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Preparation of Collagen/Poly(L-lactic acid) Composite Material for Wound Dressing

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Abstract: Collagen is the major structural protein of connective tissues. It can be used as a prosthetic biomaterial applicable to artificial skin, tendon, ligaments, and collagen implants. The objective of this study is to investigate the possibility of realizing wound dressing medical products by the synthesis of composite materials with collagen and a biodegradable polymer, PLLA, via a surface modification process. Type I collagen was obtained from pig skin by a separation process. The structural characteristics of the extracted collagen were confirmed by SDS-polyacrylamide (PAcr) gel electrophoresis (PAGE) and FTIR. Also, PLLA-*g*-PAcr was synthesized by the radical polymerization of acrylamide initiated by AIBN in the presence of PLLA. The surface of PLLA was modified by the presence of the acrylamide residues. The structural characteristics of the copolymer were analyzed by FTIR, ^{1}H-NMR and contact angle measurements. The water uptake and WVTR of the collagen/PLLA-*g*-PAcr composite tended to increase with increasing collagen concentration and with decreasing EDC concentration.

Keywords: collagen, PLLA, acrylamide, wound dressing, biomaterial.

Introduction

Wound healing is the tissue response to injury and the process of regeneration. It is a complex biological process involving chemotaxis, cell proliferation, production of extracellular matrix (ECM) proteins, neovascularization, and so on. Impairment of one or more of these processes can lead to impaired wound healing. Many factors, both local and systemic, can contribute to impaired wound healing. One of the therapeutic options for local wound care of a healing-impaired wound is to enhance normal healing events using naturally occurring stimulatory agents, such as growth factors. Therefore, the method of drug delivery system (DDS) has been accepted for full thickness skin wound care, and antimicrobial drug impregnated wound dressings are proven effective in controlling bacterial invasion through a porous matrix.

Collagen is the major structural protein of connective tissues such as skin, tendon, cartilage, and bone. Also, it has a unique amino acid composition and structure. Many different forms of collagen products such as film, gel, sponge, and scaffolds have been fabricated and used in practice. There are many properties of collagen that make it an attractive substance for various medical applications such as implants, organ replacement, and surgical dressing for wound, burn, etc.[1] Collagen exhibits biodegradability, weak antigenecity, and superior biocompatibility compared with other natural polymers such as albumin and gelatin. The main applications of collagen in drug delivery systems are collagen shields in ophthalmology,[2,3] sponges for wound dressings,[4] a gel formulation containing liposome for sustained drug delivery, as a controlling material for transdermal delivery,[5] and as a making nanoparticles for gene delivery.[6] In addition, it is used in surgical sutures,[7] hemostatic agents,[8,9] and tissue engineering including as basic matrices for cell culture systems[10] and replacement/substitutes for artificial blood vessels and valves.[11-13] Although collagen has superior biocompatibility, its fast biodegradation rate and low mechanical strength are not appropriate to the demands of *in vitro* and *in vivo* biomaterial applications.

Synthetic polymers such as PLLA (poly(L-lactic acid)) are widely used to build three-dimensional scaffolds in the field of tissue engineering due to their relatively good biocompatibility, mechanical strength, and appropriate degradation rate[14] and their shape can be easily modified, but their surfaces are hydrophobic. Naturally derived polymers such as collagen have good and hydrophilicity but they are mechanically too weak to maintain the desired shape until newly formed tissue matures. A combination of natural and synthetic biopolymer such as collagen/silicone[15] was desirable to modify the physical properties, such as compression, flexibility, durability and compactness and may have the

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advantage of controlled release of antibacterial drugs for biomedical materials.

In the present study, with an aim to improve the biomaterial properties of collagen, we studied the formulation of a collagen/PLLA-*g*-PAcr composite through a surface modification process by acrylamide grafting. In addition, the water vapor transmission rate (WVTR) and the water uptake rate of the collagen/PLLA-*g*-PAcr composite were investigated for feasibility of wound dressing.

Experimental

Materials. Fresh pig skin was obtained from a slaughter house. The skin was stored in a refrigerator at -20 °C. Acetone, potassium hydroxide, and sodium hydroxide, used as a fatty remover, were purchased from Oriental Chemical. Pepsin, guanidine (99%), cellulose membrane dialysis tubing (M.W. 12,400), EDC {*N*-(3-dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride}, L-lactic acid (90%), and titanium (IV) butoxide (TNBT, 99.99%) were purchased from Sigma Aldrich. All other chemicals were of analytical grade.

