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Fibroblast interaction with carboxymethylchitosan-based hydrogels

Wanida Janvikul · Paweena Uppanan · Boonlom Thavornyutikarn · Rujiporn Prateepasen · Somporn Swasdison

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Abstract The interaction between L929 cells and carboxymethylchitosan (CM-chitosan)-based hydrogels, hydrogels from pure CM-chitosan and its blends, was examined in this study. Cytotoxicity of all materials was also assessed. The cellular morphology and behavior on the surfaces of the hydrogels were observed by scanning electron microscopy (SEM). The effects of various parameters, e.g., type and content of blended polymers, surface structure of hydrogels, and steaming condition used for the preparation of the hydrogels, on the cell-material response were investigated. The results of the cytotoxicity test revealed that all hydrogels were non-cytotoxic. The SEM micrographs demonstrated that the cells proliferated and spread onto a porous CM-chitosan sample. Better cell spreading was found on a flat surface of a CM-chitosan film. Rounded cells were observed when poly(vinyl alcohol) (PVA) was incorporated into CM-chitosan. Fewer cells were found when the content of PVA increased. Spherical clusters of the aggregated cells existed in the blends with ultra high viscosity carboxymethylcellulose (CM-cellulose). In contrast, with the use of low viscosity CM-cellulose, the cells appeared more spreading. The attached cells on the CM-chitosan film steamed at the highest temperature and longest period appeared to spread the most among all tested steaming conditions.

W. Janvikul (✉) · P. Uppanan · B. Thavornyutikarn
National Metal and Materials Technology Center,
Pathumthani 12120, Thailand
e-mail: wanidaj@mtec.or.th

R. Prateepasen
Scientific and Technological Research Equipment Center,
Chulalongkorn University, Bangkok 10330, Thailand

S. Swasdison
Department of Oral Pathology, Faculty of Dentistry,
Chulalongkorn University, Bangkok 10330, Thailand

Introduction

It is essential to evaluate in vitro biocompatibility of newly developed biomedical materials. Several techniques have been established to determine biosafety and biofunctionality of such materials [1]. The principal of biosafety encompasses both cytotoxicity and the complicated field of mutagenesis and carcinogenesis. Cytotoxicity can be assessed by qualitative and quantitative methods, e.g., direct contact test, agar diffusion test, elution test, MTT test, DNA analysis, and membrane integrity test [2]. Biofunctionality of biomaterials is investigated through functional characteristics of attached cells, e.g., anchoragement, attachment, adhesion, spreading and growth of the cells. The adhesion and proliferation of cells can be directly affected by the properties of adjoined biomaterials [3], so various materials were developed to promote their ability to support the attachment and proliferation of cells [4, 5]. In addition, it was known that the surface morphology of substrates essentially governed the cell behaviors [6, 7]. The proper selection of a suitable material surface would be markedly beneficial for the interaction of cells with a biomaterial.

Chitin is an abundant biopolymer obtained from renewable resources, while chitosan is a partially or fully deacetylated form of chitin. These biopolymers possess fascinating characteristics and find uses in a

variety of application fields, such as agricultural, biotechnological, and biomedical fields [8]. Clinical studies on chitin and chitosan have been well established. It was found that they could promote wound healing and reduce scar formation by stimulating human skin fibroblast proliferation [9]. Carboxy methylchitosan is a water-soluble chitin/chitosan derivative that was proved to be antibacterial and non-cytotoxic and has also found uses in biomedical applications [10, 11]. In addition, it could promote the proliferation of the normal skin fibroblasts [12].

In this study, the responses of L929 mouse fibroblasts to the hydrogels of pure sodium salt of carboxymethylchitosan (CM-chitosan) and its blends prepared by the steam treatment [13] and unpublished results] were evaluated. Various parameters such as the type and amount of blended polymers used, surface structure of hydrogels, and hydrogel-preparation condition were studied to elucidate their effects on the cellular morphology and behavior of the L929 cells on the hydrogels, using cell culture and scanning electron microscopy (SEM). The blended polymers used here were poly(vinyl alcohol) (PVA) and sodium salt of carboxymethylcellulose (CM-cellulose). The hydrogels were prepared in two different surface structures, i.e., porous (sponge) and dense (film) forms.

