J Mater Sci: Mater Med (2007) 18:951–957
DOI 10.1007/s10856-006-0093-y

In vitro biodegradation and biocompatibility of gelatin/montmorillonite-chitosan intercalated nanocomposite

Hong Zhuang · Jun Ping Zheng · Hong Gao · Kang De Yao

Received: 10 October 2005 / Accepted: 15 February 2006 / Published online: 13 January 2007
© Springer Science+Business Media, LLC 2007

Abstract The intercalated nanocomposite of gelatin/montmorillonite-chitosan (Gel/MMT-CS) was prepared via the solution intercalation process. In vitro degradation tests showed that the nanocomposite had a lower degradation rate than Gel-CS composite. And the introduced intercalation structure endowed Gel/MMT-CS nanocomposite with a controllable degradation rate when changing the MMT content. Cells attachment, spread and proliferation on the Gel/MMT-CS membranes were investigated by scanning electron microscopy (SEM) and mitochondrial activity assay. The results provided evidences of good adhesion, proliferation and morphology of rat stromal stem cells on Gel/MMT-CS membranes compared to the tissue culture plates (TCPs), making the Gel/MMT-CS nanocomposite a promising candidate towards tissue engineering.

Introduction

The rapid development of tissue engineering involving the artificial manipulation of cells requires novel design of the scaffold and membrane for guided tissue regeneration. Cells once implanted need a biocompatible substrate to maintain their tissue-specific functions and support their organization. The biocompatibility of a biomaterial is closely related to the behavior of the cells in contact with them, such as cell adhesion, morphology, and proliferation [1]. Recently, the development of biomaterials is focus on the biodegradable materials which could degrade in response to the cellular environments. In particular, tissues are allowed to grow into the matrix while eliminating the need for a second surgery to remove the implants, preventing potential chronic problems with the presence of implants [2, 3].

Gelatin, a denatured derivative of collagen, is known to have no antigenicity and can be completely absorbed in vivo [4]. Chitosan is a linear polysaccharide consisting of β $(1 \rightarrow 4)$ linked D-glucosamine residues with a variable number of randomly located *N*-acetylglucosamine groups that has structural characteristics similar to GAGs [5, 6]. Its cationic nature and high charge density allow chitosan to form polyelectrolyte complexes (PECs) with anionic polymers e.g. poly (acrylic acid) [7], sodium alginate [8], gelatin [9] and pectin [10], etc. In vivo tests have proven that chitosan-based biomaterials do not have any inflammatory or allergic reaction following implantation, injection, topical application, or ingestion in the human body [11]. Recently, much attention has been paid to utilize gelatin-chitosan complexes in biomedical applications. Lu et al. has prepared the chitosan-gelatin network coated with hydroxyapatite block to simulate the bimodal structure of natural bone, which has potential application in the construction of cages for spinal operations [12]. Rao et al. used collagen and chitosan co-crosslinked with glutaraldehyde to prepare films for epidermis cell culture in vitro [13]. MMT [$Na_{0.7}(Al_{3.3}\ Mg_{0.7})\ Si_8O_{20}(OH)_4 \bullet nH_2O$] is a kind of layered silicate. Polymer/MMT nanocomposites have been reported a lot

H. Zhuang · J. P. Zheng (✉) · H. Gao · K. De Yao
College of Materials Science and Engineering, Tianjin University, Tianjin 300072, P.R. China
e-mail: jpzheng@tju.edu.cn

these years [14, 15]. With only a low content of MMT, the strength, Young's modulus, and solvent resistance of the composites can be greatly improved [16, 17]. As a bioinert clay mineral, MMT has been applied in cosmetic and pharmaceutical industries [18]. However, there are few reports about biodegradable polymer/MMT nanocomposites [19], and no study on the cytotoxicity of polymer/MMT composite has been reported yet.

In this work, we report a novel biomaterial-Gel/MMT-CS nanocomposite. In vitro biodegradability and biocompatibility of the Gel/MMT-CS nanocomposite are investigated. The present work could provide potential prospects of polymer/MMT nanocomposite for biomedical applications.

