Collagen and Collagen/Hyaluronan Complex Modifications

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Chemical analogues of extracellular matrix are mainly graft copolymers of collagen and glycosaminoglycans. The new complex matrix based on collagen and hyaluronan was used to develop a synthetic derma. The chemical treatment with hexamethylene diisocyanate with poly(oxyethylene glycol) resp. with poly(oxypropylene glycol) was chosen for improving mechanical properties. The study of mechanical properties, enzymatic degradation, and cytotoxicity after chemical treatment was first performed with pure collagen membranes and subsequently optimized to the complex membrane of collagen with hyaluronan. We suppose that the combination of excellent biological properties of both the polymers with lower enzymatic degradation and decreasing swelling after chemical modification can result in a valuable biomaterial for several medical applications.

The skin is the largest organ in the body, and the loss of extensive areas of skin represents a serious threat to life. Early surgical wound excision at patients with extensive burns has been a major advance in burn care and the rapidity of wound closure has been shown to correlate with the ultimate survival of the patient. The practice of excisional therapy leads to an understanding that excised wounds must be immediately covered with autologous skin or with an appropriate biosynthetic covering. Skin bioengineering is complicated because there are multiple components of the skin which need to be put together to make a "skin equivalent" to be used as a skin graft for patients. When considering tissue engineering and wound repair, several different approaches are available. Products and potential therapies currently being investigated generally fall into three categories. Epidermal replacements consist of keratinocytes grown either alone (on the surface of a tissue culture flask), or in close association with carrier vehicle such as a polymeric film or bioresorbable matrix. Dermal replacements consist of a support structure able to support neo-matrix production by fibroblasts. Skin substitutes are a combination of the above, able to support both dermal and epidermal components. Formulation of a suitable support structure able to facilitate several biological processes is a major issue for groups developing dermal replacements and skin substitutes.

A variety of synthetic wound dressings and skin substitutes have been introduced during the past three decades [1—3]. The ideal properties of such dressings and skin substitutes were listed by *Pruitt* and *Levine* [4]. They are: absence of antigenicity, tissue compatibility, absence of local and systemic toxicity, water vapour transmission similar to normal skin, impermeability to exogenous microorganisms, rapid and sustained adherence to wound surface, inner surface structure that permits ingrowth of fibrovascular tissue, flexibility and pliability to permit conformation to irregular wound surface, elasticity to permit motion of underlying body tissue, resistance to linear and shear stresses, prevention of proliferation of wound surface flora and reduction of bacterial density of the wound, tensile strength to resist fragmentation and retention of membrane fragments when removed, biodegradability (important for "permanently" implanted membranes), low cost, indefinite shelf life, minimal storage requirements, *etc.*

The inoculation of glycosaminoglycans chains into collagen and the formation of precipitate in acidic support is the most common method used [5]. The new material does not lose the biological activity typical for both components and therefore it is possible to select desired mechanical properties of a new complex matrix by alternation of the ratio of both the components and/or by structure modification [6]. Resorption control of collagen membranes is essential where it is used for tissue regeneration controlled by varying the cross-link density of the biomaterial. Cross-linking is an effective method to control resorption rate of collagen-based biomaterials and to prevent a rapid elution of the material into wound fluids. This is important for synchronizing degradation of biomaterial with wound healing, as well as for increasing tensile properties and flexibility of the membrane to the level necessary for application as a synthetic derma for biosynthetic skin

substitute. However, cross-linked biomaterial is more resistant to enzymes, with a higher elasticity modulus and a lower degree of swelling.

Until now, the predominant convenient cross-linking used for collagen biomaterials was with water-soluble glutaraldehyde [7, 8]. However, its cytotoxicity has motivated the search for alternative reagents [9, 10]. Residual glutaraldehyde remaining in the collagen implant gradually would modify tissue reactions to the implant.

Currently, there are two different tendencies aimed at improving the properties of biological materials: chemical procedures and physical procedures. The latter involve the use of photooxidation, dehydrothermal treatments, and ultraviolet radiation, designed to avoid introducing potentially toxic chemical agents [11].

