In vitro aging of mineralized collagen-based composite as guided tissue regeneration membrane

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Abstract

The technique of guided tissue regeneration (GTR) has been developed for the regeneration of periodontal tissues, bone around natural teeth and dental implants. The aim of this study is to investigate the biodegradability and mechanic behavior of a novel mineralized nano-hydroxyapatite/collagen/poly (lactic acid) (nHAC/PLA) composite as GTR membrane in vitro. The elastic modulus and maximum tensile strength of GTR film samples with different nHAC/PLA ratio were measured to get an optimal nHAC/PLA ratio. Thermogravimetric analysis was conducted to evaluate the change of the inorganic component in the samples during the process of in vitro aging. Morphology of samples was checked by using scanning electron microscopy. On the basis of the above results, it can be concluded that the GTR membranes maintained integrity and the original appearance throughout the 1-month in vitro aging. There is an active dissolution and deposition process of crystals which is propitious to the bone formation on the surface of the composite membrane. The optimal nHAC/PLA ratio of the novel membrane is 0.4:1. For a longer period of bone repair, PLA with higher molecular weight should be chosen as the scaffold for the GTR membrane.
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1. Introduction

Guided tissue regeneration (GTR) [1] was proposed as a method for regenerating neurons and later, bone and periodontium. Physical barriers in the form of a membrane are placed between the mucogingival flap and the bone and tooth surfaces during surgery. The membrane acts as a barrier. It deflects the gingival tissue away from the root surface and creates a protected space over the defect that allows the remaining periodontal ligament fibroblasts to selectively repopulate on the root surface. These fibroblasts are thought to account for the formation of a new fibrous attachment which prevents the epithelial migration.

In addition to periodontal defects, this GTR principle has also been used in the treatment of bone defects and alveolar ridge augmentation. The concept of GTR in these applications is to exclude the rapidly repairing tissues (epithelium and gingival connective tissue) and to allow the migration of regenerative bone cells from the surrounding alveolar bone into the defect [2]. Various GTR membranes have been developed and used in the interface between soft tissue and restoration areas to prevent soft tissue invasion from normal tissue healing. Although various GTR membranes have been tested and applied clinically, only few membranes have reached the stage of routine clinical application [3–5]. Recently, a novel GTR membrane, which contains mineralized nano-hydroxyapatite/collagen/poly (lactic acid) (nHAC/PLA), has been biomimetically fabricated to obtain excellent biocompatibility, biodegradability and mechanical properties [6]. In this study, we investigate the aging process in vitro and evolution of mechanic property of the GTR membranes based on nHAC/PLA composite.

2. Materials and methods

2.1. Preparation of nHAC/PLA GTR membrane

Synthesis of nHAC powder has been reported previously [7–9]. Type I collagen solution (CELLON Company) was

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adjusted to a concentration of 0.67 g collagen/l. Solutions of $CaCl_2$ and H_3PO_4 (Ca/P = 1.66) were then added separately by drops. The solution was gently stirred and titrated at room temperature with sodium hydroxide solution to pH 7.4. After 48 h, the nHAC deposition was harvested by centrifugation and freeze-dried.

Nano-HAC/PLA membrane was made by the following procedure: PLA (Chengdu Institute of Organic Chemistry, Chinese Academy of Sciences) of molecular weight 125,000, 150,000 and 200,000 Da was dissolved in dioxane to final concentrations of 10% (m/v). The solution was then stirred gently at room temperature for 4–6 h, and the nHAC powder was added at different nHAC/PLA weight ratio (0.2:1, 0.4:1, 0.6:1, and 0.8:1). The solution was then ultrasonicated, poured into a mold, frozen at a temperature between 0 and −20 °C overnight, and then lyophilized to remove dioxane. The inorganic phase in the membrane is carbonate-substituted HA with low crystallinity and nanometer size. PLA was used as scaffold to support the nHAC grains. The thickness of the GTR membrane is 0.3 mm. Pure PLA membranes were prepared as controlled group.

All groups of the film samples are listed in Table 1. The formed nHAC/PLA membranes were opalescent, translucent, and flexile membranes.

