STUDY ON THE CROSSLINK-INDUCED RADICAL INTERCOUPLING BETWEEN ATELOCOLLAGEN AND *IN SITU*-GENERATED (CO)POLYMERS♦

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Abstract: The paper presents a study on the obtaining of collagen-based interpenetrated and intercoupled networks, involving short chains of (co-)polymers as linking bridges, in order to generate loose hydrogels with a definite rheological personality, enabling convenient handling for cell culture techniques. The steps involved are: (**i**) obtaining tactoidal atelocollagen entities, by transglutaminase assisted cross-linking, (**ii**) atelocollagen conjugation with glycidil methacrylate (**GMA**), in order to attach ethylenically unsaturated functions and (**iii**) involving these functions in radical (co-)polymerization with GMA or other biotolerable monomers. The (co-)polymerization process is induced at low temperature (below 6 ˚C), using a ternary initiator mixture including H_2O_2, ascorbic acid (**AA**) and uric acid (**UA**). The optimal composition of the mixture, which assure a minimal gelation time (640 min) and a maximal hydrogel consistency (0.61 Pa·s), comprises 0.67 H_2O_2, 0.22 AA, 0.11 UA expressed as mass fractions.

Keywords: *atelocollagen, hydrogel, glycidyl methacrylate, grafting, low temperature, biotolerable redox system*.

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INTRODUCTION

Hydrogels composed of, or including type I atelocollagen, are versatile substrates in 3D cell cultures with applications in tissue engineering [1], or in cell behaviour investigation [2]. The main reason why scleroprotein isolation and purification is still in use is that them surpass the novel synthetic biocompatible polymers performances in cell-related applications, as it concerns the fibroblasts ability to differentially recognize the particular reactivity and nano-scale morphology of collagen structures. All the fibroblasts cell-life cycle processes are promoted or facilitated in the presence of collagen supramolecular aggregates, even *in-vitro*, but are inhibited or substantially impaired by the majority of the man-made biopolymers [3]. The molecular species that determine wether a (bio)chemical environment is cyto-friendly are located at the cell membrane level and comprise the integrins and the syndecans [4]. Precisely located portions of type I collagen molecules act as very specific ligand for $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins, being able to signalise the full biocompatibility of a substrate [5]. To date, no synthetic macromolecular structure can satisfactorily mimic the functionality of integrins-complementary collagen sites. This is why, at least for a medium time horizon, fibrilar collagens remain the preffered components in producing scaffolds for cell-related applications.

In this paper we present an engineer's approach to the design and validation of a technique for the obtaining of collagen-based scaffolds, by crosslinking intercoupling of type I atelocollagen aggregates through short (co)polymeric chains generated *in situ*, following a radical mechanism. In this way, hydrogels with imposed rheological personality can be generated, able to resists, in an unmodified state, to the attack of non-specific proteases. If appropriate nano- and micro-scale morphology are also induced, the resulted hydrogels will support cell adhesion and proliferation.

PRINCIPLES OF OBTAINING PARTICULAR HYDROGELS

Strategy for the generation of hydrogel-type scaffolds with the above described properties mainly involves the following stages:

- *generation of quasi-ordered atelocollagen supramolecular aggregates*, having tactoidal nano-scale morphology and a sufficiently stable, compactly packaged structure;
- *chemical functionalization of the tactoid superficial molecules*, as prerequisite for the (co)polymerization – mediated cross-linkage;
- *long-range hydrogel structuration*, by intercoupled and inter-penetrated networks generation, including the functionalized tactoids, preferably in a controlled manner. As a mandatory condition, all the necessary operations must be conducted so that the atelocollagen component preserves its quasi-native state. In this respect, working at low temperature, in the presence of protein protectants, must be considered.