Preparation of Collagen. Acid soluble pig skin type I collagen was used as the starting biomaterial in this study. The widely employed chemical procedure used to remove hair and fat from pig skin by alkali solution and acetone was employed as the separating process. The fragment tissue residues were recovered by centrifugation. Recovered samples were suspended in guanidine solution included Tris-buffer (pH 7.4) for 24 h. After separation by centrifugation, the residue samples were rinsed with pure water and then digested in a mixed solution of sodium chloride (0.2 M), acetic acid (0.5 M), and pepsin (500 mg) to remove undigested material. The collagen solution was precipitated by repeated dialysis in a sodium chloride and acetic acid solution for 24 h. Finally, the collagen solution was dialyzed against an acetic acid solution at 4 °C to remove salt. Type I collagen was subsequently obtained by a lyophilizing process and stored in a freezer.

Synthesis of PLLA-*g*-PAcr Copolymer. Lactic acid (200 g) was charged and titanium(IV) butoxide (0.08 mL) was added as a catalyst in a three-neck flask equipped with a mechanical stirrer and a reflux condenser connected with a vacuum system through a cold trap. The mixture solution was heated at 180 °C with stirring on a mechanical stirrer. After esterification reaction, the pressure of the reactor was reduced stepwise to 500 torr, and then reduced to 1 torr and maintained at this condition for 40 h. The polymerized products were then cooled to room temperature and dissolved in chloroform. The polymer product was precipitated by addition of excess methanol for removal of residual monomers. The product, PLLA, was filtered and dried in a vacuum oven.[16-18] The molecular weights of the synthesized PLLA were measured using Waters GPC (Gel Permeation Chromatography) equipped with 510 differential refractometer and Viscotek T50 differential viscometer. Universal calibration curve was made using ten PS standard samples (Polymer Laboratories, UK) with molecular weights of 580-7,500,000 g/mole. The PLLA dissolved in THF was injected at a flow rate of 1.0 mL/min.

PLLA-*g*-PAcr copolymer was synthesized by the suspension polymerization method. As the suspension polymerization medium, electrolytes of sodium phosphate dibasic and sodium phosphate monobasic were dissolved in pure water and poly(vinyl alcohol) was added. Acrylamide solution was then dissolved in chloroform. PLLA (5 g) dissolved in chloroform (30 mL) and AIBN were mixed together in this medium solution. The copolymer was synthesized from the suspension mixture heated at 70 °C for 24 h. The copolymer was precipitated by pouring the polymerized solution into an excess of methanol for removal of residual monomers, filtered, and dried in a vacuum oven.[19]

Preparation of Composite Material. The synthetic procedure of composite collagen/ PLLA-*g*-PAcr was as follows. The lyophilized collagen was dissolved in 0.5 M acetic acid at concentration range of 0.1-1.0 wt% and the PLLA-*g*-PAcr copolymer was dissolved in DMSO at a concentration range of 1-10 wt%. The samples were mixed by a homogenizer for 3 min at varying molar ratio of collagen: PLLA-*g*-PAcr (7:3, 5:5, 3:7; groups: CP73, CP55, and CP37). The samples were added to the EDC at a concentration range of 0.1-0.5 wt% and were then shaken in a refrigerator at 4 °C for 24 h. After freeze-drying, the samples were repeatedly washed with water for removal of EDC and freeze-dried again.

Results and Discussion

Characterization of Extracted Collagen. The extracted collagen and standard sample (Type I, commercial pig collagen) were analyzed by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) method (Figure 1). The electrophoresis patterns of the extracted collagen were significantly similar to those of the standard collagen sample. All collagen samples displayed $\alpha 1$ and $\alpha 2$ bands. These results are consistent with the fact that the helical structure of type I collagen is composed of three polypeptide chains, namely, two $\alpha 1$'s and one $\alpha 2$.[20]

Collagen hydrogel solution has viscoelastic and flow properties similar to those of most polymer materials. Figure 2 shows the viscosity change as a function of temperature for various concentrations of collagen. It was observed that the viscosity of the collagen solution tended to slightly decrease with increasing temperature. However, beyond 46 °C, no viscosity change in accordance with temperature could be observed. This indicates that the collagen properties were changed by thermal energy. It was called the denaturation temperature of collagen solution (about 45-48 °C). Thermal denaturation of collagen is related to the thermal stability of collagen by amino acid content and hydroxyproline con-

