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Cytotoxicity evaluation of gelatin sponges prepared with different cross-linking agents

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Abstract—Gelatin is a natural polymer used in pharmaceutical and medical applications, especially in the production of biocompatible and biodegradable wound dressings and drug delivery systems. Gelatin granules hydrate, swell and solubilize in water, and rapidly degrade *in vivo*. The durability of these materials could, however, be prolonged by cross-linking by aldehydes, carbodiimides, and aldose sugars, but the biocompatibility of collagenous biomaterials is profoundly influenced by the nature and extent of cross-linking. In this study, gelatin sponges were prepared by using various cross-linkers such as glutaraldehyde (GA), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC), and D-fructose. The effects of the type and the amount of cross-linker on thermal and mechanical properties, stability, and cytotoxicity were investigated. The mechanical analysis data showed that an increase in the amount of GA in the sponge structures caused a slight increase in the modulus of elasticity but had almost no effect on the tensile strength. Increase in the EDAC concentration produced a maximum in the modulus of elasticity and tensile strength values. The stability of the sponges and the time required for complete degradation in aqueous media increased in parallel with the cross-linker content. *In vitro* studies carried out with fibroblast cells demonstrated a higher cell viability for the samples cross-linked with low concentrations of GA than for those cross-linked with EDAC.

Key words: Gelatin sponge; cytotoxicity; cross-linking agents; glutaraldehyde; carbodiimide; fructose; solubility; degradation.

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INTRODUCTION

Gelatin, the major constituent of skin, bone, and connective tissues, is generally obtained from collagen by controlled hydrolysis. The biological origin and biocompatibility of gelatin has led to wide-ranging applications in the pharmaceutical and medical fields; for example, as sealants for vascular prostheses [1, 2], bone-repairing matrices, controlled drug release systems [3–5], ligament substitutes, wound dressing materials [6–8], wound healing agents [9, 10], and scaffolds for tissue engineering purposes [11].

Gelatin granules, films, and sponges quickly hydrate in aqueous media and rapidly degrade *in vivo*. Cross-linking the structure can improve the mechanical properties, increase stability, and prolong durability. A number of cross-linking methods have been described, such as treatment with heat [12], or ultraviolet or gamma irradiation [13, 14], and by adding chemical agents such as sugar [15], epoxy compounds [16, 17], formaldehyde [18], glutaraldehyde [19, 20], genipin [21], and carbodiimides [2, 22, 23]. However, it must be remembered that the use of cross-linkers can lead to toxic side-effects due to the unreacted residues.

Glutaraldehyde (GA) is the most commonly used cross-linking reagent in the preparation of bioprostheses (such as heart valves, vascular grafts, elastic cartilage, and artificial skin), in cell and enzyme immobilization, and in protein and polysaccharide stabilization. GA is presumed to cross-link by inter- and intramolecular covalent bonds. This can occur in two ways: by the formation of a Schiff base by the reaction of the aldehyde group with the amino group of lysine or hydroxylysine (Fig. 1A), or by aldol condensation between two adjacent aldehydes. The Schiff base linkage is not very stable, whereas the aldol condensation product is. GA not only interacts with amino groups, but can also react with carboxy, amido, and other groups of proteins. GA cross-linking of collagenous tissues significantly reduces biodegradation, making them biocompatible and non-thrombogenic while preserving anatomic integrity, strength, and flexibility. Among aldehydes, which are used to cross-link a protein matrix, GA is advantageous because its reaction is rapid; it is less expensive; it is readily available and highly soluble in aqueous solution; and it reacts with a large number of available amino groups present in proteins. The polymerization of GA takes place quickly under alkaline conditions, but even at pH 5, aqueous solutions of GA contain polymeric moieties. Compared with formaldehyde and other aldehydes, the cross-links are more stable when GA reacts with proteins such as albumin, collagen, and mucopolysaccharides including heparin [24].

Carbodiimides belong to the class of zero-length cross-linkers, in contrast to bifunctional reagents such as GA or polyfunctional reagents such as polyepoxides. Carbodiimides activate the carboxylic acid groups of glutamic or aspartic acid residues and form bonds with the amino groups of another polypeptide chain (Fig. 1B), but they do not remain in the linkage and are released as substituted urea molecules [2]. They are available in a variety of molecular structures and the most widely used is 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC).

