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Studied on a novel human keratinocyte membrane delivery system *in vitro*

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The culture of keratinocytes on flexible membranes has been proposed as a means to simplify, accelerate and improve the efficiency with which proliferating cells are delivered to full thickness or non-healing skin defects. The purpose of this article was to study the ability of chitosan-gelatin manbranes to facilitate the growth of human keratinocytes. The membranes with different chitosan contents were studied. The surface properties of chitosan-gelatin membranes were investigate by SEM, and water contact angle test. The mechanical property of the membranes was tested. Data implied that gelatin could make the membranes more flexible and hydrophilic than chitosan membranes, which may regulate the seeded cells behavior. Loading human keratinocytes on chitosan-gelatin membranes, cells attachment, spread, and growth were investigated by light microscopy, SEM, and MTT test. The results suggested that the adhesion and proliferation of keratinocytes seeded on chitosan-gelatin membranes were same as on tissue culture plate, in which gelatin could modify the interaction between keratinocytes and chitosan membranes. Therefore, chitosan-gelatin membrane is a good candidate for keratinocytes delivery system.

1. Introduction

In a third degree burn, the conventional treatment is to remove a split-thickness skin sheet from the patient's own body and graft it on the burned area. In cases of burns covering 50% or more of the body surface area, this method is limited by the availability of donor site. To circumvent this deficiency cultured keratinocytes sheets have become a recognized method of skin replacement [1–3]. But there are some practical difficulties made such that they are not routinely used by many burn surgeons [4, 5]. First, it is need 3–5 weeks to product sufficient areas of grafts, during which time the patients are susceptible to sepsis, the major cause of death in badly burned patients who have survived the initial injury. Second, released from the plastic with the enzyme dispase, the confluent sheets are difficult to handle, very delicate and vulnerable to disruption. Strategies to make cultured grafts more appealing are aimed at reducing the time spent in culture and improving the handling properties [6].

A keratinocytes membrane delivery system is a means of support, chiefly mechanical one, for culturing keratinocytes in culture or in the early post-transplantation period. These methods can be used to successfully transfer pre-confluent cultures to patients within 3–4 days [7]. Several techniques have been described using a polyurethane membrane [8], acellular porcine dermis [9] and hyaluronic acid membrane as the delivery systems [10, 11].

Chitosan is a polysaccharide obtained from N-deacetylation of chitin, which exhibits numerous interesting physicochemical and biological properties [12]. Chitosan is a biocompatible polymer. *In vivo* tests have proved that chitosan-based biomaterials do not have any inflammatory or allergic reaction following implantation, injection, topical application or ingestion in the human body [13]. Chitosan can be hydrolyzed by lysozyme [14]. Its degredation leads to the release of aminosugars, which can be incorporated into glycosaminoglycans and glycoproteins metabolic pathways, or excreted. Chitosan is an antimicrobial polysaccharide having an amino group at the C-2 position of the glucosamine residue [15].

Gelatin is obtained by a denatured partially of the fibrous insoluble protein collagen, which is widely found in nature and is the major constituent of skin, bones and connective tissue. Being a protein, gelatin is composed of a unique sequence of amino acids.

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Structurally, gelatin molecules contain repeating sequences of glycine-X-Y triplets, where X and Y are frequently proline and hydroxyproline [16]. While collagen, also known to have wide biomedical applications, expresses antigenicity in physiological condition, gelatin is known to have no such antigenicity [17]. Recently, gelatin has shown to exhibit activation of macrophages and high hemostatic effect [18]. Gelatin is completely resorbable *in vivo*; its physicochemical properties can be suitable modulated due to the existence of many functional groups. Gelatin can form polyelectrolyte complex (PEC) with chitosan at the suitable pH value [19].

According to these properties, chitosan and gelatin appears to be good candidates for keratinocytes delivery system. In this study, the effect of chitosan content in the chitosan-gelatin membranes on the biological and mechanical properties was considered. Human keratinocytes adhesion and proliferation on these different culture supports were investigated.

