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Evaluation of the release rate of bioactive recombinant human epidermal growth factor from crosslinking collagen sponges

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Abstract The purpose of this study was to prepare recombinant human epidermal growth factor (rhEGF) collagen sponges for topical applications and investigate the effects of different types of crosslinked collagen sponges as platforms for the controlled release of rhEGF. The microstructure and the drug release rates of collagen sponges were modified through treatment with different types (glutaraldehyde (GTA), genipin and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)), different concentrations of crosslinking agents and various preparation conditions. A controlled release profile was observed for the crosslinked collagen sponges as compared to the non-crosslinked ones. The results indicated that the GTA crosslinked sponges have the most potent controlling effect. As the amount of GTA increased, a greater rigidity of the collagen sponge structure combined with a lower hydrophilicity was observed, leading to a decreased drug release rate and an increased water uptake. This study also demonstrated that a good correlation was obtained for in vitro release rates of rhEGF using the power model. The crosslinked rhEGF collagen sponges showed a successful delivery of rhEGF in bioactive form to stimulate cell proliferation.

1 Introduction

The epidermal growth factor (EGF) is a single-chain polypeptide of 53 amino acid residues (M_w = 6045) containing three intramolecular disulfide bridges required for biological activity [1]. EGF stimulates the initiation of DNA synthesis, cell replication, activation of RNA and protein synthesis [2]. In addition, EGF can inhibit gastric acid secretions in the stomach [3], enhance the proliferation and keratinization of epithelial tissues [4, 5] and accelerate wound healing [6, 7]. Due to its wound healing properties, EGF is an attractive candidate for a therapeutic drug. Studies have demonstrated that topical applications of EGF promote wound healing in healthy and impaired healing animals [8–11]. Since Carpenter and co-workers first reported that for a mitogenic effect of EGF, a continuous exposure of the target cells to EGF was required for a minimum of 6–12 h, maintaining an effective topical concentration at the wound site for a certain period of time has become vital in the application of EGF [6, 8, 9, 12–14].

Collagen is a major constituent of the connective tissue and is potentially a highly useful biomaterial. It has characteristics that are suitable in medical application, such as biodegradability and weak antigenicity, and it has been used in resorbable surgical sutures, hemostatic agents, and wound dressings for many years [15–17]. Several factors, such as the type used, the amount and the concentration of the collagen, treatment with cross-linking agents, as well as the preparation methods, can affect both the structural integrity of the collagen sponges and the diffusion rate of the drugs through this form of application collagen [18–21]. In general, inducing additional crosslinking agents into the native collagen by means of chemical or physical treatment is the main factor influencing the modification of the collagen structure and the rate of drug release from the collagen matrices.

In this study, collagen sponges were prepared for topical applications. The sponges' release rates were modified by treating the collagen with various types and concentrations

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of crosslinking agents, such as glutaraldehyde (GTA), genipin and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), and by employing different preparation conditions. An in vitro controlled release study was conducted to investigate the mechanism of recombinant human epidermal growth factor (rhEGF) release from the different degree of crosslinked collagen sponges. The in vitro cell stimulation ability of rhEGF-collagen sponges was also evaluated to determine their clinical usefulness.

2 Materials and methods

2.1 Materials

Highly purified recombinant human epidermal growth factor (rhEGF of more than 99% purity) was purchased from Pepro Tech Inc. (Rocky Hill, NJ). Glutaraldehyde (GTA), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), collagenase (*Clostridium histolyticum*, EC 3. 3. 24. 3) and trinitrobezenesulfonic acid (TNBS) were obtained from Sigma Chem. Co. (St. Louis, MO). 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2*H*-5-tetrazolio]-1,3-benzene disulphonate (WST-1), was purchased from MK400, Takara Biomedicals (Japan). Type I collagen from pig skin was purchased from the Animal Technology Institute Taiwan (Miaoli, Taiwan). Genipin was obtained from Wako Pure Chemical Industries (Osaka, Japan). ^{125}I-labeled rhEGF was purchased from Amersham Pharmacia Biotech (UK). All other reagents used are commercially available and of standard analytical grade.

