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Stability of Collagen Scaffold Implants for Animals with Iatrogenic Articular Cartilage Defects

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Abstract

Synthesis and characterization of biodegradable hydrogels based on collagen modified by addition of synthetic biodegradable copolymer intended for preparation of porous scaffolds for mesenchymal stem cells used for possible implantation to animals with articular surface defects was investigated.

The synthetic biodegradable tri-block copolymer used was the block copolymer of polyethylene glycol (PEG), polylactic acid (PLA), polyglycolic acid (PGA) (PEG-PLGA) endcapped with itaconic acid (ITA). The water-soluble carbodiimide and N-hydroxysuccimide system (EDC-NHS) was chosen as the cross-linking agent used to control the rate of hydrogel resorption. Dependence of the physical properties of the prepared hydrogels on the concentration of the EDC-NHS cross-linker, reaction time and concentration of PEG-PLGA-ITA copolymer was examined. Swelling behaviour, thermal stability, surface morphology and degradation rate were also characterized.

Based on the obtained results, it can be concluded that increase in concentration of the cross-linking agent, as well as prolonged cross-linking time and increased amount of synthetic copolymer lead to enhanced thermal stability of the gels together with a reduced swelling ratio and degradation rate in saline. The resorption rate of these gels used in preparation of cartilage scaffolds can be controlled over a wide time interval by varying the collagen/(PEG-PLGA-ITA) blend composition or the conditions of the cross-linking reaction.

Collagen, PEG, PLGA, ITA, cross-linking, EDC, scaffold

Collagen extracted from bovine skin is one of the most often used biopolymers in various medical applications due to its inherent biocompatibility, ability to support cell growth and differentiation and negligible immuno-reactivity. On the other hand, collagen exhibits poor mechanical properties and low stability in various physiologically important solvents. To overcome its shortcomings, collagen can be blended with other polymers, filled with solid inclusions or cross-linked (Langer and Vacanti 1993).

Cross-linking of collagen using the reactive hydroxy-, amino- and carboxy- groups on the collagen molecule has been described previously (Herink and Van Nostrum 2002; Marquie 2001; Lynn et al. 1998; Zeeman 1998; Damink et al. 1996; Purna and Babu 2000). So far, various aldehydes have been used as the cross-linking agents. The main shortcoming of aldehydes is their toxicity and poor reactivity requiring lengthy removal of the residues prior to use in experimental animals or patients (Henink and Van Nostrum 2002; Marquie 2001). Recently, non-toxic cross-linking agent has been described based on water-soluble carbodiimide and N-hydroxysuccimide (EDC-NHS) (Zeeman 1998; Olde Damink et al. 1996; Purna and Babu 2000).

Polylactide acid (PLA), polyglycolide acid (PGA) or their copolymer (PLGA) are the best known synthetic biodegradable polymers considered in medicine (Hennink and Van Nostrum 2002). Polyethylene glycol (PEG) is a hydrophilic and non-degradable biomaterial. Block copolymerization of PEG and polyesters (PEG-PLGA) is a way to

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combine advantages of biodegradable polyesters and polyethylene glycol (Metters et al. 2000). Reactive double bonds and carboxyl groups can be added to PEG-PLGA copolymer through addition of itaconic anhydride (ITA). The carboxyl groups result in increasing hydrophility, reactivity, biocompatibility, bioinduction and material adhesion. Double bonds can be used for radical cross-linking of the hydrogels using various techniques employing electromagnetic radiation. Further, the carboxyl groups can react with chemical compounds such as drugs and can be used in controlled drug delivery systems (Vojtová et al. 2006). Most of the synthetic polymer biomaterials exhibit good mechanical properties but their biological properties are poor. On the other hand, natural biopolymers exhibit excellent biological properties, however, they lack the biomechanical properties required by the specific medical applications. In order to obviate these shortcomings, synthetic and natural biopolymers, e.g. PEG-PLGA polyesters and collagen, can be combined in blends, inter-penetrating networks or copolymers.