In the first stage, zero-length cross-linking bridges are enzymatically induced, using transglutaminase (EC 2.3.2.13). By controlling the cross-linking density, supramolecular aggregates with adequate spatial expansion can be obtained. For this, it is essential that atelocollagen species bearing enough amide groups (able to be linked to lysine amino groups, by transglutaminase) are used. As a second condition, a high local

density of atelocollagen molecules must be assured, in order to provide the spatial crowding conditions necessary for transglutaminase to act. The solution proposed by the authors consists in inducing a preliminary labile spatial aggregation, mediated by Ca^{2+} salt bridges, stable enough to temporarily generate short fibrilar dense structures. Besides, the Ca^{2+} ions acts as transglutaminase activators, triggering the covalent cross-linking of adjacent atelocollagen molecules, which result in a compact tactoidal structure, with limited spatial extension. If a subsequent transglutaminase cross-linking action must be prevented, specific inhibitors will be used (*i.e.* 10^{-3} M β-phenyl-propionyl-thiocholine, or the more expensive, but more efficient, N-benzyloxycarbonil-L-phenylalanyl-6-dimethylsulfonium-5-oxo-L-norleucine).
To assure the integrity of the obtained atelocollagen tactoids, all the subsequent operations in aqueous medium must be conducted in the presence of a native-state protein protective.
The second stage, aiming the tactoid superficial functionalization, involves grafting of a sufficient number of ethylenically unsaturated functions, able to participate in the (co)polymerization process; for this, the glycidyl-methacrylate (GMA), a bio-tolerable monomer, was chosen. To limit the GMA oxyrane group inactivation in aqueous medium, a diluted acetone solution was used (1 : 3 acetone in water). The addition of 0.2% Triton X 100 is needed to assure a rapid dispersion / solubilization of GMA in the aqueous medium.
As a third stage, a radical induced (co)polymerization, involving the grafted unsaturated functions, will generate, *in-situ*, short chains acting as cross-linking bridges. Bio-tolerable species must be used as initiator system. The ability to trigger radicalic processes at low temperature represents another important restriction in the initiator recipe formulation. Complex redox systems including at least three components (an oxidizing agent, an activator and an efficient promoter) are able to fulfill both conditions. The authors have tested the radical initiating capacity of the ternary system: H_2O_2, ascorbic acid (AA) and uric acid (UA), for the polymerization of acrylamide in the presence of N,N'-methylene-bis-acrylamide, at 4 … 6 °C, in order to generate covalent cross-linked hydrogels [6]. The objective of the present paper is to generate collagen-based interpenetrated and intercoupled networks, using the above-mentioned redox initiator system.

MATERIALS AND METHODS

The atelocollagen colloidal solution was obtained from calf skin, through a pepsin solubilization technique. A short-time, strong alkali post-treatment was applied to inactivate prions and viruses (two hours, at *pH* 12.5). The final solution characteristics are: 1.4 % atelocollagen in 0.5 M acetic acid solution containing 0.2 M NaCl; isoelectric *pH* in the domain 8.2 ... 8.6; 2.66% dry matter; 0.58% amide content.
The cross-linking enzyme, lyophilized pure guinea pig liver transglutaminase (CAS 80146-85-6), was purchased from Sigma-Aldrich Corporation, in ampoules containing 2 units / 1.3 mg, solid diluted with sodium caseinate and maltodextrin. The product was kept in refrigerated state (at – 20 °C). Before use, it was gentely grinded in its ampoule, on a water bath, as near as possible to 0 °C. Minimal quantities of powder were drawn out, weigth, and immediately disolved in a cooled solution of 10 mM 1,4-dithiothreitol

in HEPES buffer (*pH* = 6.8). The prepared solution can be stored at 4 °C for maximum 48 hours (for longer times, the enzyme activity in solution tends to fade).