2.2 Preparation of collagen sponges incorporating rhEGF

One percent collagen was dissolved in 0.05 M acetic acid. Various types and concentrations of crosslinking agents were added and kept at 4 °C for 24 h. The solutions were then cast into Teflon molds. Immediately after being frozen to –20 °C, the sponges were freeze-dried. The resulting crosslinked sponges were washed three times with double distilled water (DDW) at 37 °C, so as to exclude any residual crosslinking agents. This was followed by freeze-drying to obtain crosslinked collagen sponges. After that, 10 μL solution containing 10 ng/mL rhEGF and 1 μCi/mL ^{125}I-labeled rhEGF in phosphate-buffer saline (PBS, pH 7.4) was dropped on the collagen sponges. The sponges were left for 12 h at 4 °C. The rhEGF solution soaked completely into the collagen sponge, because the solution volume was much less than what was theoretically required for equilibrated swelling of the sponge. As a control, an rhEGF-free collagen sponge was prepared by the addition of PBS without rhEGF.

2.3 Characterization of the rhEGF-collagen sponges

2.3.1 Determination of the degree of crosslinking

The extent of the crosslinking of the collagen sponges was determined by UV assay of non-crosslinked ε-amino groups before and after crosslinking [22]. Following the reaction with 0.5% TNBS, the collagen sponge was hydrolysed with 6 M HCl, and extracted with ethyl ether. The absorbance of the diluted solution was measured at 346 nm using a U2000 spectrophotometer against a blank. The crosslinking degree could then be obtained from the differences between the absorbance values before and after the crosslinking. The equation is as follows:

$$\text{Cross - linking degree } (\%) = [1 - (\text{absorption}_s/\text{mass}_s) \times (\text{absorption}_{ncl}/\text{mass}_{ncl})^{-1}] \times 100, \quad (1)$$

where s is the sample and ncl is non-crosslinked.

2.3.2 Water-binding capacity

To test the water uptake ability, the GTA-crosslinked sponges were immersed separately in distilled water at room temperature for 5 min. They were then removed from the water, hung over a table for 5 min to drip dry until no more water dripped from them, and then weighed. The water uptake of the collagen sponges was calculated using the following equation:

$$\text{Water uptake } (\%) = [(W_s - W_d)/W_s] \times 100, \quad (2)$$

where W_d is the weight of the dry sponge and W_s is the weight of the swollen sponge.

2.3.3 Mechanical properties

The mechanical properties of a sponge in tissue-engineering application is of great importance due to the necessity of structural stability to withstand stress incurred during culturing in vitro and implanting in vivo [23]. Stress–strain analysis of sponges was performed by uniaxial measurements using a NEXYGEN^{TM} mechanical tester (Lloyd Instruments Ltd., England). The elastic modulus was calculated from the inclination of the stress-strain graph, and the tensile strength was monitored at the rupture of the sponges.

2.3.4 Morphological characterization

The cross-sections and surface structures of crosslinked collagen sponges with various degrees of porosity were

characterized under the scanning electron microscope (SEM, JEOL TSM-5300, Japan) after crosslinking with various amounts of GTA using an image analyzing system. Sponges were mounted on stubs and coated with an ultra-thin layer of gold in a Polaron E5100 SEM coating system. Specimens were studied with a JEOL TSM-5300 SEM apparatus operating at 10 kV. The image analysis program was used to determine the average diameter of the pores, and at least 40 pores were assessed.

2.4 In vitro rhEGF release test

In vitro release studies of rhEGF from collagen sponges were performed using collagenase (*C. histolyticum*, EC 3. 3. 24. 3), and each collagen sponge incorporating ^{125}I-labeled rhEGF was used as a trace. Briefly, collagenase (0.2 mg/mL) was dissolved in 0.1 M tris-buffer (pH 7.4) containing 10 mM $CaCl_2$ and 0.05 mg/mL sodium azide. Sponges (15 mm in diameter) were placed in 2 mL of enzyme solution and incubated at 37 °C under constant shaking at 50 rpm. The release medium was changed every 24 h and the radioactivity was measured on a gamma counter. The percentage of rhEGF released from the collagen sponge was calculated, and the samples were tested in triplicate.