2.2. Mechanical property of the GTR membranes

Selected samples were made into $0.5 \times 5.0 \times 0.03$ cm³ rectangle pieces, and the elastic modulus and tensile strength of these samples were measured with mechanic test machine (DCS-5000, SHIMADZU Co., Tokyo Japan).

2.3. In vitro aging of the GTR membranes in simulated body fluid

All samples were cut into 5×5 mm² round pieces and were put into 37 °C simulated body fluid. In the beginning 24 h, the pH of the simulated body fluid was 5 to mimic the acid environment in the body after surgery, and then the pH was adjusted to 7 with trishydroxymethylaminomethane (Tris) and hydrochloric (HCL). All samples were put into 37 °C water bath shaker and mildly shaken. Simulated body fluid was changed every 48 h to keep the stability of concentration of ions. 5 samples of each membrane were taken out after 5, 10, and 30 days, rinsed with deionized water, dehydrated with 100% ethanol, desiccated in 45 °C oven, and the corresponding dry weights of each sample before and after being put into simulated body fluid (W_o, W_t) were recorded for further analysis. At the same time, the pH
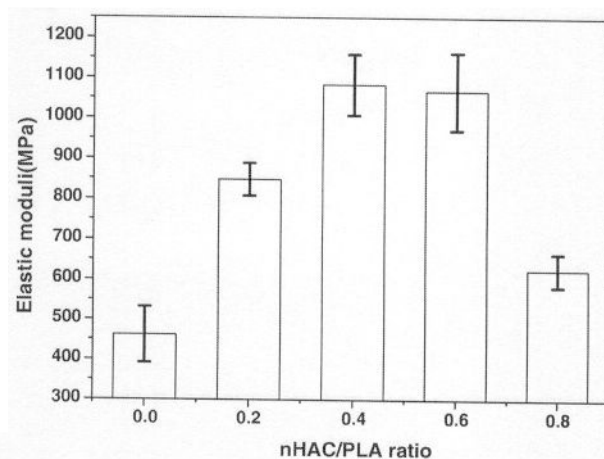

Fig. 1. Elastic modulus of the GTR membranes with different nHAC/PLA ratio (molecular weight of PLA = 125,000 Da).

values of the residual solution were measured with the pH test paper.

$$\text{Weight loss}(\%) = (W_o - W_t)/W_o \times 100$$

Morphology of randomly chosen samples in different aging time was inspected with scanning electron microscopy (SEM, JSM-6460).

2.4. Change of composition

A group of the samples were heated to 450 °C. The result of thermogravimetric analysis (Universal V2.3C TA Instrument) of these samples provided the percentage of hydroxyapatite (HA) in these membranes.

3. Results

3.1. Elastic modulus and maximum tensile strength of the membrane

The mechanical properties are very important for guided tissue regeneration. As shown in Figs. 1 and 2, when nHAC/
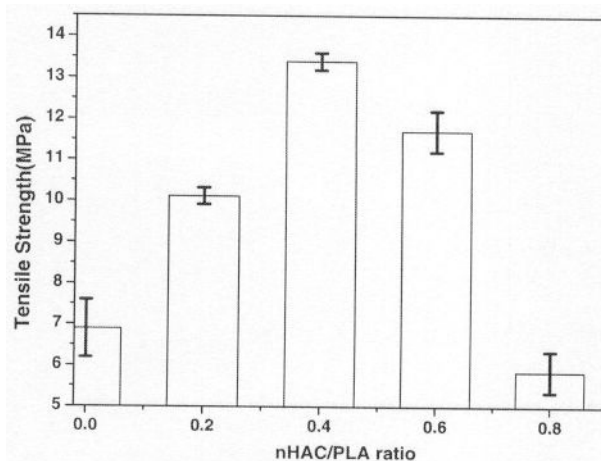

Fig. 2. Tensile strengths of the GTR membranes with different nHAC/PLA ratio (molecular weight of PLA = 125,000 Da).

