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Crosslinked collagen/chitosan matrix for artificial livers

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Abstract

Matrices composed of collagen and chitosan may create an appropriate environment for the regeneration of livers. In this study, we have prepared, characterized and evaluated a new collagen/chitosan matrix (CCM). The CCM was made by using crosslinking agent 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in *N*-hydroxysuccinimide (NHS) and a 2-morpholinoethane sulfonic acid (MES) buffer system. The chemical characteristics were evaluated by Fourier-transformed infrared (FTIR) spectroscopy and X-ray photoelectron spectroscopy (XPS). The mechanical strength was measured by tensile tests. The platelet deposition and hepatocyte culture experiments show that CCM has excellent blood and cell compatibility. The results suggest that the CCM is a promising candidate matrix for implantable bioartificial livers.

Keywords: Collagen/chitosan matrix (CCM); 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC); Hepatocyte; Liver

1. Introduction

The severe donor shortage of orthotopic allogeneic livers and the extremely high transplantation cost have stimulated continuing efforts to develop a tissue-engineered liver. During the last several decades, a variety of hepatic tissue-engineered systems have been studied using both isolated hepatocytes (the major parenchymal cells of the liver) and biodegradable polymers, to develop liver substitutes that restore, maintain or improve hepatic functions [1]. Finding a suitable method to attach the cells to a proper matrix to maintain the liver-specific gene expression has proven to be a significant challenge. Several synthetic polymers, such as poly(lactic acid) [PLA] and poly(lactic acid-co-glycolic acid) [PLGA], are currently being investigated as bioartificial liver scaffolds on account of their biodegradability, good mechanical properties and proper degradation rate. However, a serious limitation of these synthetic polymers in artificial liver systems is the lack of biocompatibility with cells and blood. For example, 90–95% of the hepatocytes died within 24 h after implantation in a PLGA scaffold [2].

It is well known that the liver is a complex internal organ to which a large blood supply is essential. Hepatocytes in an implant will work poorly or die if they are further than 500 μm away from blood vessels or other sources of nourishment—a distance roughly equivalent to the diameter of the graphite in a mechanical pencil [3]. Therefore an ideal matrix for an artificial liver, in which patient blood can circulate through the vessels that houses metabolically active liver cells, and in which the complex tissue architecture of the liver with all of its miraculous functions is partly recreated, must have good cell and blood compatibility.

Collagen is a structural protein that is said to be one of the most suitable materials for constructing artificial substitutes for diseased or damaged tissues and organs [4]. It is a natural polymer that contains both acid and basic amino acid residues and may bear either a positive or negative charge depending on the environment [5]. The chemical and physical structural characteristics of collagen, in forms like fibrils, native or denatured, crosslinked or not, has a marked influence on the

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morphology and physiology of cells [6–8]. Non-crosslinked collagen has been reported as a good matrix for endothelial cell seeding in vitro [9]. Collagens combined with proteoglycans have been reported to be metabolically stable when implanted in vivo and have been used as artificial skin, which accelerates the wound healing [10]. However, collagen fibrils induce slim and rodlike shapes for fibroblasts in vitro and enhance the expression of collagenase genes in fibroblasts [11]. The rates of proliferation for both fibroblasts and epithelial cells are markedly decreased when cultured in contact with collagen fibrils. Furthermore, collagen is a highly thrombogenic material, as is demonstrated by its use as a hemostatic power or sponge [12]. Collagen induces platelet adhesion and aggregation as well as activation of intrinsic blood coagulation.

Chitosan, an amino polysaccharide (poly-1,4-D-glucoamine) derived from chitin by deacetylation, has been widely applied in biomedical applications, such as wound dressings and drug delivery systems [13,14] on account of its non-toxic and biocompatible nature. Since chitosan, chemically, has both reactive amino and hydroxyl groups that can be chemically modified and, physically, is relatively easy to manipulate for different pore structures [15,16], it has a high potential in tissue engineering applications. One of the most interesting effects of chitosan on wound healing is the formation of granulation tissue with angiogenesis [17]. It is reported that chitosan induces fibroblasts to release interleukin-8, which is involved in migration and proliferation of fibroblasts and vascular endothelial cells [18]. However, chitosan also promotes surface-induced thrombosis and embolization [19,20]. The high thrombogenetic property has limited its applications in blood-contacting biomaterials.

Matrices composed of collagen and chitosan may create an appropriate environment for the regeneration of liver cells but as described above, both of these materials are hemostatic. Besides that, the mechanical properties and biodegradation rates of the two natural materials, especially in aqueous media, are not particularly good. A common approach for changing the properties of these materials is by crosslinking them by various processes [21,22]. Collagen and chitosan should be crosslinked to make them applicable as biodegradable materials. Both physical and chemical approaches for crosslinking of collagen and chitosan have been carried out, such as thermal heating [23], ultraviolet irradiation and treatment with glutaraldehyde or polyepoxy compounds [24]. Water-soluble 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) was recently known to be non-toxic and biocompatible, because it is not incorporated directly into the crosslinked sponge structure, but rather changed to water-soluble urea derivatives. The cytotoxicity of the urea derivative was found to be quite low compared with that of EDC [25]. In our former studies, an implantable bioartificial liver with multichannel and multilayer architecture was developed by crosslinking the collagen-based materials with EDC in a *N*-hydroxysuccinimide (NHS) and 2-morpholinoethane sulfonic acid (MES) buffer system. Most of the layers were made of collagen and chitosan, which has been proven to be a suitable material for a bioartificial liver. The goal of this study was to show the synergetic effects in blood and cell compatibility by describing some essential properties of the CCM.

