PRELIMINARY STUDY ON A BIOTOLERABLE TERNARY SYSTEM, ACTING AS REDOX INITIATOR♦

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Abstract: This paper studies radical initiation of the crosslinking process within a model mixture of 19 : 1 acrylamide : N,N'-methylene-bis-acrylamide, carried out at low temperature (below 6 ˚C), using a ternary initiator mixture containing *hydrogen peroxyde* (H_2O_2) as oxidizing agent, *ascorbic acid* (AA) as activating agent and *uric acid* (UA) as promoter and modulator of the radical centers development. The initiator system contains only biotolerable components and can be used for the obtaining of crosslinked protein molecular composites hydrogels, containing *in-situ* generated oligomers, able to be used in tissue engineering. The study establishes *(i)* the optimal composition of the initiator mixture (H_2O_2 : AA : UA molar ratio of 1 : 1 : 0.1), *(ii)* the monomer : initiator mixture molar ratio (1 : 0.021) and *(iii)* the lowest feasible concentration of H_2O_2 that simultaneously determines maximal hydrogel consistency and minimal volume of water extracted by syneresis. The hydrogel consistency was modeled using a mixture experimental design.

Keywords: *redox initiator, ternary mixture, low temperature, biotolerable, acrylamide system, hydrogel*.

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INTRODUCTION

Extracellular matrix mimicking represents one of the central tasks in tissue engineering. Artificial scaffolds based on scleroprotein nano- and micro-structured hydrogels are accepted as substrates for cell culture, both in regenerative medicine (cell-based implants) [1,2] and in anchorage-dependent cell banking (3-D culture systems) [3]. Certain hydrogels can be obtained as intercoupled and / or semi-interpenetrated networks, through *in-situ* crosslinking between protein aggregates and synthesis olygomers. Ideally, the artificial scleroprotein-based scaffolds must be generated (**i**) using biotolerable (co)monomers, (**ii**) in the absence of additives (initiators, catalysts etc.), (**iii**) in non-denaturating conditions (*inter-alia*, in the absence of radiation energization), (**iv**) by controlling the grafting and crosslinking degrees, (**v**) providing a controllable kinetics. To date, there are no methods that satisfy all the above-mentioned conditions, but a compromise can be obtained by slow rate radical initiation of (co)polymerization.

The crosslink-induced (co)polymerization in systems with high content of protein fractions must be carried out in such conditions that the protein constituent is minimally affected, mainly as it concerns the protein aggregates destructuration, protein denaturation and polypeptide chains break-down. Precisely, at least the following working conditions must be met: low temperature (below 10 ˚C) and initiator systems containing only biotolerable components, with no adverse effects neither on the protein constituent, nor on the physiology of cells designated for cell culture.

Controlled free-radical generation at low temperature can be conducted using sophisticated initiator systems (alkoxyamines and nitroxides [4], bromomethyl-dimethylsilyl propargyl ethers (BMDMS-PE) [5], hematin complexes, 2,4-pentanedione and horseradish peroxidase (HRP) [6]), but all of them develop secondary non-cyto-friendly products, or necessitate non-biotolerable adjuvants.

The objective of the present paper is the design and testing of a completely biotolerable redox initiator system, able to efficiently trigger radical cascade (co)polymerization at low temperature. The initiation efficiency of the designed system has been assessed by conducting crosslinking-(co)polymerization reactions in a model monomer mixture, able to generate hydrogels having similar characteristics to the scleroprotein hydrogels.

THE COMPOSITION OF THE REDOX INITIATOR SYSTEM

Usually, an efficiently controllable redox initiator system contains three types of chemical species: (**i**) radical generators, (**ii**) promoters and / or activators of radicalic processes and (**iii**) modulators of radical reactivity. When sensitive constituents are present in the radical (co)polymerization medium, a fourth type must be added, *i.e.* (**iv**) protectants against free radical damaging. Because in the free radical processes each component induce a large amount of variability, it is mandatory to simplify the initiator composition, by selecting species able to induce more than one individual efect.

