Optimization of the Properties of Chitosan Lactate/Hyaluronan Film

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ABSTRACT: Polyelectrolyte complexes represent attractive class of polymer-based materials, finding an irreplaceable role in biomaterial preparation for tissue engineering or drug delivery beads. Mechanical properties, physical properties, and enzymatic degradation of the film prepared from chitosan lactate/hyaluronan polyelectrolyte complex, crosslinked with starch dialdehyde derivatives, were studied to optimize its composition. This work represents an example demonstrating how a minor modification of the modified complex composition changes final properties of the prepared film and emphasizes enormous variations in complex formation by crosslinking. To obtain sufficiently useful information, experimental design was employed.
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Key words: polyelectrolytes; biopolymers; films; crosslinking

INTRODUCTION

Biodegradable and biocompatible materials based on natural polymers occupy prominent place in substitutive medicine, at present. These materials are effectively used in tissue engineering for a replacement connective tissue or as drug delivery materials. Chitin and chitosan have many distinctive biomedical properties and have been applied in many different industrial areas. Chitosan and some of its derivatives and complexes have been studied for use in different biomedical applications. Chitosan is currently of interest also because of its effect on phenotypic expression of fibroblasts, one of the key cell types involved in wound healing. The charge density allows chitosan to form insoluble ionic complexes or complex coacervates with a wide variety of water-soluble anionic polymers.[1]

Polyelectrolyte complexes represent an attractive class of polymer-based materials, finding an irreplaceable role also in the preparation of 3D membranes.[2,3] Numerous factors affect the properties of the polyelectrolyte complex. Besides selected polyelectrolytes and their properties, preparation conditions (concentration, reaction time, temperature, ionic strength, pH, and presence of other polyelectrolytes) are substantial complex-forming factors. From the point of view of polymer hydrogels, the polyelectrolyte complexes belong to the category of the physically crosslinked gels with the crosslinks of small but finite energy and of finite lifetime.[4]For example, alginate-chitosan microcapsules with alginate as the core material were thoroughly investigated as the bioadhesive drug delivery system to prolong the residence time of a drug carrier in the gastrointestinal tract.[5–7]

Several studies are focused on complexes of chitosan with glycosaminoglycans (GAGs), which could have interesting biological properties such as improving the wound-healing acceleration and the cellular assistance for skin and cartilage recovery. The formation of polyelectrolyte complexes between chitosan and hyaluronan was characterized by different physical–mechanical methods: pH-metry, conductometry, IR-spectrometry, and X-ray analysis. Ionic complex results from strong electrostatic interaction between positive $—NH_3^+$ groups of chitosan and negative $—COO^-$ groups of hyaluronan in acidic environment.[8–10] Similarly, biological properties such as their hydrolysis by hydrolytic enzymes, cell adhesion, and viability on the materials based on these polyelectrolyte complexes have been studied.[11] It was found that chitosan has a protective effect on specific enzymes of GAGs, though at the different pH, as it is optimal for a complex formation.

Lim et al.[12] studied the character of chitosan–hyaluronan complex set for adhesive preparation, because both components have good mucoadhesive properties, which depend on a mutual ratio of polymers.[13] Borchard et al.[14] showed interaction between the opposite charged chitosan and cell membrane, such that

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the combination of chitosan and hyaluronan guarantees higher complex adhesion to sinus nasal epithel.

Mao et al.[15] studied properties of lyophilized chitosan–gelatin membranes modified with hyaluronan. The complex was modified by two methods: the first one was crosslinking of chitosan–gelatin membrane in solution of hyaluronan, and the second one was preparation of the membrane after mixing all polymers together before lyophilization.

This work represents an excellent example demonstrating how a minor modification of the complex composition modifies the final properties of the prepared film and emphasizes an enormous variation in film formation conditions if the complex chitosan lactate/hyaluronan is crosslinked with starch dialdehyde derivatives.

MATERIALS AND METHODS

Polymers

Bacterial hyaluronic acid (HA) produced biotechnologically by *Streptococcus zooepidermicus* was used in the form of sodium salt with molecular weight 1.40 MDa.