In this paper, preparation and cross-linking of transparent biodegradable hydrogels based on collagen modified by addition of PEG-PLGA-ITA copolymer was investigated. Dependence of the physical properties of the prepared hydrogels on concentration of EDC-NHS, cross-linking reaction time and concentration of the copolymer was investigated. Swelling behaviour, thermal properties surface morphology and degradation rate of the prepared hydrogels were characterized. Use of these gels in preparation of lyophilized porous cartilage scaffolds with controlled pore size has also been investigated.

Materials and Methods

Polyethylene glycol (PEG) (Sigma-Aldrich, USA) was re-crystallised from dimethylmethane solution. Lactide anhydride (LA) and glycolide anhydride (GA) (Polysciences Inc., USA) and 95 wt% solution of stanous-2-ethylhexanoate were used as received. Bovine collagen I was supplied in the native form as the 8 wt% aqueous solution (VUP, a.s., Czech Republic), M_w = 300 000. The 1-ethyl-3-(3-dimethylaminopropyl)–carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) (Sigma-Aldrich, USA) were used as received. The copolymers based on PEG-PLGA (M_n = 4843) and PEG-PLGA-ITA (M_n = 4944) (Fig. 1) were prepared by ring-opening copolymerization (Hubbell 1995). The 1 wt% solution of collagen and 2.5 wt% solution of prepared copolymer of PEG-PLGA or PEG-PLGA-ITA were mixed in various mass ratio in a mixer (IKA Turrax Basic T18) and air-dried in Petri dishes.

Molar and weight ratios between GA and LA, molar ratio between PEG and PLGA and total molecular weight were determined using ^{1}HNMR analysis (^{1}HNMR, Bruker 500 MHz, USA). The measurements were performed in *CDCl*$_3$ solution using 500 MHz and 128 scans. Molecular weight and polydispersity of both PEG-PLGA and PEG-PLGA-ITA copolymers were determined using GPC analysis (Agilent Technologies 1100 Series, USA). Tetrahydrofuran was used as the mobile phase (1 ml·min^{-1}) and polystyrene weight fractions as the standards. Thermal behaviours of pure and modified collagen films were measured employing differential scanning calorimeter (DSC TA Instruments 2920, USA) over the temperature range from 35 to 140 °C at the heating rate of 5 °C/min. Prior to each measurement, the dry film samples were swelled in saline (0.9 wt% NaCl solution) for one hour.

The modified collagen films were cross-linked using the EDC-NHS, where the molar ratio of EDC and NHS in the cross-linking solution was 2:1. Two different types of cross-linking conditions were chosen. First, the EDC concentration was varied (5, 25 and 50 mmol/l) and the cross-linking time was kept constant at 4 h. Secondly, the cross-linking time was varied (0.25, 1 and 4 h) and the EDC-NHS concentration was kept constant equal to 50 mmol/l.

After completing the cross-linking reaction, samples were washed for 2 h with 0.1 M Na_2HPO_4 solution to remove unreacted EDC, and then washed for 2 h in distilled water. Finally, the samples were air-dried. After these steps, the transparent, cross-linked and modified collagen films were obtained. Prior to measurements, thickness and weight of the sample films has been determined.

Aqueous solutions containing 20 wt% of the PLA-PGA-PEG or PLA-PGA-PEG-ITA were mixed with 5 wt% collagen solution. The mixtures were homogenized for 5 min and then centrifuged to remove air bubbles. The homogeneous solution was frozen at -35 °C for 24 h. Then, samples were lyophilized at -55 °C and 15Pa for 24 h. Nucleating agents were used to control the size of water crystals in order to prepare porous scaffolds with the desired pore size.