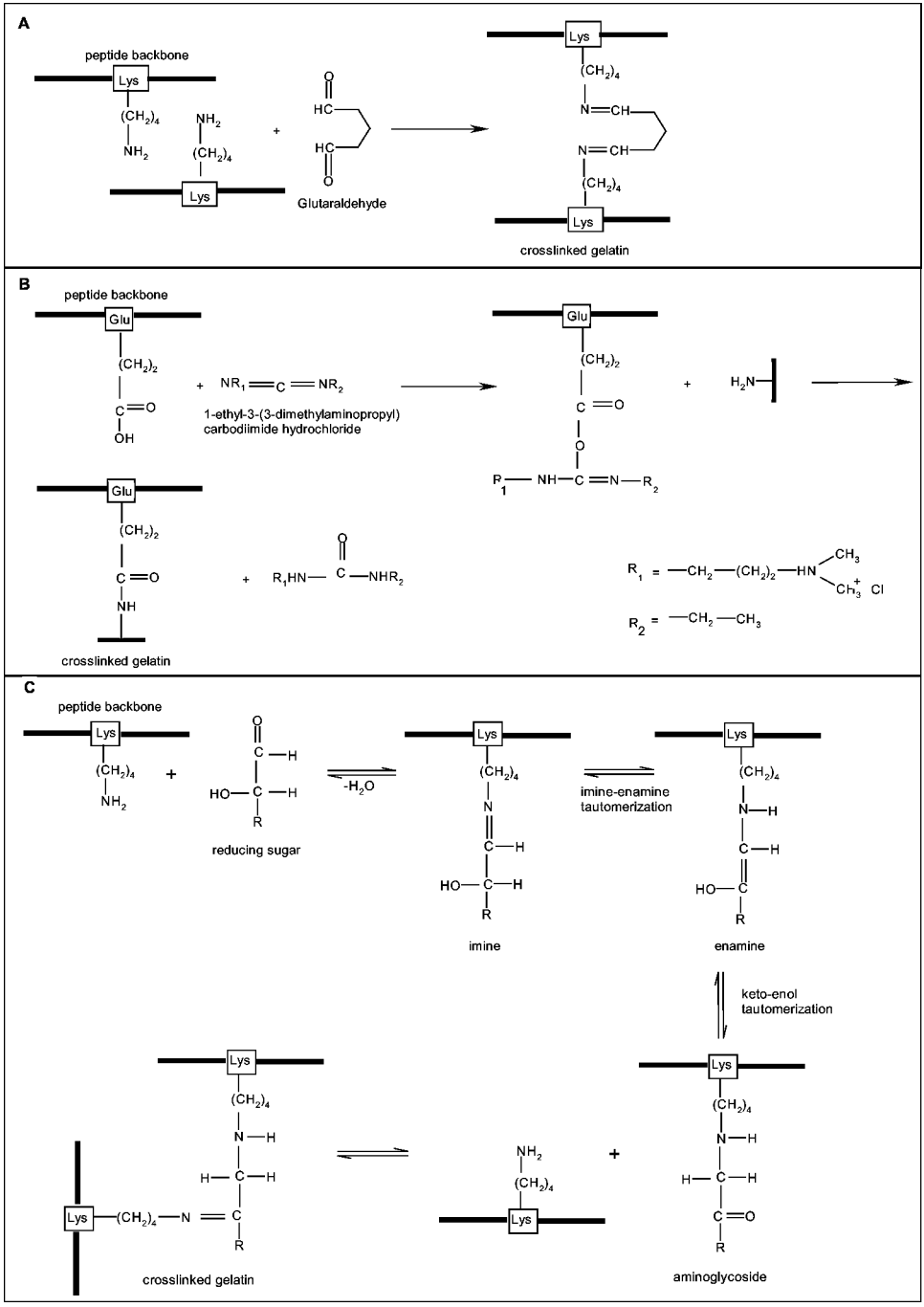

Figure 1. Schematic presentation of gelatin cross-linkage with (A) glutaraldehyde, (B) carbodiimide, and (C) fructose.

Glucose or other aldose sugars may provide another possibility for gelatin cross-linking by one or several reactions. One possible mechanism involves aldose sugars. In this case, the aldehyde group of the reducing sugar (e.g. glucose or fructose) can react with the free amino group of the gelatin molecule to form aminoglycoside, which can react further with another amine group, leading to a cross-linked network structure (Fig. 1C).

The most important requirement for the clinical usefulness of cross-linked gelatin or collagen is their non-toxic properties. It is known that gelatin itself is not toxic, but the cross-linkers used for the preparation of stable structures may create toxicity. In the literature, it has been reported that GA-related molecules could be released from cross-linked collagen-based biomaterials and lead to toxicity [25, 26]. The released molecules may be either unreacted GA present in the sample or products of hydrolytic or enzymatic degradation of collagen [27], and may contribute to the toxicity observed *in vivo* and *in vitro* [28, 29]. It was reported that materials cross-linked with GA at concentrations higher than 0.05% (v/v) are highly cytotoxic [28]. It was also reported that a GA concentration of 3.0 ppm released upon biodegradation is toxic [30]. Other cross-linkers such as genipin, epoxy compounds, carbodiimides, and sugars have been evaluated in an attempt to eliminate the cytotoxicity associated with the use of aldehydes and they were reported to be significantly less cytotoxic than GA [31–33]. EDAC is known as a non-toxic and biocompatible cross-linker, since it is not incorporated into the cross-linked structure, but is simply transformed into a water-soluble urea derivative during the production process. The cytotoxicity of urea derivatives has been found to be quite low compared with EDAC and in the literature, EDAC-crosslinked collagen has been reported to be biocompatible in both *in vitro* and *in vivo* studies [34–36]. It was reported that sugar moieties did not lead to inhibition of cell growth *in vitro* assays [37].

In this study, gelatin matrices were prepared in sponge form by using various cross-linkers. The physical and mechanical properties of the prepared matrices were examined and cytotoxicity experiments were carried out *in vitro* in order to study the cell viability of fibroblast cells.

MATERIALS AND METHODS

Materials

Bacto gelatin was purchased from Difco Laboratories (USA). 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDAC), D-fructose, 10% calf serum, and Dulbecco's modified eagles medium F12 (DMEM-F12) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Glutaraldehyde was from BHD Limited (Poole, UK). Giemsa was from Merck (Germany). The human skin fibroblast cell line (HSAn2) was kindly supplied by HUKUK FMD Institute (Ankara, Turkey). All other reagents were of analytical grade.

