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Guided bone regeneration by poly(lactic-*co*-glycolic acid) grafted hyaluronic acid bi-layer films for periodontal barrier applications

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Abstract

A novel protocol for the synthesis of biocompatible and degradation controlled poly(lactic-*co*-glycolic acid) grafted hyaluronic acid (HA-PLGA) was successfully developed for periodontal barrier applications. HA was chemically modified with adipic acid dihydrazide (ADH) in the mixed solvent of water and ethanol, which resulted in a high degree of HA modification up to 85 mol.%. The stability of HA-ADH to enzymatic degradation by hyaluronidase increased with ADH content in HA-ADH. When the ADH content in HA-ADH was higher than 80 mol.%, HA-ADH became soluble in dimethyl sulfoxide and could be grafted to the activated PLGA with N,N′-dicyclohexyl carbodiimide and N-hydroxysuccinimide. The resulting HA-PLGA was used for the preparation of biphasic periodontal barrier membranes in chloroform. According to in vitro hydrolytic degradation tests in phosphate buffered saline, HA-PLGA/PLGA blend film with a weight ratio of 1/2 degraded relatively slowly compared to PLGA film and HA coated PLGA film. Four different samples of a control, OSSIX^{TM} membrane, PLGA film, and HA-PLGA/PLGA film were assessed as periodontal barrier membranes for the calvarial critical size bone defects in SD rats. Histological and histomorphometric analyses revealed that HA-PLGA/PLGA film resulted in the most effective bone regeneration compared to other samples with a regenerated bone area of 63.1% covering the bone defect area.

Keywords: Hyaluronic acid; Poly(lactic-*co*-glycolic acid); Periodontal barrier membrane; Controlled degradation; Bone regeneration

1. Introduction

A variety of membrane materials has been developed for guided bone regeneration (GBR) and guided tissue regeneration (GTR) [1–6]. The materials that are used as a barrier membrane for GBR/GTR procedures should meet several prerequisites. As the membrane is supposed to be implanted in the body, it must be biocompatible, non-immunogenic, and non-toxic. To avoid the removal of the membrane after healing, it would be better to be composed of biodegradable materials. The degradation time should be long enough to achieve bone regeneration before membrane disintegration. Other properties such as tissue integration, cell occlusivity, nutrient transfer, space making ability and ease of use in the clinic are also of interest [7]. There are various commer-

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cially available products, ranging from non-resorbable materials such as expanded polytetrafluorethylene (e-PTFE) to bioabsorbable membranes composed of poly(lactic acid), poly(glycolic acid), polyurethane, and so on [7–11]. More recently, many investigations focused on the use of products derived from type I and type III porcine or bovine collagen [12]. Some advantageous properties of collagen over other materials include homeostatic function to allow early wound stabilization, chemotactic properties to attract fibroblasts, and semi-permeability to facilitate nutrient transfer [13]. However, the porcine and bovine collagens are known to have a major drawback of immunogenicity in the body.

Poly(lactic-*co*-glycolic acid) (PLGA) has been extensively investigated and used for various medical applications for a few decades due to its biodegradability and biocompatibility [14]. The biodegradation of PLGA can be controlled by changing its molecular weight, composition (the ratio of LA to GA in PLGA), crystallinity and other parameters [14]. More significantly, PLGA has the outstanding biocompatibility with bio-absorbable and non-toxic degradation products. PLGA exhibits a wide range of physicochemical diversities depending on the structural characteristics. For example, high-molecular-weight crystalline PLGA can be fabricated into surgical sutures, bone fixation nails and screws with a feasible mechanical strength. On the other hand, low molecular weight amorphous PLGA is found to be useful for controlled drug delivery applications [15]. Recently, hyaluronic acid (HA) and modified HA have been used for various medical applications such as drug delivery and tissue engineering [16–21]. As a natural linear polysaccharide, HA is biodegradable, biocompatible and non-immunogenic [22]. HA is also known to be osteoconductive, promote angiogenesis, and moderate immune responses [22]. A number of strategies for the chemical modification of HA through the functional groups of carboxyl and hydroxyl groups have been reported as described elsewhere [23–27]. Most of HA chemical modifications have been carried out in aqueous solution. In order for the chemical modification of HA in an organic solvent, such as dimethyl sulfoxide (DMSO), tetrabutyl ammonium (TBA) salt of HA was prepared in aqueous solution using ion-exchange resins [23]. For example, benzyl ester of HA, Hyaff®, has been synthesized by the esterification of TBA salt of HA with benzyl bromide in DMSO [23].

In this work, we have developed a novel biocompatible and degradation-controlled HA-PLGA for the applications to periodontal barrier membranes. HA was chemically modified with adipic acid dihydrazide (ADH) in the mixed solvent of water and ethanol. The addition of ethanol resulted in highly modified HA-ADH, which exhibited the enhanced stability to enzymatic degradation by hyaluronidase. Interestingly, when the ADH content in HA-ADH was higher than 80 mol.%, HA-ADH became soluble in DMSO and could be grafted to the activated PLGA with N, N′-dicyclohexyl carbodiimide (DCC) and N-hydroxysuccinimide (NHS). The resulting HA-PLGA was used for the preparation of amphiphilic bi-phasic films. After in vitro degradation tests in phosphate buffered saline (PBS), four different samples of a control (no treatment), OSSIX™ membrane, PLGA film, and HA-PLGA/PLGA blend film were assessed as periodontal barrier membranes for bone regeneration in the calvarial critical size bone defect of SD rats. Histological and histomorphometric analyses were carried out after hematoxylin–eosin (H&E) staining of regenerated bones in 8 and 12 weeks.