2. Materials and methods

2.1. Preparation of chitosan-gelatin PEC membranes

The basic materials are chitosan (degree of deacetylation >85%, China) and gelatin (Sigma). Chitosan with concentration of 1.5% were prepared by dissolution in 0.2 M acetic acid. Gelatin was dissolved in distilled water at about 50°C. The warm gelatin solution was added to the chitosan solution prepared above and the mixture was stirred for 30 min. The solution was added in to the wells of tissue culture plate, and dried under 37°C. After the solvent was evaporated in the air, the formed film was washed with aqueous 1% NaOH solution to remove residual acetic acid and then with a large amount of distilled water.

2.2. Mechanical properties of chitosan-gelatin membranes

The mechanical properties of the membrane strips were determined at room temperature with a strain rate of 10 mm/min on a mechanical tester (Testometric, M500-25KN). The swollen specimens were tested quickly while took out of water. The specimens were rectangular disks 4 cm × 1 cm × T, where T is the thick of the films. The fracture stress, elongation at break and Young's modulus were determined. The values were expressed as the means ± standard error ($n = 3$).

2.3. Contact angles against water

The surface morphology of the chitosan-gelatin membranes were observed on a scanning electron microscope (SEM) after the specimens were coated with an ultrathin layer of gold in a coating apparatus. The contact angles against water of all membranes were measured using a goniometer (Model JY-82; Chengde Experimental Matchine Plant, China). Deionized distilled water was dropped onto the surface of the membrane before measuring. Each experiment was repeated 3 times and the average value was taken. The values were expressed as the means ± standard error ($n = 3$).

2.4. Human keratinocytes culture

Proliferative human keratinocytes were isolated from abundant fresh human skin from plastic surgical procedures with 24 U/100 ml Dispase (Sigma) and refrigerated at 4°C for 16–24 h. Epidermal cells were then isolated into a single cell suspension by treatment with 0.05% Trypsin and 0.02% EDTA (Gibco) at 37°C for 30 min, resuspended and expanded in number in 75 cm^2 tissue culture plates (Falcon) in serum –free keratinocytes culture medium kit (Gibco) at 5% CO_2 and 37°C. The culture medium was changed 3 times a week. Subconfluent primary cultures were washed twice with phosphate-buffered saline (PBS) and incubated with trypsin-EDTA for 15 min at 37°C to detach the cells. The effect of trypsin was then inhibited by adding the complete medium at room temperature and the cells were centrifuged and resuspended in above medium for reseeding and growing in new culture plate.

2.5. Keratinocytes adhesion tests

Second passage keratinocytes were isolated into a single cell with suspension 0.25% Trypsin after having reached subconfluence of 60–70% in the culture flask. Chitosan-gelatin membranes have different chitosan content were prepared as described above, and sterilized by contact with 70% ethanol for 24 h, rinsed in a sterile phosphate-buffered saline (PBS) solution. Before used, the membranes were equilibrated in the culture medium for 2 h. Keratinocytes were counted by blood plate reader and the cell suspension was diluted with fresh medium to the final concentration, then seeded directly onto the membranes at a density of 5×10^4 cell/cm^2. Then they were incubated at 37°C for 4 h in a humidified atmosphere of 5% CO_2. After the removal of the culture medium, the materials were washed twice with a sterile PBS solution to eliminate free cells. Then, a MTT test, modified from the method of Mosmam [20], was carried out to quantify the viability of the cells, which adhered on chitosan-gelatin membranes. Briefly, 100 μl MTT (5 mg/ml, Sigma) solution was added to each well. After 4 h incubation at 37°C, DMSO (Sigma) 200 μl was added to dissolve the formazan crystals. The solution was jogged by the shaker for 15 min. The absorbance of the solution was measured at 490 nm using an ELISA (Biorad). It was compared to the cell adhesion observed on the tissue culture plates.

2.6. Proliferation tests of keratinocytes grown on the chitosan-gelatin membranes

The membranes were treated as above. Second passage keratinocytes were seeded on the membranes with different chitosan content. The cell loaded membranes were incubated at 37°C in a humidified atmosphere

containing 5% CO_2. Medium was replaced every 2–3 days of tissue culture. At timed intervals, three separate samples from each membrane group were rinsed twice with a sterile PBS solution to remove dead cells. Then a MTT test was performed to quantify the cell viability as above.

