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Collagen/elastin hydrogels cross-linked by squaric acid

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ABSTRACT

Hydrogels based on collagen and elastin are very valuable materials for medicine and tissue engineering. They are biocompatible; however their mechanical properties and resistance for enzymatic degradation need to be improved by cross-linking. Up to this point many reagents have been tested but more secure reactants are still sought. Squaric acid (SqAc), 3,4-dihydroxy 3-cyclobutene 1,2-dione, is a strong, cyclic acid, which reacts easily with amine groups.

The properties of hydrogels based on collagen/elastin mixtures (95/5, 90/10) containing 5%, 10% and 20% of SqAc and neutralized via dialysis against deionized water were tested. Cross-linked, 3-D, transparent hydrogels were created. The cross-linked materials are stiffer and more resistant to enzymatic degradation than those that are unmodified. The pore size, swelling ability and surface polarity are reduced due to 5% and 10% of SqAc addition. At the same time, the cellular response is not significantly affected by the cross-linking. Therefore, squaric acid would be regarded as a safe, effective cross-linking agent.

1. Introduction

Hydrogels are a class of materials that have been used widely in medicine for many years. They are applied as drug delivery systems, dressings for wound healings and now, increasingly often, in tissue engineering. Various types of hydrophilic polymers, both synthetic and natural, are used for the preparation of hydrogels [1,2]. Biopolymers such as proteins, due to the presence of a large number of functional groups, are also easily wettable by polar solvents. Collagen and elastin are widely distributed in human tissues. They are the main components of an extracellular matrix (ECM) most of the connective tissues. Collagen has a triple-helical structure which gives it unusual strength. Elastin forms a very elastic, extensively cross-linked continuous network of fibers. Both proteins, collagen and elastin, occur together in tissues, especially in load bearing tissues such as the bones, tendons, lungs, skin, and arteries; ensuring their elasticity and resilience. As biomaterials, these polymers improve the cell adhesion and promote the production of extracellular matrix from proliferating cells [3–7].

The cross-linking of proteins allows us to create the supramolecular network capable of swelling. The formation of bonds between two different macromolecules stabilizes the structure. Usually, it improves the mechanical properties of the materials, their stiffness and degradation resistance [8]. One of the many possibilities of the protein chains cross-linking is the use of bifunctional reagents containing reactive groups. Many various cross-linking agents have been tested over the last number of years, e.g. dialdehydes, carbodiimides, isocyanates, tannic acid, divinylsulphone, diglycidylether, and others. Many of these compounds are effective for cross-linking proteins [3,9,10]. However, researchers are still looking for new, more secure reactants.

Squaric acid (SqAc), 3,4-dihydroxy 3-cyclobutene 1,2-dione, is a very strong acid which possesses a cyclic, symmetrical, planar and rigid structure. The molecule has a significant aromatic character. Due to the presence of two carboxyl groups there is an extensive π-electron delocalization over all the oxygen and carbon atoms. Squaric acid is a highly acidic molecule because the negative charges are equally distributed between oxygen atoms in the completely symmetrical dianion. Therefore, the squaric acid willingly reacts with amino groups (Fig. 1) [11–13]. In our best knowledge it has not been used before as a cross-linking agent for protein materials.

The main purpose of this study was to investigate the influence of the addition of squaric acid on the chemical, physical and biological properties of collagen/elastin materials.

2. Materials and methods

2.1. Materials preparation

Collagen was isolated from the tail tendons of young albino rats (Collegium Medicum of Nicolaus Copernicus University, Bydgoszcz, Poland; Local University Ethics Committee, permission no. 32/2012). Tendons were separated from adhering tissues, washed in deionized water and