2.4.1 Release kinetics

To determine the possible release mechanism, drug release from collagen sponges was fitted to the following power model [24]:

$$M_t/M_\infty = kt^n \quad (3)$$

where M_t/M_∞ is the fractional drug release percentage at time t, and k is a constant related to the properties of the drug delivery system and n is the diffusional exponent which characterizes the drug transport mechanism. The value of n = 0.45 indicates Case I (Fickian) diffusion, $0.45 < n < 0.89$ indicates anomalous (non-Fickian) diffusion and n = 0.89 indicates Case II transport.

2.5 In vitro cell proliferation ability of the rhEGF-collagen sponge

The in vitro cell proliferation ability of the sponges was observed on the basis of cell morphology, viability and proliferation. Fibroblast proliferation was assessed by measuring mitochondrial dehydrogenase activity using tetrazolium salt WST-1 according to the manufacturer's protocol. Sponges (15 mm) were placed in 6 cm culture dishes. Fibroblasts of 1×10^5 were seeded onto the sponges and cultured for 3 and 7 days at 37 °C/5% CO_2 in proliferation medium consisting of 10% (v/v) fetal bovine serum, 4 mM glutamine, penicillin (100 U/mL) and streptomycin (100 μg/mL) in Dulbecco's Minimum Essential Medium (DMEM). After the addition of 20 μL WST-1 per well, and subsequent incubation for 2 h at 37 °C/5% CO_2, the absorption (optical density) was measured by an enzyme-linked immunosorbent assay (ELISA) reader at 450 nm. Enzyme activity ratio was expressed as a percentage of the ratio of cells grown on the non-crosslinked collagen sponges. The cell proliferation stimulation index (CPSI) was calculated according to:

$$\begin{aligned} \text{CPSI} = &(\text{enzyme activity of cells in cultures with crosslinked sponge})/ \\ &(\text{enzyme activityof cells in culture without crosslinked sponge}) \times 100\% \end{aligned} \quad (4)$$

2.6 Data analysis

Results were expressed as the means ± standard deviation ($n = 3$). The statistical difference was assumed to be significant when $P < 0.05$ by the two-sided Student's *t*-test.

3 Results and discussion

3.1 rhEGF release from collagen sponges

Figure 1 shows the release profiles of rhEGF from collagen sponge at 37 °C in PBS with/without collagenase solution. Under the condition of without collagenase, the burst release value from collagen crosslinked with 0.2% GTA is 7.2 ± 2.3%. In addition, up to about 8 ± 2.4% rhEGF is released within 1 day. Relatively little further release was observed over the next 2-day period. We suppose that under the in vitro non-degradation conditions, rhEGF was initially released by diffusion. Generally speaking, since collagen is enzymatically degraded, low final release values are expected in the absence of any enzymes. Therefore, collagenase was employed for the model of the in vitro rhEGF release study.

The microstructure and characterization of collagen matrices are modified by treatment with a crosslinking agent [18–21]. Therefore, the influence of the types and the concentrations of the crosslinking agents and the preparation conditions on the structures and characteristics of collagen sponges, and the rhEGF release from collagen

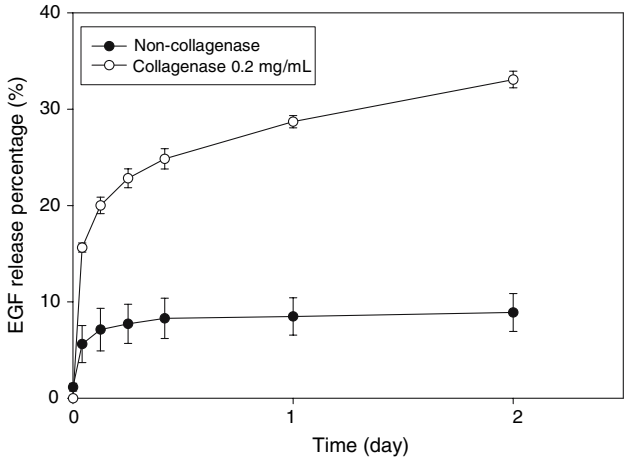

Fig. 1 With/without collagenase for the release of rhEGF from collagen sponges. ●: without collagenase; ◯: with 0.2 mg/mL collagenase