Table 1
Composition of GTR membranes

Molecular weight of PLA	nHAC/PLA ratios				
200,000 (group 1)	0:1	0.2:1	0.4:1	0.6:1	–
150,000 (group 2)	0:1	0.2:1	0.4:1	0.6:1	0.8:1
125,000 (group 3)	0:1	0.2:1	0.4:1	0.6:1	0.8:1

a

In Vitro Aging Time (days)

nHAC/PLA
ratio

☐ (a)
■ (b)
■ (c)
■ (d)

b

In Vitro Aging Time (days)

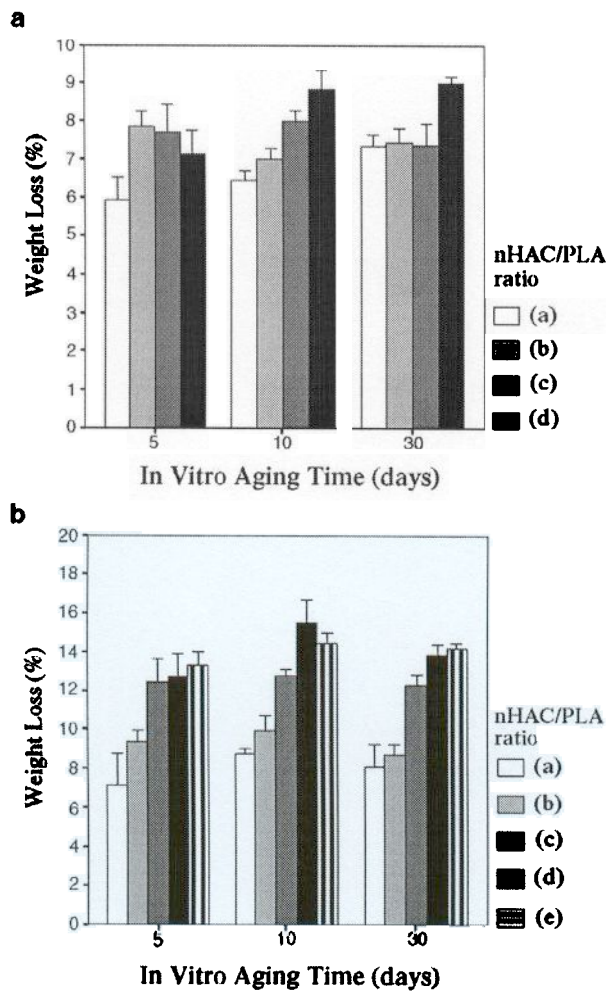nHAC/PLA
ratio

☐ (a)
▨ (b)
■ (c)
■ (d)
▨ (e)

Fig. 3. Weight loss of nHAC membrane with different nHAC/PLA ratio after 5, 10 and 30 days in 37 °C simulated body fluid. (a) Molecular weight of PLA=200,000 Da, (b) molecular weight of PLA=125, 000 Da. (a) nHAC/PLA ratio=0:1; (b) HAC/PLA ratio=0.2:1; (c) nHAC/PLA ratio=0.4:1; (d) nHAC/PLA ratio=0.6:1; (e) nHAC/PLA ratio=0.8:1.

PLA ratios were 20% and 40%, the tensile strength increased from 6.9 MPa to 10.12 and 13.4 MPa, and the elastic module increased from 462 MPa to 848 MPa and 1084 MPa, respectively. It indicated that HA grains can improve the mechanical strength of PLA films. However, if the nHAC/PLA ratio further increased, the mechanical properties began to decrease, which meant that the content nHAC grains was too much to destroy the PLA structure. The results indicated that nHAC/PLA membrane which has 40% nHAC/PLA ratio might be a suitable GTR candidate.

3.2. Weight loss of the nHAC/PLA membrane

The general shapes of all nHAC/PLA samples were maintained throughout the experimental period. Some of the pure PLA membranes showed distortion in the 30-day group (group 3: molecular weight of PLA=125,000 Da). Weight losses of samples in two groups (group 1: molecular weight of PLA=200.000 Da; group 3: molecular weight of PLA=125,000

Da) were shown in Fig. 3(a) and (b). The figures of group 2 (molecular weight of PLA=150,000 Da) was analogous to that of group 3, the only differences between them were that the weight losses of samples in group 2 was a little lower than those in group 3, so the figure of group 2 is not shown in this article. From Fig. 3 we can get the following results: the higher the molecular weight of the PLA is, the lower the weight loss of the film is. In the end of the in vitro aging period, the weight loss of group 1 (molecular weight of PLA=200,000 Da) was 7.33–8.97%, and those in the other two groups were 7.33–14.82% (molecular weight of PLA=150,000 Da) and 8.08–14.31% (molecular weight of PLA=125,000 Da).