Materials and methods

Materials

Water-soluble CM-chitosan was directly prepared in our laboratory [13]. The molecular weight of the product was found to be as follows: Mw = 1,307,000 g/mol and Mn = 65,000 g/mol (determined by gel permeation chromatography with pullan as standard). PVA (Mw ≈ 30,000–70,000 g/mol) and two different grades of CM-cellulose (a highly purified ultra high viscosity grade and a highly purified low viscosity grade) were purchased from Sigma and Fluka, respectively, and used as received. De-ionized water was employed as a solvent in the fabrication of all hydrogels.

Preparation of CM-chitosan-based hydrogels in a sponge form

CM-chitosan and its blends with different weight ratios of CM-chitosan to PVA and CM-chitosan to viscosity-varied CM-cellulose (e.g., 80:20 and 60:40) were primarily dissolved in de-ionized water. Each viscous solution was subsequently poured into molds and lyophilized to produce water-soluble sponge-like pads. To obtain hydrogels, the sponges were then exposed to saturated steam at 115°C for 15 min.

Preparation of CM-chitosan hydrogels in a film form

To fabricate CM-chitosan hydrogel in a film form, the viscous solution of CM-chitosan was typically cast in a petri-dish, dried at 40°C overnight, and exposed to saturated steam at given temperatures and times, i.e., 100°C/10 min, 115°C/15 min, and 130°C/30 min.

Cell culture

The cell line used in the assay was L929 (ECACC No. 85011425), mouse connective tissue, fibroblast-like cells. The growth medium used was Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), together with penicillin (100 units/ml) and streptomycin (100 μg/ml). The cells were maintained at 37°C in a 5% CO_2 atmosphere. Once 80% confluence was reached, the cells were subcultured for cytotoxicity study and SEM observation. The hydrogels tested were sterilized by ethylene oxide gas.

Cytotoxicity test

The hydrogels were cut into small circular 5-mm diameter discs and saturated with growth medium. The discs were then placed in the middle of a 35-mm dish. L929 cells were seeded onto the dish at a density of 6×10^4 cells/dish and incubated for 48 h. Cell morphology and the toxic zone were evaluated by inverted phase contrast light microscopy after a 48-h exposure to the cells. The cells were stained with 0.01% neutral red in phosphate buffer saline (PBS) for membrane integrity. High-density polyethylene (HDPE) and natural rubber containing carbon black were used as negative and positive controls, respectively. Each sample was tested in triplicate, and the test was repeated twice.

Scanning electron microscopic study

The hydrogels in film and sponge forms were cut into square pieces and saturated with growth medium before being placed onto a 35-mm dish. Control was performed using a coverslip. L929 cell suspension (2×10^5 cells/dish) was directly seeded onto the surfaces of the samples. At 14-day incubation period, the samples with the attached cells were fixed with 2% (v/v) glutaraldehyde in 0.1 M phosphate buffer (PB) pH 7.2