Materials and methods

Materials

Gelatin (Type B, extracted from bovine skin) and lysozyme (8×10^4 U/mg) were purchased from Sigma Chemical Co. (St. Louis, MO). Chitosan (degree of deacetylation: 95%) was supplied by Haihui Bioengineering Co. (Qingdao, China). MMT (the particle size is 40 μm) was obtained from Huate Chemical Co. (Zhejiang, China). All other reagents used were of reagent grade.

Preparation of Gel/MMT-CS membrane

The required amount of MMT suspension which was ultrasonically pre-treated was added dropwise to the 6 wt% gelatin solution at 70°C under agitation for 1 h. Then, chitosan dissolved in 1% acetic acid was added to the above homogeneous mixture at 40°C. After vigorous agitation for 6 h, the mixture was placed stilly to be degassed. Then 5 ml of 0.5% glutaraldehyde solution was dropped into the mixture and slowly stirred at 40°C for another 20 min. The product was poured into the 75 mm^2 Petri dishes (Falco, USA) and dried at 50°C. The membrane obtained was treated with 1% sodium hydroxide aqueous solution and 3% sodium borohydride aqueous solution to remove excessive acetic acid and non-reacted aldehyde groups, then rinsed with a good deal of distilled water and dried.

X-ray diffraction(XRD) Characterization

To measure the change of gallery distance of MMT before and after intercalation, XRD patterns were recorded at 2°/min between 1°and 30° on a Rigaku DMAX-RC diffractometer using CuKα radiation (λ = 0.154 nm) at a generator voltage of 50 KV and a generator current of 180 mA.

Scanning electron microscopy (SEM)

The membrane surfaces were sputter-coated with a thin layer of gold and then observed on a Philips XL-30 environmental scanning electron microscope.

In vitro degradation

The in vitro degradation experiments were carried out by incubating the samples (1×1 cm^2) in phosphate-buffered saline (PBS, pH = 7.4) and PBS with 8×10^4 U/ml of lysozyme in an incubation tube at 37°C. At regular intervals, three specimens of each group were taken out from the PBS medium, rinsed with distilled water and dried in vacuo thoroughly. The degradation percentage D was determined by the following weight equation: $D = (W_0–W_t)/W_0 \times 100\%$, where W_0 represents the initial weight and W_t is the weight at time t. The degradation percentage D was expressed as the mean ± standard deviation (n = 3).

Stromal stem cells culture

The rat stromal stem cells TC1 (supplied by Union Stem Cell Gene engineering Co. Ltd., Tianjin, China) were cultured in IMDM (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA) and gentamicin (2×10^4 units/L). Cells were incubated at 37°C in a 5% CO_2 incubator (Thermo Forma, USA) and the culture medium was changed every 3 days. Cells reached 80% subconfluent were detached by incubating with 0.25% Trypsin (Sigma, USA) and 1 mmol/L EDTA (Gibco, USA) at 37°C for 15 min. The effect of Trypsin was then stopped by adding the complete medium at room temperature. Cells were resuspended and counted by blood plate reader; the cell suspension was diluted with fresh medium to the final concentration for reseeding and growing.

Cell morphology

Gel/MMT-CS membranes sterilized by ^{60}Co γ irradiation were cut into circular pieces (10 mm in diameter) by a cork borer and equilibrated in the culture medium for 1 h before used. Then they were placed in a 24-well cell culture plate (Nunc., Denmark). A concentration of 8×10^4 cells/well was seeded on the membranes and then incubated at 37°C in 5% CO_2 and 100% humidified

atmosphere. The cell morphology was observed with SEM after fixation by 2.5% glutaraldehyde in 0.1 M PBS for 2 h at 4°C and dehydration with graded ethanol (30, 50, 70, 90, 100%) each for 2 × 20 min. The samples were dried at critical point and gold-sputtered prior to observation.

