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Collagen tissue treated with chitosan solutions in carbonic acid for improved biological prosthetic heart valves

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ABSTRACT

Calcification of bovine pericardium dramatically shortens typical lifetimes of biological prosthetic heart valves and thus precludes their choice for younger patients. The aim of the present work is to demonstrate that the calcification is to be mitigated by means of treatment of bovine pericardium in solutions of chitosan in carbonic acid, i.e. water saturated with carbon dioxide at high pressure. This acidic aqueous fluid unusually combines antimicrobial properties with absolute biocompatibility as far as at normal pressure it decomposes spontaneously and completely into H_2O and CO_2. Yet, at high pressures it can protonate and dissolve chitosan materials with different degrees of acetylation (in the range of 16–33%, at least) without any further pretreatment. Even exposure of the bovine pericardium in pure carbonic acid solution without chitosan already favours certain reduction in calcification, somewhat improved mechanical properties, complete biocompatibility and evident antimicrobial activity of the treated collagen tissue. The reason may be due to high extraction ability of this peculiar compressed fluidic mixture. Moreover, exposure of the bovine pericardium in solutions of chitosan in carbonic acid introduces even better mechanical properties and highly pronounced antimicrobial activity of the modified collagen tissue against adherence and biofilm formation of relevant Gram-positive and Gram-negative strains. Yet, the most important achievement is the detected dramatic reduction in calcification for such modified collagen tissues in spite of the fact that the amount of the thus introduced chitosan is rather small (typically ca. 1 wt.%), which has been reliably detected using original tritium labelling method. We believe that these improved properties are achieved due to particularly deep and uniform impregnation of the collagen matrix with chitosan from its pressurised solutions in carbonic acid.

1. Introduction

There is strong interest in improving properties of biological prosthetic heart valves, which demonstrate certain advantages as compared with mechanical heart valves [1–5]. The major functional part of the bioprosthetic valves is a soft, elastic and durable collagen tissue made of either bovine pericardium or porcine aortic valve. For surgery applications the tissue is to be crosslinked mainly by glutaraldehyde (GA) to ensure mechanical stability and absence of any foreign-body immune response of a patient [1–5]. Such a leaflet of the valve is akin in its structure and properties to the native one being replaced. As far as bioprosthetic valves better emulate haemodynamic 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 [1–5].

Nevertheless, still in the majority of surgery cases mechanical heart valves remain to be the best or the only possible choice. Indeed, according to the recent review of Siddiqui et al., more than 250,000 substitute valves are implanted each year worldwide, of which roughly 55% are mechanical heart valves and only 45% are biological prosthetic heart valves [6]. A decisive drawback of the bioprosthetic valves is too short service life therefore they are mainly not recommended for young patients [1–8]. Calcification of the collagen tissue is among the main causes of failure of the bioprosthetic valves [9,10]. This calcification is considered to be induced by residual unreacted aldehyde groups remaining in the crosslinked tissue and interacting with the components of the blood plasma, though the total concentration of such groups is not really high [11]. GA-crosslinked collagen is a spongy-like matrix with many internal voids and cavities, which tend to be filled with the calcium-containing deposit [12,13] mainly in the form of calcium phosphate salts [14].

Many efforts were directed towards the development of methods to mitigate the calcium deposition. In order to reduce susceptibility of the GA-crosslinked collagen tissue to calcification, researchers in this area

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mainly aimed at masking or inactivating of residual free aldehyde groups. In some cases they also tried additionally to fill the interstitial (inter-fibrils or inter-tropocollagen) space with some filler. Both low-molecular-weight and polymer materials were tested. Among successfully tested low-molecular-weight anticalcification agents one should mention amino-containing α-amino oleic acid (AOA) [15–17] and L-arginine [18], amino-containing [19] and other diphosphonates [20], urazole with sodium borohydride [21] as well as citric acid [22] and some surfactants [23]. Among tested polymer materials promising results were obtained with generally biocompatible poly(ethylene oxide) [24–26], other biocompatible polymers [27], amino-containing polyacrylamide [28] as well as with glycosaminoglycans such as hyaluronic acid [29] and heparin [30,31]. In general, according to subcutaneous implantation express-tests in rats, polymeric modifiers [24–31] demonstrated higher efficiency in the reduction of calcification in comparison with their low-molecular-weight counterparts [15–23]. Yet, the treatment procedure with polymers was typically rather complex and in some cases an additional step of a special chemical modification was required to ensure polymer grafting [24,25,29].

