaction residual unreacted aldehyde groups remaining in the crosslinked tissue with the components of the blood plasma (Schoen et al., 1986).

To reduce calcification, researchers are trying to mask the free aldehyde groups in the collagen tissue. It was shown that the most straight forward way to reduce calcification under this paradigm is the deposition of chitosan on the collagen tissue (Chanda, 1994; Nogueira et al., 2010).

Chitosan is a linear polysaccharide composed of β 1/4-Dglucosamine and β 1/4 N-acetyl D-glucosamine units. The specific structure and unique properties of this material has involved significant interest in a broad range of scientific areas. Chitosan has been under extensive investigation for various biomedical and pharmaceutical applications because of low toxicity, excellent biocompatibility, biodegradability and polycationic properties (Domard, 2011; Jayakumar, 2010; Casettari et al., 2012).

It is known that chitosan can be dissolved in acidic aqua media (pK of chitosan is 6.1) (Roberts,1992; Yi et al., 2005). One of the most widely used solvents of chitosan is acetic acid (Rinaudo et al., 1999a, 1999b). However, the use of acetic acid as a solvent of chitosan is not optimal in some cases. Indeed, the residual solvent in the modified chitosan coating may cause to allergic reactions or other adverse responses of an organism by virtue of the possible individual hypersensitivity or intolerance (Wuethrich, 2011; Przybilla et al., 1986). Also the traces of residual solvent associated with the chitosan chains acetic acid anions may lead to excessive swelling and delamination of chitosan with subsequent contact with the aqueous environment of the body.

Our task was to find a solvent capable of dissolving chitosan and which residual traces were harmless to the human body. Previously, we studied the possibility of using biocompatible supercritical (sc) CO_2 as a solvent for chitosan, but the solubility was too low for real applications (Chaschin et al., 2012).

Further, we found out rather good solubility of chitosan in water saturated with pressurised CO_2, i.e. in carbonic acid (~10 g/L) (Khokhlova et al., 2012). In fact, before, it was already confirmed (Sakai et al., 2002) that it is probable to dissolve chitosan with different degree of acetylation in carbonic acid. The observed values of the chitosan solubility in solutions in carbonic acid allow expecting the possibility of the practical use of such a "green" solvent chitosan in biomedical applications. Specifically, the carbonic acid solvent looks very promising from the viewpoint of modifying of the collagen tissue of bovine pericardium.

In our previous work we deposited chitosan from phase of liquid water saturated CO_2 under high pressure (P=30 MPa) and temperature 25 °C on the collagen matrixes of bovine pericardium. Under this condition the system of H_2O/CO_2 is liquid CO_2 with density about 0.9 g/cm^3 which located on the top half of reactor and liquid water saturated CO_2 (carbonic acid) with density about 1.0 g/cm^{3}which located on the bottom half of reactor. We found that GA-stabilised collagen tissue of bovine pericardium post-treated with chitosan from solutions in carbonic acid demonstrates even more advanced and highly promising properties (Gallyamov et al., 2014).

It is known there is a region of phase space pressures and temperatures at which the mixture of H_2O/CO_2 in the liquid phase coexist to the phase of clathrate hydrates. From the literature it is known that the mixture of H_2O/CO_2at temperatures below 10 °C and pressures above 2.1 MPa forms clathrate hydrates, with the approximate chemical formula $CO_2{\cdot}6H_2O$ (Manakov et al., 2009; Wendland et al.,1999; Spycher et al., 2003; Anderson, 2002). Clathrate hydrates are crystalline compounds of inclusion of guest molecules in the framework formed by hydrogen-bonded water molecules. Between the guest molecules (CO_2) and hosts molecules (H_2O) present only the van der Waals interaction. Typically, clathrate hydrates existing at pressures of less than 100 MPa, belong one of three structural types: cubic structure I (CS-I), the cubic structure II (CS-II), or a hexagonal structure III (HS-III or SH). CO_2 saturated water clathrates, and liquid CO_2 (also saturated with water) may coexist in a temperature range from about −1 °C to 10 °C. Mole fraction CO_2 under pressures and temperatures corresponding to phase separation into liquid CO_2 and H_2O is 2–3%, but under the conditions of existence of clathrates, having the structural formula $CO_2{\cdot}6H_2O$, the mole fraction of CO_2 is 1/7, which is about 14%. Under formation of clathrates boundary between water, liquid and solid phase CO_2 becomes dynamically fluctuating and poorly visualized (Manakov et al., 2009; Wendland et al.,1999; Spycher et al., 2003; Anderson, 2002).

The aim of our work is to study the impact of such an unusual media as a biphasic system of H_2O/CO_2 with clathrates on the structure and properties of collagen tissue by direct deposition of chitosan from this media. The impact of this unusual solvent of chitosan on the structure and properties of the collagen tissue is quite difficult to predict. On the one hand a high percentage of carbon dioxide in a medium (14% compared to the carbonic acid of about 3%) may lead to a better solubility of chitosan that as a consequence may lead to an increase in the amount of chitosan deposited onto the collagen tissue. However, on the other hand, a high degree of fluctuation of the environment and the existence of the clathrates may lead to degradation of the collagen tissue, and chitosan coating.

2. Experimental

2.1. Materials

For our research we used the chitosan of a “low molecular weight” grade supplied by Sigma-Aldrich (catalogue number: #448869). Using gel permeation chromatograph (Agilent 1200) calibrated with pullulan standards (from 1.08 to 710 kg mol^{-1}) we determined the molecular weight of this chitosan sample: M_w=210 kg/mol, M_n=80 kg mol^{-1} (25 °C, aqueous buffer solution, 0.2 M acetic acid, 0.15 M ammonium acetate, 1 ml min^{-1}), which correlated well with Mη=80 kg/mol as determined by us from viscosity measurements (25 °C, aqueous solution, 0.3 M acetic acid, 0.2 M sodium acetate, using Mark–Kuhn–Houwink equation with previously described coefficients according to Ref. (A. Gamzazade et al., 1999). The chitosan sample had DA of 16–24% according to our data of potentiometric titration and IR spectroscopy (Thermo Nicolet IS5 FT-IR).

In the experiments we used CO_2 of high purity (> 99.997%; Linde Gas Rus, Russia) and freshly purified Milli-Q water (Milli-Q Synthesis).

Collagen matrices of bovine pericardium were picked out, GA-stabilised and sterilised in accordance with technological regulations approved for surgery practice in A.N. Bakulev Scientific Center for Cardiovascular Surgery. In general, this treatment includes washing of collagen tissue in 0.9% sodium chloride with heparin (5000 U/L) and stabilization by 0.625% GA aqueous solution. The stabilization of collagen tissue is carried out in two stages with intermediate washing with aqueous solution of 1% sodium dodecyl sulphate and with HEPES buffer. After washing with sterile saline solution, the collagen samples were immersed into a high pressure vessel for subsequent coating with chitosan.