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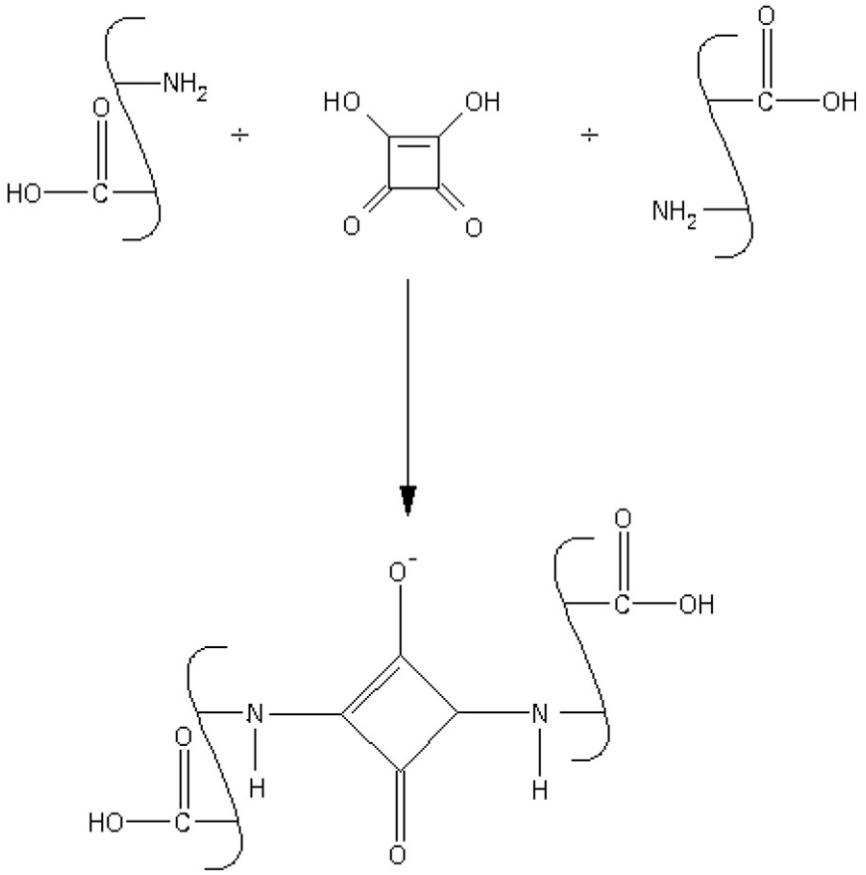

Fig. 1. Scheme of reaction of squaric acid with amine groups of peptides.

placed in 0.1 M of acetic acid for 72 h at 8 °C. The impurities and insoluble parts were separated by centrifugation at 10,000 rpm in an Eppendorf Centrifuge. The collagen solution was then freeze-dried. The method used was the same as previously employed [4].

Elastin from porcine aorta was purified by Lansing's method as it has been described [6]. Pig aortas, obtained from a local butcher, were cleaned from adhering tissues and cut into small pieces (roughly 0.5 cm wide rings). All remaining fat was removed by sequential extractions in ethanol (twice), a mixture of ethanol/ether (50/50) (twice) and ether (also twice). The 50 g of de-fatted tissue was placed in 100 ml of 0.1 M NaOH and heated to 95 °C for 50 min in a water bath under continuous stirring. After cooling at room temperature the samples were washed twice with cold 0.1 M NaOH in a Buchner funnel and then with deionized water. Dry material was minced in liquid nitrogen. The elastin powder (1 g) was suspended in a mixture of 50 ml of tert-butanol and 50 ml of 1 M KOH and was stirred for 48 h at room temperature. After this procedure, 50 ml of water was added and the resulting solution was neutralized with acetic acid. The solution of elastin hydrolysates was then dialyzed against deionized water and then lyophilized. All the used reagents were supplied from Avantor (Poland; purity: p.a.).

The squaric acid was purchased from Sigma-Aldrich (USA, 99% purity) and was used as a cross-linking agent.

2.2. Hydrogel preparation

1% solution of collagen in 0.1 M acetic acid and 1% solution of elastin hydrolysates in water were prepared and mixed in appropriate volume ratios (95/5, 90/10). Then the 5%, 10% or 20% (weight percent based on the dry weight of the protein) of the cross-linking agent (squaric acid) was added. The blends were mixed for 30 min in a magnetic stirrer and placed in dialysis tubes (Servapor dialysis tubing, MWCO 12 000–14 000RC). The dialysis against deionized water was carried out for 7 days (until the moment when the pH of surrounding solution was not changing). During this time spontaneous formation of hydrogel via neutralization process took place — Fig. 2, [14].

The names of all obtained hydrogels are shown in Table 1.

2.3. Infrared spectroscopy

The thin slides of collagen/elastin gels were dried in air. The FTIR-ATR spectra of samples were obtained using the spectrophotometer Mattson Genesis II (USA) equipped with an ATR tool with the resolution 4 cm^{-1}. The FTIR spectra were compared using the program supplied by the manufacturer.

2.4. Thermal analysis

The thermal properties of unmodified and cross-linked collagen/elastin materials were studied using a differential scanning calorimeter (DSC 204 F1 Phoenix, Netzsch, Germany). Standard aluminum DSC pans were used with about 1.5 mg of the dried material. All samples were held at 20 °C for 3 min and then scanned from 20 °C to 250 °C at a heating rate of 10 °C/min in the atmosphere of nitrogen.

2.5. SEM images

The images of morphology of lyophilized collagen/elastin biomaterials were obtained by a scanning electron microscope made by LEO Electron Microscopy Ltd., England, model 1430 VP. All samples were coated with gold.