2. Experimental

2.1. Materials

PLGA with a molecular weight (MW) of 66,000 was obtained from Wako Pure Chemicals Co. (Osaka, Japan). HA with MW of 20,000 and 132,000 was purchased from Lifecore Co. (Chaska, MN). Adipic acid dihydrazide (ADH), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC hydrochloride), N-hydroxysuccinimide (NHS), N,N′-dicyclohexyl carbodiimide (DCC), and PBS tablet were purchased from Sigma–Aldrich (Milwaukee, WI). Ethanol, hydrochloride (HCl), sodium hydroxide, acetonitrile, dimethyl sulfoxide (DMSO) and chloroform ($CHCl_3$) were obtained from Junsei Chemicals (Tokyo, Japan), and hyaluronidase SD (*Streptococcus dysgalactiae*) from Seikagaku Biobusiness Co. (Tokyo, Japan). All reagents were used without further purification.

2.2. HA-ADH synthesis

To increase the degree of ADH modification in HA-ADH, the protocol for HA-ADH preparation by Luo et al. was slightly modified as follows [27]. HA (100 mg, 250 μmol) was dissolved in 20 ml of water to prepare HA solution of 5 mg ml^{-1}. Forty times molar excess of solid ADH (10 mmol) was added to the solution and dissolved completely by mixing for 10 min. The pH of the mixed solution was adjusted to 4.8 by the addition of 1.0 N HCl. Then, ethanol (20 ml, 50 vol.%) was added and mixed for 30 min. After that, four times molar excess of EDC (1 mmol) was added in a solid form. The pH of the mixed solution was maintained at 4.8 by the addition of 1.0 N HCl. The reaction was stopped in 2 h by raising the pH of reaction mixture to 7.0 with 1.0 N NaOH. The reaction solution was poured into the pre-washed dialysis membrane tube (MWCO of 7000) and dialyzed against a large excess amount of 100 mM NaCl solution, followed by the dialysis against 25 vol.% ethanol and pure water. The resulting solution was finally lyophilized for 3 days. The purity of HA-ADH was determined by gel permeation chromatography (GPC, Waters, Milford, MA) and the degree of ADH modification was measured by ^1H nuclear magnetic resonance (NMR, DPX300, Bruker, Germany) analysis [27].

2.3. In vitro degradation tests of HA-ADH

HA with a MW of 132,300 (0.8 mg) and HA-ADH with three different degrees of ADH modification (24, 57, and 80 mol.%, 0.8 mg) were dissolved in 0.4 ml of water, respectively. After complete dissolution, 0.4 ml of water containing 0.08 U of hyaluronidase SD was added to each solution. Then, the solutions were incubated at 37 °C for the predetermined time (0–48 h). At the sampling time, 50 μl of each solution was collected and analyzed by GPC. GPC analysis was performed using the following systems: Waters 1525 binary HPLC pump, Waters in-line degasser AF, Waters 2424 ELS detector, Waters 717 plus autosampler, Ultrahydrogel 250, 500, and 1000 columns (7.8 mm × 30 cm) (Milford, MA, USA). The eluant was 10 mM (pH 5.8) ammonium acetate buffer/methanol with a volume ratio of 80/20 and the flow rate was 0.5 ml min^{-1}. ELS parameters were set to a drift tube temperature of 50 °C and a nitrogen gas pressure of 30 psi. Triplicates were carried out for the in vitro degradation tests.

2.4. Conjugation of HA-ADH with PLGA

PLGA (200 mg, 10 μmol) was dissolved in DMSO (5 ml) and activated by the addition of DCC (3.1 mg, 15 μmol) and NHS (1.73 mg, 15 μmol). Then, HA-ADH (5 mg, 10 μmol) with 83 mol.% ADH content was dissolved in 5 ml of DMSO and mixed with the activated PLGA solution for 12 h. The resulting HA-PLGA was recovered by the dialysis against excess amount of water and freeze-dried for 3 days. The degree of PLGA modification in HA-PLGA was determined by ^{1}H NMR analysis in comparison with PLGA peaks.

2.5. Preparation and characterization of HA-PLGA/PLGA films

HA-PLGA was re-dissolved in DMSO and dialyzed again for the preparation of HA-PLGA nano-particles, which were analyzed by scanning electron microscopy (SEM, ×15,000, Hitachi S-4200, Tokyo, Japan). HA-PLGA/PLGA blend films were fabricated by the solvent casting method. HA-PLGA and PLGA were dissolved in chloroform (5 ml) at a concentration of 10 mg ml^{-1}, respectively. The weight ratio of HA-PLGA to PLGA was changed from 1/0, 1/1, 2/1, 1/2 to 0/1. The solution was mixed for the blending of two polymers for 5 h. After filtration, the solution was poured into poly(tetra-fluoroethane) petri dish and dried at room temperature for 2 days. HA-PLGA/PLGA blend films were analyzed with a SEM (×3000) and a contact angle analyzer (Face contact angle meter, Kyowa Kaimenkagaku, Tokyo, Japan). For comparison, HA coated PLGA films were prepared by putting the PLGA film in HA-ADH aqueous solution (2 wt.%) containing four equivalent amount of EDC to carboxyl groups of PLGA. Triplicates were carried out for the film preparation and characterization.

2.6. In vitro degradation tests of HA-PLGA/PLGA films

Three kinds of films, PLGA, HA coated PLGA, and HA-PLGA/PLGA (blending weight ratio of 1/2) films, were prepared with a dimension of 1 × 1 cm and a weight of ~10 mg and put into vials for in vitro degradation tests, respectively. PBS (0.2 M, pH 7.4) was added to each vial, which was incubated at 37 °C for 8 weeks. At the sampling time, the recovered film was washed with distilled water and dried to measure the remaining weight of the films. The degree of film degradation was represented by a weight ratio (%) of the remaining film to the original film. Triplicates were carried out for the in vitro degradation tests.