With the increase of the nHAC/PLA ratio, the weight loss increased. In group 1, at the end of in vitro aging, the weight loss of the nHAC:PLA ratio=0.6:1 was 22% higher than that of the pure PLA. In group 3, the weight loss of the nHAC:PLA ratio=0.6:1 was 76% higher than that of the pure PLA.

There are significant weight losses in the first 5 days, and then the rate of weight loss decreased significantly. There are even light weight increases in the late part of the in vitro aging period in some samples.

3.3. Change of inorganic composition (HA)

The change of the weight percentage of the HA is shown in Fig. 4. The percentage of the inorganic component changed with aging time. In the original membranes, the higher the nHAC/PLA ratios are, the higher the weight percentages of HA in the membranes are. During the process of in vitro aging, the samples with higher nHAC/PLA ratio still have the higher inorganic component percentage. In the first 10 days, the HA percentage of the samples increased slightly, and then from 10 to 30 days, the weight percentage of HA increased at a higher rate. At the end of the in vitro aging, the increase of weight percentage of HA in the membranes with different nHAC/PLA
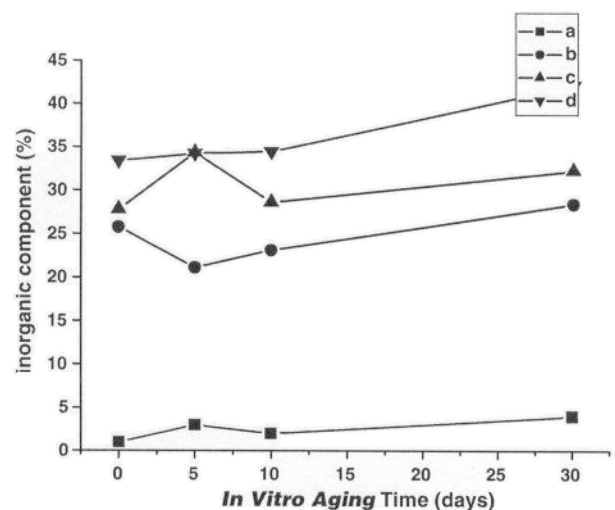

In Vitro Aging Time (days)

Fig. 4. Change of inorganic component (HA) in different nHAC/PLA ratio membranes after 5, 10 and 30 days in 37 °C simulated body fluid (molecular weight of PLA=150,000 Da); (a) nHAC/PLA ratio=0.2:1; (b) nHAC/PLA ratio=0.4:1; (c) nHAC/PLA ratio=0.6:1; (d) nHAC/PLA ratio=0.8:1.
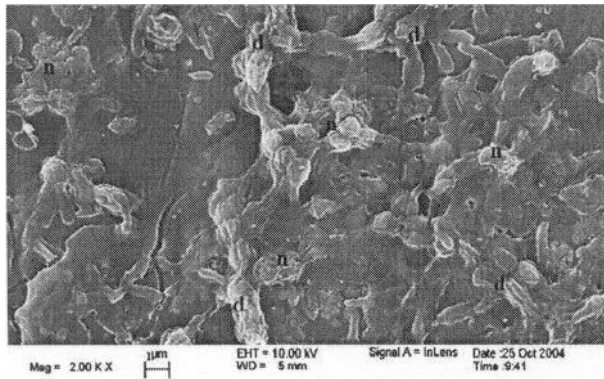
Fig. 5. SEM picture of the nHAC/PLA membrane (molecular weight of PLA=150,000 Da, nHAC/PLA ratio=0.4:1, in vitro aging time: 5 day). Crystal depositions are around and near the nHAC grains in the membrane. n: nHAC grains in the membrane; d: crystal deposition on the membrane.

ratio ranged from 2.89% to 8.82%. The higher the nHAC/PLA ratios are, the higher the increases of weight percentages of inorganic component in the membranes are.