The protectant of the collagen native-state consists in a mixture comprising [7]: 136 mM NaCl, 2.7 mM KCl, 0.42 mM NaH_2PO_4, 12 mM $NaHCO_3$, 5.5 mM glucose, 5 mM HEPES (*pH* 7.4). The salts act as cosmotropes and ionization regulator [8]. Glucose significantly delays the spontaneous atelocollagen aggregation [9]. The protein quasi-native local conformation and aggregation state were improved by adding 10 mM Triton X 100 [10]. To prevent excessive generation of spatially extended micelles around the atelocollagen aggregates by the surfactant, 25 mM 1,2-ethandiol were added [11]. Because no sterility precautions were taken, 0.5 mg/mL Gentamycin were added in all the aqueous solutions used in atelocollagen aggregates processing. The preparation prescriptions are described in reference [6].

The free radical initiator mixture, comprising H_2O_2, ascorbic acid and uric acid, was prepared according to [6] and observing the mass fraction ratios given in Table 2.

The experimental design used a mixture design matrix with the parameters given in Table 1. The placement of the experimental points in the ternary diagram [12], coresponding to the restricted mixture design, can be seen in Figure 1.

Table 1. *The parameters of the applied experimental design*

The structure of the mixture design matrix			
Number of mixture components: 3	Imposed restrictions to mixture components		
Design degree: 3	Variable	Lower value	Upper value
Design type: extreme vertices	H_2O_2	0.5000	0.9500
Design augmenting: central point, axial points	ascorbic acid	0.0500	0.5000
A priori model: third order, full cubic	uric acid	0.0000	0.1500

Table 2. *The restricted mixture design matrix*

Exp. no.	**Order in the experiment**	**Design point (Fig. 1)**	**Mixture components (mass fractions)**		
			H_2O_2	**ascorbic acid**	**uric acid**
1.	10	1	0.50000	0.50000	0.0000
2.	3	2	0.50000	0.35000	0.1500
3.	5	3	0.95000	0.05000	0.0000
4.	12	4	0.80000	0.05000	0.1500
5.	9	5	0.50000	0.42500	0.0750
6.	4	6	0.87500	0.05000	0.0750
7.	7	7	0.72500	0.27500	0.0000
8.	1	8	0.65000	0.20000	0.1500
9.	6	9	0.68750	0.23750	0.0750
10.	2	10	0.59375	0.36875	0.0375
11.	8	11	0.59375	0.29375	0.1125
12.	13	12	0.81875	0.14375	0.0375
13.	11	13	0.74375	0.14375	0.1125

The general experimental procedure aims to establish the optimal composition of the initiator mixture, which simultaneously determines maximal hydrogel network consistency and minimal gelification time. In this regard, the following steps were carried out:

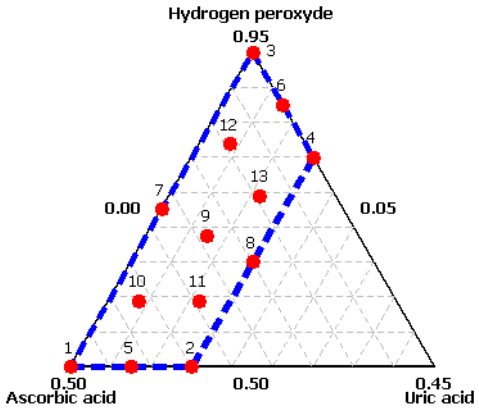

Figure 1. *Points location in the restricted experimental design of the ternary radical initiator mixture*

1. – **preparation of calcium atelocollagenate**: for each trial, the *pH* of of 100 mL atelocollagen solution was adjusted to 9.2, at 4 °C, with NH_4OH 25%, then 0.183 g of freshly oven-dried (105 °C, 6 hours) $CaCl_2 \cdot 6\ H_2O$ (approx. 0.8 mM) were added grain by grain, under vigorous agitation; after a 6 hours maturation under slow agitation at 4 °C, cloudy friable flocks appeared; under rapid mixing, flocks disruption occured, and a fine, white, very stable suspension resulted; after another 18 hours maturation at 4 °C, no settling tendency could be noticed; the suspension was three-fold diluted using NH_4OH solution with *pH* 9.2; the denser fraction was separated by centrifugation (10^3 g, 10 minutes), and then resuspended in 100 mL NH_4OH solution with *pH* 9.2, in ultrasonic field (350 W, 22 kHz), at 4 °C; a long-term stable suspension was obtained, released by any Ca^{2+} ion excess; prior to further use, the centrifugation –resuspension was repeated once more and the final calcium atelocollagenate slurry was resuspended in 50 mL solution of protein protectant;