2. Materials and methods

2.1. Materials

Chitosan (80% deacetylated, M_w: 5.3×10^4) was purchased from Shandong medical appliance factory, China. Acetone, ammonia, type I collagen, heparin sodium, 1-ethyl-(dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), 2-morpholinoethane sulfonic (MES) acid and other chemicals were purchased from Beijing Chemical Co. Ltd., China.

2.2. Preparation and characterization of collagen/chitosan matrices and controls

One percent insoluble type I collagen acetic acid, 1% chitosan acetic acid solution and a mixture of the 1% insoluble type I collagen and 1% chitosan (1:1 v/v) solution were cast on a Teflon plate and air-dried at room temperature. Half of the films were used as controls. After the films were air-dried, the collagen and collagen/chitosan (CC) samples were put into an EDC/NHS/MES solution for crosslinking 4 h before they were washed by distilled water thoroughly and air-dried again. The chitosan film was immersed in a solution of dilute ammonia in methanol (15 ml NH_4OH 33%/35 ml distilled water/500 ml MeOH) to regenerate the free amino form of chitosan.

Characterization of the CCM and the controls was performed using methods such as Fourier-transformed infrared (FTIR) spectroscopy, X-ray photoelectron spectroscopy (XPS) and mechanical property measurement. XPS analysis was performed using a Perkin-Elmer 5600 electron spectroscope for chemical analysis (ESCA). Infrared (IR) spectra were recorded from the films with a Bio-Red FIS135 FTIR spectrometer. Mechanical properties were measured on an M500-10AX testing machine (Testometric Universal Tester, England). The average tensile strength, the breakage stress, and the Young's modulus were calculated from three specimens (thickness: 1.3 μm, width: 1.5 cm and length: 4.5 cm) in both the dry and wet states after submersion in distilled water for 20 min. The loading rate for dry samples was 100 mm/min and for wet samples was 10 mm/min.

2.3. In vitro platelet adhesion experiment

The immobilized samples and the control were cut into disks of 10 mm in diameter. Ten milliliter fresh whole blood with a little citron was centrifuged at 1300 rpm for 20 min. The samples were immersed in 2 ml of the above suspension for 2 h. After washing with 0.9% NaCI solution, the samples were fixed using 2.5% glutaraldehyde solution for 30 min, washed again with 0.9% NaCI for three times and then subsequently immersed in 50%, 60%, 70%, 80%, 90% and 100% ethanol solution and dried in a dessicator. The dried samples were sputter-coated with gold before being imaged by scanning electron microscopy (SEM, AMRAY, USA).

2.4. Cytocompatibility tests

The hepatocytes were isolated from the liver of an adult male rat (8 weeks, 200 g) by liver perfusion using 0.05% IV collagenase. The isolated hepatocytes were dispersed in a Hepatocyte Attachment Medium (HAM, without fibronectin, Gibco BRL) and were plated onto 24-well culture dishes with the chitosan, EDC crosslinked collagen and collagen/chitosan films at a density of 4×10^3 cells/cm^2. The cells were cultured in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The culture medium was changed routinely every 3 days with HepatoZYME-SFM (Gibco BRL).

The morphology of the cells was examined by phase contrast microscopy. Cells cultured on the CCM films and the controls were examined by SEM. The cells adhering to the films were washed with phosphate-buffered saline (PBS) after 15 days of culture, and then fixed with 2.5% glutaraldehyde in the PBS for 0.5 h. After being thoroughly washed with PBS, the cells were dehydrated through a series of graded alcohols and vacuum-dried. The films were then gold sputtered in vacuum and examined by SEM.

The percentages of viable cells were determined using trypan blue exclusion (0.4% trypan blue solution). Assay was performed at the end of 1, 4 and 7 days. At each time interval, cells were isolated using 0.25% trypsin to single cells. Cell suspensions were diluted with 0.4% (w/v) trypan blue; and the effect of trypsin was inhibited by adding the control medium at room temperature. Then the number and viability of nucleated cells were determined under a light microscope using a haemacytometer over a 3 min period.