3.4. The pH value

We measured the pH value at the point just before replacing the old simulated body fluid with the new one during the 30-day period. The pH value remained constant at 7.0 (except the first 24 h in which period the pH value was 5.0). The residual solution showed no distinct changes in either color or transparency.

3.5. Membrane morphology

Under the SEM, membranes in different stage were inspected. There are 6 main characteristics of the morphology of different membrane.

1. With nHAC/PLA ratios increased, the nHAC observed in membrane increased.
2. The longer the aging time, the more deposits of crystals on the surface of membrane (Figs. 6 and 7).
3. The areas of crystal deposits were on or around the nHAC grains in the membrane (Fig. 5).
4. There are more deposits of crystals on the membranes with higher nHAC/PLA ratios.
5. There are some rules of the deposition of crystals on the membranes. In the beginning (5 days), the crystal deposits on the membranes were dispersive (Fig. 7a), and the dimension of these crystals was about $1.0 \times 0.2 \times 0.2$ μm. Under the SEM, these crystals can be distinguished from one to another (Fig. 7a). Sometimes, there can be two or several club-shaped connected together with the nHAC in the membrane (Fig. 5). With the aging time increased (10 days and 30 days), the number of crystals on the surface of membrane increased remarkably. The crystals deposited on the surface of membrane began to connect with each other, and formed conglomeration. Layers of depositions of crystals could be observed (Figs. 6 and 7). There were holes in the bulks of crystal deposits, and the holes could be connected with each

other (Fig. 6). We show a typical process of crystal deposition on the nHAC membrane in Fig. 7.

6. There were no evident perforations or breakage on the membrane in 3 aging stages. But with the aging time increased, there were more nHAC grains exposure on the surface of membrane.

4. Discussion

The aim of our study was to get a novel GTR membrane which has better bone conductivity and tissue biocompatibility by adding nHAC into PLA. It is considered that the addition of nHAC has the following advantages:

1. nHAC can be released from the GTR membrane to give the local environment higher Ca, P ion concentration which is propitious to the bone formation.
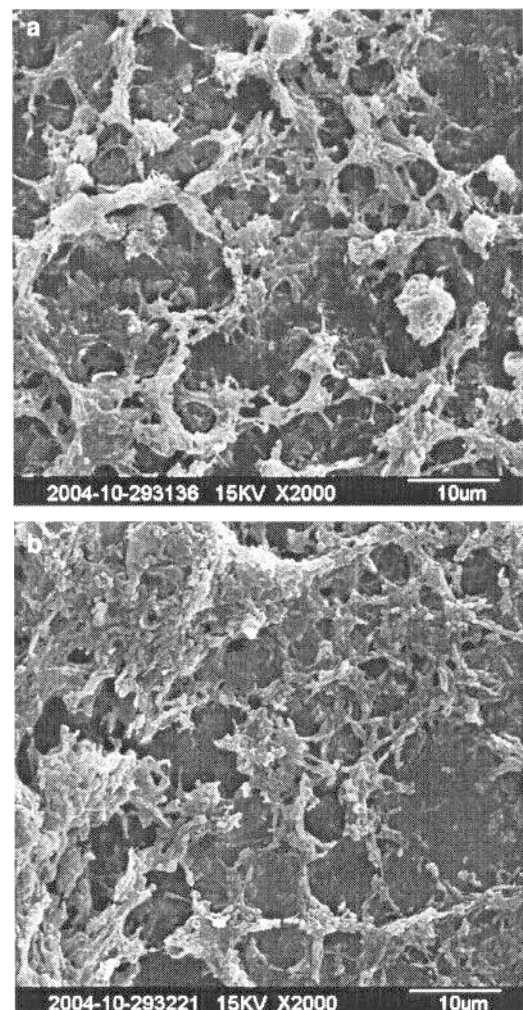