Designing the redox initiator system has taken in account three types of restrictions, related to its final application in producing scleroprotein scaffolds applicable in tissue engineering:

- restrictions related to the chemical aggressivity towards the protein constituents:
  - *minimal quantity of radical generator*,
  - *minimal quantity of radical processes activator* and
  - *maximal quantity of radical reactivity modulator*,

  in order to limit the excess of free radicals able to disrupt or chemically modify the protein chains;
- restrictions related to the maintaining of a low kinetics of the radical processes:
  - *minimal quantity of radical processes promoter* and
  - *a simple mechanism of free radical generation*,

  in order to limit the occurence of uncontrolled olygomer chain growing-up and ramification;
- restrictions related to the necesity of preserving the native state of proteins:
  - *working at low temperature* (below 6 ˚C),
  - *working in conditions near to physiologic ones* and
  - *working in the presence of protein protectant species*,

  in oder to assure the scleroprotein conformational stability and the three-dimensional aggregation morphology.

In order to meet these requirements, the following reagents were chosen:
- as **radical generator**: the *hydrogen peroxyde* (**H_2O_2**);
- as **radical processes activator**: the *ascorbic acid* (**AA**);
- as **radical processes promoter and modulator**: the *uric acid* (**UA**);
- as **protein protectant**, an aqueous solution of: 136 mM *NaCl*, 2.7 mM *KCl*, 0.42 mM *NaH_2PO_4*, 12mM *$NaHCO_3$*, 5.5 mM *glucose*, 5mM *HEPES*, 10 mM *Triton X 100*, 25 mM *1,2-ethandiol*, 0.5 mg/mL *Gentamycin*.

All the components are bio-tolerable, and do not generate secondary non-bio-tolerable products. The chemical species that dictate the restrictions in the final formulation of the initiator mixture are: *uric acid*, due to its very low water solubility [7] (70 μg/mL in distilled water, at 20 ˚C) and *ascorbic acid*, due to its high sensitivity to aerobic oxidation and inactivation [8], even at low concentrations, in physiological aqueous systems (slightly alkaline, pH 7.2 ... 7.8).

MATERIALS AND METHODS

The model monomer : crosslinker system used in radical-induced polymerization and crosslinking study is the mixture of 19 : 1 acrylamide : N,N'-methylene-bis-acrylamide (the commercial product Rotiphorese Gel 40, Carl Roth GmbH). Such a mixture provides a final hydrogel with rheological and physico-chemical properties very close to those of the collagen hydrogels used in 3-D cell culture systems.

The experimental design uses a mixture design matrix with the parameters presented in table 1. Due to the restrictions on the mass ratios between the radical generator, activator and modulator, a constrained location of the experimental points must be considered. A complex *apriori* model has been postulated (third order, full cubic) in order to allow the use of a stepwise algorithm for the selection of the final model

structure. Figure 1 depicts the placement of the experimental points in the ternary diagram, coresponding to the restricted mixture design.

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	0.0500	0.9500
Design augmenting: central point,axial points	ascorbic acid	0.0500	0.9500
A priori model: third order, full cubic	uric acid	0.0010	0.1000

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	0.050000	0.949000	0.00100
2.	3	2	0.050000	0.850000	0.10000
3.	5	3	0.949000	0.050000	0.00100
4.	12	4	0.850000	0.050000	0.10000
5.	9	5	0.050000	0.899500	0.05050
6.	4	6	0.899500	0.050000	0.05050
7.	7	7	0.499500	0.499500	0.00100
8.	1	8	0.450000	0.450000	0.10000
9.	6	9	0.474750	0.474750	0.05050
10.	2	10	0.262375	0.711875	0.02575
11.	8	11	0.262375	0.662375	0.07525
12.	13	12	0.711875	0.262375	0.02575
13.	11	13	0.662375	0.262375	0.07525

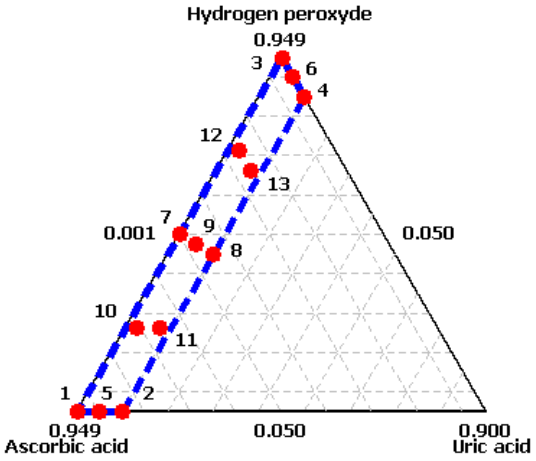

Figure 1. *Points location in the restricted experimental design of the ternary radical initiator mixture*