Table 1.
Densities of the gelatin sponges ($n = 9$)

Sample	Type of cross-linker	Amount of cross-linker	Cross-linker (mmol)	Density (g/cm^3)
GS	—	—	—	0.0070 ± 0.0004
GS-GA1	GA	5 ml of 0.5% (v/v)	0.25	0.0064 ± 0.0004
GS-GA3	GA	1 ml of 5% (v/v)	0.50	0.0081 ± 0.0015
GS-EDAC1	EDAC	1 ml of 0.5% (w/v)	0.026	0.0062 ± 0.0011
GS-EDAC4	EDAC	1 ml of 5% (w/v)	0.26	0.0060 ± 0.0004
GS-EDAC6	EDAC	5 ml of 5% (w/v)	1.30	0.0076 ± 0.0008
GS-F	Fructose	0.075 g	0.42	0.0077 ± 0.0009

Preparation of gelatin sponges

Aqueous gelatin solutions (3% w/v, 50 ml) were heated to 60°C and cross-linking agents were added to the solutions under continuous stirring at 2000 rpm for 30 min. Foaming solutions were poured into Petri dishes (1.5 cm thick) and freeze-dried [6, 38]. The compositions of the prepared sponges are given in Table 1.

Density analysis

The prepared sponges were cut into cubes (*ca* 1 cm on each side) with extreme care, the dimensions were carefully measured with a micrometer, and the sponges weighed. Sponge densities were calculated from the mass to volume ratio. At least nine measurements of each type of sponge were made and averages were calculated.

Mechanical analysis

The mechanical properties of the gelatin sponges were investigated by using a Lloyd® mechanical testing machine (Lloyd Instruments, LRX) at 23 ± 2°C. For this purpose, rectangular prism sponge samples (approximately 20 mm × 50 mm × 10 mm) were placed on the fixed and movable member of the mechanical testing machine by special clamps. The applied extension rate was 2 mm/min. For each type of sponge, at least nine samples were subjected to tensile testing. One-way ANOVA was applied to determine the average, the standard deviation, and the significance of the difference.

Thermal analysis

The thermal properties of the gelatin sponges were examined by a DuPont 2000 differential scanning calorimeter. Measurements were carried out under a nitrogen atmosphere, using about 3–5 mg samples with a heating rate of 10°C/min up to 300°C.

In vitro *degradation of gelatin sponges*

In order to evaluate the amount of degraded gelatin, sponges (1 cm^3) were immersed in phosphate buffer solutions (0.01 M, 10 ml, pH 7.4) and incubated at 37°C in a

constant-temperature shaking bath. Sodium azide was added to the released medium to prevent microbial growth. At certain time intervals, 500 μl solutions were withdrawn, filtered through 0.45 μm Millipore filters, and the amount of solubilized gelatin was determined spectrophotometrically at $\lambda = 660$ nm by applying the Lowry protein analysis method. Percent degradation values were calculated using the following equation, $\%D = (m_o - m_t/m_o) \times 100$, where m_o and m_t are the amounts (in g) of the initial and solubilized sponge, respectively.

Cytotoxicity analysis

The cytotoxicity of the gelatin sponges was tested *in vitro* by using the human skin fibroblast cell line HSAn2, which was obtained from the Animal Cell Culture Collection (HUKUK, Ankara). The viability of the cells was measured by the trypan blue dye exclusion method [39] and cell morphologies were monitored by the Giemsa staining method. In brief, the procedure was as follows. Gelatin sponges (10 of each type) in cube shapes (1 cm $\times$ 1 cm $\times$ 1 cm) were weighed and sterilized by UV application for 5.5 h prior to cell culture experiments. Fibroblast cells [resuspended in Dulbecco's modified Eagle's medium and Ham's F12 nutrient mixture, 1 : 1 (DMEM/F12) supplemented with fetal calf serum (10%), gentamycin, and vancomycin] were seeded into 24-well tissue culture plates (1 ml growth medium in each well) at an initial density of 2×10^4 cells/cm^3 and incubated at 37°C in a humid (95–99%) atmosphere with 5% CO_2. After 24 h, the medium was replaced with fresh medium and sterilized sponge samples were added to the wells. In the control plates, no sponge was added. After 48 h of incubation, the total content of the medium was removed. Some plates were stained with Giemsa and microphotographs were obtained under a light microscope in order to study the morphological appearance of the cell monolayer. Viable cell numbers were obtained as follows. Cells were dispersed with trypsin–EDTA solution, centrifuged, resuspended in phosphate-buffered saline, stained with trypan blue dye, and counted with a haemocytometer. Negative controls were also counted and compared with others. The generation number of the cells (n) was calculated using the equation $n = (\log C_T - \log C_o)/\log 2$, where C_T is the number of living cells in the plate and C_o is the number of seeded cells. Doubling time (d) values were calculated from the ratio t/n, where t is the duration of the experiment (chosen as 48 h) and n is the generation number. Percent death values were calculated from the equation % death $= (C_{control} - C_{test}/C_{control}) \times 100$, where C_{test} and $C_{control}$ are the average total numbers of viable cells in the test and in the control plates, respectively, after 48 h of incubation. Cytotoxicities were recorded according to the numbers of viable fibroblast cells obtained by trypan blue exclusion.

RESULTS AND DISCUSSION

Gelatin sponges

The use of gelatin in pharmaceutical and biomedical applications is particularly attractive because of its biocompatibility and biodegradability, together with the total absence of toxicity or allergic problems generally associated with the use of synthetic polymers. Furthermore, gelatin is generally obtained from the collagenous parts of tissues and is more convenient practically than collagen itself, because it has no antigenicity while collagen expresses some under physiological conditions [40]. In this study, gelatin sponges were prepared at ambient temperature using rather simple methods, avoiding the use of surfactants or organic solvents. The sponges were white in color, very soft, porous, and had highly elastic structures (Fig. 2).