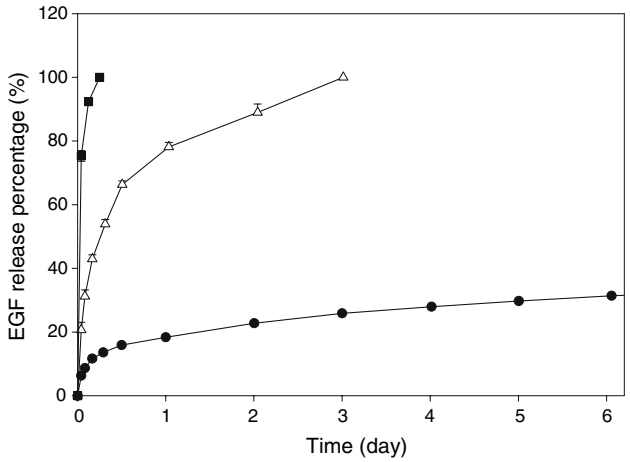

Fig. 2 The drug release patterns from crosslinked collagen sponges treated with various types of crosslinking agents. ●: 0.05% GTA; Δ: 0.05% genipin; ■: 0.05% EDC

sponges, was investigated in this study. Three different types of crosslinking agents, GTA, genipin and ECD were used to prepare crosslinked collagen sponges. The rhEGF release patterns from collagen sponges are shown in Fig. 2. The drug release rate from crosslinked collagen sponges treated with ECD was the fastest, followed by collagen sponges treated with genipin and GTA, respectively. The ECD crosslinked collagen showed no release control effect, which was probably due to the fact that ECD increased the water-solubility and lowered the viscosity of collagen (data not shown). GTA crosslinked collagen showed the most potent release control effect than the other two (EDC and genipin). Earlier reports [19, 20] indicated that the control effect of collagen matrices was mainly due to the reduction in the hydrophilicity of collagen fibrils, with the result that most of the hydrophilic ε-amine groups of lysine residuals

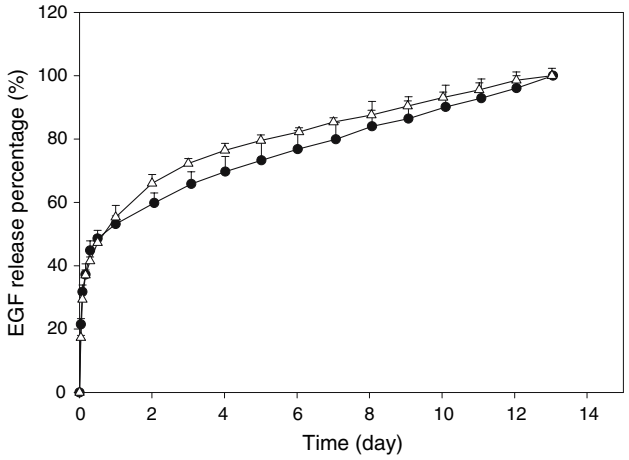

Fig. 3 The drug release patterns from crosslinked collagen sponges with 0.01% GTA prepared at 4 and 25 °C. Δ: 4 °C; ●: 25 °C

are crosslinked with a hydrophobic crosslinker of GTA, resulting in decreased drug release rates.

The influence of crosslinking temperatures (4, 25 and 37 °C) on drug release was evaluated, as shown in Fig. 3. Results show that collagen with a 0.05% concentration of GTA can not form a sponge at 37 °C. In addition, variations in the preparation temperatures of 4 and 25 °C of crosslinked collagen sponges did not appear to modify the drug release (P (0.05). We presume that raising the preparation's temperature will enhance the crosslinking efficiency, but too high a temperature will destroy the protein.