3. Results and discussion

3.1. Study of the matrices by IR spectra

The FTIR spectra obtained from pure collagen, EDC crosslinked collagen and CCM are given in Fig. 1. In the spectrum of pure collagen (Fig. 1c), four amide bands at the frequencies of 3324, 3082, 1659 and 1553 cm^{-1} can been observed. Generally, amide I bands (1659 cm^{-1}) originate from C=O stretching vibrations coupled to N–H bending vibration. The amide II bands (1553 cm^{-1}) arise from the N–H bending vibrations coupled to C–N stretching vibrations. The other two amines, arising from the stretching vibrations of N–H group, of a medium to weak intensity, appear at 3324

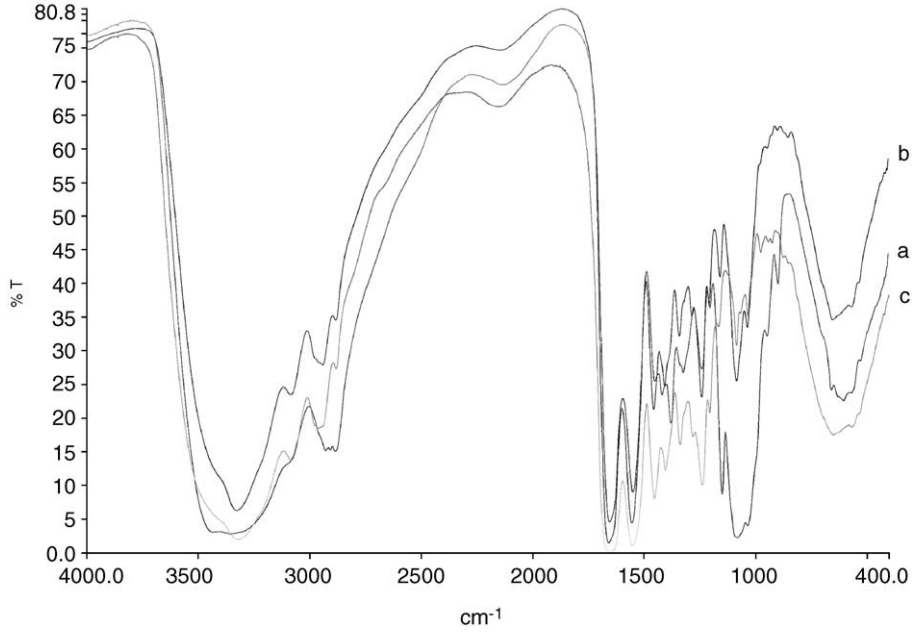

Fig. 1. FTIR spectra of: (a) non-crosslinked collagen; (b) crosslinked collagen and (c) crosslinked CCM.

and 3082 cm^{-1}, respectively. After crosslinking with EDC (Fig. 1b), the values of frequencies of these bands differ a little. Amide II bands decrease obviously. Since the intensity of $–NH_2$ band in collagen molecules is stronger than that of N–H and amidation has no effect on the intensity of C=O band, the change of amide II bands means that the free $–NH_2$ groups in collagen molecules were changed to N–H groups. The intermolecular or intramolecular amide linkages of collagen formed.

It is well known that the characteristic absorption bands of chitosan appear at 1655 (C=O stretching), 1585 ($–NH_2$ bending) and 1380 cm^{-1} ($–CH_2$ bending). And the absorption bands at 1152 (anti-symmetric stretching of the C–O–C bridge), 1084 and 1040 cm^{-1} (skeletal vibrations involving the C–O stretching) are characteristics of saccharide structure of chitosan [26]. In the spectrum of EDC crosslinked collagen/chitosan (Fig. 1a), the amide II bands (1552 cm^{-1}) decreased greatly. This result suggests that the $–NH_2$ (1585 cm^{-1}) groups of chitosan were also changed into N–H groups (i.e. intermolecular crosslinkages between chitosan and collagen or within collagen moleculars formed). The symmetric stretching bands of carboxylate salts (near 1404 cm^{-1}), appear in the collagen spectrum, decrease in the EDC crosslinked collagen spectrum and totally disappear in the EDC crosslinked collagen/chitosan spectrum, indicates that all the carboxylate salts took part in the crosslinking reaction. Furthermore, EDC seems to mediate acid anhydride formation between two carboxyl groups belonging to the same or different collagen molecules. The resultant acid anhydride may readily react with a hydroxyl group (mostly in chitosan) to yield an ester bond (1152 cm^{-1}), with functions as another crosslinking of collagen and chitosan. Therefore, IR spectrum of the EDC crosslinked collagen/chitosan suggests that carboxyl groups of collagen reacted with amino groups of chitosan and with hydroxyl groups of chitosan resulting in the formation of amide and ester linkages. By the way, the strong absorption bands between 800 and 1200 cm^{-1} and the large increased absorption bands of –OH (Fig. 1a near 3363 cm^{-1}) come mostly from the saccharide structure of chitosan.