Chitosan lactate from biotechnological processing (89.9% deacetylated) was as 0.5% solution in 1% acetic acid with viscosity 3.4×10^{-2} m^2/s. Both polymers were obtained from CNP (Ústí nad Orlicí, Czech Republic).

Starch dialdehyde derivatives were prepared by periodic oxidation of starch, according to CS Patent 193,058 (1982).[16]

Preparation of chitosan lactate—hyaluronan film

Films from the complex of chitosan lactate–hyaluronan were prepared from gel originated from mixing water solutions of chitosan lactate and acidified (pH 4.5) hyaluronan solution. The gel of the complex was intensely mixed with the crosslinking agent (starch dialdehyde derivatives). The experimental design was used to optimize all three main polymeric components. Films were prepared by drying hydrogel on a tetrafluorethylene plate.

Insoluble fraction

The insoluble fraction was evaluated gravimetrically. After conditioning, the samples were weighted and leached in physiological solution for 6 h. Then, the samples were dried for 2 h at the temperature of 37°C, conditioned, and weighted again. The insoluble fraction was determined and used for the corrected calculation of the maximum swelling degree.

Swelling measurements

Swelling kinetics was followed using Dogadkin apparatus.[17] This method is based on the measurement of volume of low-molecular solution penetrating into the film. The measurements were performed at the temperature of 23°C.

Enzymatic degradation

Bacterial hyaluronidase (EC 3.2.1.35-Sigma, activity 290 units/mg) 0.1 wt % in the solution of phosphate buffer at pH 7.4 was used for enzymatic degradation of the samples at 37°C for 2 h. Concentrated sulfuric acid was added to filtrate and tempered in boiling water bath, after separation of odd particles. Then, after cooling in an ice bath, a solution of carbazole (0.1%) in ethanol was added. The color intensity was measured at $\lambda = 530$ nm. The degradation degree was determined as a quantity of liberated glucuronic acid calculated to the initial amount of hyaluronan in the sample (mg GA/g H).

Mechanical properties

The tensile strength, elongation at break, and toughness were evaluated from the dried films, using Instron 5565 Tensometer. The samples (10×70 mm^2) were conditioned for 24 h at 50% relative humidity and 23°C before testing. The working part of the sample was 10×30 mm^2 and cross head rate was 50 mm/min. Toughness was calculated as an integral area under the tension versus elongation.

Design of experiment (DOE) method

The DOE method was used to describe mathematical–statistical data of complex formation. The output of this method is the system of regression equations, which can be used for optimization of the system. DOE was applied for the study of effects of chitosan (CH), hyaluronan (H), dialdehyde derivatives of starch (DS) on membrane properties, and the amount of solvent used (R-0.05*M* acetic acid) as well. The experimental design of the following three factors on five levels was chosen:

$$x_1 = \mathrm{CH/H} \quad x_2 = \mathrm{DS/(CH + H)}$$

$$x_3 = \mathrm{(DS + H + CH)/R}$$

The range of the factors was designed with respect to the concentration range of individual components as follows: content of CH from 14 to 30 wt % and content of GL from 11 to 23 wt %. The conditions of DOE and compositions of individual blends are shown in Tables I and II.

RESULTS AND DISCUSSION

Mechanical properties, physical properties, and the enzymatic degradation of the membrane, based on the

TABLE I
Conditions of DOE—Recalculation of Encoded Levels of Factors to Real Values

Factor	−1.682	−1	0	1	1.682	Step
$x_1 = \frac{CH}{H}$	3.3	6.7	12	16	20	4.6
$x_2 = \frac{DS}{(CH+H)}$	0.00080	0.011	0.025	0.040	0.050	0.015
$x_3 = \frac{(DS+H+CH)}{R}$	0.020	0.025	0.031	0.037	0.042	0.0063

chitosanlactate/hyaluronan complex chemically modified with starch dialdehyde derivatives, were evaluated to identify the parameters influencing the quality and their mutual interactions in the system. The structure of membranes was homogeneous with a smooth surface. The swollen membranes were flexible, with a very good adhesion to surfaces.