The effect of crosslinking times on drug release is shown in Fig. 4, indicating that the release rate decreases with increases in the crosslinking time for up to 24 h. Results show no significant difference between after 24 h and after 48 h ($P > 0.05$). Figure 5 shows the effect of various concentrations of GTA on rhEGF release from the collagen sponges. It was found that as the amount of GTA increased, the release rate decreased. These results could be attributed to the fact that the hydrophobic characteristics of collagen sponges increase as more GTA is added, resulting in decreased permeability [19, 20]. In addition, it was found that the collagen sponges exhibited sustained release for 1–3, 7–21 and >30 days when the GTA concentrations were below 0.0085%, from 0.01% to 0.02%, and above 0.025%, respectively (See Figs. 5A and B).

The release mechanism of rhEGF from collagen sponges was evaluated by a power equation [24]. The n values of all sponges containing various amounts of GTA were from 0.3034 to 0.3764 (Table 1), indicating that the release mechanism is not affected by the concentration of GTA. An n value below 0.5 means that its release is faster than that of the Fickian diffusion in the early stage of release, and suggests that the release of rhEGF from the sponges occurs by means of a combined mechanism consisting of diffusion through a swollen matrix, diffusion through

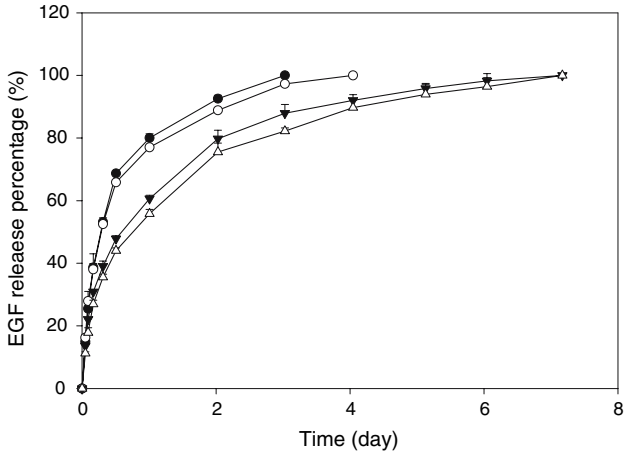

Fig. 4 The drug release patterns from crosslinked collagen sponges with 0.01% GTA at various crosslinking times. ●: 6 h; ◯: 12 h; ▼: 24 h; Δ: 48 h