Chemical treatment may perhaps be the alternative preferred by the different research teams. In addition to the routine use of glutaraldehyde, more recently cross-linking techniques involve among other agents acyl azide, carbodiimide, hexamethylene diisocyanate, diamines or polyepoxidic resins [12].

Therefore, in developing the synthetic derma we have chosen chemical treatment with hexamethylene diisocyanate [13]. This cross-linking agent, in which the isocyanate groups react with amino, amide, and guanidine groups of polypeptide chain, forms bonds stable in water environment [14]. Since the cross-linked collagen membranes seemed to be rather stiff, we have tried to improve flexibility and mechanical properties of the material by combining this cross-linking agent with poly(oxyethylene glycol) resp. with poly(oxypropylene glycol). The study of mechanical properties and cytotoxicity after chemical treatment was first performed with pure collagen membranes and subsequently optimized to the complex membrane prepared by precipitation of collagen with hyaluronan (HA) in acidic environment.

EXPERIMENTAL

Preparation of Membranes

Fibrous insoluble atelocollagen (HYPRO Ltd., Otrokovice, Czech Republic) was mechanically fibrilized and the suspension was dried on the teflon support at the temperature 37 °C. The cross-linked membranes were prepared by modification with hexamethylene diisocyanate (HMDIC) (Bayer AG, Germany) in combination with poly(oxyethylene glycols) (PEG) and poly(oxypropylene glycols) (PPG) (CHZ Nováky, Slovak Republic). The membranes were immersed in solution of the calculated amount of PEG in 270 cm^3 of chloroform for 4 h. Then the related amount of HMDIC was added and the mixture was kept to react at room temperature for 24 h. Finally the samples were thoroughly rinsed with water and dried at 37 °C. For cytotoxicity evaluation the samples were sealed in polyethylene bags and sterilized by ^{60}Co radiation source with the dose 30 kGy.

The complex collagen-hyaluronan membranes were prepared as follows. Insoluble atelocollagen was mechanically treated in 0.5 M solution of acetic acid (pH 3.2). The aqueous solution of HA (CONTIPRO Ltd., Ústí nad Orlicí, Czech Republic) was added into the acid atelocollagen dispersion to form the complex. Coagulate was filtered and rinsed with deionized water. After mixing the coagulate and the rheology control, water was slowly evaporated during drying the coagulate on the teflon support at 37 °C. The hybrid membranes with bubble structure with different amount of HA in complex with collagen were prepared.

Evaluation of Cytotoxicity

The cytotoxicity of the new membranes was tested on cultures of the human larynx carcinoma cells HEp-2 in the tissue culture laboratory [15]. Briefly, 2 mm × 2 mm × 0.3 mm sterile pieces of membranes were placed in Burke chambers, covered with covering glasses containing a confluent layer of cultured cells and incubated in DMEM tissue culture media in a CO_2 thermostat at 37 °C for 6 h. The pieces of sterile rubber tubing (0.3 mm thick) were used as positive control, and the pieces of silicone nontoxic tubing of the same size as negative control. After the end of incubation time the cultures were observed by Nikon inverted microscope and photographed. The ratio of lysed cells compared to the positive and negative controls was calculated.

Testing of Mechanical Properties

Mechanical properties of the membranes were determined as a function of the extent of cross-linking. Collagen membranes were cut to strips with the size 50 mm × 100 mm, immersed in distilled water for 30 s prior to the testing. Mechanical properties of these strips were analyzed in hydrated state using the Instron Tester 1122.

Enzymatic Degradation

Enzymatic degradation of the samples was determined using bacterial collagenase (*Clostridium histolyticum*, 1.8 FALGPA/mg, SIGMA), 0.08 mass %, in the solution of TRIS-HCl, pH 7.4 and the temperature 37 °C during 5 h. After separation of undisposed particles the solutions of ninhydrine and hydrintandine (pH 5.5) were added to the filtrate and temperated in boiling water bath [16]. The mixture of propanol—water (φ_r = 1 1) was added and absorbance of samples was measured at λ = 600 nm. The quantity of escaped L-leucine was determined as a degradation degree of collagen.