The degree of maximum swelling corrected by measured insoluble fraction after dissolution in simulated body liquid was the chosen physical–chemical characteristic indirectly describing macrostructure and microstructure of the membrane. On the other hand, enzymatic degradation with hyaluronidase as a model for material bioresorption in tissue can indirectly give a picture of film structure accessibility to enzymes and film cohesivity. From the point of view of potential applications, an evaluation of mechanical properties (tensile strength, elongation, and toughness) was the fundamental part of the optimization of film composition.

The ranges of the individual selected factors were determined on the basis of the preliminary experiments. The range from 0.13 to 0.68% was taken for hyaluronan content (calculated to the total weight of all three polymeric components in the mixture) and the range from 113 to 116 mL for solvent content in the mixture. All complexes were prepared by the mixing of viscose solutions of the basic polymers and starch dialdehyde derivatives as crosslinking agent in the high-speed mixer.

For polyelectrolyte complex, pH value is an important characteristic of the system. The pH range between 5.2 and 8.0 is indicated as the optimum scale of pH for stable chitosan complex.[18] All complexes prepared fell within this range, except the complex from experiment number 11 (Table II).

Films prepared from polyelectrolyte complexes were highly hydrophilic, able to take liquid and increase their volume. Penetration of small molecules of solvent to the material structure is a very selective process. Therefore,

TABLE II
The Composition of Complexes of Individual Experiments of DOE

	Coded levels of factors			Real levels of factor			Complex composition (g)			
Experiment	x_1	x_2	x_3	x_1	x_2	x_3	C	H	S	R
1	−1	−1	−1	6.6	0.011	0.025	2.45	0.37	0.030	115.2
2	1	−1	−1	16	0.011	0.025	2.65	0.16	0.030	115.2
3	−1	1	−1	6.6	0.040	0.025	2.38	0.36	0.110	115.2
4	1	1	−1	16	0.040	0.025	2.58	0.16	0.11	115.2
5	−1	−1	1	6.6	0.011	0.037	3.65	0.55	0.045	113.8
6	1	−1	1	16	0.011	0.037	3.96	0.24	0.045	113.8
7	−1	1	1	6.6	0.040	0.037	3.55	0.53	0.164	113.8
8	1	1	1	16	0.040	0.037	3.85	0.23	0.164	113.8
9	−1.681	0	0	3.3	0.025	0.031	2.66	0.81	0.088	114.5
10	1.681	0	0	19.8	0.025	0.031	3.30	0.17	0.088	114.5
11	0	−1.681	0	12	0.00080	0.031	3.27	0.28	0.003	114.5
12	0	1.681	0	12	0.050	0.031	3.11	0.27	0.169	114.5
13	0	0	−1.681	12	0.025	0.020	2.12	0.18	0.0	115.65
14	0	0	1.681	12	0.025	0.042	4.24	0.37	0.117	113.3
15	0	0	0	12	0.025	0.031	3.19	0.28	0.088	114.5
16	0	0	0	12	0.025	0.031	3.19	0.28	0.088	114.5
17	0	0	0	12	0.025	0.031	3.19	0.28	0.088	114.5
18	0	0	0	12	0.025	0.031	3.19	0.28	0.088	114.5
19	0	0	0	12	0.025	0.031	3.19	0.28	0.088	114.5
20	0	0	0	12	0.025	0.031	3.19	0.28	0.088	114.5