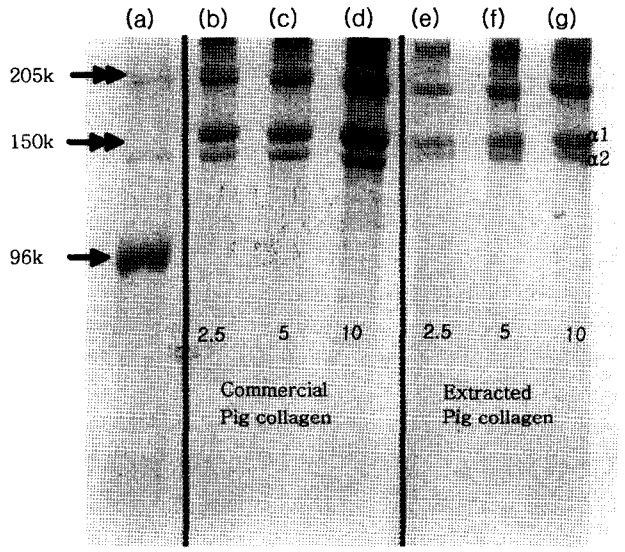


Figure 1. SDS-PAGE electrophoresis of various preparation of collagen; (a) molecular weight standard, (b) commercial pig skin 2.5 μL, (c) 5 μL, (d) 10 μL, (e) extracted pig skin 2.5 μL, (f) 5 μL, and (g) 10 μL.

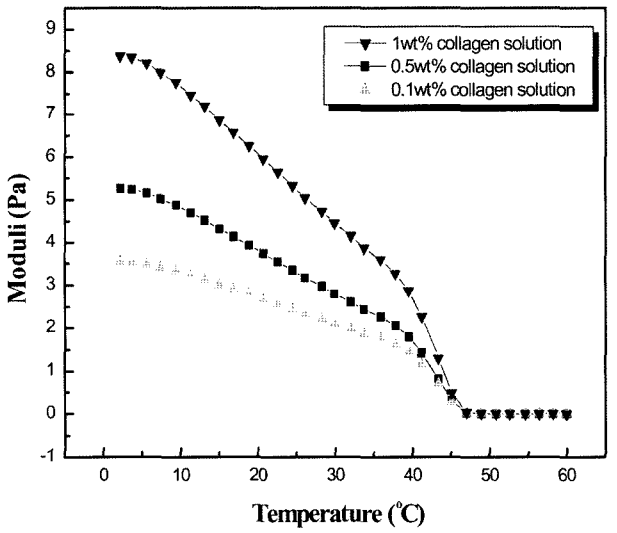


Figure 2. Viscosity as function of temperature for concentration of various collagens.

tent.[21] Denaturation temperature is known to increase with increasing amounts of amino acid residues. Hydroxyproline may be related to the stabilization of the triple-stranded collagen helix due to hydrogen bonding ability attained through its OH group.[22]

The extracted collagen was also analyzed for comparison with the other collagens by FTIR spectra (Figure 3). The amide band is associated with N-H stretching frequency and N-H stretching vibration takes places in a range of 3400-

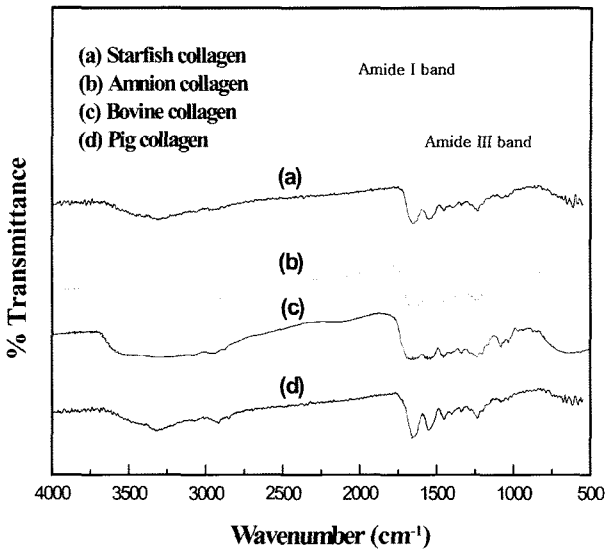


Figure 3. FTIR spectra for various species collagen.