The general experimental procedure was designed in order to establish the optimal values for: (**i**) the composition of the initiator mixture, (**ii**) the feasible ratios between monomer mixture and initiator mixture and (**iii**) the lowest feasible concentration of the

oxidant (H_2O_2), which simultaneously determines maximal hydrogel consistency and minimal volume of water expelled by syneresis (which is equivalent to the highest possible water content of the hydrogel). In this regard, the experimental procedure comprises the following steps:

1. – **preparation of protein protectant solution**: 10 mL of salt solution mixture is prepared starting from individual solutions with appropriate concentration, so that the following quantities of chemicals are found in the final mixture: 79.5 mg NaCl, 2.0 mg KCl, 0.5 mg NaH_2PO_4, 10 mg $NaHCO_3$; in the salt solution mixture 9.9 mg glucose and 15.5 mg 1,2-ethandiol are added; next, 62.4 mg Triton X 100 is added under slow agitation; right before use, 5.0 mg Gentamycin sulfate is added; the pH of the final aqueous mixture is about 8.5;

2. – **preparation of uric acid dihydrate crystals**: is done according to the protocol described by Wang and Königsberger [9], starting from anhydrous amorphous uric acid; the dihydrate crystals can be easier dissolved in water and saline solutions, comparing to the comerical anhydrous product;

3. – **preparation of free radical activator – modulator mixture**: is done right before use, by successively dissolving, in the aqueous mixture of protein protectant, the quantities of uric acid dihydrate and ascorbic acid according to the experimental matrix (see table 2); in all experiments, the reference mass faction is the value associated to the uric acid (the less soluble component); the higher participation fraction of the uric acid dihydrate is 0.1, which coresponds to 0.8 mg; the amounts of the other components are calculated taking in account this value; the total mass of the mixture is maintained constant, at 215 mg;

4. – **preparation of monomer – crosslinker – free radical initiator mixture**: is done extemporaneously, by adding 15 mL Rotiphorese Gel 40 (which include 79.05 mM acrylamide and 4.16 mM N,N'-methylene-bis-acrylamide) to the aqueous mixture of free radical activator - modulator (in protein protectant solution); after good homogenization under slow stirring and thermostating at 4 ˚C, the prescribed quantity of H_2O_2 is added (see table 2); the time of polymerization – hydrogel formation is measured from the moment of the H_2O_2 addition; after another one minute of viguros stirring, the composition is poured into the cylinder of the Höppler falling ball consistometer, previously thermostated at 4 ˚C;

5. – **hydrogel characterization**: eleven samples were prepared and characterized at successive time intervals, calculated as terms of a decreasing geometric series obeying the following mathematical law:

$$t_{polymerization} = 640 - \frac{1280}{5 \times 2^{(n-1)}} + 32 \times n \qquad (1)$$

where $n = 0 \ldots 10$ min; the polymerization – crosslinking process is conducted at 4 ˚C; a « blind » sample is characterized immediately after preparation; as a *first test*, the water expelled by synthesis is absorbed in a bundle of four filter paper strips (completely dried and weight after conditioning at 60 % R.H.), placed on the surface of the hydrogel and maintained until the water is entirely collected; the water quantity is then determined gravimetrically; the *second test* consists in the hydrogel consistency determination, using the Höppler falling ball consistometer (with a ball diameter of 16 mm and cylinder diameter of 20 mm, under a compression force of 1.226 N); the obtained values of consistency and expelled water are given in table 3.

The experimental data processing was performed according to the algorithms described in reference [10]. Briefly, experimental data were represented for each variable as function of time and the optimal duration was determined. Then, for each variable (hydrogel consistency and mass of expelled water), a model has been derived, diagnosed (and errors, if any, corrected by repeating the measurements) and represented in ternary diagrams.