TABLE III
Measured and Calculated Parameters for Individual Experiments of DOE

Experiment	Non-soluble fraction (%)	$Q_{max(corr)}$ (mL/g)	Amount of liberated D-glucuronic acid (mg/1 g chitosan)	Tensile strength (MPa)	Elongation at break (%)	Toughness (MPa)
1	90.4	3.13	0.0618	44.3	13.6	4.83
2	91.9	2.11	0.1995	51.5	15.5	6.51
3	84.9	1.38	0.0390	21.8	6.7	1.06
4	83.5	0.68	0.1041	33.4	10.7	2.76
5	93.5	0.82	0.0343	30	3.7	0.49
6	94.4	0.81	0.0941	43.5	10.9	3.02
7	76.4	1.35	0.1494	31.5	9.2	2.17
8	78.4	1.25	0.0576	30.25	7.7	1.59
9	83.0	1.07	0.0407	27.15	17.9	3.75
10	80.9	1.32	0.1135	49.7	28.3	10.98
11	6.9	5.96	0.1645	36.4	4.73	0.78
12	76.5	1.35	0.1222	29.8	26.93	5.16
13	83.3	1.07	0.0798	32.3	8.43	1.94
14	85.6	0.98	0.0384	39.8	14.4	4.28
15	82.0	1.37	0.0803	44.6	17.24	5.93
16	80.0	1.54	0.0439	49.8	17.2	6.50
17	82.1	1.47	0.0501	40.6	24.12	7.29
18	84.9	1.45	0.0464	42.4	23.3	7.39
19	81.5	1.51	0.0509	38.8	18.6	6.89
20	81.9	1.49	0.0379	41.8	17.6	6.60

swelling parameter is a very important characteristic of the material, which is applied in wet state. The study of swelling process is also important because of a possible evaluation of chemical crosslinking.

The typical shape of the swelling curve was observed for all the films prepared. After quick surface solvatation, diffusion of the liquid into the film induces solvatation of further locations in the crosslinked structure of film till the amount of water taken is constant, which corresponds to the maximum swelling degree, Q_{max}. We found that during soaking of the sample in the physiological solution, a part of crosslinked film sample was washed off because of partial solubility. Therefore, we decided to correct Q_{max} by calculating the amount of absorbed liquid to dry weight of the sample reduced by an insoluble fraction to receive $Q_{max(corr)}$.

Q_{max} was determined by regression of measured data according to the eq. (1)

$$Q_{(t)} = Q_{max}\,(1 - e^{-kt}) \tag{1}$$

where $Q_{(t)}$ is the swelling degree in time t, Q_{max} is the maximum swelling degree, and k is the rate constant of the swelling process.

Subsequently $Q_{max(corr)}$ was calculated as

$$Q_{max(corr)} = (v_{sol}/w_s)(w_{s.}/w_{if}) = v_{sol}/w_{if} \tag{2}$$

where v_{sol} is the volume of absorbed solution in sample, w_s is the weight of dry sample before swelling, and w_{if} is the weight of insoluble fraction.

The measured parameters of films were statistically evaluated according to the DOE method[19] using STA-

TABLE IV
Results of Variance Group Analysis for Evaluated Parameter

Statistic parameter	Non-soluble fraction (%)	$Q_{max(corr)}$ (mL/g)	Amount of liberated D-glucuronic acid (mg/1 g chitosan)	Tensile strength (MPa)	Elongation at break (%)	Toughness (MPa)	$F_{k,0.05}$
F_{S1}	8.9	765.6	13.0	14.6	4.0	26.6	5.4
F_{S2}	16.2	526.1	24.9	4.2	6.4	45.3	4.9
F_{SLF}	35.7	319.4	0.954	2.7	8.3	28.2	5.1
$s_{E+/-}$	0.016	0.060	0.015	3.840	3.174	0.543	
$s_{LF+/-}$	0.096	1.067	0.015	6.363	9.132	2.885	

F_{S1}, F test value for significance testing of the linear part of regression equation; F_{S2}, F test value for significance testing of the nonlinear part of regression equation; F_{LF}, F test value for significance testing of insufficiency of regression equation; $F_{k,0.05}$, F test value on 95% probability level; S_E, mean quadratic error experimental; S_{LF}, mean quadratic error of regression estimation.