3.2. ESCA survey scan spectra

The changes in chemical structure of the matrices were further investigated by ESCA spectra (Fig. 2). The control collagen surface shows carbon (binding energy: 287.8 eV), oxygen (binding energy: 534.8 eV) and nitrogen (binding energy: 534.8 eV) peaks, as expected (Fig. 2a). For the crosslinked CCM surface, a decreased nitrogen peak and increased carbon and oxygen peaks were observed corresponding to low collagen content and the addition of chitosan. The chemical compositions of the CCM and the controls, calculated from the ESCA survey scan spectra, are shown in Table 1. The carbon content (69.57%) of the collagen film was increased (71.58%) because of the introduction of chitosan in the matrix and because of the high C/O ratio in the chitosan, whereas the nitrogen content (9.58%) decreased (5.88%) in the crosslinked CCM. These results suggest that chitosan was successfully crosslinked with collagen. In addition, element fluorine (F) in the spectra belongs to the Teflon we used for the films to dry. The more collagen contained in the mixture, the higher F peaks in the spectra. In other samples made by freeze-drying the matrices on watch-glasses, this element was not detected in the resulting spectra. This result indicates that collagen has a strong bonding ability to F.

3.3. Mechanical properties of the matrices

The mechanical properties of the matrix in tissue-engineering applications is of great importance due to the necessity of structural stability to withstand stress incurred during culturing in vitro and implanting in vivo. It can also significantly affect the specific biological functions of cells within the engineered tissue [27]. Therefore, the tensile strength and Young's modulus of the crosslinked CCM and the controls are shown in Table 2. Generally speaking, the mechanical strength of dried films is higher than that of wet films. The mechanical strength of crosslinked collagen is higher than that of crosslinked CCM. The addition of chitosan to the matrix provided many more amino groups than required for crosslinking which led to a decrease of the relative crosslinking degree and the mechanical strength.

3.4. Platelet adhesion

Contact of blood with artificial surfaces during extracorporeal circulation procedures is associated with activation of blood cells as well as plasma proteolytic enzyme systems, such as the complement, coagulation, fibrinolytic and FXII–kallikrein–kinin cascades [28,29]. Numerous attempts have been made to solve these problems by modifying the surface chemistry of blood-contacting materials in order to make them more thromboresistant [30]. In this paper, platelet adhesion tests were carried out on the samples to check for active platelets. Fig. 3 shows the platelet adhesion on collagen, non-crosslinked CCM, crosslinked CCM and chitosan. Compared to pure collagen, non-crosslinked CCM, especially crosslinked CCM, resulted in an excellent anticoagulant property. Platelet adhesion was greatly inhibited on the non-crosslinked CCM, and was totally obstructed on the crosslinked CCM. In other words, there were few platelets on the non-crosslinked CCM and almost no platelets deposited on the crosslinked CCM (Fig. 3b). The morphology of the platelets