2.7. In vivo bone regeneration tests

The skull of each SD rat was incised and two critical size bone defects with a diameter of 8 mm were made as identical as possible with a trephine bur ($d = 8$ mm). Then, three kinds of films, OSSIX™ membrane (Herzliya, Israel), PLGA film, and HA-PLGA/PLGA film, were used to cover up the bone defect regions ($n = 3$ for each sample). After 8 and 12 weeks, the rats were sacrificed for histological and histomorphometric analyses. The regenerated bone defect samples were fixed with 10% formalin for 2 days and decalcified with 10% EDTA for 2–3 weeks. The 5 μm-thick paraffin sections were prepared following the routine procedure. The degree of bone regeneration was assessed by the observation with a digital camera connected light microscope (Olympus Corporation, Tokyo, Japan) at a magnification of ×20. The histomorphometric data were collected using a picture analysis program (*i*MT image analysis software, *i*MTechnolog, Daejeon, Korea). The percentage (%) of new bone formation was presented as the ratio of new bone area versus total defect area. We complied with institutional ethical use protocols for the animals.

2.8. Statistical analysis

The data are expressed as mean ± SD from three separate experiments. Statistical analysis was carried out via *t*-test using a software of SigmaPlot 9.0 and a value for $P < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Synthesis and in vitro degradation test of HA-ADH

A novel protocol to synthesize biocompatible and degradation-controlled HA-PLGA was successfully developed using HA-ADH for periodontal barrier applications. HA was chemically modified by grafting ADH to the carboxyl group of HA in the mixed solvent of water and ethanol to prepare highly modified HA-ADH (Fig. 1a). The carboxyl group of HA is known to be the recognition site of hyaluronidase [28,29]. Previously, we have reported the effect

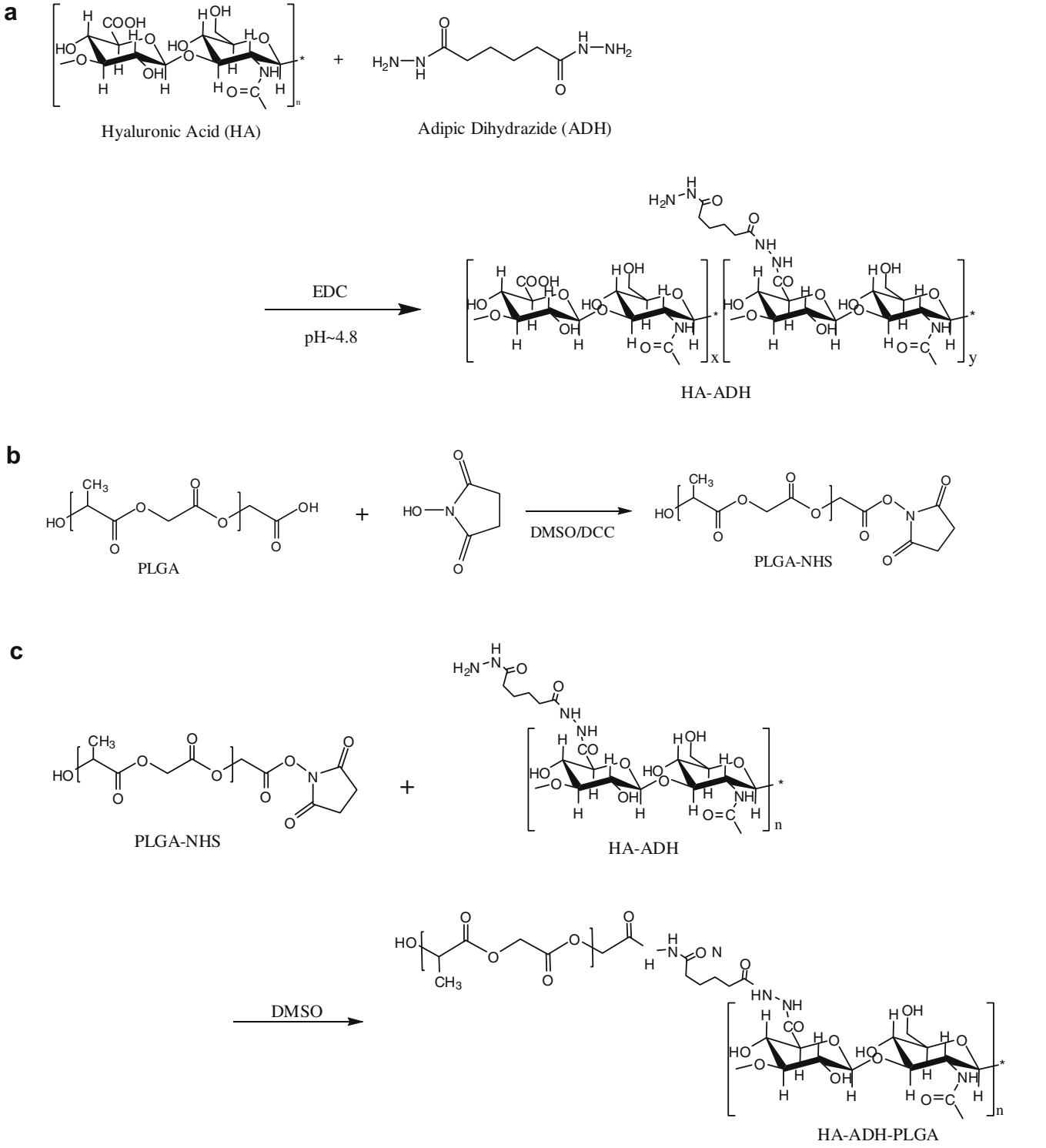

Fig. 1. Schematic representations for (a) synthesis of adipic acid dihydrazide modified hyaluronic acid (HA-ADH), (b) activation of PLGA with N,N′-dicyclohexyl carbodiimide (DCC) and N-hydroxysuccinimide (NHS), and (c) conjugation of HA-ADH with the activated PLGA–NHS to synthesize HA-PLGA.

of HA modification on its distribution in the body [28]. In addition, degradation-controlled HA hydrogels were discussed for tissue augmentation applications [29]. The peak assignment of HA-ADH in ^{1}H NMR spectra was carried out as described elsewhere [28,29]. The methyl resonance ($\delta = 1.85$–1.90 ppm) of acetamido moiety of N-acetyl-D-glucosamine residue was used as an internal standard and the degree of HA-ADH modification was determined from the peak area of methylenes of ADH at $\delta = 1.7$ and 2.4 ppm [29]. The degree of HA modification increased

up to 85 mol.% with increasing ethanol content in the mixed solvent of water and ethanol. The addition of ethanol appeared to contribute for high degree ADH modification in HA-ADH (Table 1). As discussed in our previous report [29], the higher ADH modification of HA in the mixed solvent of water and ethanol might be attributed to the fact that HA has a different conformational structure in water and in organic solvent due to the different hydrogen bonding. The helical structure of HA in water was thought to be disorganized by the addition of ethanol contributing for higher degree of ADH modification [29]. When the ADH content in HA-ADH was higher than ~80 mol.%, HA-ADH became soluble in DMSO contributing for the versatile chemical modification of HA in an organic solvent.