Experiments were run in triplicate per sample. All data were expressed as means ± standard deviation(SD) for $n = 3$. Single factor analysis of variance (ANOVA) technique was used to assess statistical significance of results. Scheffe's method was employed for multiple comparison tests at level of 95%.

2.7. Morphology of keratinocytes seeded on the membranes

As previously, keratinocytes were seeded on the membranes and cultured for 3 days to become subconfluent. Then the samples were washed with PBS buffer and fixed in the buffer containing 2.5% glutaraldehyde for 2 h at room temperature and for another 12 h at 4°C. After washing with PBS buffer to remove residual glutaraldehyde, the cell-seeded materials were dehydrated through a graded series of aqueous ethanol soaks. After the specimens were critically point dried and coated with an ultrathin gold layer, they were observed by a scanning electron microscopy (SEM, Hitachi S-3500N, Japan).

3. Result and discussion

3.1. Characterization of the membranes

Static water contact angles on chitosan-gelatin membranes with different chitosan content indicated the relative hydrophilicity of these surfaces. The results are shown in Fig. 1. All five membranes had contact angles little then 90°. The lower contact angle indicated higher hydrophilicity. As the weight rate of chitosan was decreased, the water contact angle of the membranes decreased ($p < 0.05$). The water contact angle on the chitosan-gelatin membranes demonstrated that mixing gelatin with chitosan could decrease the contact angle on the membranes. Therefore, the hydrophilicity would be improved.

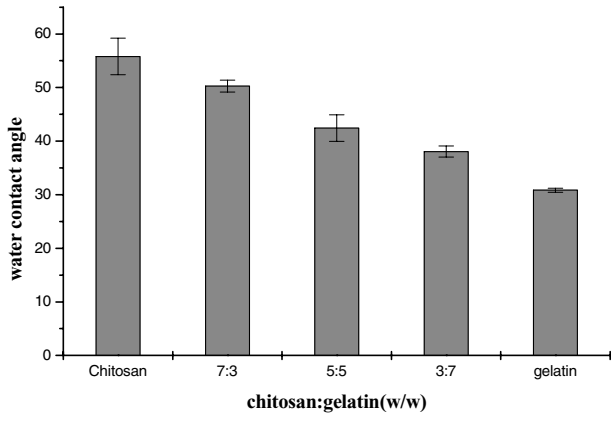


Figure 1 Water contact angle of chitosan-gelatin membranes with different chitosan content.

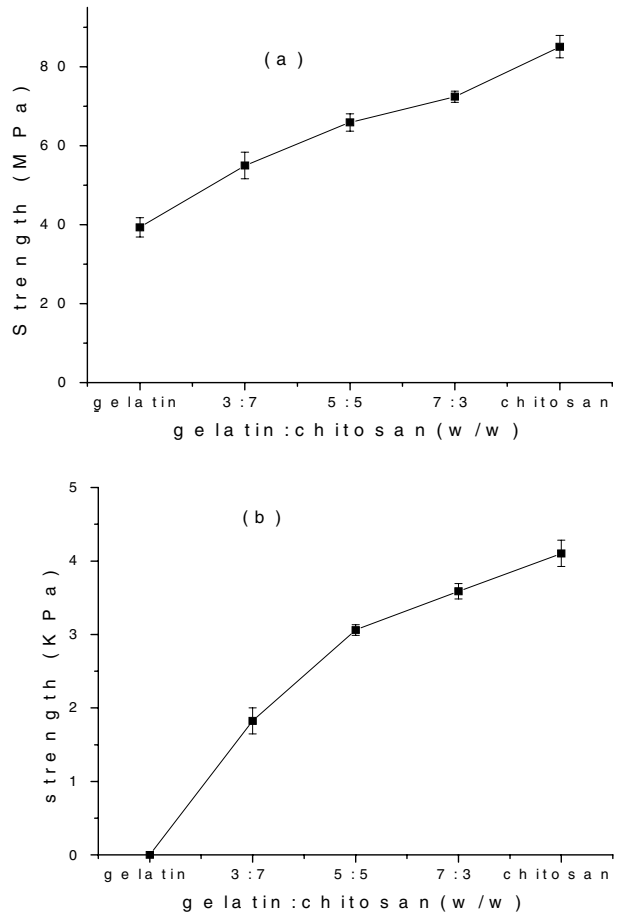


Figure 2 Tensile strength of chitosan-gelatin membranes with different chitosan content, in the dry state (a) and the swollen state (b).