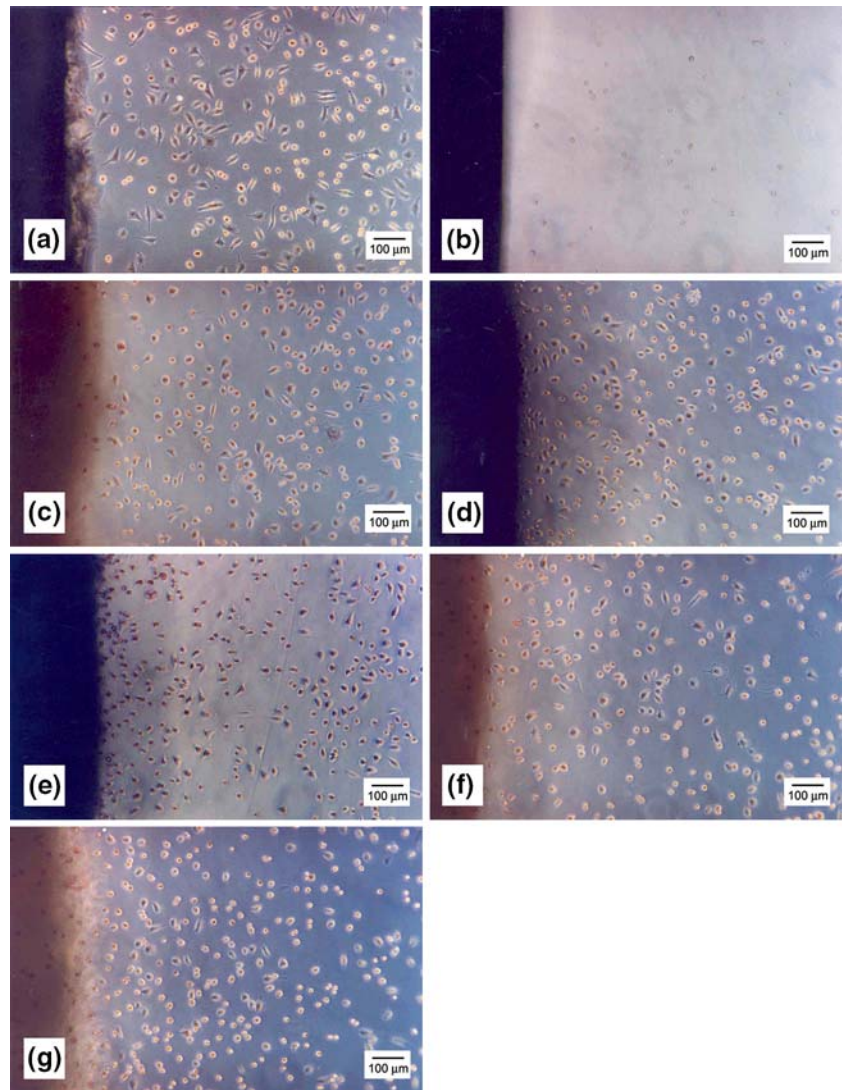

Fig. 1 Inverted phase contrast micrographs of L929 cells after 48-h incubation observed around: (**a**) HDPE, (**b**) natural rubber, (**c**) CM-chitosan hydrogel, (**d**) 80/20 CM-chitosan/PVA hydrogel, (**e**) 60/40 CM-chitosan/PVA hydrogel, (**f**) 80/20 CM-chitosan/CM-cellulose (ultra high viscosity) hydrogel, and (**g**) 60/40 CM-chitosan/CM-cellulose (ultra high viscosity) hydrogel

for 4 h at 4°C. The samples were subsequently washed with 0.1 M PB, dehydrated by graded ethanol series, and dried using a critical point CO_2 method. The samples were eventually gold sputtered in vacuum and examined by SEM to observe the cellular morphology and behavior of the L929 cells on the samples. The examination was made at 15 kV emission voltage and the specimen tilt angle of 0 degree on a Jeol JSM-5410 (Jeol, Japan). The samples were tested in triplicate.

Results and discussion

Cytotoxicity

The results of the direct contact test revealed that all CM-chitosan-based hydrogels were non-cytotoxic. The L929 cells around each hydrogel possessed normal morphology after 48-h incubation as shown in Fig. 1. They were well attached.