The swelling ratio between the sample mass after the swelling and the sample mass before the swelling was measured. The swelling ratio was calculated after the swelling pure and modified collagenous films in saline for one hour. Film surface morphology was studied using the confocal scanning laser microscope (Olympus - Lext OLS 3000 Japan). Films with varying composition which were cross-linked using 25 mmol·l^{-1} EDC solution

were observed. Before the observation, samples were washed out with acetone to remove the present synthtetic copolymer resulting in highlighting the surface. The degradation rate was examined in saline using CO_2 incubator (Sanyo MCO-18AIC, Japan) at 37 °C.

Results and Discussion

The average molecular weight, M_n, polydispersity, M_w/M_n, molar and weight ratios between the co-monomers in the copolymer obtained from the ^{1}HNMR and GPC analyses are summarized in Table 1. Based on the obtained results, we can conclude that the molecular structure of the prepared copolymers was in good agreement with the theoretically predicted molecular structure.

Table 1. GPC and ^{1}HNMR analysis results

	Theory	GPC		^{1}HNMR	
Property		PEG-PLGA	PEG-PLGA-ITA	PEG-PLGA	PEG-PLGA-ITA
Molecular weight (Mn)	5250	7108	6773	4843	4944
Polydispersity (Mw/Mn)	1	1.18	1.22	–	–
Molar ratio PLA/PGA	3	–	–	2.87	2.83
Weight ratio PLGA/PEG	2.5	–	–	2.23	2.3

Scaffold preparation

The crystallization of water is a process controlling the pore size, pore size distribution and pore connectivity in the collagen/PLA-PGA-PEG scaffolds prepared using lyophilization. In order to control the pore size, mineral additives were added to alter crystal nucleation rate. In addition, the observed differences in pore size and overall porosity of scaffolds prepared from different collagen mixtures were related to the change in hydrophylicity occurring with varying molecular structure and content of the modifying copolymer.

The collagen-PEG-PLGA and collagen-PEG-PLGA-ITA scaffolds exhibited high porosity in comparison with pure collagen scaffolds. The porosity decreased after cross-linking in the following order: collagen-PEG-PLGA > collagen-PEG-PLGA-ITA > pure collagen with the corresponding porosity of 81% > 80% > 47%, respectively. Morphology of cross-linked collagen sponges modified with different additive in concentration of 20% is shown in Fig. 9 (Plate XI).

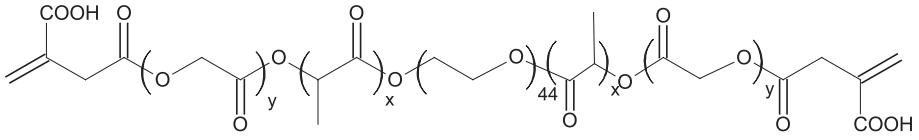

Fig. 1. Copolymer ITA - p(LA-co-GA) - b - PEG - b - p(LA-co- GA) - ITA

Thermal behaviour

Thermal behaviour of pure cross-linked collagen films and cross-linked collagen films modified with PEG-PLGA and PEG-PLGA-ITA copolymers are similar. The total amount of heat, determined as the area under the c_p vs. T plot obtained from DSC measurement decreased with increasing cross-linking time (Fig. 2) and with concentration of the EDC-NHS cross-linker (Fig. 3). Since most of the heat was consumed to evaporate water, this phenomenon can be attributed to the reduction of the swelling capability with enhanced cross-link density.

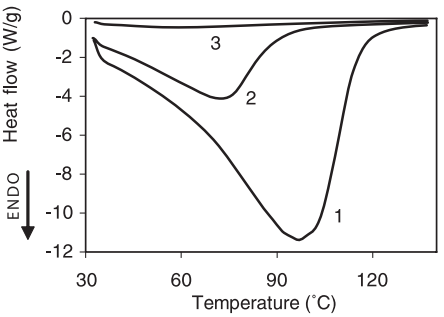

Fig. 2. Influence of cross-linking time on thermal behaviour of collagen films with 20% PEG-PLGA; 2.25 h (line 1), 1 h (line 2), 4 h (line 3)