Table 1. The Results of Mechanical Tests and Enzymatic Degradation for the Different Combinations of Cross-linking Agents

Agent	Mass fraction w(HMDIC)	Mass fraction w(spacer)	Stress	Strain	Enzymatic digestion/ leucine of collagen
	%	%	MPa	%	mg g^{-1}
Pure collagen	–		2.62	8.40	33.34
Pure	1	–	6.65	6.33	32.40
HMDIC	2	–	6.20	6.25	28.55
	5		8.97	6.20	9.41
EG	1	1	6.84	18.56	6.21
	2	2	5.38	19.15	15.63
	5	5	5.09	20.35	11.67
	10	10	4.84	19.97	9.87
TEG	0.5	2	–	–	19.56
	1	4	–	–	20.89
	2	8	4.86	19.97	15.82
	5	20	–	–	16.17
PEG 300	1	2	2.19	12.90	30.59
	2	4	2.27	17.06	30.12
	5	10	4.69	21.14	29.29
	10	20	8.55	29.09	28.54
PEG 600	1	1	4.08	18.25	30.38
	2	2	4.55	26.67	30.13
	5	5	6.11	35.96	29.72
	10	10	6.09	31.87	25.19
PEG 1500	1	0.4	2.46	14.14	31.96
	2	0.8	2.93	17.78	30.28
	5	2	3.75	19.15	28.64
	10	4	4.30	13.81	23.03
PPG 750	1	0.8	–	–	10.60
	2	1.6	–		6.70
	2	2	4.98	17.58	5.59
	5	4	–	–	5.70
	10	8	6.19	15.81	6.35
DIPG	1	1	2.96	14.73	31.17
	2	2	3.61	16.97	32.51
	5	5	4.29	17.31	28.47
	10	10	4.90	17.80	25.21

RESULTS AND DISCUSSION

The requirements for applications of the modified collagen layers in surgery include a good flexibility, as well as a high cohesivity of the material. Mechanical properties and particularly tensile strength of treated membranes nondirectly suggest a degree of chemical cross-linking. On the other hand, chemical cross-linking retards resorption of biomaterial in tissues. Therefore, the study of enzymatic degradation can be a good model for the evaluation of resorption rate in tissue [17]. The standardization of collagen treatment procedure by alternating the agents has proved appropriate. Mechanical properties measured for different combinations are summarized in Table 1. It can be seen that the samples treated with HMDIC in the combination with PEG were fairly more flexible comparing to the samples treated with pure HMDIC. The different ratios of the agents were chosen according to the molar content of the reacting groups in the system. The cross-linking with combination HMDIC and small molecular ethylene glycol (EG) was very effective in increasing strain. On the other hand, strain was just lightly influenced by changes of the concentration of agents. We have concluded that the combination of HMDIC with PEG 300 showed the optimal properties. The high values of stress and a considerable increase of strain values were observed in this case. The modification by this combination of the agents was chosen for further treatment of more complex collagen membranes with included hyaluronan.

The enzymatic degradation of the modified collagen membranes as a measure of chemical modification is expressed as the amount of escaped L-leucine from 1 g of collagen. These values for the different combinations of modifying agents used for chemical treating of collagen membranes are also shown in Table 1. It can be seen that cross-linking with HMDIC strongly inhibits enzymatic degradation. EG and also surprisingly PPG 750 showed generally strong effect. On the other hand, enzymatic degradation is slowly inhibited by triethylene glycol (TEG) and dipropy-

Table 2. The Evaluation of Specific Toxicity of the Different Membranes

Sample	Specific cytotoxicity/%
Nonmodified collagen	< 0
Collagen modif. 5 % HMDIC + 20 % TEG	< 0
Collagen modif. 10 % HMDIC + 4 % PEG 1500	< 0
Collagen modif. 10 % HMDIC + 4 % PPG 750	0.71
Collagen modif. 10 % HMDIC + 10 % EG	1.07
Collagen modif. 10 % HMDIC + 10 % PEG 600	0.07
Collagen modif. 10 % HMDIC + 10 % DIPG	< 0

Specific cytotoxicity $= -(y/N_1 - x/N_2)\ 100$, where: N_1 – the initial total number of cells in the control culture, N_2 – the initial total number of cells in the culture with tested sample, y – the number of lysed or loose cells in control culture after 6 h, x – the number of lysed or loose cells in the culture with tested sample after 6 h.