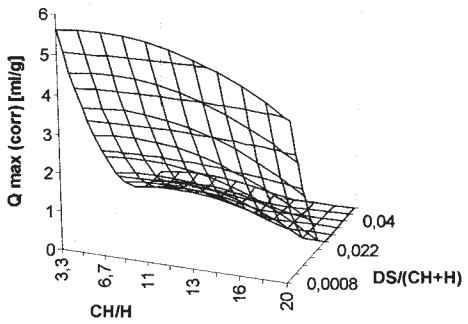

Figure 1 Dependence of response surface of the corrected maximum swelling degree on CU/H ratio (factor x_1) and DS/(CH + H) ratio (factor x_2) at the constant ratio (DS + H + CH)/R = 0.020 (factor x_3).

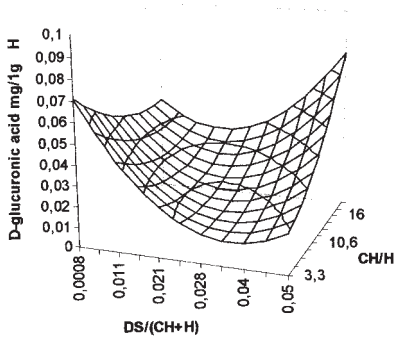

Figure 3 Dependence of response surface of the amount of liberated D-glucuronic acid calculated to 1 g of hyaluronan on DS/(CH + H) ratio (factor x_2) and CH/H ratio (factor x_1) at the constant ratio (DS + H + CH)/R = 0.031 (factor x_3).

TIS software.[20] The measured properties are summarized according to the DOE method in Table III. The regression equations used for the description of response surfaces were of the type $Y = b_0 + b_1x_1 + b_2x_2 + b_{12}x_1x_2 + b_{11}x_1^2 + b_{22}x_2^2$, where Y is the evaluated parameter, b_0, b_1, b_2, b_{12}, b_{11}, b_{22} are regression coefficients, and x_i are factors on the coded levels. The results from the regression analysis are given in Table IV. Based on the regression equations, it was possible to plot response surfaces of individual parameters at chosen conditions.

Swelling experiments

On evaluating a corrected maximum swelling degree, it is clear that film swelling is influenced by changing all three defined factors. The dependence of the response surfaces of $Q_{max(corr)}$ on the factors are showed in Figures 1 and 2. As hyaluronan has higher affinity to water, $Q_{max(corr)}$ decreases with its lower content and the response surface following this axis is only moderately curved to a maximum. Comparing these two figures with different values of the factor x_3 (relating solvent influence), we can see that $Q_{max(corr)}$ is influenced significantly with a solvent content. The formation of dry film structure is finished after losing free solvent. Therefore, density of network during drying depends on an amount of used solvent. We can also see a considerable impact of chemical modification on swelling. Starch dialdehyde derivatives used as crosslinking agent create a denser network of chemical bonds in chitosan/hyaluronan complex, which lowers swelling capacity of the film. The response surfaces (Figs. 1 and 2), which are markedly curved by this axis, passes the minimum and show significant interaction between factors x_2 and x_3.

The observed extremes can be explained by the preferred reaction of crosslinking agent with chitosan component, which after dissociation in acidic environment forms the Shiff bases with starch dialdehyde derivatives. This is a concurrent reaction to the complexation process. At a higher content of crosslinking agent, more hyaluronan remains unbound. An influence of solvent can be connected with easier diffusion of crosslinking agent, which prefers the reaction with chitosan component. These effects control the film swelling and show a necessity of the optimization of the amount of crosslinking agent used for the reaction.

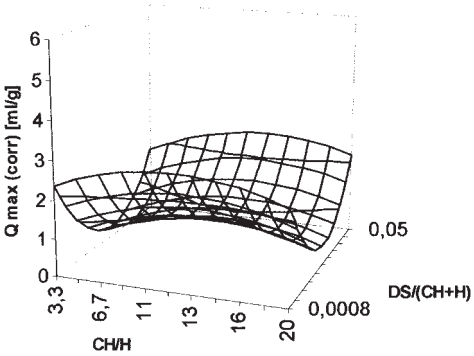

Figure 2 Dependence of response surface of the corrected maximum swelling degree on CU/H ratio (factor x_1) and DS/(CH + H) ratio (factor x_2) at the constant ratio (DS + H + CH)/R = 0.037 (factor x_3).