Sponge densities

The calculated average densities of all the sponges are given in Table 1. The values obtained are quite low, which is expected since the structures were highly porous. The density of the sponges prepared without any cross-linker (GS) was found to be 0.0070 g/cm^3. The addition of GA or EDAC cross-linkers changed this value slightly, but no direct relationship between the amount of cross-linker and the density was observed.

Thermal properties

Polymers which have hydrophilic components, such as hydroxyl or amide groups, form intermolecular bonds in the presence of water and this strongly affects their glass transition temperature. In certain proteins and polysaccharides, no glass transition or melting is observed until decomposition of the main chain, because of the high level of stabilization provided by the hydrogen bonding. The introduction of small amounts of water may disrupt the intermolecular bonds, enhancing the main chain motion. Two glass transition temperatures (T_g) were reported for gelatin and this has been explained by the block copolymer nature of gelatin due to its

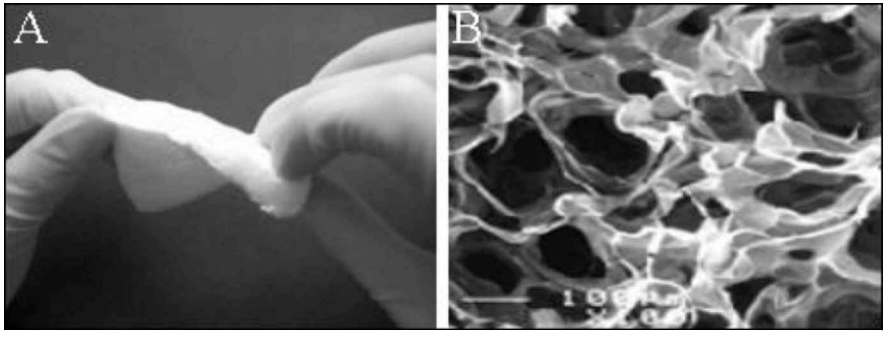

Figure 2. Gelatin sponge: (A) photograph and (B) SEM micrograph.

amino acid content [41]. The first glass transition temperature is located around 90–100°C and corresponds to the association of the glass transition of α-amino acid blocks in the peptide chain. The second glass transition temperature is located around 180–200°C, depending on the method of evaluation, and represents the blocks of imino acids, proline and hydroxyproline, with glycine. It is assumed that the latter T_g is responsible for the overall physical behavior of gelatin and is the one most cited and studied. For dry gelatin samples, the T_g was reported to be at 95°C by dilatometric [42], at 120°C by viscoelastic [43], and at 190°C by shear modulus [44] measurements. For gelatin samples containing 9.6% moisture, the T_g values were reported at −85°C, 130°C, and 180°C by measuring mechanical loss properties [45]. There are also reports citing T_g values of 175°C and 180–200°C for uncross-linked and cross-linked gelatins [41, 46, 47]. All of these values indicate the complex structure of gelatin. In this study, the first T_g of the GS samples was observed at 70°C (Fig. 3). For the GS-GA1 and GS-GA3 samples, this value shifted to 77°C, giving an endothermic peak at 90°C, mainly as a result of cross-linking with GA. With GS-GA1 and GS-GA3, a second endothermic peak was observed at 120°C and 125°C, respectively. This second endothermic absorption may be caused by the removal of bound water (at 120°C or 125°C). Two endothermic absorption peaks, at 222°C and 247°C, were observed for all samples. The one at 222°C may be related to the conversion of reinforced rubbery structures to rubbery states and the peak at 247°C could be the beginning of the degradation of gelatin structures.

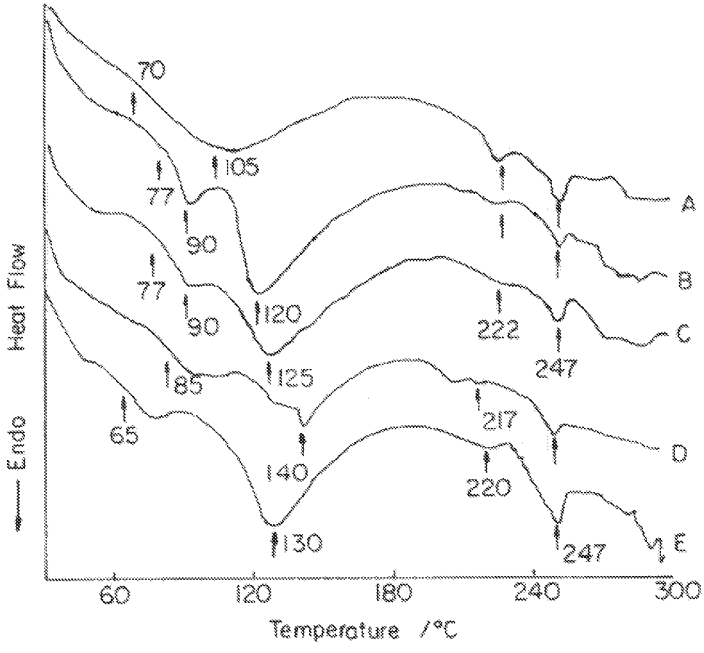

Figure 3. DSC thermograms: (A) GS; (B) GS-GA1; (C) GS-GA3; (D) GS-EDAC4; and (E) GS-EDAC6.

Similar thermal behavior was observed for the samples cross-linked with EDAC (Fig. 3). The first T_g , which corresponds to the movements of soft segments, shifted from 70°C to 85°C and 65°C, giving endothermic peaks at 95°C and 80°C for GS-EDAC4 and GS-EDAC6 samples, respectively. The endothermic peaks observed with the same samples at 140°C and 130°C may be related to the removal of bound water and the peaks observed around 220°C may be related to the conversion of reinforced rubbery structures to rubbery states, similar to the GA cross-linked samples.