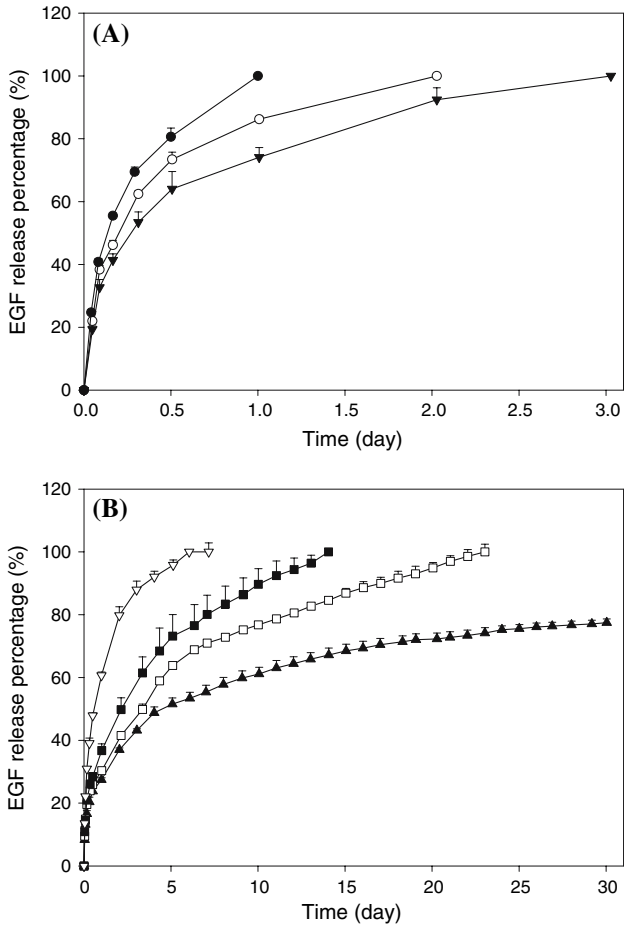

Fig. 5 The drug release patterns from crosslinked collagen sponges with various amounts of GTA. (**A**) ●: 0.005%; ◯: 0.0065%; ▼: 0.0085%, (**B**) ▽: 0.01%; ■: 0.015%; □: 0.02%; ▲: 0.025%

Table 1 The release parameter of crosslinked collagen sponges treated with various amounts of GTA

Glutaraldehyde (%)	k	n	r
0.0050	91.49	0.3437	0.9569
0.0065	92.01	0.3392	0.9337
0.0085	73.96	0.3704	0.9774
0.010	59.09	0.3034	0.9882
0.015	37.83	0.3764	0.9989
0.020	33.47	0.3548	0.9969
0.025	28.52	0.3276	0.9971

water-filled pores, and erosion of collagen from the surface [17, 18]. The initial quick release would be beneficial, as it would achieve therapeutic concentrations of the drug in the minimum amount of time, and the constant release later on would provide a sustained release of the drug.

3.2 Morphological characterization of crosslinked collagen sponges

The photographs of the surfaces and cross-sections of collagen sponges containing various amounts of GTA are shown in Fig. 6, revealing that the collagen sponges consist of pores and fibers. As expected, denser structures of collagen sponge were obtained by increasing the concentration of GTA for the same concentration of 1% w/w collagen. Fiber thickness increased and pore size decreased from 100 μm to 20 μm, while the amount of crosslinking agent increased from 0 to 0.025%. This result is in accordance with previous findings of a higher resistance of the collagen matrices with increasing amounts of crosslinking agents [17, 20].

3.3 The degree of crosslinking of sponges

The effect of GTA concentration on the degree of crosslinking between sponges is shown in Table 2. All the collagen sponges crosslinked with GTA show an increasing tendency towards crosslinking, from 40.01 to 91.75 with the increase of GTA concentration ranging between 0.0085 and 0.2% ($P < 0.05$).

3.4 Mechanical properties

The mechanical parameters evaluated from the stress-strain analysis are reported in Table 3 as a function of GTA concentration. As shown in Table 3 the stiffness and Young's modulus increased with the increase in GTA concentration.