3.2. Mechanical property of the membranes

Introducing flexible gelatin into rigid chitosan molecular chains decreased the tensile strength, both in the dry and swollen states, at the same time improved the extensity of the membranes, as shown in Fig. 2. The lower tensile strength of the swollen specimens compared with the dry state was attributed to the plasticization which is the effect of the absorbed water. Data indicated that gelatin molecular containing ─COOH and ─NH_2 groups could absorb more water than chitosan bearing ─OH and ─NH_3 groups. As gelatin was added into the chitosan, the water contents in the membranes increased significantly.

It was worth noting that there was a maximum in the curve of elongation versus the ratio of gelatin to chitosan, seen in Fig. 3. The maximum occurred at about 5:5, which corresponded to optimum compatibility between chitosan and gelatin, which has been verified by our previously studying [19].

3.3. Cell adhesion test

After human keratinocytes were seeded on the chitosan-gelatin membranes for 4 h, the MTT test was carried out. The result was displayed in Fig. 4. Data came from the average adsorbance from three different assays. They have been compared to the culture plates. The result showed that chitosan membranes could adhere much more cells than the others ($p < 0.05$). While the ratio

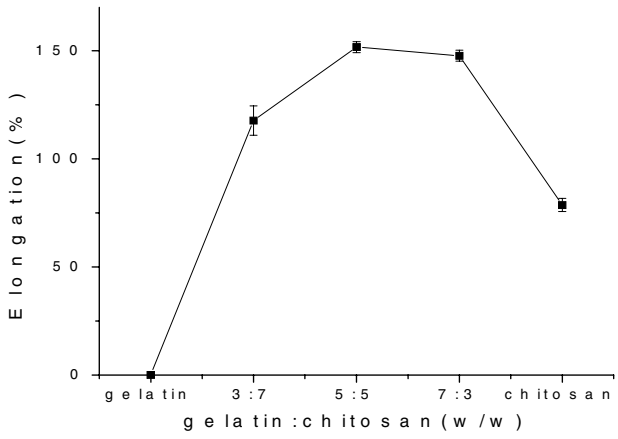


Figure 3 Elongation of chitosan-gelatin membranes with different chitosan content in swollen state.

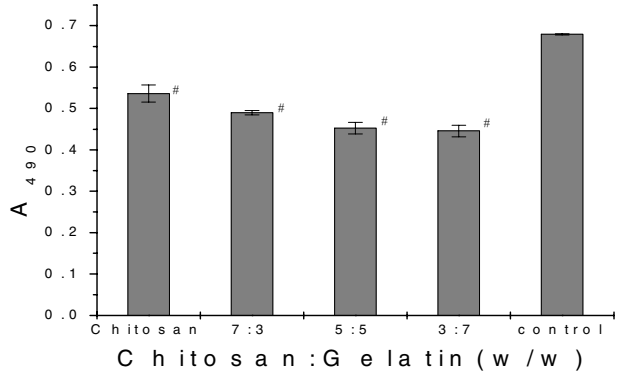


Figure 4 Adhesion of human keratinocytes on chitosan-gelatin membranes with different chitosan content. ($n = 3$). $^{\#}p < 0.05$ relative to chitosan membranes.

of chitosan to gelatin change from 7:3 to 3:7, there was no significant different among them. The number of cells attached to the culture plates was the highest one.

According to previous studies, the rate of growth, proliferation, and differentiation of cells on a material depend on the successful initial attachment, which affected by the characteristics of the surface of the membranes, such as, wettability and charge. Chitosan membranes had higher adhesion number could be explained that even at a pH close to 7, there remained sufficient amount of cationic sites on the chains of chitosan allowing electrostatic interactions with the negative charges of the surface of cell membranes [21]. Chitosan was liner polysaccharides. Cation charged chitosan could interact with an anionic polyelectrolyte, such as gelatin with isoelectric point $pH_{iso} = 4.5$, to form polyelectrolyte complexes (PEC). The amino groups on the chitosan interacted with the carboxy group of gelatin. So, while gelatin was added in the system, the charge density of the membranes decreased, and then weakened the cell adhesion.