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

Design point	Measured properties	Total polymerization – crosslinking duration (min)										
		128	**416**	**576**	**672**	**736**	**784**	**824**	**860**	**894**	**927**	**960**
1	expelled water	n.s.	2.2	5.6	12.4	27.5	33.9	48.2	60.1	63.4	71.2	76.6
	consistency	0.06	0.34	1.92	6.55	28.4	91.1	104	118	121	120	121
2	expelled water	n.s.	n.s.	2.3	5.6	9.5	13.3	18.1	20.9	23.1	25.3	26.1
	consistency	0.09	0.28	1.37	6.18	23.5	88.4	97.2	105	108	111	110
3	expelled water	n.s.	3.2	9.1	12.5	29.1	51.1	86.3	110	114	119	122
	consistency	0.05	0.41	2.11	7.30	30.3	97.2	112	139	144	152	150
4	expelled water	n.s.	1.7	3.3	5.1	11.3	19.9	38.2	55.2	57.1	60.3	62.2
	consistency	0.07	0.22	1.57	6.29	25.8	89.2	101	112	115	114	115
5	expelled water	n.s.	1.3	2.6	5.0	9.7	18.1	27.3	32.3	34.1	36.0	36.7
	consistency	0.05	0.19	1.66	6.39	26.9	90.4	99.3	108	110	112	112
6	expelled water	n.s.	2.7	5.1	16.3	29.3	40.6	66.3	82.6	84.1	86.0	87.2
	consistency	0.08	0.27	1.52	8.03	33.1	104	116	122	130	133	135
7	expelled water	n.s.	2.1	4.6	9.3	12.3	19.6	30.1	45.6	47.3	49.1	49.8
	consistency	0.06	0.20	1.38	6.29	26.8	89.7	96.5	109	112	113	112
8	expelled water	n.s.	1.4	3.3	9.3	17.6	33.6	59.7	75.3	77.1	79.3	80.6
	consistency	0.09	0.40	1.88	6.31	27.5	90.1	109	116	118	118	117
9	expelled water	n.s.	n.s.	2.7	6.1	13.3	19.3	29.7	38.3	40.3	43.3	45.6
	consistency	0.11	0.33	1.51	6.47	29.1	88.3	92.2	110	112	115	113
10	expelled water	n.s.	1.9	6.6	9.3	15.6	31.3	53.3	61.4	63.1	63.6	64.3
	consistency	0.07	0.44	1.81	8.15	36.9	92.5	120	141	148	150	150
11	expelled water	n.s.	n.s.	1.1	3.3	6.1	12.6	23.1	29.3	31.6	33.3	34.6
	consistency	0.05	0.3	1.33	6.18	24.7	77.1	88.5	102	104	105	104
12	expelled water	n.s.	n.s.	1.3	4.3	13.6	17.3	20.1	33.6	34.1	34.6	35.1
	consistency	0.08	0.46	1.61	6.28	25.8	88.1	92.5	107	105	108	106
13	expelled water	n.s.	0.4	1.6	13.1	23.3	57.6	88.3	94.6	96.3	97.6	98.1
	consistency	0.10	0.58	1.93	7.18	31.7	78.3	94.4	117	119	121	121

Notes: - the expelled water is given in miligrams;
- the consistency is given in 10^3 cP;
- acronyme **n.s.** indicate „non-significant" value, impossible to measure.

RESULTS AND DISCUSSION

Figures 2 and 3, respectively, present the evolution of the measured variables during hydrogel formation. For the clarity of graphical representation, only te first three dependencies (the first three corespondant lines in table 3) are figured. It is obvious that, at 4 ˚C, the effective gel formation occurs only after twelve hours. For a „lag time interval" of about 9 hours, the aqueous system remains unstructured, with consistency only slightly higher than those of the „blind" sample (48 cP, at 4 ˚C). During a „log period" of about 6 hours, gelation occurs as a consequence of the simultaneous processes of acrylamide polymerization and crosslinking. Practically, the gelation can

be considered finished after 15 hours, when the hydrogel consistency remains approximately constant. However, the slope of water syneresis evolution remains high, most likely due to the diffusional processes taking place within the contracted polymer network. Actually, the syneresis continue, with slowing-down kinetics, for about 36 hours, then stops. Probably this is the period needed for the structural stabilization of the crosslinked acrylamide hydrogel, at low temperature. Time evolutions of hydrogel consistency and water expelling are coherent for all 13 compositions and point out the same successive „lag", „log" and „plateau" pattern, with very similar moments of slope changing, but with different final value of the stable plateau. This indicates that the processes mechanisms are identical from the kinetics point of view, but the initiator mixture composition dictates the final rheologic properties of the hydrogel. A possible explanation lies in the fact that the polymerization and crosslinking individual progress are sensitive to the decorrelations induced by the differences in the chain-growth rate. Because the crosslinking process is not influenced by the mixture composition, for slow rates of radical generation (correlated to the ratios between the initiator mixture components), the polymer chain grow slowly and facilitates a denser crosslinking in the polymer network structure, which directly affects the final hydrogel rheological properties, including its consistency and spatial contraction magnitude. As a confirmation, an obvious correlation between hydrogel consistency and its water expelling capacity can be pointed out by comparing figures 2 and 3. Also, a time delay is present, indicating that the hydrogel contraction is triggered only after a certain correlated progress of polymerization and crosslinking processes.