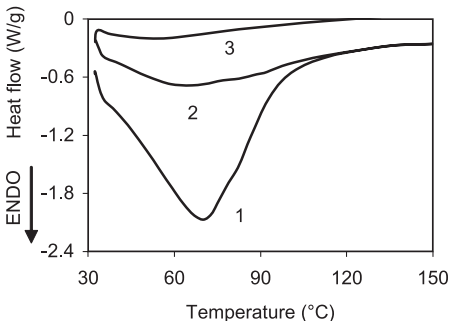

Fig. 3. Influence of cross-linking agent concentration on the thermal behaviour of pure collagen film; 5 mml·l^{-1} EDC (line 1), 25 mmol·l^{-1} EDC (line 2), 50 mmol·l^{-1} EDC (line 3)

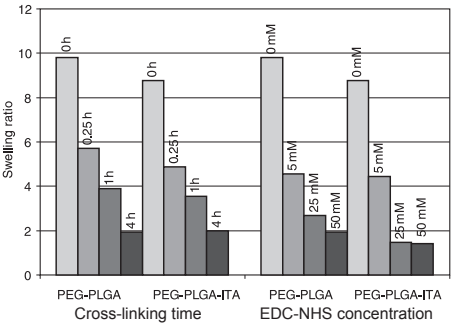

Fig. 4. Comparison of all cross-linking conditions used on collagenous film with 20% copolymer

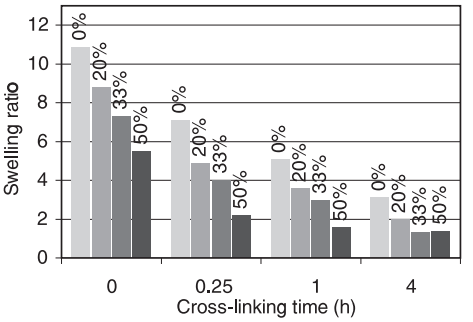

Fig. 5. Influence of copolymer amoount during various cross-linking times

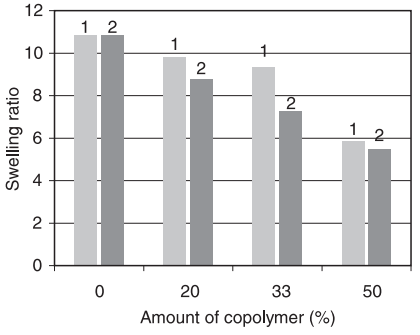

Fig. 6. Comparison of PEG-PLGA (columns 1) and PEG-PLGA-ITA (columns 2) copolymers on swelling behaviour of modified films

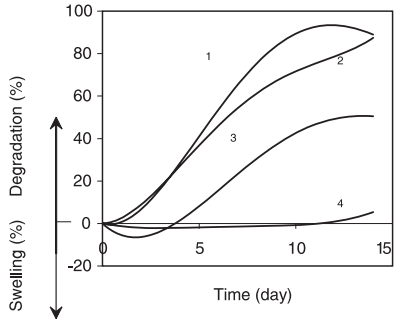

Fig. 8 Influence of degradation rate on pure collagen (line 1), collagen with 20% of PEG-PLGA (line 2), collagen with 33% of PEG-PLGA (line 3) and collagen with 50% of PEG-PLGA (line 4) cross-linked with 50 mmol·l^{-1} EDC-NHS for 15 min

Swelling behaviour

Collagen modified with both types of co-polymers, i.e., PEG-PLGA and PEG-PLGA-ITA, exhibited similar swelling behaviour. The swelling ratio decreased with increasing cross-linking time and EDC-NHS concentration as expected (Fig. 4). This phenomenon

was caused by the enhanced cross-link density. In addition, the water absorption was inversely proportional to the concentration of the block copolymer in the mixture (Fig. 5). This feature was caused by the lower water absorption of the copolymer phase. In addition, collagen modified with PEG-PLGA-ITA swelled less than that containing copolymer based on PEG-PLGA at the same weight fraction (Fig. 6). It can be assumed that the carboxyl end-groups of ITA can react with free amino groups on the collagen molecule to form a peptide bond. This peptide bond contributes to further increase in the cross-link density in modified films resulting in a decrease of the swelling ratio.