For the GS-F and GS-EDAC1 samples, DSC thermographs were not obtained, since these samples demonstrated very fast degradation (as fast as the uncross-linked GS samples) in aqueous media.

Mechanical properties

The tensile strength and modulus of elasticity of the sponges are presented in Figs 4 and 5. GS samples (containing no cross-linker) had tensile strength and elastic modulus values of 0.0135 MPa and 0.6451 MPa, respectively. The modulus of elasticity increased significantly, up to 0.855 MPa ($p < 0.001$), with an increase in the amount of glutaraldehyde (Fig. 5). This is the result of the formation of additional amide bonds with GA. On the other hand, the tensile strength values did not demonstrate a significant change upon varying the amount of GA (0.0135 MPa, $p < 0.747$).

The sponges cross-linked with EDAC demonstrated a different trend. As mentioned previously, carbodiimide helps bond formation between nitrogen and car-

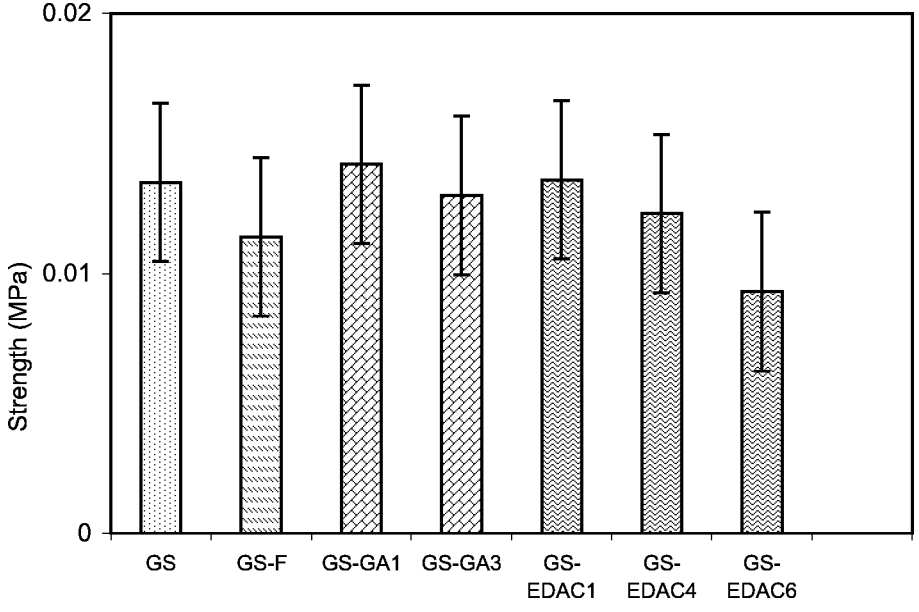

Figure 4. Tensile strength values of the gelatin sponges.

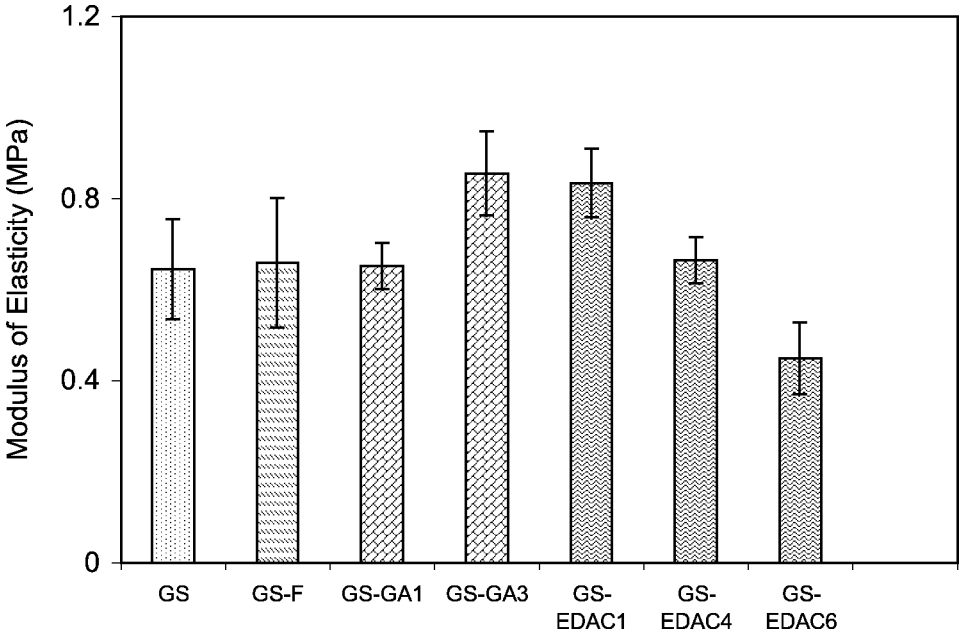

Figure 5. Modulus of elasticity values of the gelatin sponges.

bonyl residues of gelatin, but it does not remain in the structure. It was observed that with an increase in the EDAC content, the tensile strength values decreased from 0.0135 MPa to 0.0093 MPa ($p < 0.0278$) (Fig. 4). The modulus of elasticity values demonstrated first an increase and then a decrease. The highest value of the modulus of elasticity was obtained for the GS-EDAC1 sample (0.8343 MPa). With further addition of EDAC, the modulus of elasticity values decreased to 0.4500 MPa ($p < 0.0001$) (Fig. 5). These unexpected results could be explained by the plasticizing effect of the residual free carbodiimide or eliminated substituted urea in the sponge structures. For the fructose-containing gelatin sponges, modulus of elasticity and tensile strength values of 0.659 MPa and 0.0114 MPa, respectively, were obtained.