MTT assay

Cell proliferation on the Gel-CS and Gel/MMT-CS membranes was assessed by dimethylthiazol diphenyltetrazolium bromide (MTT) assay. Cells were seeded at a density of 5×10^4 cells/well onto the equilibrated membranes in 24-well cell culture plates and were incubated at 37°C in 5% CO_2 and 100% humidified atmosphere. At the time point of 2, 5, 7, 9 days, 100 μl MTT solution (5 mg/ml, Sigma, USA) was added to each well. After incubation for 4 h at 37°C, the upper medium was removed carefully and the intracellular formazan was dissolved by adding 350 μl dimethyl sulfoxide (DMSO) to each well. After jolted by a shaker for 15 min, the upper solution was transferred to a 96-well plate. The absorbance was measured at 492 nm using the spectrophotometric microplate reader (Sunrise, Tecan, Austria). Experiments were run in triplicate per sample. The cells inoculated directly on TCPs were regarded as controls. All data were expressed as the mean ± standard deviation (SD) for $n = 3$.

Results and discussion

Preparation of Gel/MMT-CS nanocomposite

According to our previous study, hydrophilic gelatin chains can insert into MMT layers via the solution intercalation process [20]. Figure 1 shows the XRD patterns of MMT and Gel/MMT-CS nanocomposite. Pure MMT exhibits the d_{001} diffraction peak at $2\theta = 7.04°$, and d_{001} is 1.25 nm according to Bragg's equation: $\lambda = 2d\sin\theta$. Comparatively, the d_{001} peak of the nanocomposite shifts to $2\theta = 1.83°$ which suggests a boarder interlayer spacing of MMT with 4.82 nm due to the intercalation. Therefore, the intercalated nanocomposite was formed.

As an amphoteric polyelectrolyte, gelatin macromolecule has many $-NH_2$ and –COOH groups on its end radicles and side chains. Chitosan, the deacetylated form of chitin, has a great deal of free $-NH_2$ groups too. Polyelectrolyte complexes (PECs) can be formed by the reaction of oppositely charged polyelectrolyte in an aqueous solution. Figure 2 shows the XRD patterns of CS, Gel and Gel-CS composite. The diffraction peaks of chitosan appear at 11.3°, 18° and 21.4°. And gelatin exhibits only one broad diffraction peak at 21°. In the XRD pattern of Gel-CS composite, the diffraction peaks of chitosan almost disappeared, and the intensity of diffraction peaks of gelatin decreased greatly. The result reveals that there exists strong interaction between the two macromolecules, which breaks the intrinsic crystal structure of chitosan. It can be concluded that the three-phase composite was obtained by the formation of PECs between gelatin and chitosan.

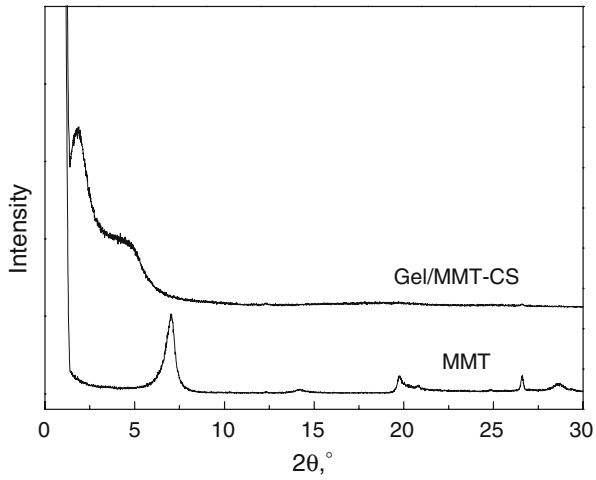

Fig. 1 XRD patterns of pure MMT and Gel/MMT-CS intercalated nanocomposite (MMT content: 15%; Gel/CS = 1:1)

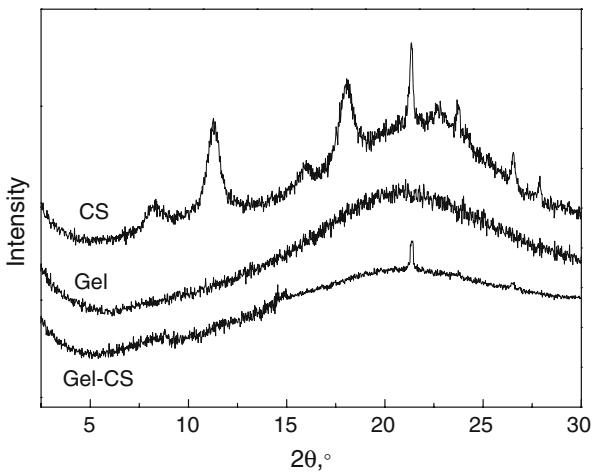

Fig. 2 XRD patterns of CS, Gel and Gel-CS composite (Gel/CS = 1:1)