On the other hands, from the results of water contact angle test, incoporated gelatin could improve hydrophilicity of the membranes. Webb *et al.* [22] reported that hydrophilicity of a membrane was a factor affecting the surface energy, which could affect the degree of cell adhesion. Cell attachment, spread, and cytoskeletal organization are greater on hydrophilic surface in comparison to hydrophobic one [23]. This can explain the fact that there was no significant difference while gelatin content enhanced.

Cell adhesion also seemed to be related to the surface morphology of the membrane. From the SEM photo, the surface of the membranes was extremely smooth, shown in Fig. 5. So we did not consider this parameter in the test.

3.4. Cell proliferation

Fig. 6 showed light microscopy photographs of keratinocytes cultured on the membranes after 24 h and 11 days of incubation. The keratinocytes grew well on chitosan-gelatin membranes. Compared to the photo in Fig. 6c and d, one could find that the keratinocytes cultured on chitosan membranes become aging.

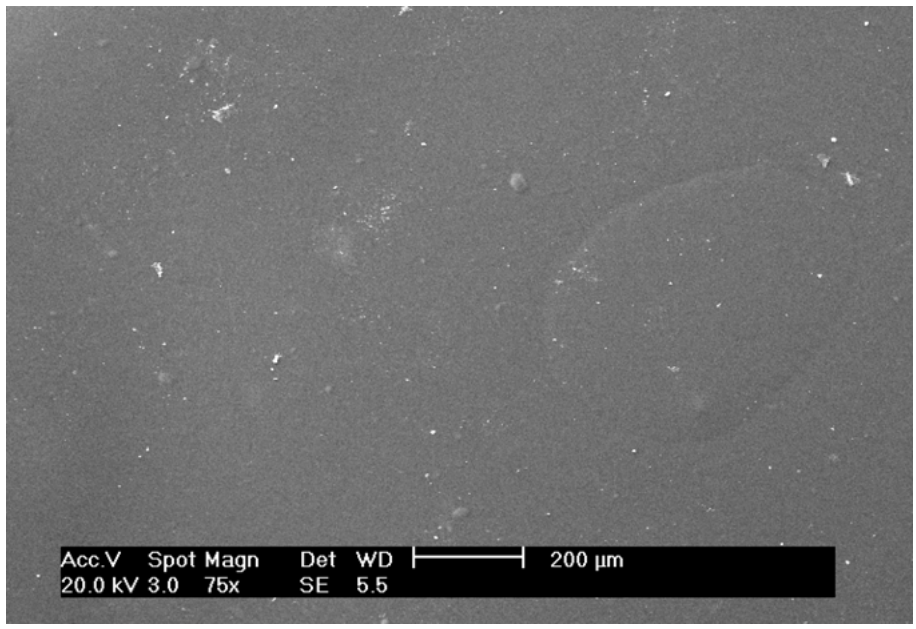
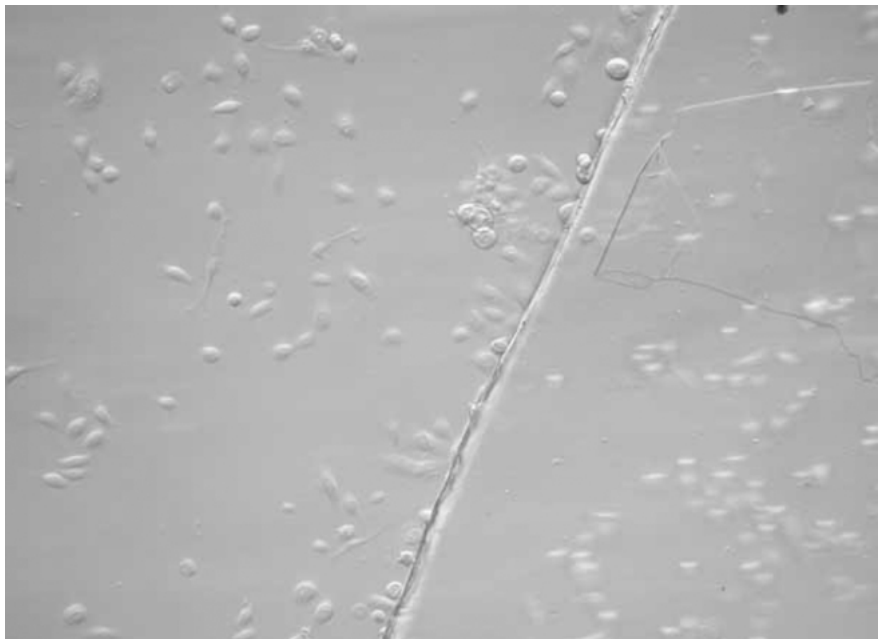
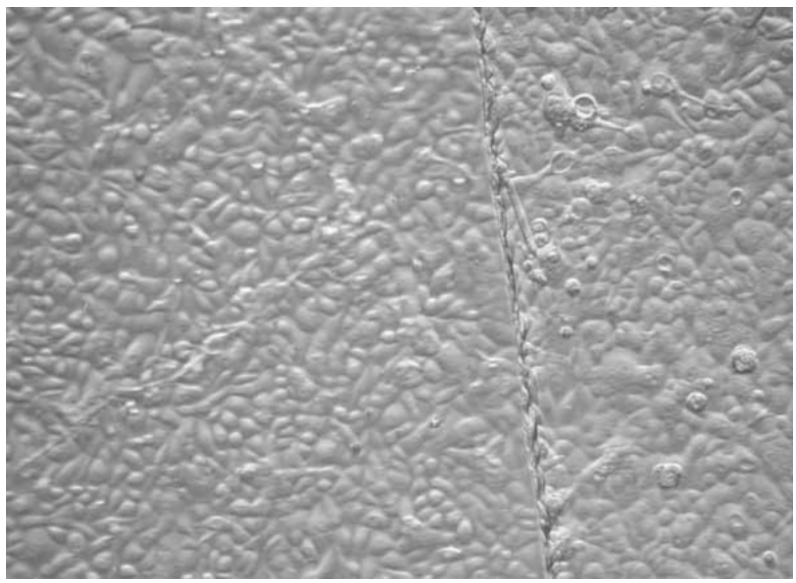