The mechanical properties of the gelatin sponges containing GA, EDAC or fructose cross-linkers were found quite close to each other.

Degradation studies

The type and the amount of cross-linker used in the production of medical materials are very important. Very low amounts of cross-linkers may cause rapid degradation, while high amounts may be toxic. The extent of cross-linking determines the stability of the biodegradable material and it can be evaluated by measuring the time required for complete solubility. Although dissolved amounts of gelatin do not give quantitative information about the extent of cross-linking at the molecular level, reduced solubility can be accepted as an indication of the level of cross-

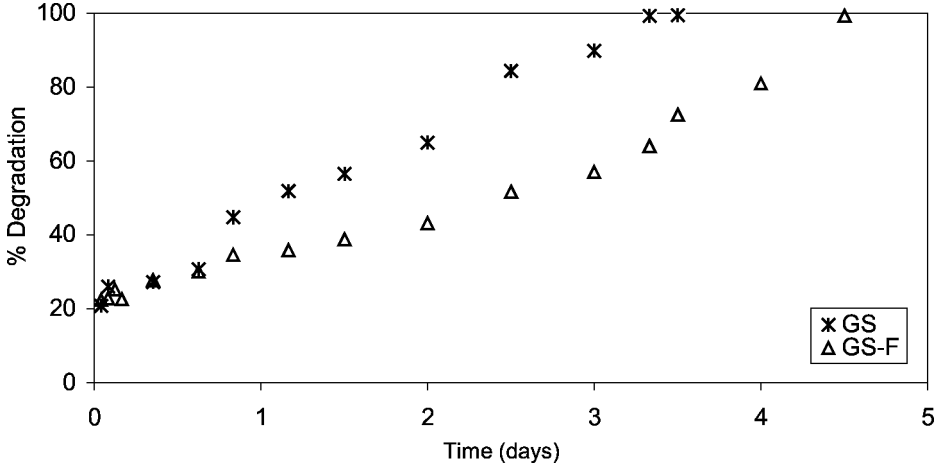

Figure 6. Degradation curves of the GS and GS-F samples.

linking. Therefore, the amount of dissolved gelatin can be used in the quantification of matrix degradation.

Percent degradation profiles of the gelatin sponges are given in Figs 6–8. Depending on the type and the amount of cross-linker used, the time for complete dissolution of the gelatin sponges varied from hours to days. For GS samples (containing no cross-linker), high degradation rates were observed. Almost half of each sponge was degraded within the first day and degradation was complete in 3.5 days (Fig. 6). The degradation rates of the GS-F samples (cross-linked with fructose) were also quite high and they demonstrated a similar behavior to that of GS samples (Fig. 6). For these samples, 30% of the sponge was degraded in 12 h and degradation was complete in 4.5 days. This rapid degradation could be a result of the high solubility of fructose in an aqueous medium, leading to rapid swelling and rapid degradation.

For the GS-GA1 samples, 50% and 100% degradation was observed on the 18th and 24th days, respectively. For the GS-GA3 samples, the same degradation profile was obtained on the 22nd and 28th days, respectively (Fig. 7). An increase in the amount of glutaraldehyde, from 0.25 mmol to 0.50 mmol, slightly delayed complete degradation from 24 days to 28 days.

Samples prepared with EDAC demonstrated very different degradation behavior, depending on the amount of cross-linker (Fig. 8). GS-EDAC1 degraded completely in 4 days, showing that the covalent linkages produced by the addition of 0.026 mmol of EDAC were not strong enough for long-term stability. When the amount of EDAC was increased, the sponges became more stable and the time required for complete degradation was longer. For the GS-EDAC4 and GS-EDAC6 samples, 10% degradation was observed on the seventh and tenth days, respectively. After that period, an increase in the degradation rates was observed for both samples and complete degradation was obtained on the 19th and 28th days, respectively.

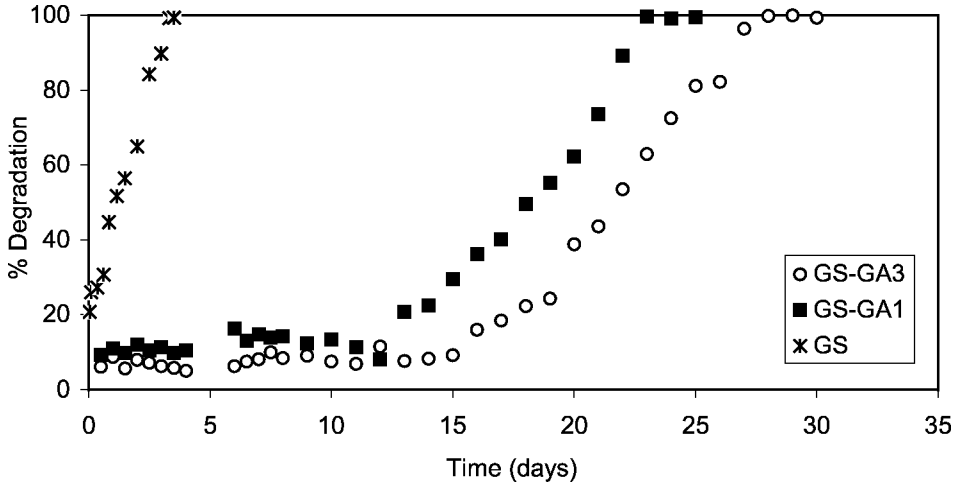

Figure 7. Degradation curves of the GA cross-linked samples.