Enzymatic degradation

The response surface of the dependence of liberated D-glucuronic acid on the followed factors is shown in Figure 3. The fact that the amount of liberated D-glucuronic acid increases with decreasing the hyaluronan content in the complex can be explained by hyaluronan releasing from the complex. Particularly, hyaluronan, which is not bound in the complex, is more easily exposed to enzymatic degradation. Enzyme accessibility can also be influenced by the crosslinking reactions of polymers in matrix. The influence of starch dialdehyde derivative content was manifested in quadratic part of the calculated regression equation (Table V). The response surface along this axis passes the minimum. Strengthening of the

TABLE V
Results of Regression Analysis

Coefficient	Non-soluble fraction (%)		$Q_{max(corr)}$ (mL/g)		Amount of liberated D-glucuronic acid (mg/1 g chitosan)		Tensile strength (MPa)		Elongation at break (%)		Toughness (MPa)	
	b_i	b_k	b_i	b_k	b_i	b_k	b_i	b_k	b_i	b_k	b_i	b_k
b_0	*0.82*	0.017	*1.49*	0.063	*0.052*	0.016	*42.9*	4.026	*19.88*	3.327	*6.82*	0.570
b_1	−0.0006	0.011	*−0.10*	0.041	*0.022*	0.010	*5.50*	2.672	2.13	2.208	*1.28*	0.378
b_2	*−0.022*		*−0.73*		−0.008		−4.65		2.04		0.007	
b_3	−0.003		*−0.24*		−0.010		−0.24		−0.36		−0.29	
b_{11}	*0.016*	0.011	*−0.21*	0.04	0.008	0.014	−1.54	2.602	−0.23	2.151	−0.11	0.368
b_{22}	*−0.021*		*0.66*		*0.032*		*−3.42*		*−2.79*		*−1.67*	
b_{33}	*0.024*		*−0.27*		0.002		−2.39		*−4.37*		*−1.62*	
b_{12}	0.002	0.015	0.03	0.054	*−0.028*	0.014	−1.30	3.490	−0.83	2.884	−0.39	0.494
b_{13}	0.003		*0.20*		*−0.029*		−0.82		−0.03		−0.18	
b_{23}	*−0.024*		*0.52*		*0.025*		*3.61*		1.74		*0.97*	

b_i, regression coefficient value; b_k, coefficient critical value on 95% probability level. Regression coefficients statistically significant are given in italics.

complex with crosslinking agent has its optimum. Factor x_3 (representing solvent influence) showed interaction with factor x_2 (representing an influence of crosslinking agent), which is connected with more or less easy diffusion of the crosslinking agent into the complex, according to the amount of solvent used.

Mechanical properties

It can be expected that the tensile strength should be higher with increasing the content of crosslinking agent. In spite of this, similarly, as we observed in swelling and enzymatic digestion experiments, tensile strength decreased with the content of starch dialdehyde derivatives (Fig. 4). The solvent content was significant only in interaction with the crosslinking agent. The response surface of the dependence of tensile strength exhibits a maximum. The influence of the factor x_1 (CH and H ratio) shows only a linear effect (according to regression coefficients—Table V) and tensile strength increases with lowering of hyaluronan content. Hyaluronan content did not affect any factors followed.

Relative elongation at break, taken in the moment of sample failure and expressed as proportion to the original length, is the further characteristic evaluated. The response surface of relative elongation at break is shown in the Figure 5. In this case, the response surface is influenced by the content of both solvent and crosslinking agent. Both components affect relative elongation separately (no interaction observed) and both factors curved the response surface and exhibit a sharp maximum. This result very clearly documents the existence of an optimum concentration of crosslinking agent, which lies somewhere in the middle of the range of DOE experiment. Small concentrations of the crosslinking agent are not sufficient for complex hardening. However, at its higher concentrations, preferential reactions of crosslink-