3440 cm^{-1}. The amide band of pig skin collagen was found at 3337 cm^{-1}, suggesting the presence of hydrogen bonding. The amide band of pig collagen was significantly similar to that of other collagen species. The amide I band position of pig collagen was found at 1653 cm^{-1}, whereas that of other collagen species is found in a range of 1650 to 1655 cm^{-1}. The helix structure of triple-stranded collagen was confirmed from the IR absorption region between 1235 cm^{-1} (amide III) and 1450 cm^{-1}.

Synthesis of PLLA-*g*-PAcr Copolymer. PLLA is a synthetic biopolymer and is widely used to build three-dimensional scaffolds in the field of tissue engineering due to their good compatibility, mechanical strength, and appropriate degradation rate. The tensile strength of a polymer is influenced by the molecular weight of the polymer in a network structure. The physical properties of a polymer such as viscosity and mechanical strength depend upon the molecular weight of the material. The synthesis of PLLA was carried out under various esterification times, duration of decompression time, and polymerization reaction conditions. It was found that the molecular weight of PLLA sensitively changed under the reaction conditions, including esterification time and duration of decompression time. The high molecular weight of the polymer could be obtained at longer decompression duration time. The characteristics of the reaction variables are summarized in Table I.

The copolymer was prepared by a grafting reaction in which the acrylamide monomer was polymerized through a radical reaction in the presence of PLLA and AIBN initiator. The grafting reaction was conducted with the acrylamide monomer in a range 5-15 g. Figure 4 shows the FTIR spectra of PLLA-*g*-PAcr prepared by a grafting reaction and the homopolymer of PLLA. The peak at 1651 cm^{-1} corresponds to the amine group of acrylamide. When the quantity of

Table I. Characteristics of PLLA

Sample	Esterification Time at 760 torr (hour)	Decompression Time from 760 to 1 torr (hour)	Polymerization Time at 1 torr (hour)	M_w (10^3 g/mole)	M_w/M_n
PLLA1	3	7	40	110	2.3
PLLA2	3	3	40	30	2.5

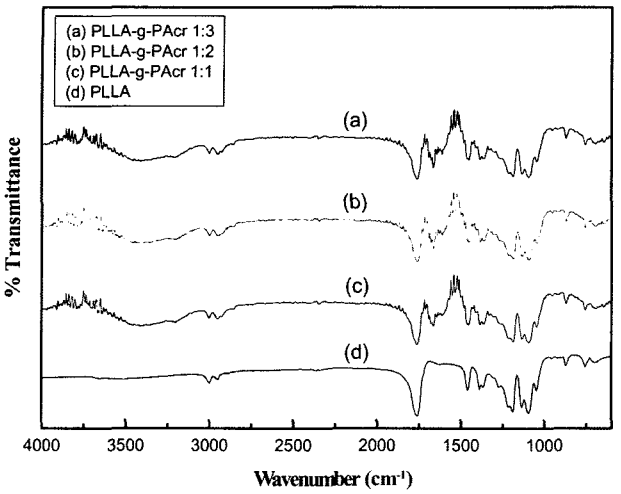


Figure 4. FTIR spectra for various ratio of synthetic PLLA-*g*-PAcr with the broad absorption at 1662~1692 cm^{-1} of NH_2.