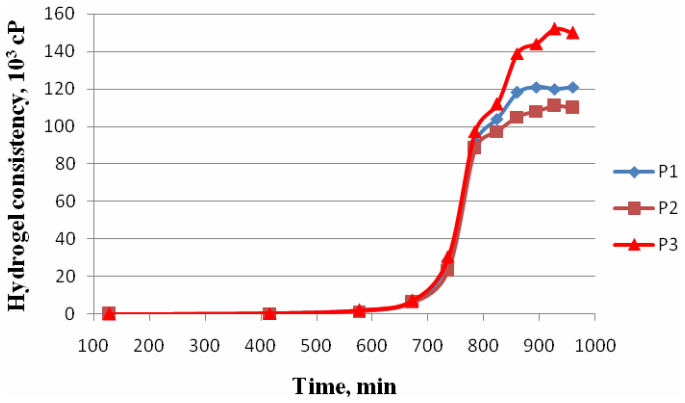

Figure 2. *The evolution of hydrogel consistency during the polymerization - crosslinking process.*

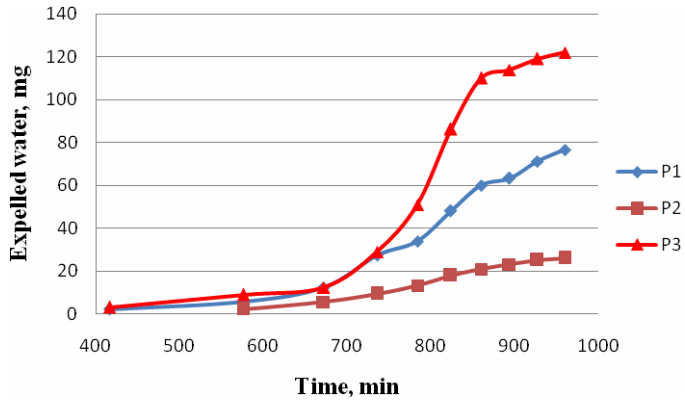

Figure 3. *The evolution of the expelled quantity of water during the polymerization - crosslinking process.*

Figure 3 illustrates the dependence of polyacrylamide hydrogel consistency on the composition of radical initiator mixture. Figure 4 indicates the same dependencies for the quantity of expelled water. Using these ternary diagrams, the individual and the correlated influences of the mixture components can be revealed, both as it concerns the precise individual quantity and the inter-components ratios.

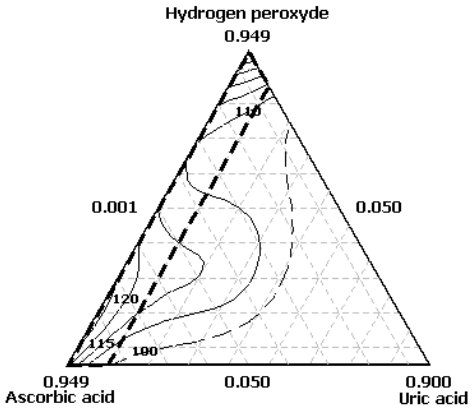

Figure 4. *The dependency of hydrogel consistency on the initiator mixture composition. (The unit of the isocurves labels: 10^3 cP).*

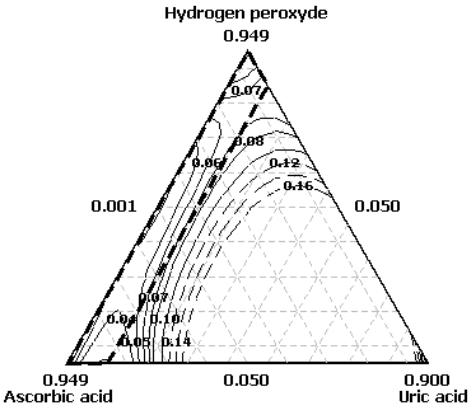

Figure 5. *The dependency of water expelling on the initiator mixture composition. (The unit of the isocurves labels: grams).*