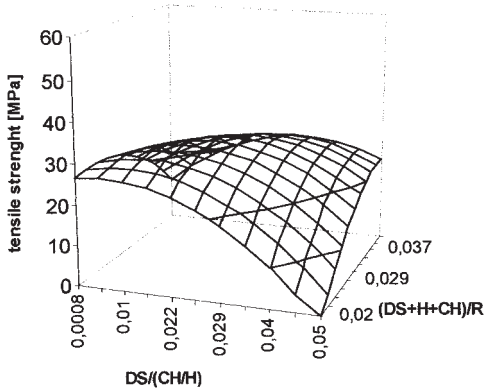

Figure 4 Dependence of response surface of tensile strength on DS/(CH + H) ratio (factor x_2) and (DS + H + CH)/R ratio (factor x_3) at the constant ratio CH/H = 3.3 (factor x_1).

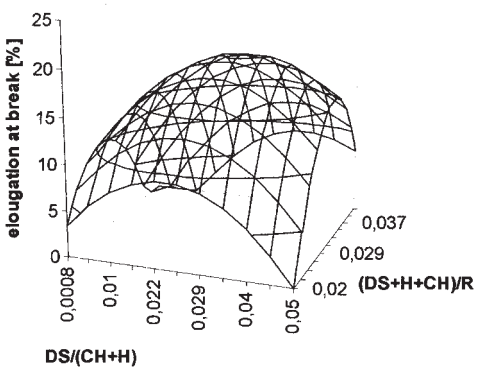

Figure 5 Dependence of response surface of elongation at break on DS/(CH + H) ratio (factor x_2) and (DS + H + CH)/R ratio (factor x_3) at the constant ratio CH/H = 3.3 (factor x_1).

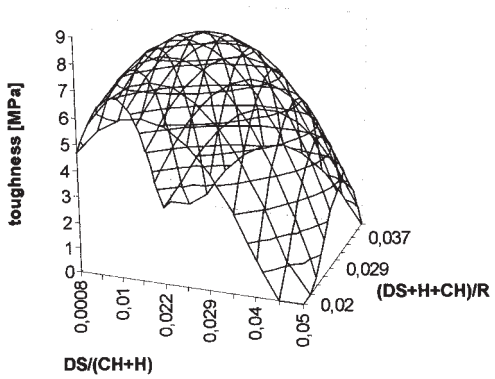

Figure 6 Dependence of response surface of toughness on DS/(CH + H) ratio (factor x_2) and (OS + H + CH)/R ratio (factor x_3) at the constant ratio CH/H =20 (factor x_1).

ing agent to chitosan component act against complex formation. Therefore, with a higher concentration of the crosslinking agent, mechanical parameters of films decrease.

The response surface of toughness is shown in Figure 6. The toughness increased linearly with decreasing hyaluronan content, but this parameter did not show any interaction. An important effect of the crosslinking agent and solvent was shown in their mutual interaction, as we can also see in Table V. Both parameters turn the response surface to the distinct maximum. This only confirms the aforementioned statements on the mutual but contrary affecting reactions of complexation and crosslinking. Therefore, a clear relation between high cohesivity and the optimum concentration of crosslinking agent is shown in this case.

CONCLUSIONS

From the point of view of receiving sufficiently useful and complex information from experiments, the use of the DOE method was efficient. The experiments enabled to obtain a basic overview on existing effects of individual factors to the evaluated properties of films. Through the regression evaluation of measured and calculated results of the DOE experiment, we obtained the regression coefficients of equations, which described relations of output parameters from mixture composition in the chosen region of concentrations. In this way, we were able to follow other characteristics important for the application of polyelectrolyte complex film as biomaterial. This approach can help to optimize the film composition to obtain a film with desired mechanical properties. As it can be seen from Table IV, the regression models (except tensile strength and liberated D-glucuronic acid) do not give a sufficiently accurate description of the response surface with respect to the experimental error. Therefore, to optimize the film preparation process on the basis of obtained results, it is necessary to specify more precise ranges of the individual factors and realize a more accurate regression experiment. Such experiment can then give the suitable regression equations for the adequately accurate model for most of the parameters followed.

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