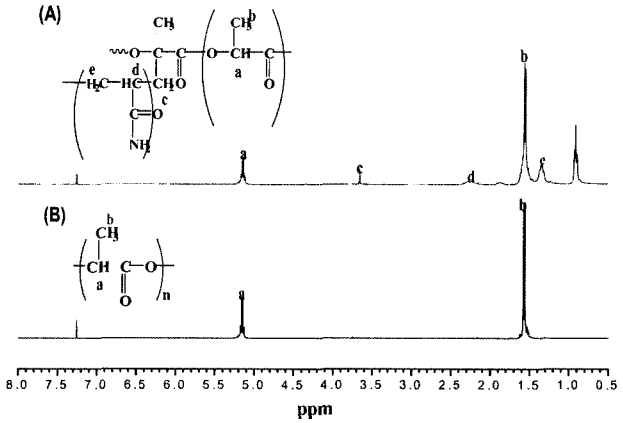
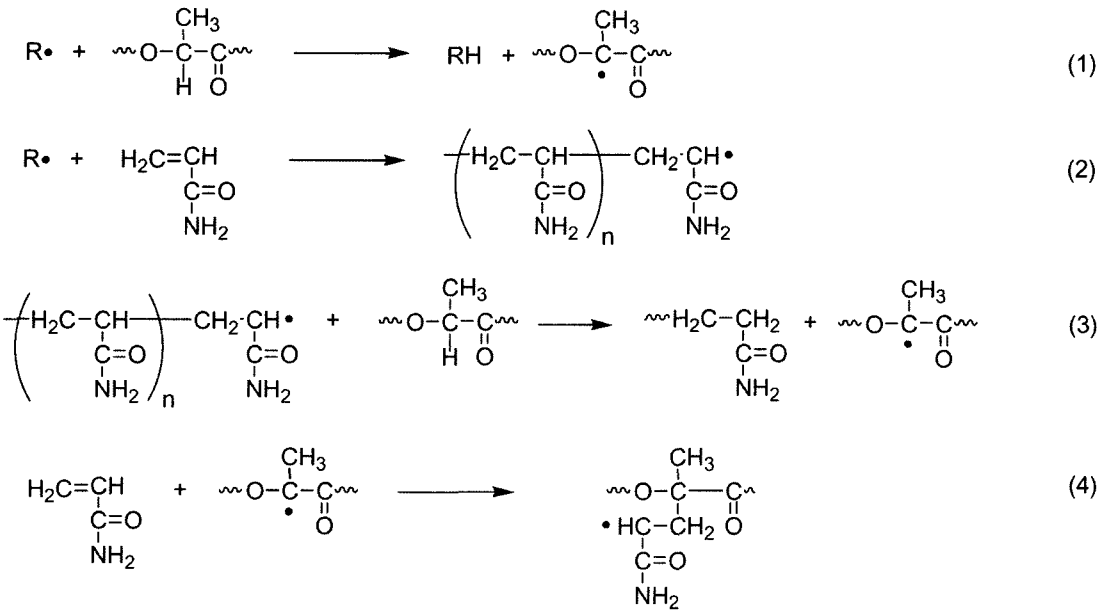


Figure 5. ^{1}H-NMR spectra of synthetic polymer (A) PLLA-*g*-PAcr and (B) PLLA.

acrylamide increased, the intensity of the amidic group bands increased. The PLLA and PLLA-*g*-PAcr copolymer was analyzed by ^{1}H-NMR. Peak at 3.66 ppm indicates the presence of a new signal, attributed to vinyl CH_2 of the PAcr grafted onto PLLA (Figure 5). It is noted that the grafting of PLLA with PAcr by radical polymerization has been reported elsewhere.[23,24] Generally the grafting yield of PAcr onto PLLA is sufficiently high (above 95%) and the reaction mechanism as illustrated in Scheme I.[24] Therefore, it is expected that the yield in this experiment is also similar to the reported value.

In general, hydrophobic polymers are known to be unfavorable for cell attachment unless modified to possess a



Scheme I. Mechanism of grafting of PAcr onto PLLA by radical polymerization.

hydrophilic surface with a higher surface energy and a corresponding lower water contact angle. To compare the hydrophilicity of the PLLA and PLLA-*g*-PAcr film surfaces, water contact angles were measured. The water contact angle of the PLLA film was 86°. With the introduction of acrylamide monomer onto the PLLA surface, the water contact angle dropped to 52° (Table II). The water contact angle decreased with increasing concentration of acrylamide monomer. Amine has a hydrophilic functional group. As a result of increasing the concentration of acrylamide monomer on the film surface, the function of the amine groups was increased. This leads to a decrease in the water contact angle. This result implies that the surface modification of PLLA by acrylamide grafting substantially improves biocompatibility.

Water Uptake and WVTR Effect of Composite Material. It was observed that water uptake and WVTR of a collagen/PLLA-*g*-PAcr sponge tended to increase with increasing collagen concentration and with decreasing EDC concentration. The aim to use EDC is to increase the mechanical strength of collagen. EDC is just used for the activation of -COOH groups in collagen. This is attributed to a decrease in the interconnected pore size of the composite material with increasing EDC concentration.

The physical properties of collagen/PLLA-*g*-PAcr composite are summarized in Table III. Water vapor transmission rate is important for the characterization of wound dressing, since it controls the accumulation of exudates in the wound area. The WVTR values of collagen/PLLA-*g*-PAcr sponge are around 298-1,298 g/m^2/day. These values are sufficiently high compared to some commercial wound dressing values (for example, Comfeel 308 g/m^2/day (Coloplast A/s), Dermiflex 90 g/m^2/day (Johnson & Johnson), Duroderm 886 g/m^2/day (Conva Tec Ltd.)). Also, water uptake shows a high swelling rate which confirms the high efficiency rate of the dressing to absorb wound exudates. The water uptake values of the collagen/PLLA-*g*-PAcr sponge were around 311-950%. The high swelling rate observed in the collagen/PLLA-*g*-PAcr sponge may be attributed to the porous structure of the material and the hydrophilic force between water and the material. Collagen solution was introduced into the PLLA-*g*-PAcr which was frozen, lyophilized, and crosslinked by EDC. Surface morphology of collagen/PLLA-*g*-PAcr sample was shown in SEM. Figure 6 shows surface morphology of three kinds of composite sponge fabricated at varying molar ratio of collagen: PLLA-*g*-PAcr (7:3, 5:5, 3:7; groups: CP73, CP55, CP37).