Fig. 6. SEM picture of nHAC/PLA membrane with molecular weight of PLA=200,000 Da after (a) 10 days and (b) 30 days in vitro aging in simulated body fluid, respectively. (a) The crystals deposited on the surface of membrane begin to connect with each other, and form conglomeration. There are holes in the bulks of crystal aggregations, and the holes can be connected with each other. (b) Layers of depositions of crystals can be observed.
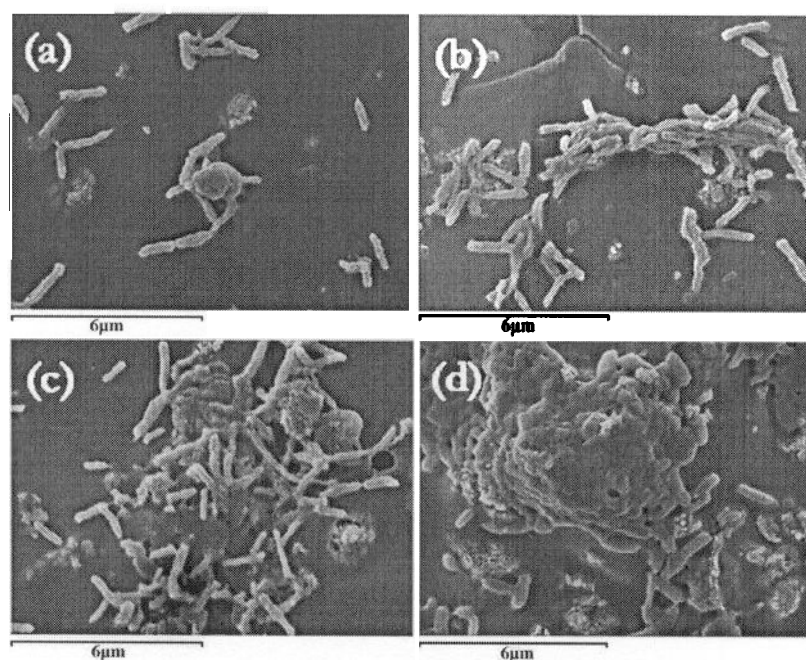
Fig. 7. The process of crystal depositions on the surface of nHAC/PLA composite membranes (molecular weight of PLA=200,000 Da). (a) Individual crystals on the membrane (5 days). (b) Crystals connection (10 days). (c) Crystals conglomeration (10 days). (d) Layers of depositions of crystal (30 days).

2. The addition of nHAC composite can give the membrane a more suitable mechanical characteristic than the pure PLA membrane. It can be used more easily in clinic and the space for the new bone tissue can be maintained for a longer time under the protection of the membrane.

4.1. The optimal content of nHAC in the membrane

Membrane used for GTR must undergo a series of physical manipulations. They are cut, shaped, and sometimes fixed in place with sutures or screws. Clinical manageability of a GTR membrane is determined largely by the ease of surgical manipulation. Optimal membrane design requires minimal difficulty in operative handling to allow the clinician to achieve proper membrane placement [1]. So the addition of the nHAC into the membrane must not impair the mechanical strength of the membrane. In the mechanical test, we add different amount of nHAC into the PLA, and we found that when the nHAC/PLA ratio was 0.4:1, the elastic modulus and maximum tensile strength of the membrane were the highest in all the samples tested. So the ratio 0.4:1 is the optimal ratio for this type of GTR membrane. If we add more nHAC into the film than the optimal ratio, the film will be too fragile to be used.

4.2. In vitro aging of the membrane

In the 30-day aging experiments in vitro, the nHAC/PLA composite membranes maintained integrity and the original appearance throughout the whole experimental period while some of the pure PLA membranes showed distortion in the 30-day group. Their weight loss was about 6–9% in group 1 (molecular weight of PLA=200,000 Da) and 8–15% in groups 2 and 3 (molecular weight of PLA=150,000 Da and 125,000 Da). In the beginning, 5 days, there were rapid weight losses, and then, from 5 to 30 days, the rate of weight loss slowed down. There were no more significant weight losses in this period. In the group of 30 days, there were even slight decreases in weight loss in many samples. As we all know, PLA is hydrophobic. Hybridization with collagen or mineralized collagen improved its wettability [9–11]. Because of the better wettability of the nHAC, the rapid increase in weight loss happened in the first few days. Another reason for the rapid weight loss in the beginning is the acidic environment of the simulated body fluid in the first 24 h; nHAC and PLA have a higher ratio of dissolution in the acidic environment.