Cell-material response

Effect of type and content of blended polymers in the sponge CM-chitosan-based hydrogels

In general, most normal cells, including L929 cells used in this experiment, spread out on a substrate in order to proliferate. Inadequate spreading due to poor adhesion will inhibit their proliferation. The SEM results, shown in Fig. 2a–e, demonstrated that the L929 cells appeared most spreading on the pure CM-chitosan hydrogel, compared to those on the blended hydrogels. Apparently, the width of the pores of the CM-chitosan sponge was not too large for the cells to bridge from one ridge of a pore to

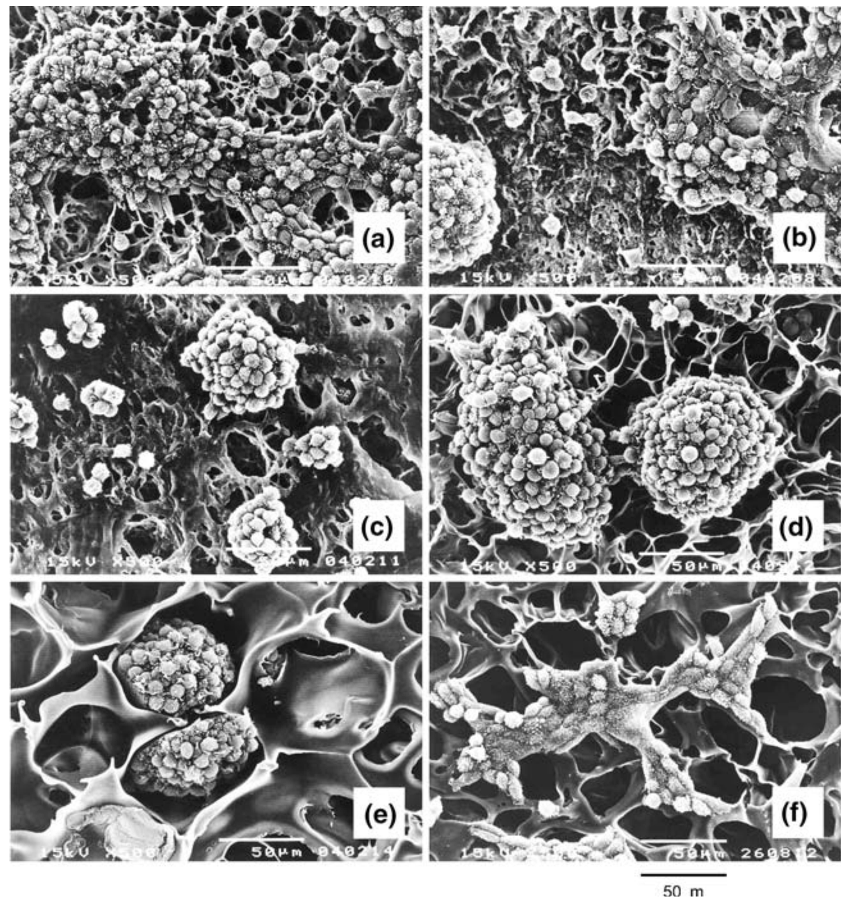

Fig. 2 SEM micrographs of L929 cells after 14-day incubation observed on: (**a**) CM-chitosan hydrogel, (**b**) 80/20 CM-chitosan/PVA hydrogel, (**c**) 60/40 CM-chitosan/PVA hydrogel, (**d**) 80/20 CM-chitosan/CM-cellulose (ultra high viscosity) hydrogel, (**e**) 60/40 CM-chitosan/CM-cellulose (ultra high viscosity) hydrogel, and (**f**) 60/40 CM-chitosan/CM-cellulose (low viscosity) hydrogel

another. After cell division, they moved to nearby areas and covered the surface of the sponge. In contrast, the attached cells in spherical clusters mixed with some spreading cells existed on the surface of the 80/20 CM-chitosan/PVA blended hydrogel. The higher the amount of PVA blended, i.e., 60/40 CM-chitosan/PVA blended hydrogel, the lower the number of the L929 cells. Moreover, the cells were rounded and exclusively aggregated. This observed cell-material response might be attributed to a difference in the chemical pattern of the CM-chitosan/PVA blended sponges. Normally, a substrate surface should be hydrophilic and correctly charged for good cell attachment [14]. The charge density of a substrate supported adhesion and function of the cells [15, 16]. Despite its hydrophilicity, PVA is essentially neutral, carrying no charge. The incorporation of PVA into CM-chitosan significantly reduced the overall charge density in the blended sponges, yielding less favorable surface for the cells to attach and spread. This result was consistent with the research of Minoura et al. [17] and Chuang et al. [18]. They reported that the attached cells on the chitosan/PVA blended hydrogels with higher PVA contents were spherical.