The effect of HA modification with ADH on HA degradation was assessed by GPC analysis after treatment with hyaluronidase SD. The amount of hyaluronidase SD was optimized to differentiate the degradation behavior of HA derivatives. Fig. 2 shows the change in elution times of HA and HA-ADH samples before and right after hyaluronidase treatments, and after 24 and 48 h hyaluronidase treatments. In all samples, the retention time increased right after hyaluronidase treatment reflecting the degradation of HA by hyaluronidase. After 24 h, however, the extent of HA degradation was different according to the degree of ADH modification. The retention times of HA-ADH with 24 and 57 mol.% ADH contents further increased from those at the previous sampling time, but that with 80 mol.% ADH content did not change as shown in Fig. 2. After hyaluronidase treatment for 48 h, HA was completely degraded to the disaccharide units, HA-ADH with 24 and 57 mol.% ADH contents were further degraded to HA fragments, but HA-ADH with 80 mol.% ADH content was not degraded any more. After confirmation of the enhanced enzymatic stability with increasing HA modification, we tried to degrade HA-ADH with 83 mol.% ADH content using excess amount of hyaluronidase. The HA-ADH was completely degraded eventually with slow degradation kinetics. A periodontal barrier membrane should be biodegradable but stable long enough to achieve bone regeneration before membrane disintegration. From the results, HA-ADH with 80 mol.% ADH content was thought to be successfully applied to the preparation of periodontal barrier membrane with a relatively good stability to enzymatic degradation by hyaluronidase.

Table 1
Effect of ethanol content in the mixed solvent of water and ethanol on the degree of adipic acid dihydrazide (ADH) modification in ADH grafted hyaluronic acid (HA-ADH) at HA concentrations of 2 and 5 mg ml^{-1}.

HA concentration	Degree of ADH modification in HA-ADH			
	Ethanol	0 vol.%	25 vol.%	50 vol.%
2 mg ml^{-1}		64.8 ± 2.3	70.9 ± 1.4	75.8 ± 3.6
5 mg ml^{-1}		69.4 ± 1.0	79.6 ± 3.2	85.0 ± 1.9

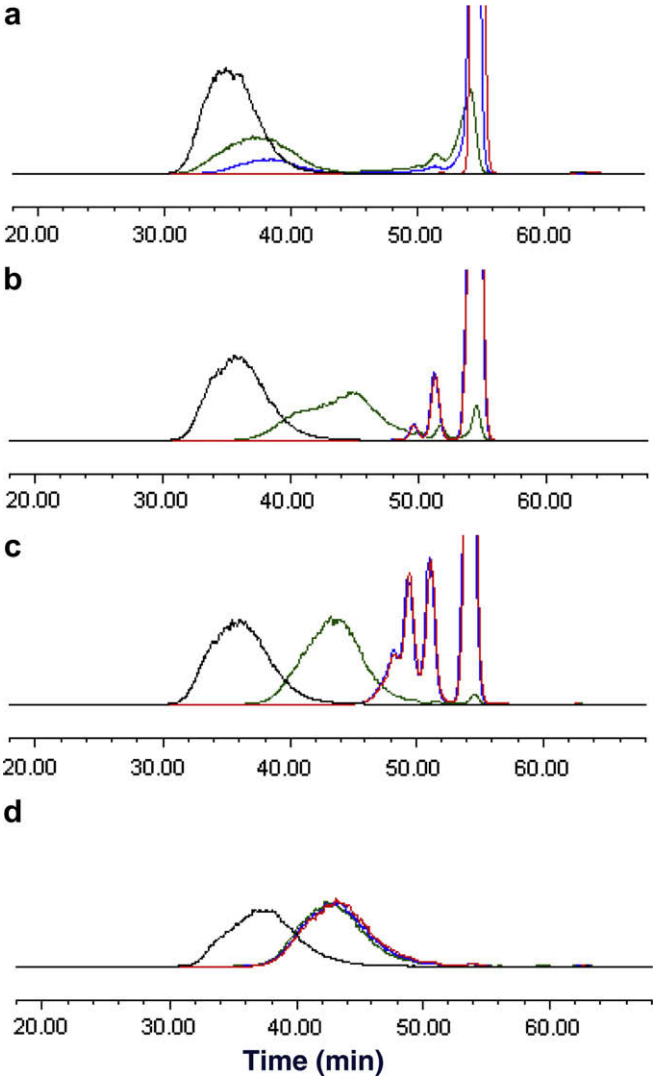

Fig. 2. Gel permeation chromatograms (GPC) of (a) hyaluronic acid (HA), (b) adipic acid dihydrazide grafted HA (HA-ADH) with 24 mol.% ADH content, (c) HA-ADH with 57 mol.% ADH content, and (d) HA-ADH with 80 mol.% ADH content before (black) and right after hyaluronidase treatment (green), and after 24 h (blue) and 48 h (red) hyaluronidase treatments (For interpretation of color mentioned in this figure legend the reader is referred to the web version of the article.).