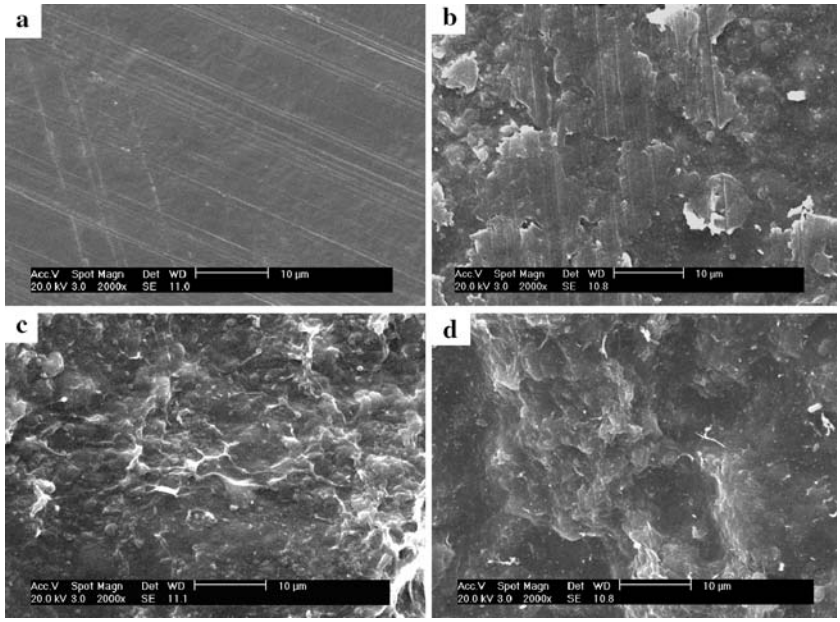

Fig. 3 SEM photographs of Gel/MMT-CS nanocomposite membranes immerged in PBS at 37°C ((**a**) initial state, (**b**) 3 days, (**c**) 6 days, (**d**) 9 days)

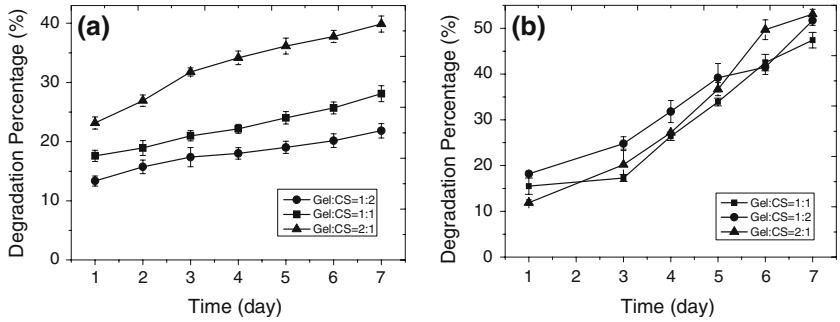

Fig. 4 In vitro degradation behaviors of the Gel/MMT-CS membranes (MMT content: 5%) with different gelatin ratios in (**a**) PBS (**b**) lysozyme

Degradation behavior in vitro

As the tissue engineering aims at the regeneration of new tissues, biomaterials are expected to be degradable and absorbable with a proper rate to match the speed of new tissues formation. And the degradation behavior of biomaterials in physiological environments plays an important role in the engineering process of a new tissue. The surface is renewed during the degradation, which provides the variational interface for the implanted cells to adhesive and grow. It may affect many cellular processes including cell growth, tissue regeneration, and host response [21]. In this work, the in vitro degradation of the Gel/MMT-CS nanocomposite was investigated.