On CM-chitosan/ultra high viscosity CM-cellulose blended hydrogels, there were only spherical clusters of the aggregated cells, as revealed in Fig. 2d, e. No spreading cells ever appeared on the blends containing 20 and 40% (by weight) ultra high viscosity CM-cellulose. Perhaps, the pore sizes of the sponges, especially for the one with 40% CM-cellulose blended, were too wide for the L929 cells to bridge from one ridge of a pore to another. Therefore, they adhered and unavoidably proliferated in huge clusters, which were found even inside the pores. To verify this speculation, a 60/40 CM-chitosan/CM-cellulose blended sponge with a smaller pore size was prepared with the use of a low viscosity CM-cellulose grade. In Fig. 2f, the cells on this blended sponge spread well over the ridges of the pores which appeared smaller than those of the sponge composed of 40% ultra high viscosity CM-cellulose. In addition, these cells spread more than those on the sponge comprising 20% ultra high viscosity CM-cellulose, which had a smaller pore size. On average, the pores of the 60/40 CM-chitosan/low viscosity CM-cellulose sponge were still wide; the cells were unable to bridge from one ridge of a pore to another. Besides the effect of the pore size, the overall charge density of the hydrogels might contribute to this

different cellular morphology. As mentioned earlier, a substrate surface should be properly charged for good cell attachment, and the charge density of a substrate played a significant role in the adhesion and function of the cells. When CM-cellulose was mixed with CM-chitosan, the extent of the amidization (crosslinking) was reduced due to a decrease in NH_2 availability, resulting in the increasing amount of free carboxylate ions. The larger the amount of the CM-cellulose incorporated, the lesser the degree of amidization attained and the greater the amount of free carboxylate groups remained. With the use of low viscosity CM-cellulose, carboxylate groups in CM-cellulose could more effectively react with available NH_2 groups in CM-chitosan. As illustrated in Fig. 2f, the pore size became smaller, compared with that in Fig. 2e, when the same weight content of two different grades of CM-cellulose was employed in both sponges. In other words, in the blend with low viscosity CM-cellulose, the less amount of free carboxylate ions existed. The difference in the content of free carboxylate ions in each blend might play a role in alteration of the cellular morphology of the L929 cells.

Effect of surface structure of CM-chitosan hydrogels

Not only chemical structure, but also surface morphology of substrates directly governs the attachment, adhesion strength, and motility of cells. Once cells are seeded on a substrate, they secrete cell-binding serum protein essential for the cell attachment [19]. Adequate adhesion yields proper cell functions. Figure 3a, b illustrated the L929 morphology on the flat surface of the coverslip at different magnifications. The cells felt so comfortable on the flat surface of the properly charged glassy coverslip that they became flat, spreading, and proliferating and covered up the entire coverslip surface. On the contrary, the cells seeded on the surfaces of CM-chitosan hydrogels (film and sponge) tended to aggregate. Both hydrogels were initially prepared from the same material and treated under the identical condition (being steamed at 115°C, 15 min), but they differed in that one was dense, and the other was porous. As revealed in Fig. 3c, most of the proliferating cells on the CM-chitosan film aggregated rather than emigrated to nearby area. Nonetheless, the cells at the bottom of the cellular clusters