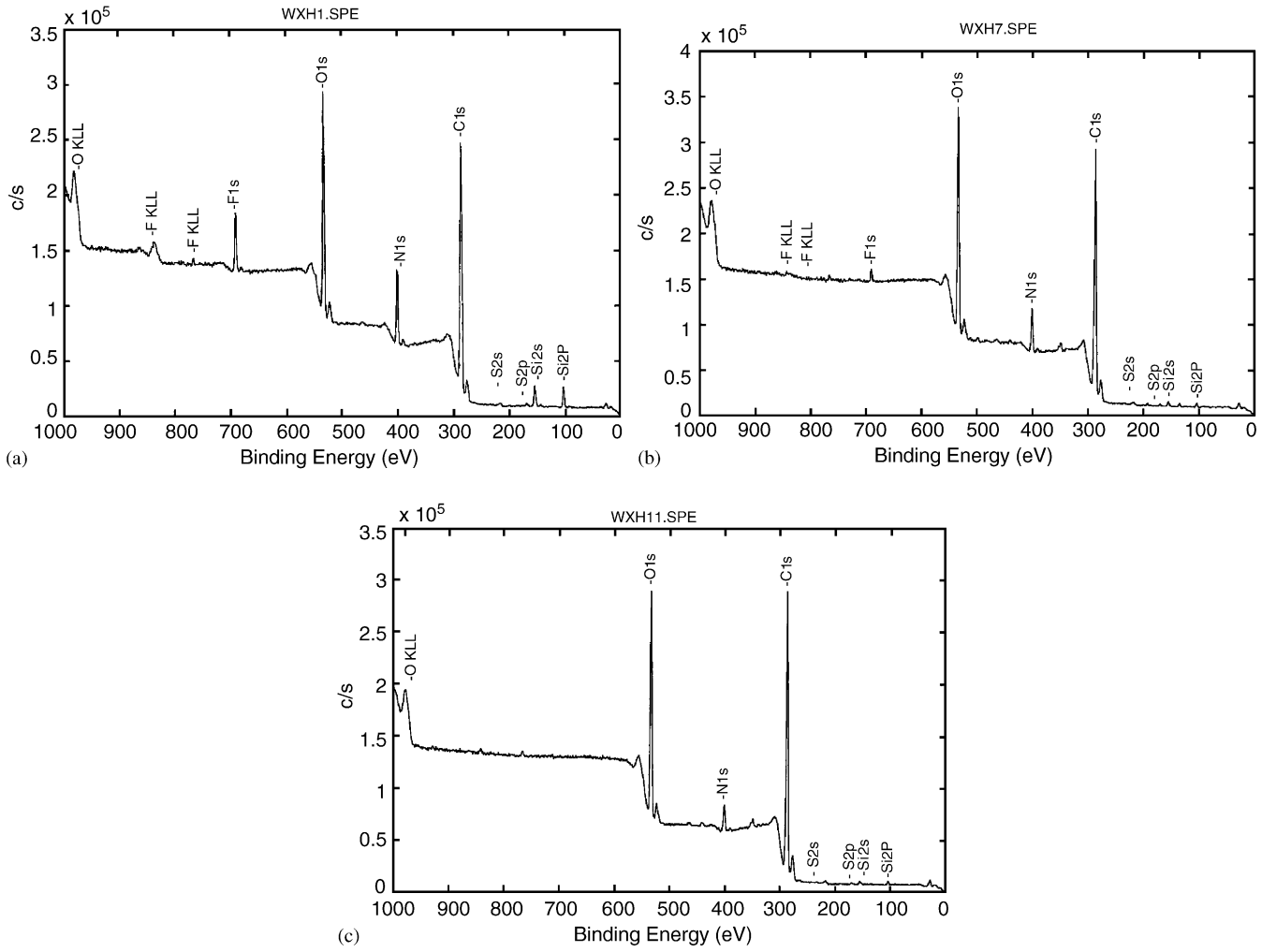

Fig. 2. ESCA spectra of collagen (a); crosslinked CCM (b) and chitosan (c).

Table 1
Elemental composition of the matrices

Sample	Composition (%)			
	C	N	O	S
Collagen	69.57	9.58	20.58	0.27
Collagen/chitosan	71.58	5.88	22.37	0.17
Chitosan	74.19	3.50	22.15	0.16

deposited on the collagen and chitosan matrices is different. Most of the adhered platelets deposited onto the collagen matrix had short pseudopodia (Fig. 3a). The adhered platelets deposited onto the chitosan matrix had such extended pseudopodia that the pseudopodia had formed a large net. The reason that crosslinking of collagen and chitosan decreased platelet deposition is due to a decrease in the number of free carboxylic acid groups of aspartic and glutamic acid residues of collagen and in the amino groups of chitosan. In our former study, an artificial liver with microchannels in which patient blood can circulate through the vessels that houses metabolically active liver cells was developed. The resulting excellent blood compatibility of the CCM is favorable for blood bypass through the microchannels of our artificial livers.

3.5. Hepatocytes culture

The extracellular matrix is composed of glycosaminoglycan, collagen, laminin, etc. This matrix controls the proliferation and differentiation of cells for maintenance of homeostasis and regeneration of damaged tissues [31]. An artificial matrix, with controlling functions like those of the normal extracellular matrix, is important for the construction of complex organs, which is the aim of tissue engineering and regenerative engineering [32]. To improve cell and blood compatibility of collagen-based materials, we tried to crosslink collagen with chitosan as discussed below. The CCM

Table 2
Mechanical properties of the matrices

Sample	Tensile strength (MPa)	Elongation at break (%)	Young's modulus (MPa)
Crosslinked collagen (dry)	33.5±1.49	2.27±0.46	1159.5±165.2
Crosslinked collagen (wet)	2.25±0.66	36.51±5.09	5.80±2.29
Crosslinked CC (dry)	11.96±1.13	2.90±0.17	422.27±230.1
Crosslinked CC (wet)	1.91±0.54	34.94±27.06	7.11±0.28
Chitosan (dry)	130.79±81.63	3.60±1.87	4429.7±2467.7
Chitosan (wet)	2.94±0.31	39.04±8.81	5.78±1.78