Figure 3 shows the morphology of Gel/MMT-CS nanocomposite membranes immerged in PBS at different periods. The initial Gel/MMT-CS membrane exhibits a smooth interface (Fig. 3a). When immerged in PBS, the water could easily permeate to the inner side of the composite, resulting in a rapid degradation from the out side to the inner side and prompting the weight loss. Macromolecules of the membrane surface behave preferential hydrolytic scission into low molecules, which can dissolve in PBS. Figure 3b indicates that localized erosion spread over the sample's surface after 3 days soakage. And with the prolongation of the degradation time, a lot of concaves appeared on the film surface because of the further erosion by PBS (Fig. 3c, d). The degradation behaviors of Gel/MMT-CS membranes are mostly the surface erosion by the environmental medium like the onion exfoliation.

Figure 4 compares the degradation rate of the Gel/MMT-CS membranes with different gelatin ratios in PBS and lysozyme aqueous. As a hydrophilic polymer, gelatin macromolecular chains hydrolyze quickly with the existence of water. So the degradation rate of Gel/MMT-CS membranes with high gelatin ratio in PBS is quicker (Fig. 4a). Lysozyme naturally exists in body fluids. The β-1, 4 *N*-acetyl-glucosamine

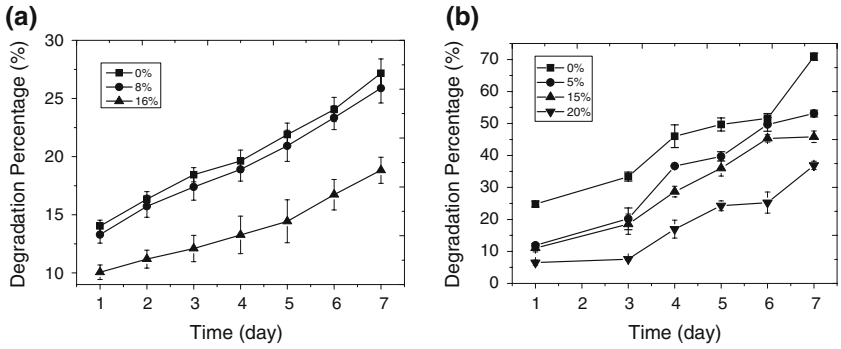

Fig. 5 In vitro degradation behaviors of the Gel/MMT-CS membranes (Gel/CS = 2:1) with different MMT contents in (**a**) PBS (**b**) lysozyme

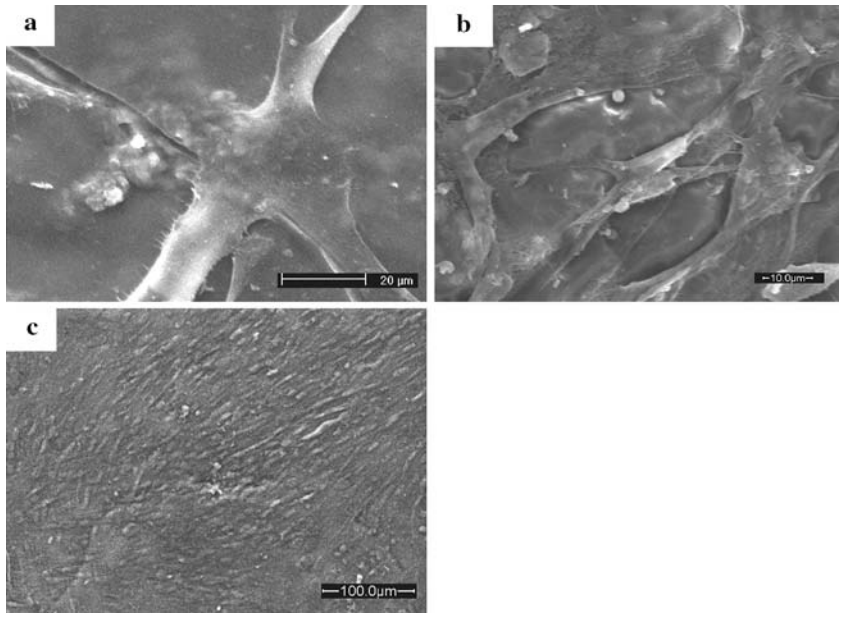

Fig. 6 SEM images of stromal stem cells cultured on the Gel/MMT-CS membranes (MMT content: 6%; Gel/CS = 2:1) for (**a**) 24 h (**b**) 2 days (**c**) 7 days; Magnification: (**a**) 1000 × (**b**) 3000 × (**c**) 400 ×