Figure 5 SEM photographs showing the morphological aspect of chitosan-gelatin membranes.



(A)



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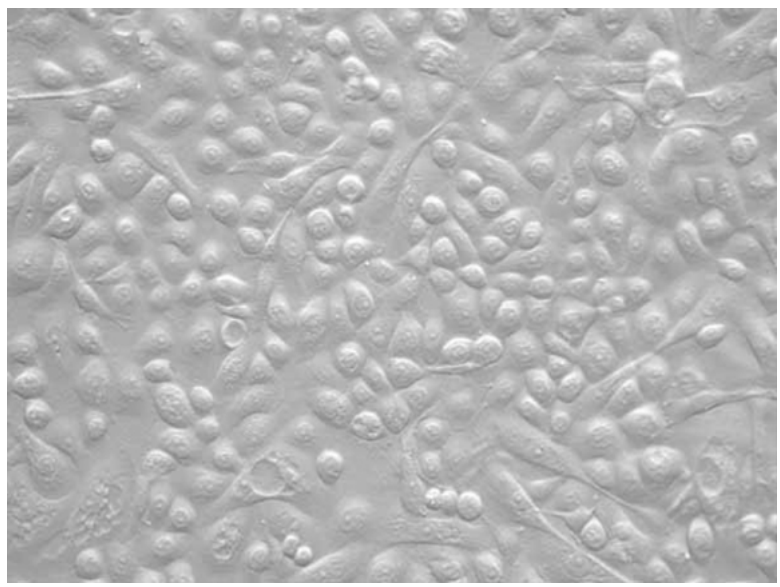

(C)

Figure 6 Light microscopy photographs of keratinocytes seeded at 3×10^4cells/cm^2 on chitosan-gelatin membranes. A: after 24 h (×40), B: after 11 days (×40), D: after 11 days (×100), C: after 11 days on chitosan membranes (×100). (*Continued*)

(D)

Figure 6 (*Continued*).