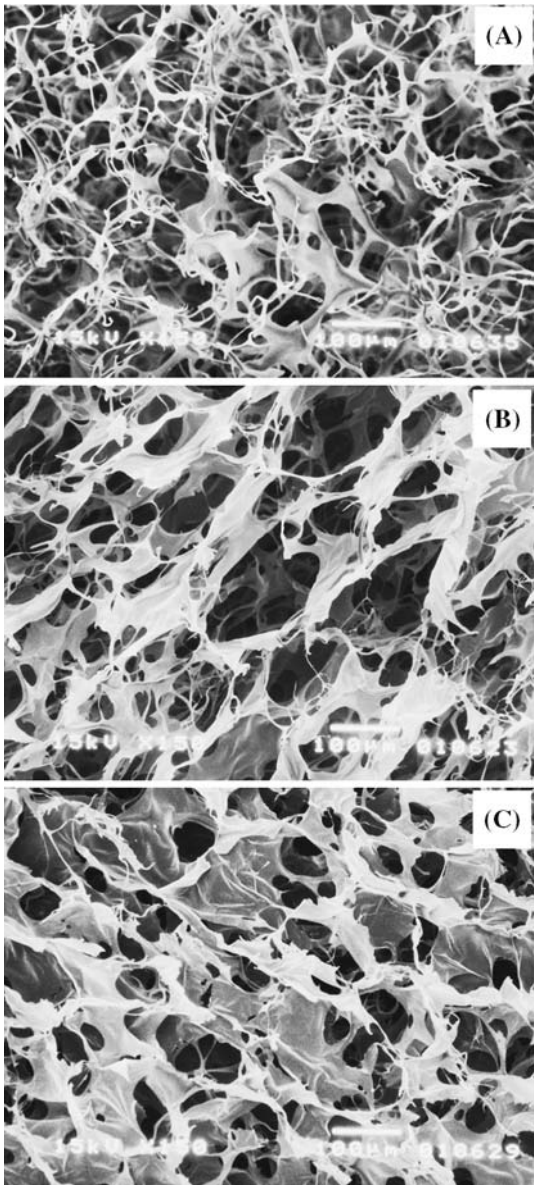

Fig. 6 Scanning electron micrographs of collagen sponges with and without various amounts of GTA. (**A**) 0; (**B**) 0.0085%; (**C**) 0.025%

Table 2 Degrees of crosslinking of collagen sponges with different concentrations of GTA

Concentration (%) of glutaraldehyde	Degree (%) of crosslinking
0.0085	40.01
0.010	52.23
0.015	64.70
0.020	68.09
0.025	82.69
0.050	84.46
0.10	88.56
0.20	91.75

3.5 Water-binding capacity

The extent of the swelling of the crosslinked collagen sponges was obvious as the amount of GTA increased (Fig. 7). Moreover, a straight line relationship was observed between the swelling and the amount of GTA used. The result was confirmed by SEM photographs (Fig. 6), indicating that a more rigid structure and a greater

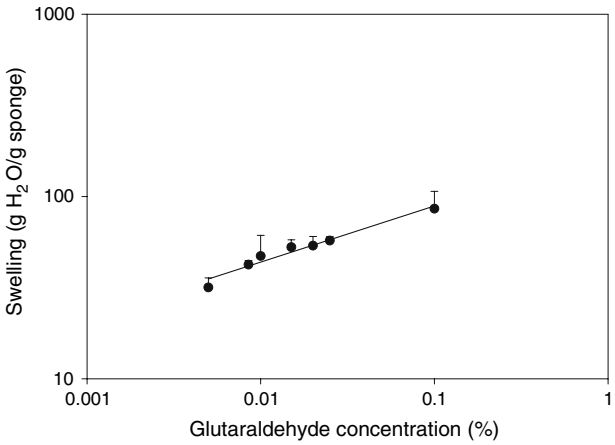

Fig. 7 The swelling of collagen crosslinked sponges with GTA concentrations from 0 to 0.25%

Table 3 Stress–strain analysis of the crosslinked collagen sponges as a function of GTA concentration

Glutaraldehyde conc. (%)	Maximum load (N)	Stiffness (N/m)	Young's modulus (MPa)
0.00	1.31 ± 0.15	746.60 ± 83.5	0.39 ± 0.05
0.0085	1.34 ± 0.18	1372.82 ± 139.5	0.51 ± 0.09
0.010	1.44 ± 0.31	1579.03 ± 197.4	0.66 ± 0.21
0.015	1.92 ± 0.34	1681.40 ± 149.6	0.96 ± 0.30
0.020	2.36 ± 0.19	1870.20 ± 519.1	1.39 ± 0.13
0.025	3.19 ± 0.05	2213.93 ± 351.2	1.78 ± 0.25
0.20	3.89 ± 0.37	4538.07 ± 623.3	3.51 ± 0.56

entanglement of fibrils was obtained in collagen sponges treated with higher amounts of GTA, resulting in an increase in water uptake.

3.6 In vitro cell stimulation ability of the rhEGF-collagen sponge

The WST-1 test is a non-radioactive cell proliferation assay that identifies living cells, and is based on the cellular conversion of a tetrazolium salt into a formazan product, a chromaphore, which can be quantified by ELISA. The ELISA reader was used to assay the cell stimulation ability of the rhEGF-collagen sponge containing varying concentrations of GTA.