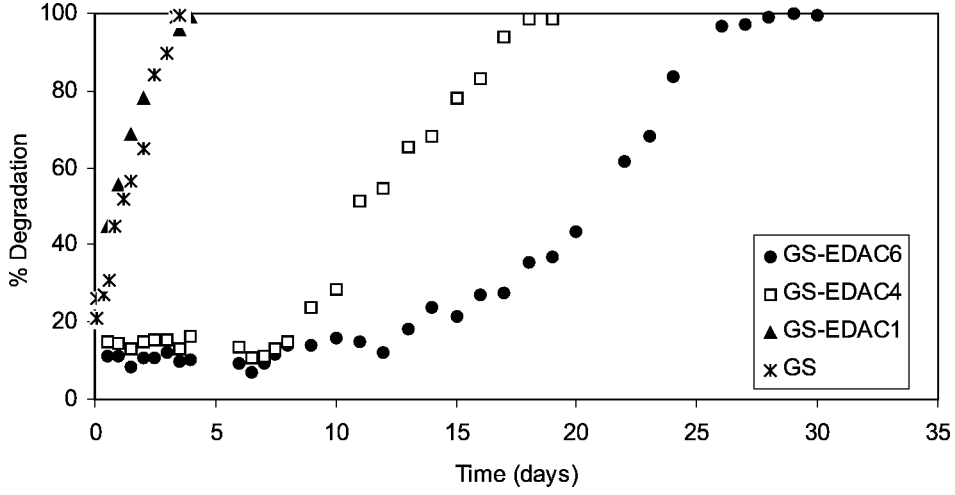

Figure 8. Degradation curves of the EDAC cross-linked samples.

On the basis of these results, it can be concluded that the type and the amount of cross-linker substantially affect the stability and it is possible to control the period in which the gelatin sponges are stable by adding various amounts of cross-linking agents, such as glutaraldehyde or carbodiimide.

Cytotoxicity

The potential source of cytotoxicity of a chemically cross-linked biomaterial may be the residues of the unreacted cross-linking agent and/or the substances leaching out as the matrix degrades. Cytotoxicity testing of chemical substances can be accomplished by either *in vivo* or *in vitro* experiments. Nishi *et al.* reported that

the results obtained in their *in vitro* experiments correlated well with those acquired in *in vivo* experiments in the cytotoxicity testing of chemical substances [48]. The cytotoxicity of a biomaterial can be evaluated *in vitro* by incubating the biomaterial in the presence of suitable target cells. If the material is cytotoxic, the target cells will be killed and the number of surviving cells will be inversely related to the cytotoxicity. Quantitative evaluation of cell survival can be carried out counting the cells after staining with trypan blue or by using the tetrazolium salt reduction assays (MTT) [49, 50].

The British Standard BS5736 part 10 (method of test for toxicity to cells in culture of extracts from medical devices) for dressing materials requires a rather simple *in vitro* testing procedure. This involves soaking the dressing in a specified culture medium for 24–72 h and then preparing a range of dilutions of this extract. One of the three specified cell lines (or any other cell fitting certain criteria) is exposed to the test agent for 24 h. The cells are then fixed, stained, and scored for survival on a 5-point scale, where 0, 1, 2, 3, and 4 stand for 0%, 0–25%, 25–50%, 50–75%, and 75–100% cell death, respectively. Although this method is not capable of detecting growth-promoting or differentiating effects, it is designed as a fast screening method of cytotoxicity [51].

In this study, the effects of different cross-linkers present in gelatin sponges on the viability and morphology of fibroblast cells were investigated *in vitro*. Cytotoxic effects of the cross-linked gelatin sponges on a human skin fibroblast cell line (HSAn2) were tested by immersing the sponges in the culture medium. Although this method is rather basic, the system allows the detection of toxicity, resulting from direct leakage of degradation products from the materials and products produced by the cell–sponge interactions.

The results for the cytotoxicity of the gelatin sponges are presented in Fig. 9 and Table 2. All the sponges, except GS-GSA1, demonstrated some cytotoxicity at varying degrees after 48 h of incubation with fibroblasts. For the control group (containing no sponge), the generation number (n), and doubling time were found to be 1.9601 and 24.48 h, respectively; the cell morphologies are shown in Fig. 9A. For the GS-containing plate, the value of n was found to be 0.8775, which is even lower than that of the control group. This unexpected result can be explained by the increase in viscosity of the feeding medium because of the fast degradation of uncross-linked GS samples, so that the higher viscosity of the medium prevents the diffusion of nutrients to the cells, causing a decrease in the number of viable cells. On the other hand, the n values for the GS-F samples were found to be even lower than those of the GS samples; this can be explained similarly to the diffusion limitations created on the nutrients. By visual inspection and from the percent death values, these two sponges were found to induce lower cell growth than the control (Figs 9B and 9C).