groups of chitosan chains can be hydrolyzed by lysozyme [22]. Its degradation leads to the release of aminosugars, which can be incorporated into glycosaminoglycan and glycoprotein metabolic pathways, or excreted. Since the chitosan chains were attacked by lysozyme continuously, the membranes with the high ratio chitosan behaved a higher degradation percentage than other groups in the initial 5 days (Fig. 4b). The cross-linked gelatin still degraded quickly because of the large quantity of hydrophilic amido and carboxyl groups. So, the membranes with the ratio of chitosan to gelatin of 1:2 presented appreciably higher degradation percentage after 5 days.

The effect of MMT contents on the degradation rate are shown in Fig. 5. With an increase in the MMT content, the degradation percentage decreases dramatically both in PBS and lysozyme aqueous solution. The layer thickness of MMT is around 1 nm and the lateral dimension of these layers may vary from 30 nm to several microns. Due to intercalation with MMT, parts of the hydrophilic groups of gelatin are shielded. Because of the barrier effect of nanosized sheets of MMT, the interaction between the macromolecules and lysozyme or water molecules is weakened, protecting the macromolecules from hydrolyzing. Meanwhile, the presence of MMT also acts as physical crosslinking sites, which enhance the stability of the networks. All above phenomena demonstrate that the degradation rate may be controllable by adjusting the MMT contents.

Cell morphology on the Gel/MMT-CS membranes

Figure 6 shows the SEM images of rat stromal stem cells grown on the Gel/MMT-CS membranes with 6% MMT content. Cells are observed to firmly attach on membranes as early as 24 h after seeding (Fig. 6a). They spread well, and the pseudopods are clearly seen. It is known that cell adhesion is an important cellular process because it directly influences cell morphology

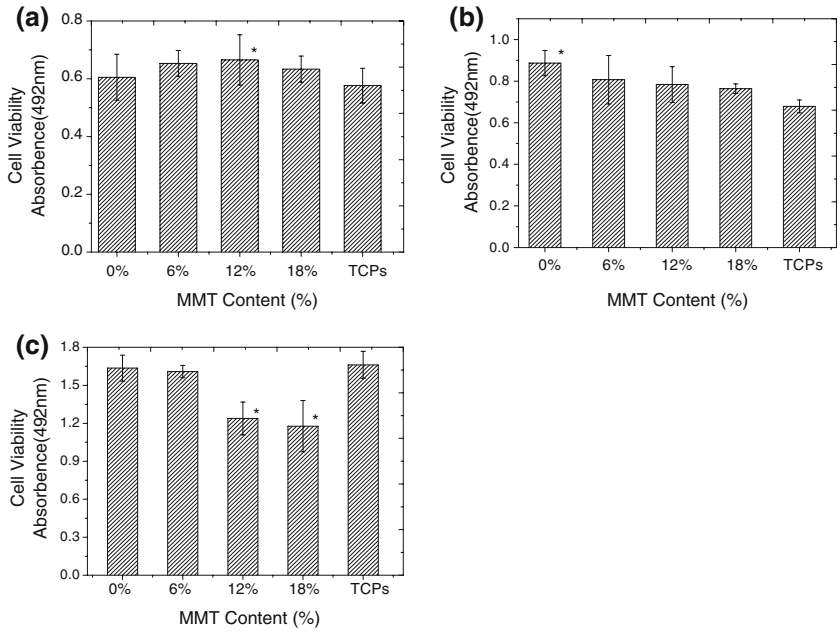

Fig. 7 The proliferation of stromal stem cells on Gel/MMT-CS membranes(Gel/CS = 2:1) with different MMT contents by MTT assay: (**a**) 2 days (**b**) 5 days (**c**) 9 days ($n = 3$, TCPs as control, $^*p < 0.01$, compared to TCPs)

and the capacity for proliferation and differentiation. Chen et al have represented that cells that spread well possess good viability and high DNA synthesis; contrarily they will easily wither when cultured [23]. The spread and growth of cells on membranes were observed in Fig. 6b; intercellular connections were maintained through the filopodia. Cells continue to proliferate on the membranes and become subconfluent, which is confirmed in Fig. 6c on the 7th day of culture.