The values of the cell proliferation stimulation index showed positive effects on the proliferation of fibroblasts for collagen sponges treated with GTA of 0–0.025% (Fig. 8A). The cell morphology of fibroblasts was observed every day during culturing, forming normal spindle-shaped cells on all sponges. No difference in morphology was found between cells cultured on non-GTA-crosslinked and on crosslinked sponges. The results show that the bioactivity of rhEGF released from crosslinked collagen sponges with GTA contents from 0 to 0.025% show a significant stimulation effect to fibroblasts, and might therefore be applied to topical wound healing ($P < 0.05$). Figure 8B shows the proliferation of fibroblasts in the presence of rhEGF-loaded/unloaded sponges. The results show that a significantly increased stimulation activity of fibroblasts in the rhEGF-loaded sponges. This is especially so when the rhEGF concentration is at 10 ng/sponge which provides the best stimulation effect ($P < 0.05$).

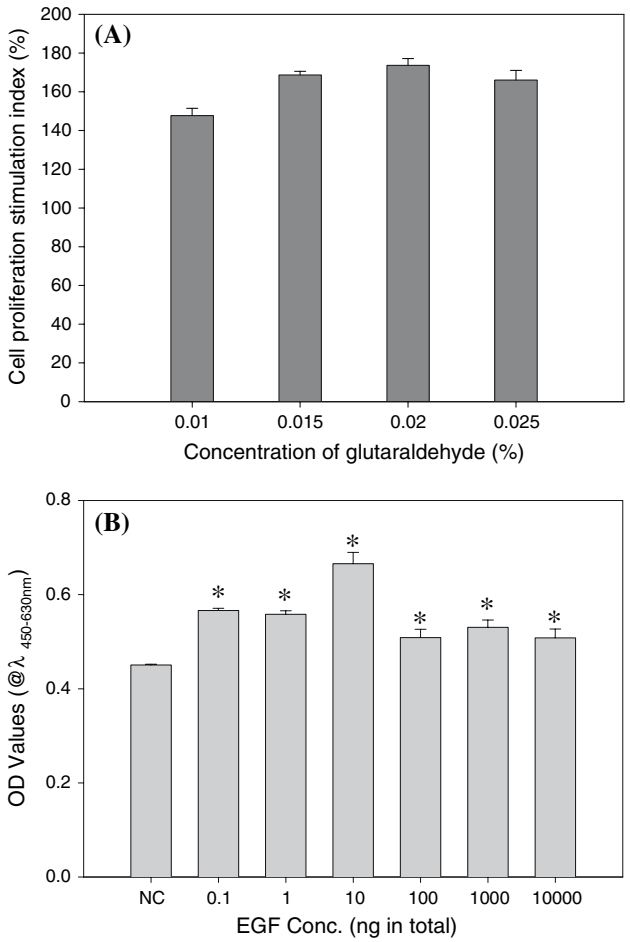

Fig. 8 (**A**) The cell proliferation stimulation index of 10 ng rhEGF-collagen sponges treated with different concentrations of GTA between 0.01 and 0.025% at day 7. (**B**) The absorption (optical density) of the cell proliferation when treated with different concentrations of rhEGF at day 7. Start (*) indicates the significance at $P < 0.05$ compared to the value of the NC group. (NC: unloaded sponges)

4 Conclusions

Crosslinked rhEGF-collagen sponges can be useful for controlling the release of rhEGF. Results have shown that upon increasing the amount of GTA, the microstructure of collagen sponges becomes more rigid, and the hydrophilicity is reduced, resulting in a decreased drug release rates and an increased water uptake. A good correlation was obtained for in vitro release rates of rhEGF from crosslinking collagen sponges using the power model. Finally, crosslinking collagen sponges with concentrations of GTA as high as 0.025% showed no evidence of cytotoxicity and delivery rhEGF in bioactive form to stimulate cell proliferation.

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