The generation number for the GS-GA1-containing medium was very close to the value of the control and the cells proliferated properly with normal morphology (Fig. 9D). It is apparent that the amount of GA in the GS-GA1 sponges does not

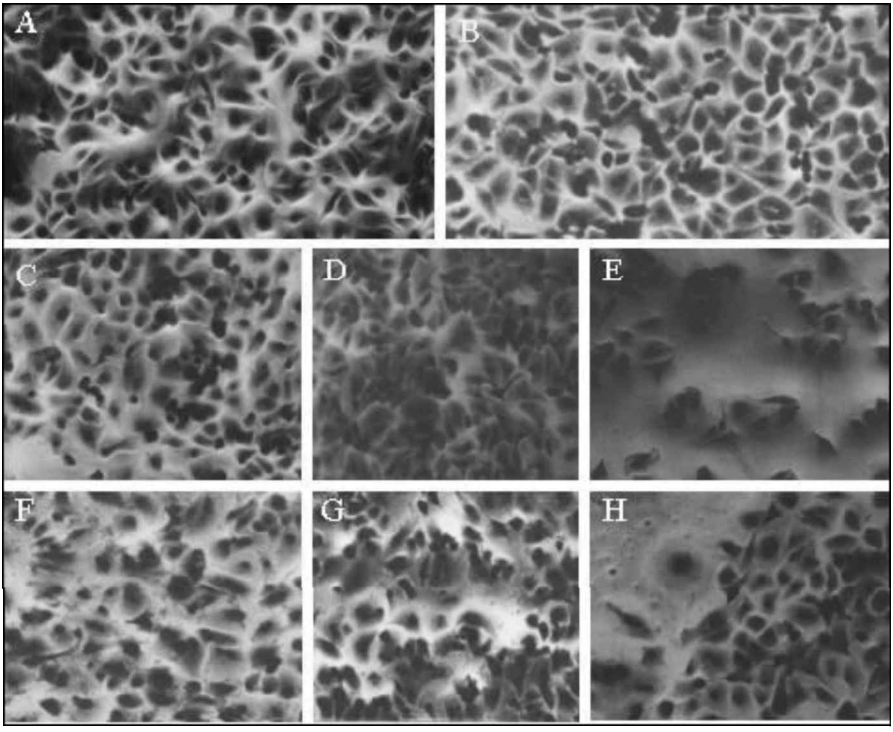

Figure 9. Microscopic examination of the cells after 48 h of exposure to gelatin sponges. (A) Control plate; (B) GS; (C) GS-F; (D) GS-GA1; (E) GS-GA3; (F) GS-EDAC1; (G) GS-EDAC4; (H) GS-EDAC6.

Table 2.
Cytotoxicity results of the gelatin sponges

Sample	Cross-linker (mmol)	Generation No. (*n*)	Doubling time (*h*)	Death (%)
Control	—	1.9601	24.48	—
GS	—	0.8775	54.70	52.7
GS-GA1	0.25	1.9764	24.29	0
GS-GA3	0.50	0.2740	175.18	68.8
GS-EDAC1	0.026	0.5462	87.88	62.4
GS-EDAC4	0.26	0.5462	87.88	62.4
GS-EDAC6	1.30	0	—	91.8
GS-F	0.42	0.7395	64.91	57.0

affect the viability of fibroblasts. However, when the GA content was increased from 0.25 mmol to 0.50 mmol, a significant drop in *n* was observed, demonstrating a moderate cytotoxic effect in GS-GA3 (Fig. 9E).

The effect of the amount of EDAC on the viability and morphology of the cells can be seen in Figs 9F–9H. Similar *n* and percent growth values were obtained for the GS-EDAC1- and GS-EDAC4-containing media. In the GS-EDAC6-containing medium, mortality after 48 h was found to be 91.8%. Since no generation was

observed and the cell numbers were even lower than the initially added values, the generation number was defined as zero. The GS-EDAC6 samples demonstrated a very high cytotoxic effect on cell cultures. In the literature, EDAC is reported to be non-toxic, because it helps bond formation between the C and N groups of gelatin and then leaves the structure by forming a urea derivative [1, 2]. It has also been reported that collagen gels prepared by the incorporation of chondroitin-6-sulfate (20%) enhanced the growth of keratinocytes but further treatment with EDAC inhibited fibroblast growth by 45% [52]. It has also been reported that extensive cleaning and washing of carbodiimide-treated gels are needed to remove the growth inhibitory effect of EDAC [53]. However, in this study, an extensive cleaning process did not apply to the prepared sponges, since they take highly hydrated, soft jelly-like forms and lose their spongy forms upon washing. Since the washing process could not be carried out, it is quite possible that urea derivatives remained in the structure and caused toxicity. The amount of cross-linker in the GS-GA1 and GS-EDAC4 samples was almost the same, but the viability of the cells and the cell morphologies in GS-GA1 were much better than those of the GS-EDAC4-containing medium. The important point is the amount of cross-linker that reacts with gelatin. It can be concluded that GA is more reactive than EDAC, so that even when similar amounts of GA and EDAC were added to the gelatin solutions (as in the GS-GA1 and GS-EDAC4 samples), more GA would go into the sponge structure by forming cross-links. Secondly, urea derivatives of EDAC might stay in the porous structure after the cross-linking reaction and might diffuse into the culture medium, causing inhibition of cell growth. Therefore, in the preparation of sponge forms of gelatin where further washing could not been carried out, GA can be considered a more suitable cross-linker than EDAC. The results obtained in this study show that the cytotoxicity of gelatin sponges depends on the type and the amount of cross-linker agent and the cross-linking process.

CONCLUSION

The effects of different cross-linkers used in the preparation of gelatin sponges on the viability of fibroblast cells were investigated in *in vitro* systems. Glutaraldehyde was found to be a better cross-linker than EDAC when used at a low concentration, because GA cross-linked sponges demonstrated a better cell morphology and a higher cell viability than those of the EDAC cross-linked ones. These samples have great potential for various applications in biomedicine, such as wound dressings or tissue engineering scaffolds.

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