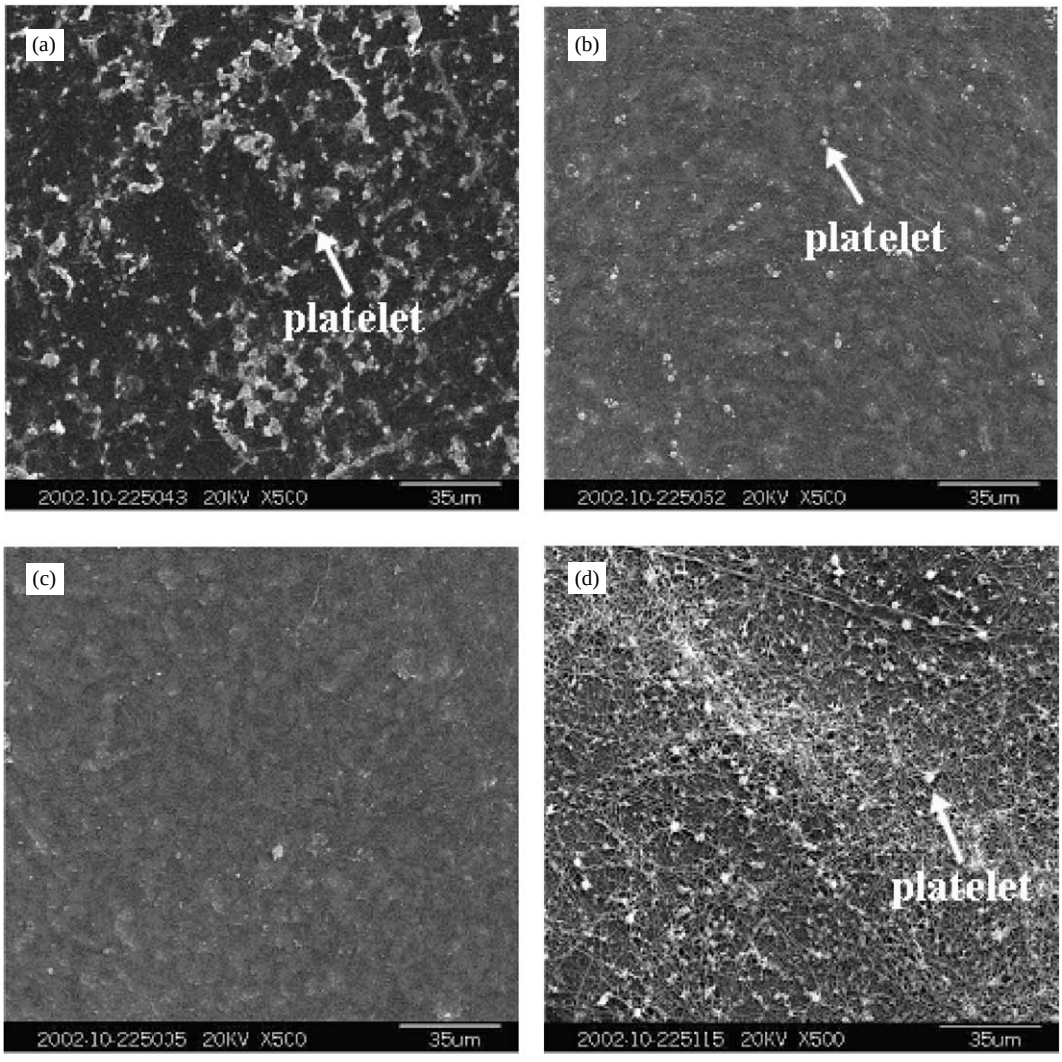

Fig. 3. SEM micrograph of platelet adhesion on collagen (a); non-crosslinked CCM (b); crosslinked CCM (c) and chitosan (d).

may create an appropriate environment for the regeneration of liver.

Hepatocytes obtained from adult rats were seeded to substrates and cultured in an HAM medium. When cultured on a pure EDC crosslinked collagen matrix, hepatocytes attached poorly, retaining their original numbers and appearing in cuboidal shapes during the 15 days. The matrix retained nearly its original shape. On the SEM pictures, a lot of ball-like secreted materials (secreta) of the hepatocytes were observed when the hepatocytes were fully washed away (Fig. 3a). This result suggests that hepatocytes have very low affinity with the crosslinked collagen matrix. In other words, the crosslinked collagen has a strong resistance to collagenase, which can be secreted by hepatocytes and can decompose the matrix or digest collagen. This, in another way, has shown that the EDC crosslinked matrix possesses a high mechanical strength. Figs. 3b

and c show that hepatocytes have dropped into the pits of biodegraded CCM and chitosan and have a very good affinity with these materials. The cell pseudopodia have connected with the matrices. The secreta (secreted by hepatocytes) are found to be round and large (ball-like, nearly 4 μm in diameter) on the crosslinked collagen, thin and long (bar or silk like) on the chitosan, and both (lies between these two—both big ball and thin bar like) on the CCM. The number of hepatocytes on the CCM made great progression in the following 2 days and remained significantly high until the end of the experiment (28 days) (Fig. 3b). There were not as many hepatocytes on the pure chitosan matrix as found on the CCM throughout the experiment (Fig. 3c). The growth and division of the hepatocytes may be inhibited by the extremely high affinity of hepatocytes with chitosan. These results indicate that the addition of chitosan can improve the adhesion and division of hepatocytes on the collagen-based materials to a certain degree. The effects of the chitosan on the cell behavior may be explained as follows. Collagen, when crosslinked by EDC, the specific cell binding amino acid motifs which interact with cell surface integrin adhesion receptors may be consumed. The addition of chitosan may provide much more amino groups for cell adhesion. The composition of CCM is closer to native tissue and has been shown to be more effective than that of collagen alone (Fig. 4).

4. Conclusion

Our primary results of the biocompatibility and mechanical strength of the EDC crosslinked collagen-based materials are encouraging. EDC crosslinked CCM has moderate mechanical strength, good