In general, cell behavior and interaction with a bioactive material surface are dependent on the properties such as topography, surface charge and chemistry [24–26]. Gelatin is a hydrophilic protein and it might be favorable for the interaction with cells. A non-specific cell interaction exists between chitosan positively charged ammonium sites at physiological pH and negatively charged cell membrane surfaces [27]. As a result, the Gel/MMT-CS membrane surface seems to be favorable for the cell adhesion in the initial stage of cell culture.

In vitro cytotoxicity test

Reduction of MTT reagent is assessed as an assay of mitochondrial redox activity of cultured cells [28]. MTT reagent is a pale yellow substance that is reduced to a dark blue formazan product when incubating with viable cells by mitochondrial succinate dehydrogenase. Therefore, the production of formazan can reflect the level of cell viability on the material. Figure 7a shows the absorbance of formazan produced by viable cells attached on Gel/MMT-CS films with different MMT contents during 2 days culture. As it shows, the absorbance values increase a little when the MMT content increases from 0 to 12%. Moreover, the absorbance values are a little higher than that of TCPs ($p < 0.01$). The absorbance values during the primary 2 days are mostly determined by the condition of cell adhesion. So, cells are possibly prone to adhere on the Gel/MMT-CS films. The potential effect of MMT on cell adhesion is under further investigation.

There is a similar trend of cell proliferation on Gel/MMT-CS films and TCPs, which is confirmed by the MTT absorbance during the 9 days cultivation (Fig. 7b, c). The increased number of cells on the membranes surfaces at the end of the culture period suggested the good affinity and biocompatibility for cells. At time point of 9 days, the cells activities in the groups with low MMT content is relatively higher and proliferate more quickly. Correspondingly, the groups with 12 and 18% MMT content still exhibit obvious proliferation, but possess lower absorbance values. However, the function of MMT on cellular behavior seems to be inexplicable.

Figure 8 compares the formazan absorbance values on Gel/MMT-CS membranes with different gelatin ratio (MMT content: 6%). The results indicate that more proliferation is found on Gel/MMT-CS membranes with high gelatin content. Gelatin is composed of a series of amino acids, such as arginine, glycin, aspartic acid, which have positive effects on cell adhesion, viability and growth. Moreover, previous

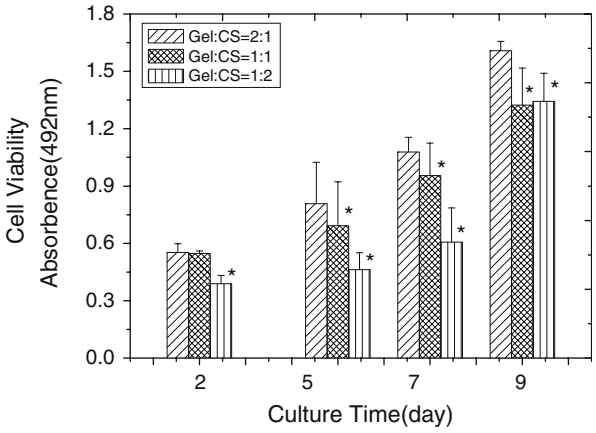

Fig. 8 The proliferation of stromal stem cells on Gel/MMT-CS membranes with different gelatin ratio by MTT assay (MMT content: 6%, $n = 3$, $^*p < 0.01$, compared to Gel:CS = 2:1)

study [29] showed that the incorporation of Gel into CS improved the hydrophilicity of the CS membranes and that the hydrophilic surface is more suitable for cell attachment and later proliferation.

Conclusions

In the present experiment, the intercalated Gel/MMT-CS nanocomposite is developed as a novel biomaterial for tissue engineering. Data reveal that the in vitro degradation rate is greatly affected by the incorporation of MMT, and it may be a controllable one when adjusting the MMT contents. MTT assay demonstrates better cells affinity and biocompatibility of Gel/MMT-CS nanomposite with 6% MMT content and the Gel/CS ratio of 2:1 compared to TCPs. The Gel/MMT-CS nanomposite has the potential application as a possible selected biomaterial. The nanocomposite technology exhibits a great potential to be applied to different systems of biodegradable polymer for the purpose of better characteristics.