Table 3. Maximum Swelling Degree, Q_{max}, for Nonmodified Samples, Samples Modified in the System with the Mass Fractions w(HMDIC) 5 % and w(PEG) 1 %, and Irradiated Nonmodified Samples

Sample w(HA)/%	$Q_{max}/(cm^3\ g^{-1})$		
	Nonmodified	Modified	Irradiated
3	14.15	10.05	
5	13.55	8.65	9.05
8	15.80	11.85	13.85
15	11.75	8.35	
20	10.5	6.05	8.40
25	9.05	5.70	

lene glycol (DIPG) acts similarly to the other types of poly(ethylene glycols). Poly(ethylene glycols) act like spacers creating more accessible structure for enzymatic digestion. These results indicate the possibility of a planned modification of collagen membranes with a required resorption rate in tissues. This can be important for the synchronization of implant material resorption with the process of tissue healing.

Evaluation of the short-term test of contact cytotoxicity confirmed that the cell culture HEp-2 was not influenced by nonmodified collagen membrane. Cells retained their epithelial-like character and viability (the number of cells in the counting areas increased, the cells during incubation exhibited regular mitoses). Cell mitoses continued during contact with the sample and divided cell grew without any change. On the other hand, the toxic effect of the catheter rubber was very clearly observed on cells already after 2 h of incubation.

The evaluation of the short-term cytotoxicity test yielded the values of specific toxicity, which are given in Table 2. It can be seen that the different modifications using HMDIC in the combinations with PEG or PPG gave generally acceptable values of toxicity because the values are under 5 % of specific toxicity. The values in the range from 5 to 30 % of specific toxicity are considered as weakly toxic.

The results and experience with the collagen membrane were used in target modification of the complex membrane collagen—hyaluronan. Besides of the composition also formation of defined structure is important for the use as a biosynthetic skin substitute. It was recognized that the structure with the air bubbles is suitable for these applications. This structure gives a better possibility for cultivation of cells *in vitro* before application. The structure of complex membrane is characterized with regularly distributed air bubbles. The structure was not changed after mild modification with the system with the mass fractions 5 % HMDIC and 1% PEG, which were selected from the previous study. The destruction of the regular bubbles was observed after a deeper modification with the mass fractions 5 % HMDIC and 10 % PEG.

The character of macromolecular cross-linking influences the swelling degree of the membrane. Therefore, the different amount of HA in the complex membrane influenced the process of swelling (maximum swelling degree). Swelling, as a measure of cross-linking efficiency, was studied in 0.5 M acetic acid. Strong swelling in acidic environment can be simultaneously influenced by at least two antagonistic factors, the polarity of the material, and hydrogen bonds formation between the macromolecular components. It is evident from Table 3 that the content of HA in the complex membrane is important for this process and influences swelling positively. Hydrogen bonds interaction between components influences the swelling degree negatively. This could explain the maximum swelling degree of the complex membrane with 8 mass % of HA. Chemical modification resulted in a general decrease of maximal swelling degree. Swelling of modified samples was not influenced after irradiation by γ-rays.

Mechanical properties of modified complex membrane collagen—hyaluronan were tested after standard wetting that simulated conditions after implantation. The results are summarized in Table 4. Tensile strength gradually increased with increasing amount of the added spacer PEG. It can be assumed that this combination of cross-linking agent and spacer is suitable for improving the complex membrane. However, the amount of added PEG is limited. Material cross-linked with the HMDIC PEG ratio 5 10 had the highest tensile strength but its use is moot because of the previous study of swelling kinetics and enzymatic degradation.

It can be assumed that the complex membrane collagen—hyaluronic acid can be prepared according to the predicted mechanical properties. The combination of excellent biological properties of both the