The tensile strength of the collagen/PLLA-*g*-PAcr sponges was higher with increasing EDC and PLLA-*g*-PAcr concentration. Although the collagen sponge showed very low mechanical strength, it could be employed to reinforce collagen/PLLA-*g*-PAcr by utilizing a cross-linked collagen sponge with added EDC.

Table II. Contact Angles of Water Droplet on the Prepared Composite Materials

Composite Material	Contact Angle
PLLA-*g*-PAcr	86°
PLLA-*g*-PAcr (1 : 1)	81.3°
PLLA-*g*-PAcr (1 : 2)	68.2°
PLLA-*g*-PAcr (1 : 3)	52°

Conclusions

In this study, a collagen/ PLLA-*g*-PAcr sponge type composite material was prepared by copolymer composed of PLLA-*g*-PAcr and its physical properties were investigated. Type I collagen was prepared by a chemical and physical separation process from pig skin and its biochemical properties were examined. A copolymer of PLLA-*g*-PAcr was

Table III. Physical Properties of Collagen/PLLA-*g*-PAcr Composite

Composite Type	Volume Ratio	EDC Conc. [wt% (w/v)]	Water Vapor Transmission Rate (g/m^{2}day)	Water Uptake (%)	Tensile Strength (MPa)
	Collgen/PLLA-*g*-PAcr				
CP73 ×1	7/3	0.1	1,298	950	0.26
CP73 ×3	7/3	0.3	980	843	0.31
CP73 ×5	7/3	0.5	789	812	0.36
CP55 ×1	5/5	0.1	967	837	0.37
CP55 ×3	5/5	0.3	883	753	0.45
CP55 ×5	5/5	0.5	672	721	0.45
CP37 ×1	3/7	0.1	798	482	0.44
CP37 ×3	3/7	0.3	387	365	0.45
CP37 ×5	3/7	0.5	298	311	0.44

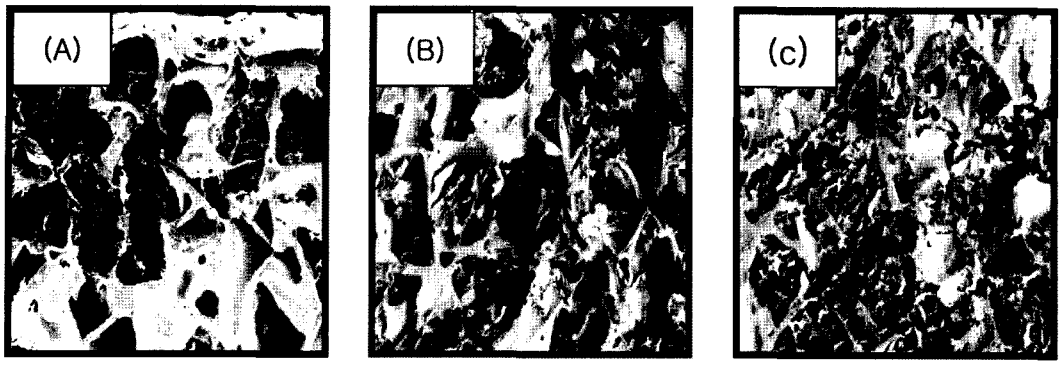


Figure 6. The morphology of collagen/PLLA-*g*-PAcr composite ratio (a) 7 : 3, (b) 5 : 5, and (c) 3 : 7.

prepared by a grafting reaction. The acrylamide monomer improved the hydrophilicity of the copolymer compared to PLLA due to the presence of an acrylamide functional group on the composite material surface. The WVTR values of the collagen/PLLA-*g*-PAcr sponge are in a range of 298-1,298 g/m^2/day. The water uptake values were around 311-950%. Good WVTR and water uptake properties of the composite collagen/PLLA-*g*-PAcr sponge were achieved compared to those of contemporary commercial wound dressings.

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