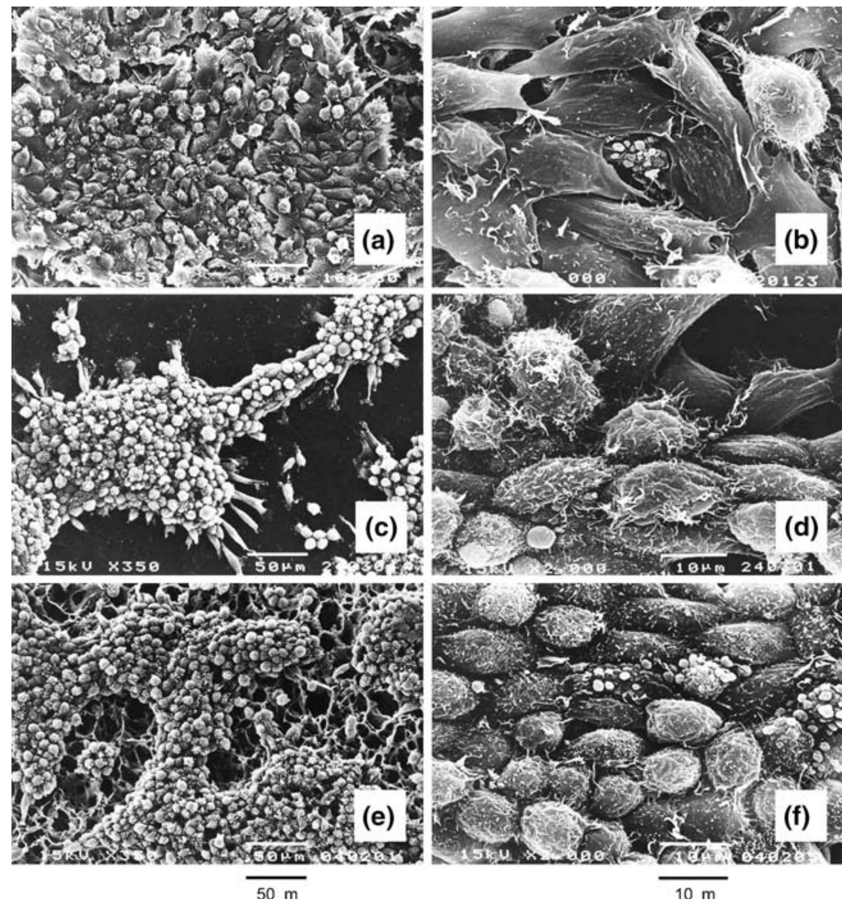

Fig. 3 SEM micrographs of L929 cells after 14-day incubation observed on: (**a, b**) coverslip; (**c, d**) film CM-chitosan hydrogel; and (**e, f**) sponge CM-chitosan hydrogel (original magnification ×350 (left) and ×2000 (right))

moved out by protruding their filopodia or lamellopodia to attach the adjacent areas, more vividly seen at a higher magnification in Fig. 3d; the attached cells close to the film surface became flat and well spread. In Fig. 3e, f, the cellular morphology observed on the porous surface of the CM-chitosan sponge was quite similar to that on the flat surface of the CM-chitosan film, except that the motility of the cells at the bottom was hindered on the rough, porous surface of the sponge CM-chitosan hydrogel. Although it was relatively difficult for those cells to anchor, they could fairly proliferate. Overall, the cells did not grow throughout on the entire surfaces of the hydrogels. As a matter of fact, this was not surprising because hydrogels with high water content were found to be difficult for cells to adhere to [4].

Effect of steam-treatment conditions used for the preparation of CM-chitosan-based hydrogels

Figure 4a–f show the cellular morphology and behavior of the L929 cells on the flat surfaces of the film CM-chitosan hydrogels prepared under different steaming conditions. A few rounded cells were observed on the surface of the film steamed at 100°C for 10 min. A higher number of the cells and more improved cellular morphology of the cells were found on the substrates treated at increasing steaming temperature and time. The attached cells on the film steamed at 130°C for 30 min appeared to spread and proliferate the most among three film hydrogels. Different steaming conditions used gave rise to the different chemical and physical properties of the prepared hydrogels. The higher the steaming temperature and the longer steaming time employed, the higher the density of hydrogel crosslinking. As a consequence, the swellability of these hydrogels was absolutely different. It was revealed that the moderate wettable surface provided the best environment for the cell adhesion and spreading [3]. Among these three steaming conditions, the wettibility of the sample steamed at 130°C for 30 min might be most suitable for the cell attachment and growth. In addition to the wettibility, the charge density might be also attributed to this different cellular morphology and behavior. As mentioned earlier, the content of free carboxylate ions in the