To test the proliferation of cells on the membranes, second passage human keratinocytes were cultured on the membranes with different chitosan contents for 11 days. The curves of cell proliferation were shown in Figs 7 and 8 respectively. The keratinocytes on all chitosan-gelatin membranes grew well, and had the same proliferation curve as the control. However, the number of the cells seeded on the chitosan membranes drooped. Data implied that gelatin could promote cell proliferation on chitosan membranes significantly ($p < 0.05$).

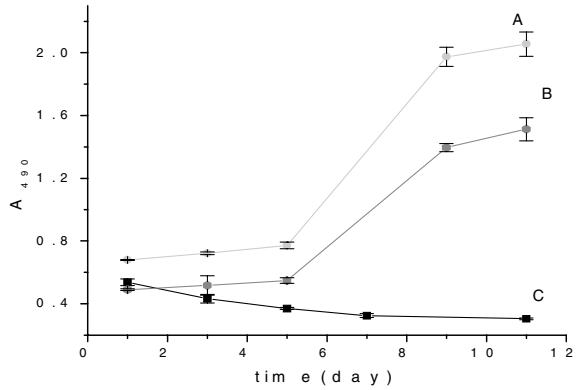

Figure 7 Growth of human keratinocytes on chitosan-gelatin membranes (B, C) and tissue culture flasks (A). B: chitosan/gelatin = 7/3, C: chitosan.

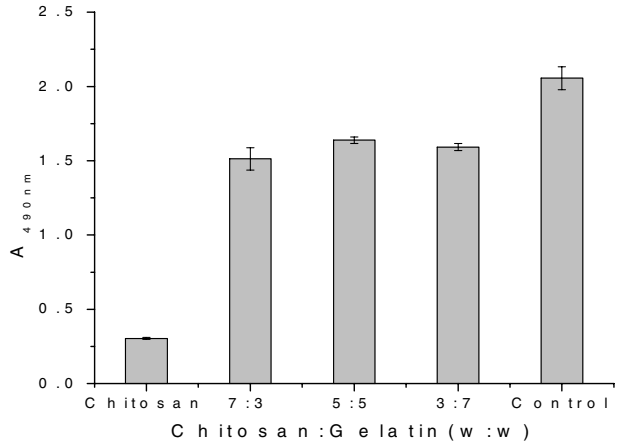

Figure 8 Cell viability (A_{490}) of keratinocytes seeded on chitosan-gelatin membranes with different chitosan content for 11 days. ($n = 3$). $P < 0.01$ related to chitosan membranes.

It appeared that keratinocytes did not proliferate on the chitosan membranes, confirmed by the morphology and MTT test performed on the 11th day of incubation (Fig. 8). The low values obtained revealed poor cell viability. Chitosan membranes had high cell adhesion but low cell proliferation. Izume *et al*. [24] suggested that an extremely high adhesion of fibroblasts on chitosan-based materials would alter their growth. Thus, chitosan seemed to be cytostatic towards human keratinocytes: it is not cytotoxic, but inhibits cell proliferation. This result may due to the degree of acetylation and the source of chitosan [25].

In contrast, keratinocytes grew well on chitosan-gelatin membranes as same as on tissue culture plate. We assumed that this result was related to the chemical components of gelatin such as repeated amino acid sequence, and the flexible property of the membranes. The amino acid sequence may affect keratinocytes growth on the membranes. On the other hand, the membranes became flexible while gelatin blend with chitosan. Mooney *et al*. [26] reported that the mechanical properties of materials to which cells are adherent can profoundly affect the function of the cells. The external mechanical forces are transmitted across the cell surface and through the cytoskeleton via transmembrane cell adhesion molecules such as integrin. Thus, from our test, human keratinocytes proliferated better on the 'soft' substrate than on the 'hard' one, the chitosan membrane.

3.5. Cell morphology

Fig. 9 shows the morphology of keratinocytes cultured on the chitosan-gelatin membranes for 24 h.