3.2. Synthesis and characterization of HA-PLGA

HA-ADH with an ADH content of 83 mol.% was used for the conjugation with PLGA. Fig. 1b and c shows schematic representations for HA-PLGA synthesis in DMSO. The carboxyl groups of PLGA were first activated with DCC and modified with sulfo-NHS (Fig. 1b). HA-ADH in DMSO could be conjugated to the activated PLGA through the formation of amide linkage between the hydrazide group of HA-ADH and the carboxyl terminal group of PLGA (Fig. 1c). This chemistry is well established for the conjugation of biological molecules such as proteins and peptides [30]. The resulting HA-PLGA was recovered in the form of nano-particles by the dialysis against distilled water. The morphology of HA-PLGA nano-particles was analyzed by SEM (Fig. 3a). They were in a spherical

shape with a mean particle size of ~150 nm. The novel HA-PLGA polymeric micelle might be used as a novel drug carrier, especially for cancer drugs. As is well known, a drug delivery system with a mean particle size of 100–200 nm can be selectively delivered to cancer cells by the enhanced permeation and retention (EPR) effect [31,32]. After freeze-drying, HA-PLGA was dissolved in DMSO and analyzed by ^{1}H NMR (Fig. 4A). According to the analysis method of Kim et al. [33], the peak assignment of PLGA in HA-PLGA was carried out in comparison with PLGA peaks in Fig. 4B. The methyl resonance ($\delta = 1.85$ ppm) of acetamido moiety of N-acetyl-D-glucosamine residue was used as an internal standard and the degree of PLGA modification in HA-PLGA was determined from the methylene peak area of GA at $\delta = 5.2$ ppm. The degree of HA modification with PLGA was quantitatively dependant on the added amount of PLGA. The ^{1}H NMR spectra showed the characteristic peaks of HA and PLGA, which confirmed the successful synthesis of HA-PLGA. HA-PLGA with a PLGA conjugation degree of 37.4 mol.% to the ADH in HA-ADH was used for the preparation of periodontal barrier membranes.

3.3. Preparation and characterization of HA-PLGA films

The recovered HA-PLGA was soluble in chloroform and used for the preparation of HA-PLGA film by the solvent casting method. HA-PLGA solution was also blended with PLGA in chloroform to make HA-PLGA/PLGA blend films. There was a phase separation between hydrophilic HA and hydrophobic PLGA domains in HA-PLGA/PLGA film. The surface analysis by contact angle measurement confirmed that top layer (~40°) was relatively hydrophilic HA domain and the bottom layer (~78°) was hydrophobic PLGA domain on poly(tetra-fluoroethane) petri dish. The film became smooth and transparent with increasing PLGA content. Fig. 3b shows the SEM image of inverted HA-PLGA/PLGA blend film with a weight ratio of 1/2, which clearly reveals the bi-layer structure of the HA-PLGA/PLGA film. The film thickness was estimated to be ~33 μm. The novel amphiphilic biphasic film was thought to be used as a periodontal barrier membrane. The hydrophobic PLGA-rich layer, which faces the soft tissue, may be cell-occlusive and prevent the invasion of soft tissue cells into the film-protected space [31,32]. The hydrophilic and loosely arranged HA-rich layer, facing the bony defect, may stabilize clots and enamel bone cells to be integrated into the barrier membrane [34,35].

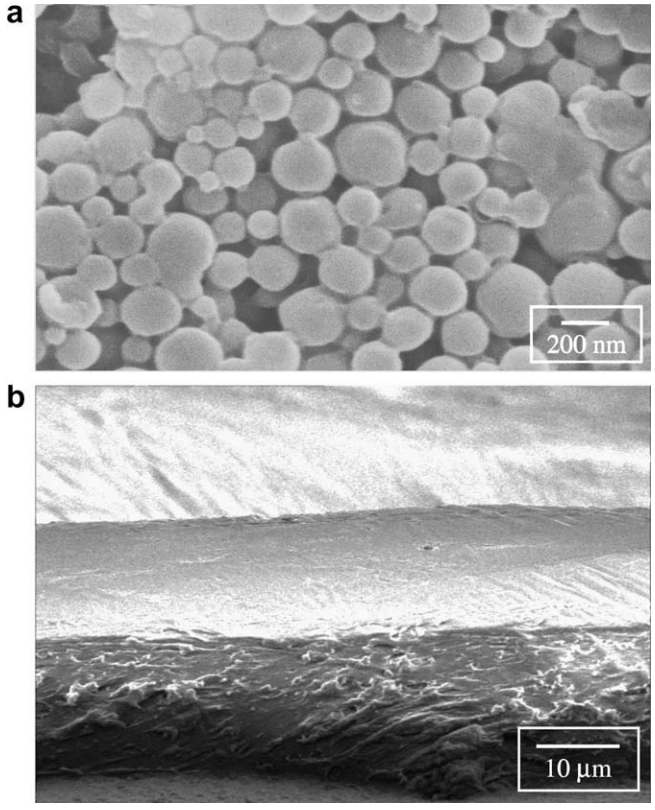

Fig. 3. Scanning electron microscopy of (a) poly(lactic-*co*-glycolic acid) grafted hyaluronic acid (HA-PLGA) nano-particles and (b) HA-PLGA/PLGA (1/2 weight ratio) blend film with a PLGA top layer and an HA-rich bottom layer.

3.4. In vitro degradation tests of HA-PLGA/PLGA film

In vitro hydrolytic degradation tests of PLGA film, HA coated PLGA film, and HA-PLGA/PLGA film were carried out in PBS. As shown in Fig. 5, all three films were degraded slowly until 6 weeks. While PLGA film and HA coated PLGA film were degraded significantly in 7 weeks due to the hydrolysis of ester linkage in PLGA, HA-PLGA/PLGA film were degraded relatively slowly. There were no remaining recoverable film fragments for PLGA film and HA coated PLGA film in 8 weeks. According to *t*-test, the biodegradability of HA-PLGA/PLGA film was significantly different from those of PLGA film and HA coated PLGA film ($P < 0.05$). Highly modified HA-ADH, which is rarely degraded in the PBS solution, might contribute to maintain the HA-PLGA/PLGA film morphology. The PLGA linked with HA-ADH by amide bond formation in HA-PLGA might be also more hydrolytically stable than PLGA with hydrophilic carboxyl groups. Furthermore, HA-PLGA/PLGA film was expected to be degraded slowly in the periodontal bony area with little hyaluronidase. On the basis of in vitro degradation test results, we decide to apply the biocompatible and degradation-controlled HA-PLGA/PLGA film to a periodontal barrier membrane for bone regeneration.