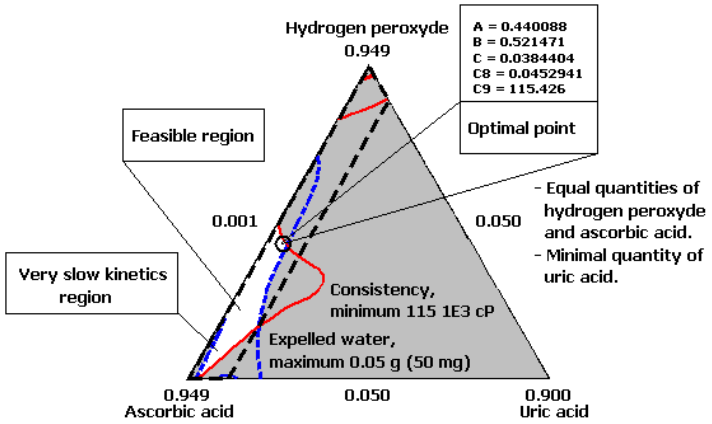

Figure 6. *The feasible region and the optimal point for conducting acrylamide hydrogel formation by using the biotolerable radical initiator mixture.*

Figure 6 presents the feasible region for conducting the acrylamide hydrogel generation by using the bio-tolerable initiator mixture, and the identified optimal point, which achieves the best compromise between the need for a maximal hydrogel consistency and a minimal quantity of water expelled during hydrogel volume contraction.
The dependencies expressed by the ternary diagrams are strongly nonlinear, which means that the polymerization – crosslinking processes are very sensitive to the variation of the radical initiator recipe. For both hydrogel consistency and expelled water, the strongest nonlinearities are induced by uric acid ratio in the mixture. Higher quantities of H_2O_2 and ascorbic acid favour the increase of hydrogel conssistency, but an excess of H_2O_2 can increase the water eliminated by syneresis, accelerating and augmenting the hydrogel contraction. On the contrary, in the presence of an ascorbic acid excess, syneresis can be diminished.
Starting from the fitted models for hydrogel consistency and for the water expelled by syneresis (resumed in table 4), the mixture composition was optimized, using the freeware software application AMPL with LOQO solver; the results are given in figure 6. A maximum consistency of 115,426 cP and a minimum of 45.29 mg of expelled water can be obtained, when the the mass fractions in the initiator mixture are: 0.44 H_2O_2, 0.52 ascorbic acid and 0.04 uric acid. This optimal point considers the necessity to assure the shorter possible duration of the gelation process, which has been deduced by processing all the columns of table 3, using the same algorithm (according reference [10]). Recalculating the optimal mixture ratio, the following molar ratios result: H_2O_2 : ascorbic acid : uric acid → 1 : 1 : 0.1. According to the experimental procedure, the molar ratios reported to the monomer (acrylamide) are: acrylamide : H_2O_2 : ascorbic acid : uric acid → 1 : 0.01 : 0.01 : 0.001.

Table 4: *Coefficients of the mathematical models fitted to the experimental data generated by the restricted mixture design (tables 2 and 3)*

The structure of fitted extended, third order, full cubic models			
Measured variables		Consistency	Expelled water
Main effects	[A]: H_2O_2	149.4	0.125
	[B]: Ascorbic acid	116.2	0.073
	[C]: Uric acid	-391.5	-0.289
Interraction effects	[A] · [B]	-72.4	-0.241
	[A] · [C]	628.2	0.814
	[B] · [C]	754.3	2.121
Extended effects	[A] · [B] · ([A] – [B])	-165.4	-0.091
	[A] · [C] · ([A] – [C])	-511.5	-1.320
	[B] · [C] · ([B] – [C])	-570.1	-3.137
Overall adequacy of the models, r^2 (multiple determination coefficient)		0.971	0.953

CONCLUSIONS

The paper proves the possibility to induce gelation in aclylamide – N,N'-methylene-bis-acrylamide systems at low temperature (typically 4 ˚C), using mixtures of biotolerable components as radical initiator. The respective mixtures contain hydrogen peroxyde as

radical generator, ascorbic acid as radical process activator and uric acid, as radical process promoter (buster) and modulator. If the aqueous medium in which gelation occurs also contains a protein protectant system, the optimal mixture composition comprises the following molar ratios: H_2O_2 : ascorbic acid : uric acid → 1 : 1 : 0.1.
The studied radical initiator system can be used to promote (co)polymerization of various monomers, in nondenaturant conditions, into aqueous protein systems. If unsaturated grafted proteins are involved in (co)polymerization, crosslinked networks (of interconected and / or semi-interpenetrated type) can be obtained.

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