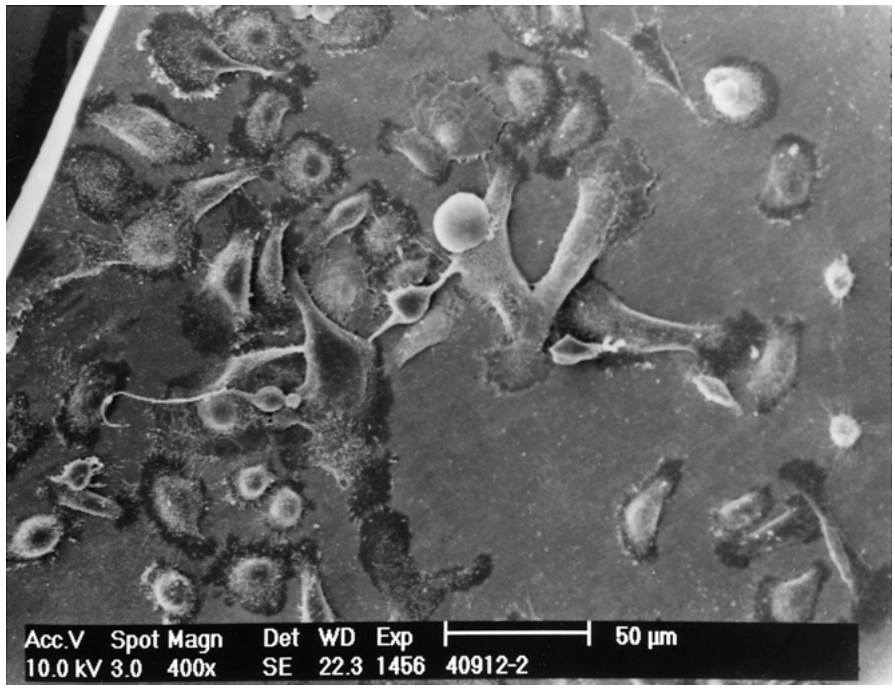

Figure 9 SEM photo of the morphology of keratinocytes cultured on the chitosan-gelatin membranes for 24 h.

The cellular behavior on a biomaterial is an important factor determining the biocompatibility of a biomaterial. After cells contact biomaterials, cells will undergo their morphological changes to stabilize the cell-material interface. The whole process of adhesion and spreading consists of cell attachment, filopodial growth, cytoplasmic webbing, flattening of the cell mass and the ruffling of peripheral cytoplasm progressing was in a sequential fashion [27]. It can be seen that the cultured cells show clear morphologies in different progress. In Fig. 9, the morphological type of keratinocytes on the surface of the membrane is in adhesion stage. At this time, SEM examination shows that most of keratinocytes with flattened morphology are in the stage of ruffling of peripheral cytoplasm on the surface of the membrane, only a few are still spherical with microvilli-like projections in appearance. Qualitatively, the chitosan-gelatin membrane is favorable for the human keratinocytes culture. Although exactly what stimuli are responsible for the observed phenomenon remains to be determined, the effect of the addition of gelatin into the chitosan membranes on the kerotinocytes culture must be involved.

4. Conclusion

In this study, a polyelectrolyte complex of chitosan and gelatin via interaction between a rigid polyaminosaccharide chitosan with flexible ampholytic gelatin chains was developed. Water contact angle test had shown the change of surface hydrophilicity, indicating chitosan-gelatin membranes were more wettable than chitosan one. While gelatin was mixed with chitosan, the hydrophilicity of the membranes could be increased due to polyelectrolyte complex formation. The mechanical properties of the membranes mostly provided by the rigid chain of chitosan, especially at the swollen state. The maximum elongation occurred at about the rate of chitosan to gelatin was 1:1.

Human keratinocytes affinity of chitosan-gelatin membranes, which was mainly evaluated by cell attachment and growth, was studied from photographs of cell culture and MTT results. The results of 24 h cell culture reflected mainly the attachment and spread of cells, whereas the results of 11 days reflected mainly cell growth. Chitosan membranes adhered more cells than the others. This result may be explained by the physicochemical properties of materials surface. Materials properties such as hydrophilicity and positive surface charges contribute to this physicochemical interaction. However, it appears that cells on the chitosan membrane were able to attach but unable to follow this attachment with spreading and growth. In contrast, when the membranes were blended with gelatin, the adhesion and growth of the kerotinocytes on them were much like the controls. Therefore, in physico-chemical and biological properties, chitosan-gelatin membrane is a good candidate for keratinocytes delivery system.

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