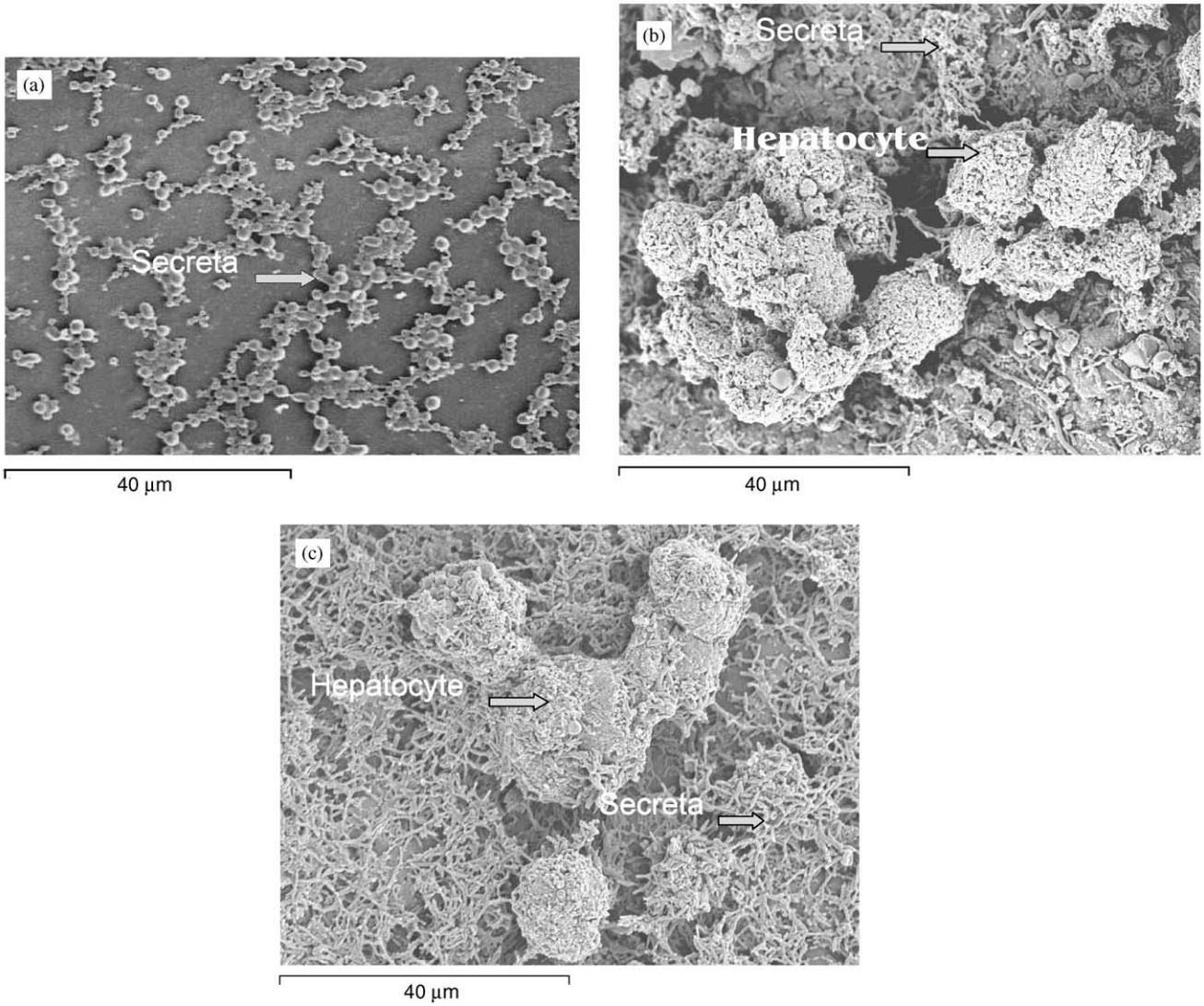

Fig. 4. SEM micrograph showing hepatocytes seeded on the matrices after 15 days of culture: (a) crosslinked collagen; (b) crosslinked CCM; (c) chitosan.

hepatocyte compatibility as well as excellent blood compatibility. These properties make CCM a promising biomaterial for the implantable bioartificial livers with microchannels.

References

[1] Tokiwa T, Kano J, Kodama M, Matsumura T. Multilayer rat hepatocyte aggregates formed on expanded polytetrafluoroethylene surface. Cytotechnology 1997;25:137–44.

[2] Mooney DJ, Sano K, Kaufmann PM, Majahod K, Schloo B, Vacanti JP, Langer R. Long-term engraftment of hepatocytes transplanted on biodegradable polymer sponges. J Biomed Mater Res 1996;37:413–20.

[3] Lysaght MJ, Aebischer P. Encapsulated cells as therapy. Sci Am 1999;4:76–82.

[4] Chvapil M, Kronenthal RL, Winkle Jr WV. Medical and surgical applications of collagen. In: Hall DA, Jackson DS, editors. International review of connective tissue research, Vol. 6. New York: Academic Press, 1973. p. 1–61.

[5] Nezu T, Winnik FM. Interaction of water-soluble collagen with poly(acrylic acid). Biomaterials 2000;21:415–9.

[6] Yoshizato K, Taira T, Shioya N. Collagen-dependent growth suppression and changes in the shape of human dermal fibroblasts. Ann Plast Surg 1984;13:9–14.

[7] Yoshizato K, Makino A, Nagayoshi K. Regulation of morphology and physiology of epithelial cells by collagen fibrils. Biomed Res 1988;9:33–45.