3.5. In vivo bone regeneration by HA-PLGA/PLGA barrier membrane

In comparison with a control (no treatment), OSSIX™ membrane, PLGA film, and HA-PLGA/PLGA film were assessed as periodontal barrier membranes for guided bone regeneration in the calvarial critical size bone defects of SD rats. Fig. 6 shows the photomicrographs of recovered calvarial critical size bone defect regions after bone regener-

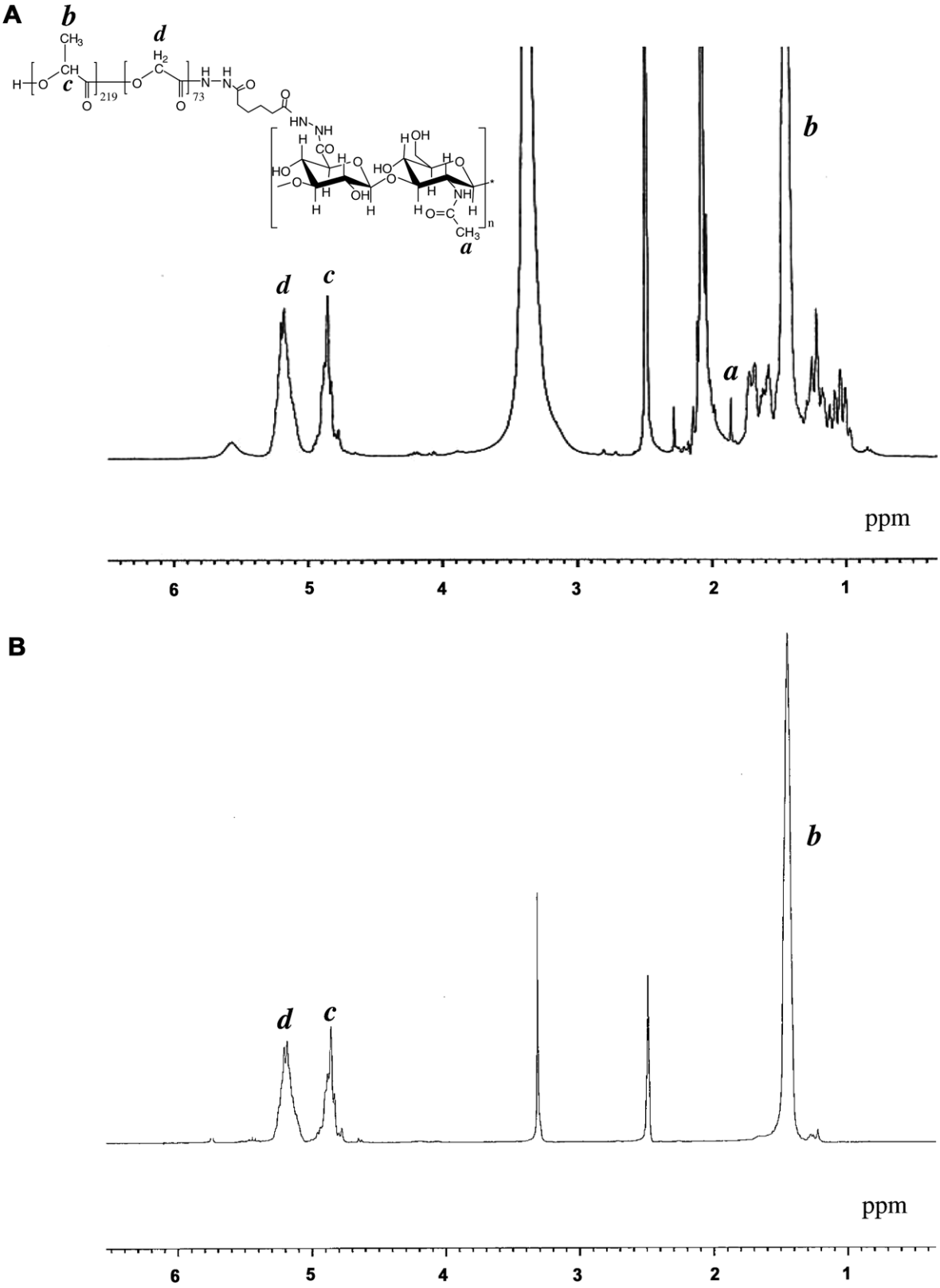

Fig. 4. ^{1}H nuclear magnetic resonance (NMR) spectra of (A) poly(lactic-*co*-glycolic acid) grafted hyaluronic acid (HA-PLGA) and (B) poly(lactic-*co*-glycolic acid) in dimethyl sulfoxide (d_6 DMSO).

ation for 8 weeks. PLGA film was degraded and absorbed completely, HA-PLGA/PLGA film was degraded significantly, but OSSIX^{TM} membrane was not degraded at all as indicated with green arrows in Fig. 6. The results were well matched with those of the in vitro degradation tests. In case of the control, bone regeneration was negligible. The bone