But why there were slight decreases in the weight loss in the 30-day group in many samples? Firstly, we know that PLA is a kind of inert substance which may break down slowly in the in vitro environment, so there was no significant weight loss in the end of the in vitro aging time. This is the main reason for the question. Secondly, from SEM picture, we find that after aging in the simulated body fluid there were crystal deposits on the surface of the membranes, and the number and size of the crystal particles increased as time went on. So we think that the crystals which contain Ca and P ions deposited on the membranes are another reason for the decrease of weight loss in the 30-day group. Simulated body fluid is an instable solution system, when the concentration of Ca, P ions approaches the threshold of deposition, the Ca, P composite will deposit on the surface of the materials. Murphy WL had tested the PLGA; he found that there was bone-like apatite deposited on the materials [12]. Kasuga T and Ladron de Guevara-Fernandez also have the same tests [13,14]. Usually,

the process of aging of PLA is a kind of bulk decomposition [15], only when the molecular weight of the polymer decreases to a critical value, can the degraded production be released. We can also see the rough surface of nHAC/PLA membrane in the 10- and 30-day groups. In this period, the weight loss was not significant, because of the deposition of inorganic crystals, the weight of the membranes even slightly increased. In fact, the decomposition of the film continued under the crystal deposits. From the results of the thermogravimetric analysis (Fig. 4), we can also see that the percentage of inorganic components in the nHAC/PLA composite membrane increased in the end of the in vitro aging process.

From the weight loss figures (Fig. 3), we also found that the higher the nHAC/PLA ratio, the more weight loss in the membranes. There was more weight loss in the membrane with higher nHAC/PLA ratio because there were nano-HA particles released from the membranes with the aging of the nHAC/PLA composite membranes. Another obvious result we got from the experiment is that the composite membrane with higher molecular weight of PLA has a lower aging rate. In oral bone defect restoration and oral implantology, it needs more than 6 months to get a stable new host bone formation around the implant [16,17]. So we should choose PLA with higher molecular weight to fulfill this requirement.

4.3. SEM analysis

SEM results of the membranes also gave us some important arguments. In the stage of 5 days, there are crystal deposits on the surface of the membrane. The formation of the crystal deposits was near the nHAC grains in the composite membrane, and there is much less crystals in the areas between the nHAC grains. In the stage of 10 days and 30 days, there are more and more crystals on the membrane. The reason for this phenomenon maybe that the nHAC in the composite membrane was released from the membrane, and increased the concentration of the Ca^{2+}, PO_3^{2+} in the local area. The formation of the crystal deposits on the membrane may become easier and more rapid in such an environment. This is also favorable for the bone formation in the clinical patients. It is an evident advantage for the new composite membrane compared with other degradable GTR membranes.

In the SEM of the membrane in the 10 days and 30 days, there are bulks of crystal depositions, and there are holes in the bulks of depositions, and the holes can be connected with each other. These holes should be very suitable for the ingrowth of the surrounding tissue, and can help the integration of the membrane with the surrounding tissue. This can result in a

more mechanically stable (and therefore predictable) wound healing environment [1].

During the whole period, the pH value of the residual simulated body fluid remained constant at 7.0 (except the first day on which we adjust the pH of the simulated body fluid to 5.0 to mimic the acid environment in the human body after surgery). This may result from the neutralization effect of the nHAC on the acidic decomposed products of PLA.

5. Conclusion

NHAC/PLA composite membranes maintained integrity and the original appearance throughout the 1-month in vitro aging. There is active dissolution and deposition process of crystals which is propitious to the bone formation on the surface of the composite membrane. The optimal nHAC/PLA ratio of the novel membrane is 0.4:1. For a longer period of bone repair, PLA with higher molecular weight should be chosen as the scaffold for the GTR membrane.

Acknowledgement

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