Degradation rate

The surface morphology of various collagen films was correlated with the amount of copolymer added. The pure cross-linked collagen films exhibited a smooth surface while addition of the copolymer resulted in coarser surface morphology, suggesting poor miscibility between collagen and the modifying copolymers. After dissolving the copolymer phase using acetone, coarse collagen fibrils were observed on the surface of the film with 50 wt% of the copolymer (Plate XI, Fig. 7) which was attributed to the self-assembly process of collagen triple-helices into microfibrils approximately 15 mm in diameter. This process leads to formation of microfibrils in living tissues, however, its kinetics and thermodynamic features are not well described. It seems that the reactive carboxyls on the tri-block copolymer can occupy the N-termini of collagen and, thus, effectively reduce its ability to cross-link. Consequently, the collagen undergoes "crystallisation"-like process resulting in microfibril formation as predicted theoretically more than 50 years ago.

The increasing amount of both PEG-PLGA and PEG-PLGA-ITA copolymers lead to a decreasing degradation rate. In other words, the addition of the PEG-PLA-PGA copolymer can enhance the stability of the scaffold. We assumed that higher hydrophobicity of both copolymers is responsible for increasing resistivity to water uptake and hydrolysis (Fig. 8).

It can be concluded that an increase in concentration of the cross-linking agent, as well as prolonged cross-linking time and increased amount of synthetic copolymer lead to enhanced thermal stability of the gels together with reduced swelling ratio and degradation rate in a saline. The resorption rate of these gels used in preparation of cartilage scaffolds can be controlled over a wide time interval by varying the collagen/(PEG-PLGA-ITA) blend composition or the conditions of the cross-linking reaction. Composition of the blend has a strong effect on the supermolecular structure of the collagenous phase. Above certain concentration of PEG-PLGA-ITA copolymer, strong trend towards self-assembly of collagen molecules into microfibrils was observed. These findings can be helpful in preparation of porous scaffolds for mesenchymal stem cells (MSCs) used in animals and humans. *In vivo* properties after surgical implantation of the scaffolds seeded with MSCs to experimental animal model are currently being investigated in miniature pigs with articular cartilage defects.

Stabilita kolageních skafoldů určených pro implantaci do iatrogenně vytvořených defektů kloubních chrupavek u zvířat

Práce se zabývá syntézou a popisem biodegradabilního hydrogelu vyrobeného modifikací kolagenu přidáním syntetického biodegradabilního kopolymeru. Tento hydrogel byl určen pro přípravu porézního skafoldu, který po osazení mesenchymálními kmenovými buňky může být využit pro transplantace do defektů kloubních chrupavek u zvířat.

Třírozměrný syntetický biodegradabilní kopolymer byl tvořen kopolymerem polyethylen glykolu (PEG), polymeru mléčné kyseliny (PLA), polyglykolové kyseliny (PGA) (PEG-PLGA) ukončených kyselinou itakonovou (ITA). Pro vyhodnocení míry resorpce hydrogelu byl použit ve vodě rozpustný carbodiimide and N-hydroxysuccimide systém (EDC-NHS). Byla zhodnocena závislost fyzikálních vlastností připraveného hydrogelu na koncentraci EDC-NHS, reakčním času a koncentraci PEG-PLGA-ITA kopolymeru, rovněž

bylo sledováno bobtnací chování, teplotní stabilita, morfologie povrchu a rychlost degradace hydrogelu.

Na základě námi získaných výsledků můžeme tvrdit, že zvýšená koncentrace EDC-NHS, tak jako množství syntetického kopolymeru, vede k zlepšení teplotní stability gelů společně se snížením stupně otoku a rychlosti jejich degradace ve fyziologickém roztoku. Resorpční rychlost u těchto gelů použitých k přípravě skafoldů do chrupavek může být řízena během širokého časového intervalu složením směsi kolagen/(*PEG-PLGA-ITA*), nebo změnou podmínek síťovací reakce.