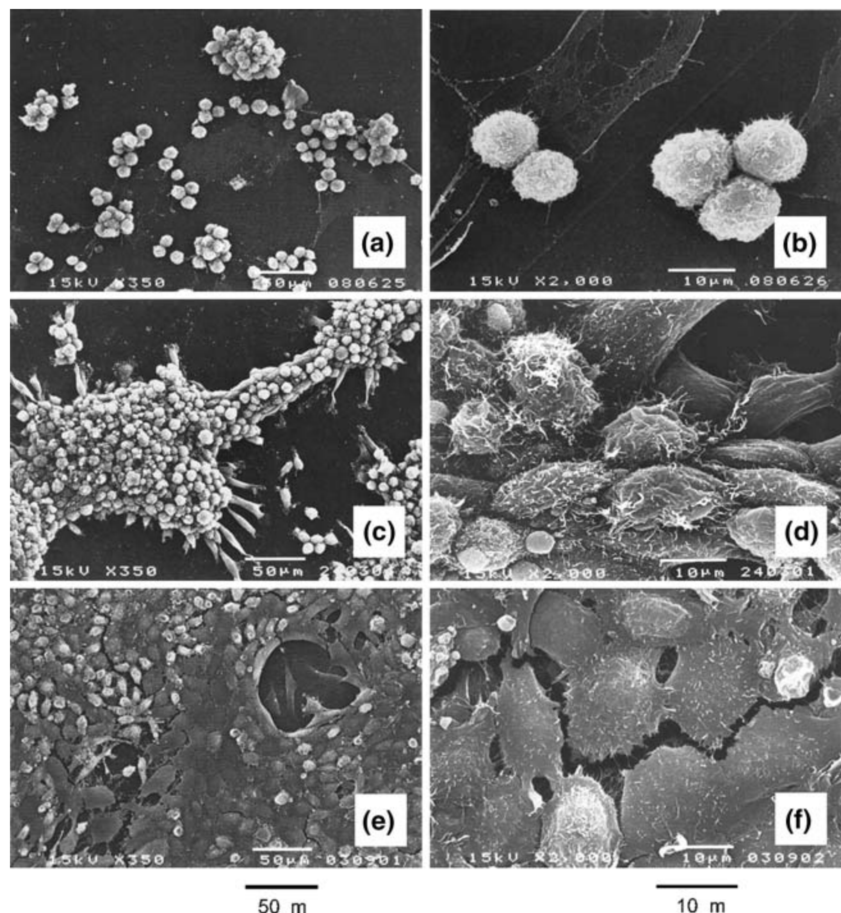

Fig. 4 SEM micrographs of L929 cells after 14-day incubation observed on film CM-chitosan hydrogels prepared under various steaming conditions: (**a**, **b**) 100°C, 10 min; (**c, d**) 115°C, 15 min; and (**e, f**) 130°C, 30 min (original magnification ×350 (left) and ×2000 (right))

hydrogels was suspected to influence the cellular morphology of the L929 cells. When the film hydrogels were prepared with low extents of crosslinking, they might possess too high contents of free carboxylates, hindering the cell adhesion and growth.