2. – **preparation of atelocollagen tactoidal aggregates**: starting from the previously obtained calcium atelocollagenate, if not otherwise specified, all the subsequent operation were conducted at 4 °C; the suspension *pH* was lowered to 6.8 with 0.5 M acetic acid, and then 0.3 M urea was added, to prevent the excessive self-assembly of calcium atelocollagenate during the transglutaminase treatment; the extemporaneous prepared transglutaminase solution was added in an equivalent quantity of 400 U/g collagen, under vigorous stirring; the resulting aqueous system was warmed at 20 °C, with a rate of 0.5 °C/min and kept at this temperature for 12 hours, in static conditions; in this interval, a gelation process was initiated; after cooling again at 4 °C with the same rate of 0.5 °C/min, the obtained loose gel was subjected to ultrasonication for 15 minutes (100 W, 22 kHz); then, the ultrasonication was repeated for another 5 minutes under vigorous mixing, till a viscous suspension was obtained; during ultrasonication, 2.4 mM EDTA and 0.1 mM iodoacetamide were added; the tactoid suspension was three-fold diluted and subjected to maturation for 48 hours, under slow mixing; the centrifugation - resuspension sequence was repeated for five times, using protein protectant solution as aqueous medium; the final suspension was subjected to another 48 hours static maturation;

3. – **superficial functionalization of atelocollagen tactoids**: was conducted at 4 °C, after the *pH* was rised to 9.0, with a 0.05 M NaOH solution and after vacuum degasing; 3 mM of glycidyl methacrylate were diluted in 1 mL acetone, and then 2 mL

of 0.2% Triton X 100 aqueous solution were added, under vigurous agitation; the GMA dispersion was added to the atelocollagen tactoid aqueous system immediately after preparation, under stirring; the grafting process was completed in about 120 minutes;

4. – **radical (co)polymerization of the grafted atelocollagen tactoids with an unsaturated monomer**: was done extemporaneously, at 4 °C, by adding 4 mM of GMA (or another ethylenic monomer) and the prescribed quantities of initiators (see Table 2); after complete homogenization under slow stirring and thermostating, the reaction mass was divided in two portions; 30 mL were poured into the cylinder of the Höppler falling ball consistometer, previously thermostated at 4 °C, and another 20 mL were introduced in a 20 mm diameter test tube, and maintained at 4 °C; gelation in the test tube was surveyed, at every 10 minutes; when the gel passed the „tube reversing test", the gelation time was recorded, and the gel consistency was determined on a Höppler falling ball consistometer (ball diameter of 16 mm; cylinder diameter of 20 mm; under a compresion force of 1.226 N); the obtained values of gelation time and consistency are given in Table 3.

Table 3. *Experimental data obtained according to the restricted mixture design*

Measured properties	**Design point**												
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
gelation time [min]	730	420	560	310	660	470	840	470	530	800	710	560	520
consistency [Pa · s]	0.61	0.67	0.44	0.79	0.65	0.67	0.55	0.84	0.63	0.66	0.69	0.66	0.72

RESULTS AND DISCUSSION

Figures 2 and 3 present the influence of the redox initiator recipe on the gelation time and the hydrogel consistency, respectively. As it concerns the gelation time, an important antagonistic interaction between the mixture components can be noticed. This is due to the inhibitory effect induced by the uric acid, obvious at low peroxide concentration. By increasing the uric acid concentration, its inhibitory effect can be reversed and a promotion activity appears. The hydrogel consistency shows a relatively monotonic dependence on the initiator mixture composition. The promoting effect of uric acid became significant when its concentration exceeded 0.1, as mass fraction.