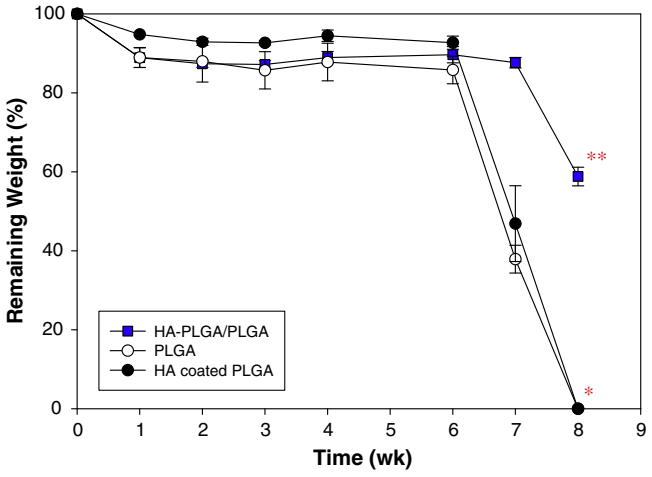

Fig. 5. In vitro hydrolytic degradation of poly(lactic-*co*-glycolic acid) grafted hyaluronic acid (HA-PLGA)/PLGA (1/2 weight ratio) blend film, PLGA film, and HA coated PLGA film in PBS.*There were no remaining recoverable film fragments for the measurement of the remaining weights of PLGA film and HA coated PLGA film in 8 weeks. **Significantly different ($P < 0.05$) biodegradability according to the *t*-test.

regeneration by PLGA film and HA-PLGA/PLGA film was comparable to that by OSSIX^{TM} membrane. Nevertheless, the bone regeneration was not complete in 8 weeks.

Fig. 7 shows the photomicrographs of recovered calvarial critical size bone defect regions after bone regeneration for 12 weeks. The bone regeneration by the control was just a little as shown in Fig. 7a. HA-PLGA/PLGA film resulted in the most effective bone regeneration followed by OSSIX^{TM} membrane and PLGA film. OSSIX^{TM} membrane was not degraded even after 12 weeks as indicated by the green arrows in Fig. 7b, whereas HA-PLGA/PLGA film was degraded and absorbed completely contributing for the formation of thick and almost complete bone plate except the central area (Fig. 7d). The regenerated new bone was well integrated with the original bone.

The bone regeneration by the periodontal barrier membranes was quantified by histomorphometric analysis and represented in Fig. 8. In the case of HA-PLGA/PLGA film, the regenerated bone appeared to cover 63.1% of the bone defect area in 12 weeks. The bone regeneration by PLGA film was not significant compared to that by HA-PLGA/PLGA film. The results might be ascribed to the relatively fast degradation of PLGA film before the formation of thick bone plate. From the results, it was thought that periodontal barrier membrane should be stable for a certain period, about 8 weeks in this study, to be effective for the formation of thick bone plate. As mentioned above, the amphiphilic bi-layer film of HA-PLGA/PLGA must be advantageous for the prevention of soft tissue invasion by PLGA-rich layer as well as guided bone regeneration under the HA-rich layer. The degradation product of HA-PLGA did not cause any negative effect in the body. HA is well known to be biocompatible, angiogenic, and osteo-conductive. Considering all these results, the biocompatible and degradation-controlled HA-PLGA/PLGA film was thought to be successfully

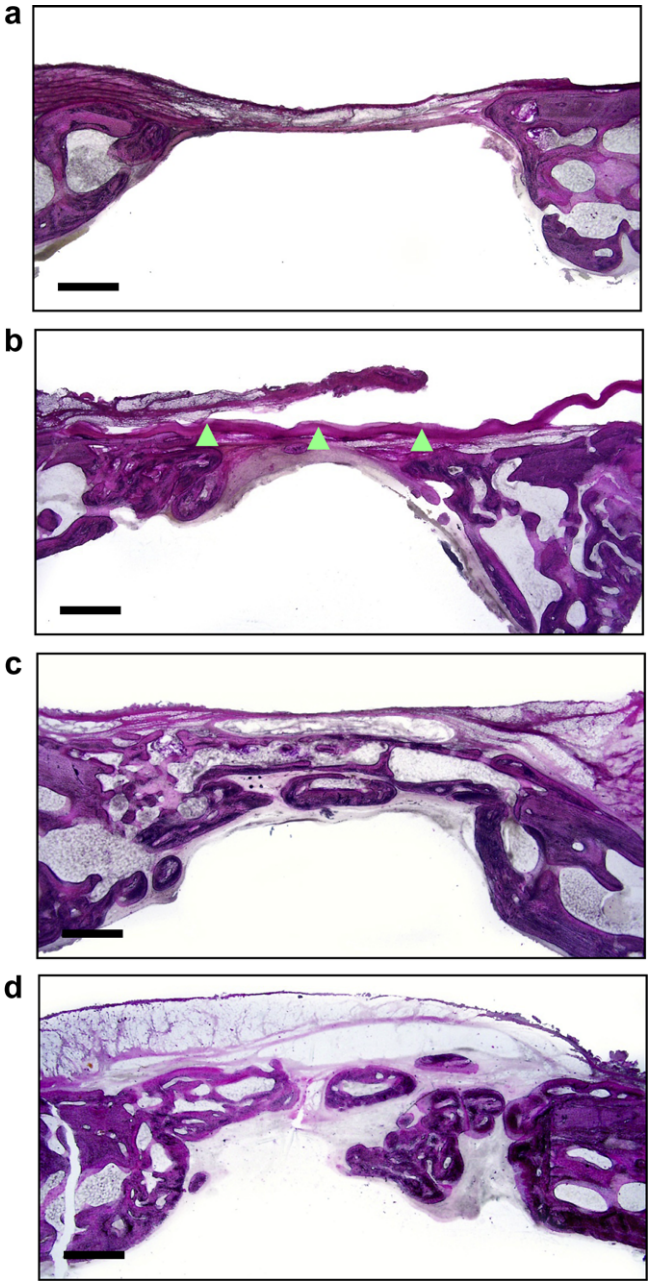

Fig. 6. Photomicrographs of the calvarial critical size bone defect regions of SD rats after bone regeneration for 8 weeks: (a) control, (b) OSSIX^{TM} membrane, (c) poly(lactic-*co*-glycolic acid) (PLGA) film, and (d) PLGA grafted hyaluronic acid (HA-PLGA)/PLGA (1/2 weight ratio) blend film, respectively (scale bar: 1 mm).

applied as a novel periodontal barrier membrane for guided bone regeneration. Furthermore, the amphiphilic HA-PLGA/PLGA bi-layer film would be usefully exploited for various biomedical applications.