Conclusion

The results obtained from this study indicated that the cell-CM-chitosan-based hydrogel response was strongly affected by the following parameters : type and content of the blended polymers, surface structure of hydrogels, and steaming condition used for the preparation of hydrogels. The hydrogels with PVA blended possessed the low charge density, deteriorating the cell adhesion, spreading, and proliferation. Although CM-cellulose carries negative charges, i.e., carboxylate ions, the L929 cells differently responded to the blended hydrogels of CM-chitosan and CM-cellulose when different grades of CM-cellulose were employed. Properly charged hydrogel was achieved when low viscosity CM-cellulose was used. Porosity also influenced the cellular morphology. The cells appeared more comfortable to attach, spread, proliferate, and be flatten on the flat surface than the rough, porous surface. The cells on the film CM-chitosan hydrogel steamed at 130°C for 30 min were more biocompatible than those on the other films treated under different steaming conditions. Even though the L929 cells interacted with the CM-chitosan-based hydrogels differently, they could be safely used as biomaterials due to their non-cytotoxicity to the L929 cells.

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References

1. C. J. KIRKPATRICK, F. BITTINGER, M. WAGNER, H. KOHLER, T. G. VAN KOOTEN, C. L. KLEIN and M. Otto, *Proc. Instn. Mech. Engr*. **212 (Part H)** (1998) 75
2. B. D. RATNER, A. S. HOFFMAN, F. J. SCHOEN and J. E. LEMONS, in ‘‘Biomaterial Science: Introduction to Materials in Medicine’’ (Academic Press, New York, 1996) p. 215
3. T. GROTH and G. ALTANKOV, *Biomaterials* **17** (1996) 1227
4. T. KOYANO, N. MINOURA, M. NAGURA and K. I. KOBAYASHI, *J. Biomed. Mater. Res.* **39** (1998) 486
5. S. TANODEKAEW, M. PRASITSILP, S. SWASDISON, B. THAVORNYUTIKARN, T. POTHSREE and R. PATE-EPASEN, *Biomaterials* **25** (2004) 1453
6. E. T. DEN BRABER, J. E. DE RUIJTER, L. A. GINSEL, A. F. VON RECUM and J. A. JANSEN, *Biomaterials* **17** (1996) 2037
7. A. CURTIS and C. WILKINSON, *Biomaterials* **18** (1997) 1573
8. R. A. A. Muzzarelli, in ‘‘Chitin’’ (Pergamon Press, New York, 1977)
9. G. I. HOWLING, P. W. DETTMAR, P. A. GODDARD, F. C. HAMPSON, M. DORNISH and E. J. WOOD, *Biomaterials*. **22** (2001) 2959
10. R. A. A. MUZZARELLI, *Carbohyd. Polym*. **8** (1988) 1
11. X. F. LIU, Y. L. GUAN, D. Z. YANG, Z. LI and K. D. YAO, *J. Appl. Polym. Sci*. **79** (2001) 1324
12. X.-G. CHEN, Z. WANG, W.-S. LIU and H.-J. PARK, *Biomaterials.* **23** (2002) 4609
13. W. JANVIKUL and B. THAVORNYUTIKARN, *J. Appl. Polym. Sci.* **90** (2003) 4016
14. R.I. Freshney, in ‘‘Culture of animal cells : a manual of basic technique’’ (4th edn, A John Wiley & Sons, 1994)
15. G. B. SCHNEIDER, A. ENGLISH, M. ABRAHAM, R. ZAHARIAS, C. STANFORD and J. KELLER, *Biomaterials* **25** (2004) 3023
16. S. F. ROSE, A. L. LEWIS, G. W. HANLON and A. W. LLOYD, *Biomaterials* **25** (2004) 5125
17. N. MINOURA, T. KOYANO, N. KOSHIZAKI, H. UMEHARA, M. NAGURA and K.-J. KOBAYASHI, *Mat. Sci. Eng. C-Bio. S*. **6** (1998) 275
18. W.-Y. CHUANG, T.-H. YOUNG, C.-H. YAO and W.-Y. CHIU, *Biomaterials* **20** (1999) 1479
19. S. VERRIER, R. BAREILLE, A. ROVIRA, M. DARD and J. AMEDEE, *J. Mater. Sci-Mater M* **7** (1996) 46