Further study will be performed to identify the biocompatibility and biodegradation of the Gel/MMT-CS nanocomposite in vivo.

Acknowledgment The authors wish to thank the National Natural Science Foundation of China, through Grant No. 20204008, for supporting this research.

References

1. D. F. WILLIAMS, 1999, The Williams dictionary of biomaterials, (Liverpool, UK: University Press)p. 40
2. L. J. CHEN and M. WANG, *Biomaterials* **23** (2002) 2631
3. J. CRESPO, M. A. SATORRE, J. A. QUINTANA and F. ANIA, *J. Mater. Sci.* **30** (1995) 6145
4. R. A. A. MUZZARELLI, *Carbohydrate Polym.* **20** (1993) 7
5. Y. L. CUI, A. D. QI and W. G. LIU, *Biomaterials* **24** (2003) 3859
6. S. HSU, S. W. WHU and C. L. TSAI, *J. Polym. Res.* **11** (2004) 141
7. A. BORZACCHIELLO, L. AMBROSIO, P. A. NETTI and J. SAN ROMAN, *J. Mater. Sci.* **12** (2001) 861
8. H. TAKEUCHI, T. YASUJI H., YAMAMOTO and Y. KAWASHIMA, *Pharm. Res.* **17** (2000) 94
9. K. LI, Y. WANG, Z. C. MIAO and M. F. FENG, *Biotechnol. Lett.* **26** (2004) 879
10. K. D. YAO, H. L. TU, F. CHENG and J. LIU, *Angew. Makromol. Chem*. **63** (1997) 245
11. E. PIANIGIANI, A. ANDREASSI and P. TADDEUCCI, *Biomaterials* **20** (1999) 1689
12. W. W. LU, F. ZHAO, K. M. C. CHEUNG and K. D. YAO, *J. Mater. Sci. Mater. Med.* **14** (2003) 1039
13. N. SHANMUGASUNDARAM, P. PAVICHANDRAN, P. NEELAKANTA REDDY and P. RAO, *Biomaterials* **22** (2001) 1943
14. A. USUKI, Y. KOJIMA, M. KAWASUMI and O. KAMIGAITO, *J. Mater. Res*. **8** (1993) 1179
15. M. KAWASUMI, N. HASEGAWA and A. OKADA, *Macromolecules* **30** (1997) 6333
16. Y. KOJIMA, A. USUKI, M. KAWASUMI, A. OKADA, Y. FUKUSHIMA and T. KURAUCHI, *J. Mater. Res.* **8** (1993) 1185
17. K. YANO, A. USUKI and A. OKADA, *J. Polym. Sci. [A1].* **35** (1997) 2289
18. F. H. LIN, Y. H. LEE and C. H. JIAN, *Biomaterials* **23** (2002) 1981
19. J. H. LEE, T. G. PARK and Y. K. LEE, *Biomaterials* **24** (2003) 2773
20. J. P. ZHENG, L. F. Xi, H. L. ZHANG and K. D. YAO, *J. Mater. Sci. Lett.* **22** (2003) 1179
21. J. E. BABENSEE, J. M. ANDERSON, L. V. MELNTIRE and A. G. MIKOS, *Adv. Drug. Deliv. Rev*. **33** (1998) 111
22. A. DOMARD and M. RINAUDO, *Int. J. Biol. Macromol.* **5** (1983) 49
23. C. S. CHEN, M. MRKSICH and D. E. INGBER, *Biotechnol. Prog.* **14** (1998) 356
24. S. VERRIER, J. J. BLAKER, V. MAQUET and A. R. BOCCACCINI, *Biomaterials* **25** (2004) 3013
25. K. ANSELME, *Biomaterials* **21** (2000) 667
26. M. HOTT, B. NOEL, D. BERNACHE-ASSOLANT and P. J. MARIE, *J. Biomed. Mater. Res.* **37** (1997) 508
27. C. CHATELET, O. DAMOUR and A. DOMARD, *Biomaterials* **22** (2001) 261
28. T. MOSMANN, *J. Immunol. Methods* **65** (1983) 55
29. J. S. MAO, X. H. WANG and K. D. YAO, *J. Mater. Sci.* **38** (2003) 2283