Starting from the fitted models for gelation time and hydrogel consistency, the mixture composition was numerically optimized, using the freeware software application AMPL with LOQO solver; the results are given in Figure 4. Two feasibility domains were identified, one for minimal duration of the gelation process (for a composition comprising 0.5 H_2O_2, 0.35 AA and 0.15 UA mass fractions), and one for maximal attainable consistency (located at 0.84 H_2O_2, 0.05 AA, 0.11 UA mass fractions). Unexpectedly, the two specified domains have a very dissimilar placement on the ternary diagram, indicating important interaction effects between the components of the radical initiator. The global identified optimum corresponds to the following mixture composition: 0.67 H_2O_2, 0.22 AA and 0.11 UA (mass fractions). This composition has the advantage to include a low quantity of uric acid, which is extremely difficult to dissolve in aqueous systems, especially at high ionic strength.

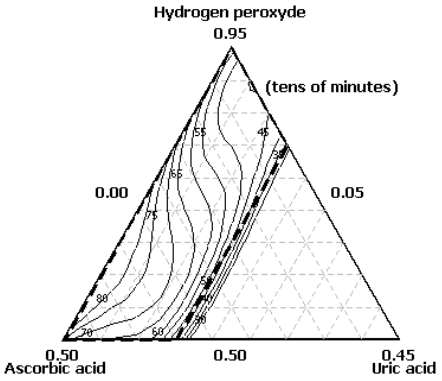

Figure 2. *The dependency of the gelation time on the initiator mixture omposition. (The unit of the isocurves labels: tens of minutes).*

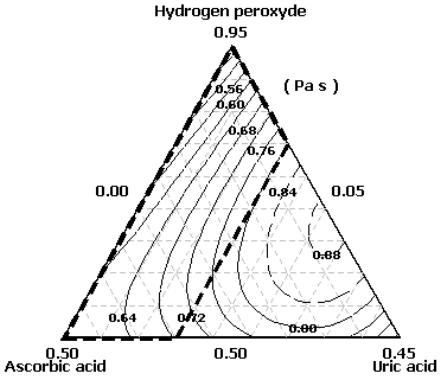

Figure 3. *The dependency of hydrogel consistency on the initiator mixture composition. (The unit of the isocurves labels: Pa·s).*

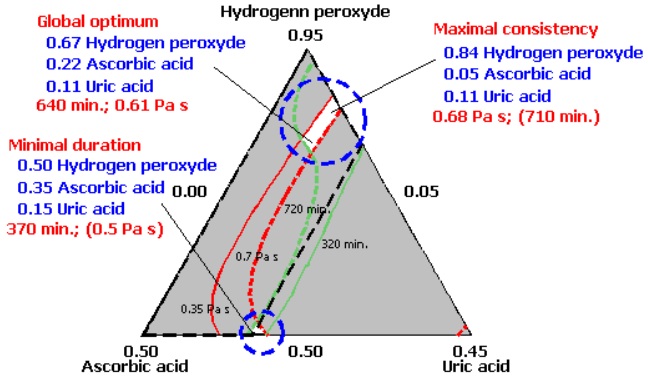

Figure 4. *The feasible region and the optimal point for conducting atelocollagen hydrogel formation by using the biotolerable radical initiator mixture.*

CONCLUSIONS

Sophisticated protocols can be used to generate mixed macromolecular networks of intercoupled and interpenetrated type, starting from atelocollagen supramolecular aggregates and ethylenic monomers. The final stage involves a radical polymerization which can be conducted at low temperature (tipically 4 °C), using a ternary mixture of H_2O_2, ascorbic acid and uric acid. When the monomer is glycidyl methacrylate, the

optimal composition of radical initiator mixture comprises 0.67 H_2O_2, 0.22 ascorbic acid and 0.11 uric acid, as mass fractions. A hydrogel having a consistency of 0.61 Pa·s was obtained after a gelation time of 640 minutes.

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