Following this general paradigm, in a set of papers Chanda proposed and successfully implemented with co-workers simpler procedure of covering the bioprostheses surface with a chitosan film. The procedure is based on direct exposure of a collagen tissue in solutions of chitosan assuming binding between chitosan amino-groups and residual free aldehyde groups in the crosslinked matrix [32–38]. But Chanda worked with water-soluble chitosan [32] such as a material with degree of N-acetylation (DA) of ca. 50% [39–41]. Yet, commercially available chitosans with normal DAs of ca. 15–35% are soluble only in acidic media [42]. There are many possible ways to prepare water-soluble chitosan materials (at neutral pH conditions). But a chitosan film adsorbed from such solutions would hardly be sufficiently stable in a blood plasma stream (pH value of ca. 7.4) and most probably should excessively swell there and eventually delaminate.

Other authors formed chitosan films on collagen matrices from commonly used solutions in acetic acids with subsequent alkaline neutralisation. Its purpose is to convert chitosan into a water-insoluble form thus ensuring mechanical stability of the adsorbed films [43–46]. But there are some possible disadvantages of using acetic acid as a solvent for treatment of implants. First of all, acetic acid deeply impregnates a collagen tissue and may even disturb organization of collagen fibrils [47–49]. Consequently, deterioration of mechanical properties of the matrix may be expected. This is why Shanthi and Rao [43] attempted to use the lowest possible concentrations of acetic acid solutions. Further, residual traces of this solvent in the modified collagen tissue are not only cytotoxic [45] but potentially allergenic. Indeed, it is well-known that in some occasional cases surprisingly strong quasi-allergenic reactions may be observed, when acetic acid acts most probably as a hapten [50–55]. In these cases it is responsible for pronounced intolerance or hypersensitivity observed, including positive skin-prick tests [50–55].

Our task was to find another solvent, which is capable to dissolve chitosan and yet its residual traces are absolutely compatible with the human body. Previously, we tested the possibility to apply biocompatible and non-allergenic supercritical (sc) CO_2 as a solvent for chitosan, but the achieved solubility was too low for real applications [56].

Further, working with the mixtures of water and CO_2 in a closed vessel at high pressure we found out rather good solubility of chitosan not in CO_2 saturated with water, but, on the contrary, in water saturated with pressurised CO_2, i.e. in carbonic acid [57]. Indeed, previously, authors of Ref. [58] have already demonstrated that it is possible to dissolve chitosan with different DAs in carbonic acid.

The aim of the present work is to develop a procedure of the formation of chitosan coatings on a bovine pericardium tissue from solutions of chitosan in carbonic acid as well as to study the structure and main properties of the modified collagen tissue including stress–strain behaviour and affinity towards calcium deposits, bacteria and cells. Taking into account very peculiar properties of this polysaccharide [59], we expected not only mitigation of calcification, but also improved biocompatibility and introduced remarkable antimicrobial activity (including suppression of possible biofilm [60–62] formation) of the collagen tissue with the formed chitosan coating to be used as a material for biological prosthetic heart valves.

2. Experimental

2.1. Materials

We tested several chitosan samples with different DAs supplied by Sigma-Aldrich: #448869 (16–24% DA), #419419 (29–30% DA), #448877 (26–27% DA), #417963 (30–31% DA), #c3646 (28–33% DA), #48165 (26–29% DA). All of them demonstrated certain solubility in carbonic acid with the achievable concentrations of up to a few g L^{-1}. The best solubility was detected for the chitosan of a "low molecular weight" grade (catalogue number: #448869), which was selected for the further experiments and used without any purification.

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^{-1}, $M_n = 80$ 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 $M_\eta = 80$ kg mol^{-1} 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. [63]). 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 stabilisation by 0.625% GA aqueous solution 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.

2.2. High pressure setup

The experimental setup was the same, as it was previously used for deposition of chitosan from solutions in $scCO_2$ or carbonic acid onto mica as described before [56,57]. Briefly, the setup consists of a high pressure generator equipped with pressure sensors and a thermostatically controlled stainless steel high pressure vessel (inner volume of 30 ml), which are connected together by a set of capillaries. The setup can sustain the pressures of up to 80 MPa (as limited by the vessel).

2.3. Preparation of chitosan solution in carbonic acid

The preparation of chitosan solution in carbonic acid was performed as described before [57] and illustrated in Fig. 1. We put 150 mg of chitosan powder into the high pressure vessel. The half of the vessel (15 ml) was filled with freshly prepared Milli-Q water. Then it was closed and filled with liquid CO_2 up to high pressure (30 MPa) at room temperature (23–25 °C) and left at these conditions for dissolution and equilibration of CO_2 in water and chitosan in the thus generated carbonic acid with periodic agitating by a magnetic stirrer. The total time of dissolution was selected in the range of several days (7–14 days, but no significant difference was observed regarding the properties of the obtained solutions).