[8] Nishikawa A, Taira T, Yoshizato K. In vitro maturation of collagen fibrils modulates spreading, DNA synthesis, and collagenolysis of epidermal cells and fibroblasts. Exp Cell Res 1987;171:164–77.

[9] Schor AM, Schor SL, Kumar S. Importance of a collagen substratum for stimulation of capillary endothelial cell proliferation by tumor angiogenesis factor. Int J Cancer 1979;24:225–34.

[10] Heimbach D, Luterman A, Burke J, Cram A, Hunt J, Jordan M, McManus M, Solem L, Warden G, Zawacki B. Artificial dermis for major burn: a multi-center clinical trial. Ann Surg 1998; 208:313–20.

[11] Biswas C, Dayer JM. Stimulation of collagenase production by collagen in mammalian cell cultures. Cell 1979;18:1035–41.

[12] Stemberger A, Ascherl R, Blumel G. Kollagen, ein Biomaterial in der Medizin. Hämostaseologie 1990;10:164–76.

[13] Kratz G, Arnander C, Swedenborg J, Back M, Falk C, Gouda I, Larm O. Heparin–chitosan complexes stimulate wound healing in human skin. Scand J Plast Reconstr Surg Hand Surg 1997; 31:119–23.

[14] Aiedeh K, Gianasai E, Orienti I, Zecchi V. Chitosan microcapsules as controlled release systems for insulin. J Microencapsul 1997;14:567–76.

[15] Yagi K, Michibayashi N, Kurikawa N, Nakashima Y, Mizoguichi T, Harada A, Higashiyama S, Muranaka H, Kawase M. Effectiveness of fructose-modified chitosan as a scaffold for hepatocyte attachment. Biol Pharm Bull 1997;20:1290–4.

[16] Madihally SV, Matthew HWT. Porous chitosan scaffolds for tissue engineering. Biomaterials 1999;20:1133–42.

[17] Okamoto Y, Watanabe M, Miyatake K, Morimoto M, Shigemasa Y, Minami S. Effects of chitin/chitosan and their oligomers/monomers on migrations of fibroblasts and vascular endothelium. Biomaterials 2002;23:1975.

[18] Mori T, Okumura M, Matsuura M, Ueno K, Tokura S, Okamoto Y, Minami S, Fujinaga T. Effects of chitin and its derivatives on the proliferation and cytokine production of fibroblasts in vitro. Biomaterials 1997;18:947–51.

[19] Amiji MM. Permeability and blood compatibility properties of chitosan-poly(ethylene oxide) blend membrane for hemodialysis. Biomaterials 1995;16:593–9.

[20] Beena MS, Chandy T, Sharma CP. Heparin immobilized chitosan-poly ethylene glycol interpenetrating network: antithrombogenicity. Artif Cells Blood Subs Immob Biotech 1995;23: 175–92.

[21] Weadock K, Olson R, Silver H. Evaluation of collagen crosslinking techniques. Biomater Med Dev Artif Organs 1983–84; 11:293–318.

[22] Draget K, Varum K, Moen E, Gynnild M, Smidsrod O. Chitosan cross-linked with Mo (VI) polyoxyanions: a new gelling system. Biomaterials 1992;13:635–8.

[23] Ma XH, Noishiki Y, Y amane Y, Iwai Y, Marato D, Matsumoto A. Thermal crosslinking for biologically degradable materials preliminary report. ASAIO J 1996;42:866–71.

[24] Noishiki Y, Miyata T, Kodaira K. Development of a small caliber vascular graft by a new cross-linking method incorporating slow heparin release collagen and natural tissue compliance. ASAIO Trans 1986;32:114–9.

[25] Tomihata K, Burczak K, Shiraki K, Ikada Y. Cross-linking and biodegradation of native and denatured collagen. In: Shalaby SW, Ikada Y, Langer RS, Williams J, editors. Polymers of biological and biomedical importance. American Chemical Society Symposium Series, 1994. p. 540.

[26] Peniche C, Elvira C, Roman JS. Interpolymer complexes of chitosan and polymethacrylic derivatives of salicylic acid: preparation, characterization and modification by thermal treatment. Polymer 1998;39(25):6549–54.

[27] Ingber D, Karpp S, Plopper G, Hansed L, Mooney D. Physical forces and the mammalian cell. New York: Academic Press, 1993 [Chapter 2].

[28] Wan S, Leclere JL, Vaincent JL. Inflammatory response to cardiopulmonary bypass: mechanisms involved and possible therapeutic strategies. Chest 1997;112:676–92.

[29] Hill GE. Cardiopulmonary bypass-induced inflammation: is it important? J Cardiothorac Vasc Anesth 1998;12:21–5.

[30] Wendel HP, Ziemer G. Coating-techniques to improve the hemocompatibility of artificial surfaces used for extracorporeal circulation. Eur J Cardiothorac Surg 1999;16:342–50.

[31] Hay ED. Cell biology of the extracellular matrix, 2nd ed. New York: Plenum Press, 1991. p. 8.

[32] Langer R, Vacanti JP. Tissue Eng Sci 1993;260:920–6.