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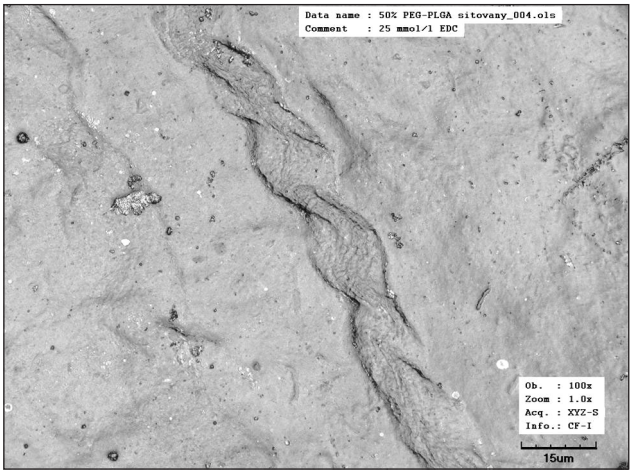

Fig. 7. Collagen fibril on the surface of collagen film containing 50 wt. % of PEG-PLGA cross-linked with 25 mmol·l^{-1} EDC. Film was etched in acetone for 24 h at 25 °C.

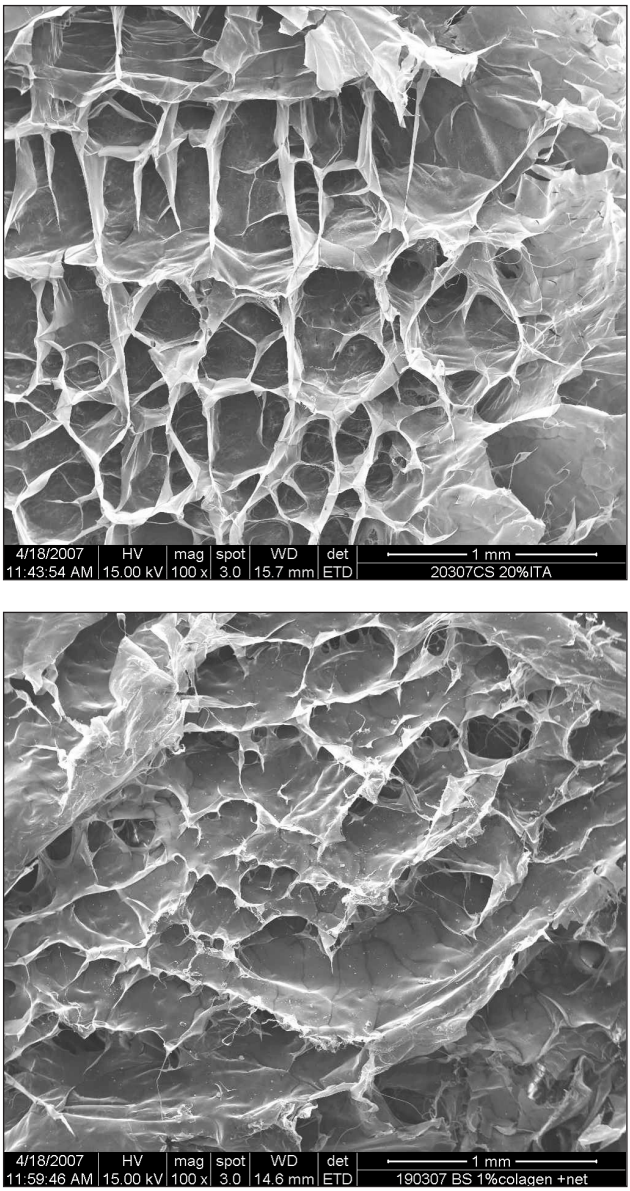

Fig. 9. Morphology of scaffolds based on cross-linked collagen with 20 wt. % of a) PEG-PLGA and b) PEG-PLGA-ITA observed using SEM.