4. Conclusions

We have developed a novel biocompatible and degradation-controlled HA-PLGA by the conjugation of HA-ADH with PLGA for the applications to periodontal bar-

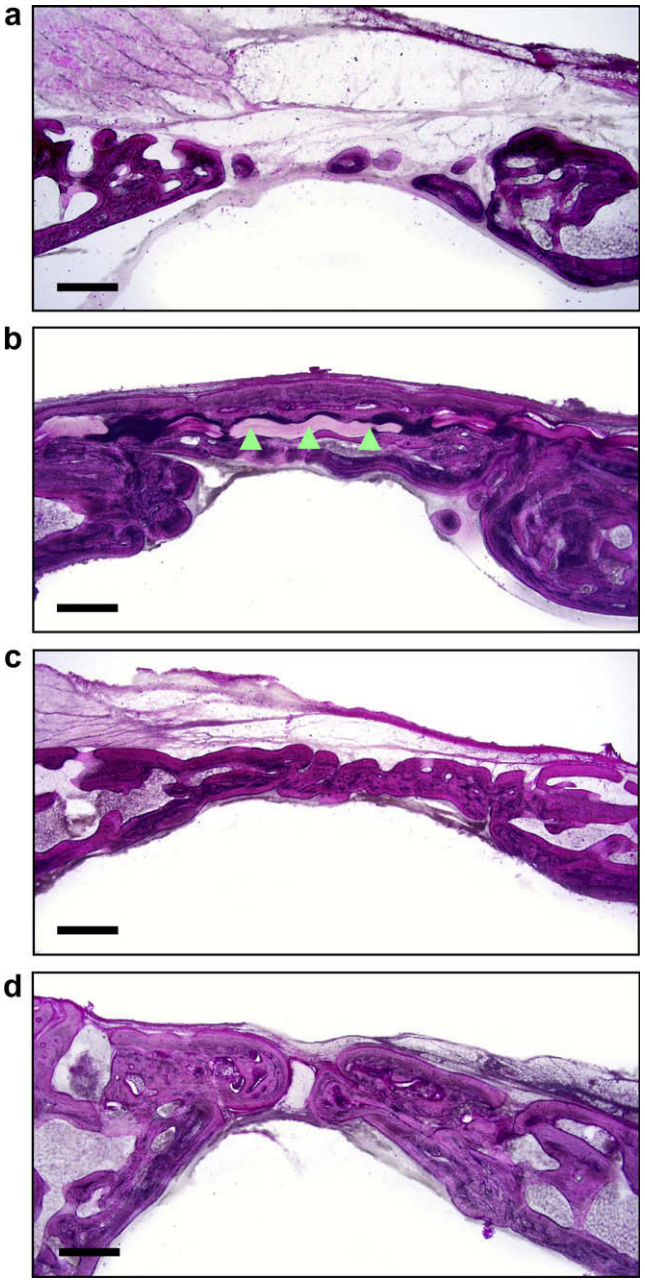

Fig. 7. Photomicrographs of the calvarial critical size bone defect regions in SD rats after bone regeneration for 12 weeks: (a) control, (b) OSSIX™ membrane, (c) poly(lactic-*co*-glycolic acid) (PLGA) film, and (d) PLGA grafted hyaluronic acid (HA-PLGA)/PLGA (1/2 weight ratio) blend film, respectively (scale bar: 1 mm).

rier membranes. The degree of HA modification with ADH could be increased up to 85 mol.% in the mixed solvent of water and ethanol. Highly modified HA-ADH appeared to be soluble in DMSO with an enhanced stability to enzymatic degradation by hyaluronidase. When HA-PLGA was blended with PLGA in chloroform, amphiphilic biphasic films were obtained with hydrophilic HA and hydrophobic PLGA layers. According to in vitro degradation tests in PBS, HA-PLGA/PLGA (weight ratio of 1/2) film degraded relatively slowly compared to PLGA film and HA coated PLGA film. Among four different samples

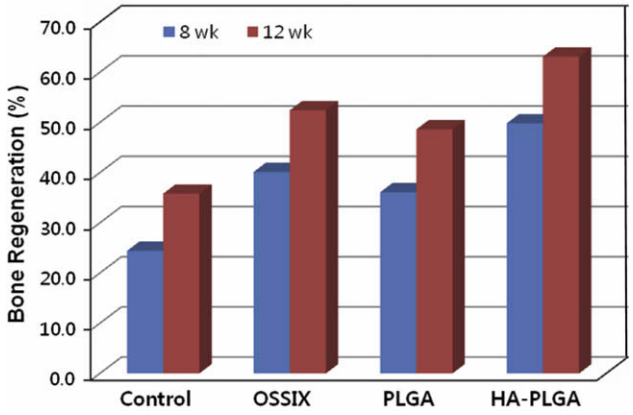

Fig. 8. Histomorphometric analyses of regenerated bones and soft tissues in the calvarial critical size bone defects of SD rats after implantation of control (no treatment), OSSIX™ membrane, poly(lactic-*co*-glycolic acid) (PLGA) film, and PLGA grafted hyaluronic acid (HA-PLGA)/PLGA (1/2 weight ratio) blend film, respectively.

of a control, OSSIX™ membrane, PLGA film and HA-PLGA/PLGA film implanted to cover the calvarial critical size bone defects in SD rats, HA-PLGA/PLGA film resulted in the most effective bone regeneration followed by OSSIX™ membrane and PLGA film. The regenerated bone covered 63.1% of the bone defect area in 12 weeks. The results might be ascribed to the biocompatible and degradation controlled characteristics of HA-PLGA/PLGA film with an enzymatic stability up to 8 weeks in the bone defect area. The novel biphasic HA-PLGA/PLGA film will be investigated further as a periodontal barrier membrane for clinical applications.

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