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## Direct chitosan scaffold formation via chitin whiskers by supercritical carbon dioxide method : A green approach

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**Abstract :** *The wide array of tissue engineering applications exacerbates the need for biodegradable materials with broad potencial. Chitosan, the partially deacetylated derivative of chitin, may be one such material. This research studies the morphology and characterization of porous structures produced from chitin for use as scaffold for cell culture. Chitosan scaffold was prepared by reaction of chitin with hydrochloric acid using supercritical $CO_2$. The prepared hydrogel was subjected to solvent exchange. The material was characterized via Fourier transform infrared spectroscopy (FTIR), scanning electron microscopy (SEM), transmission electron microscopy (TEM). BET measurement was applied to evaluate the scaffold for its porosity and surface area. With supercritical carbon dioxide method, porous chitosan scaffold was successfully fabricated.*

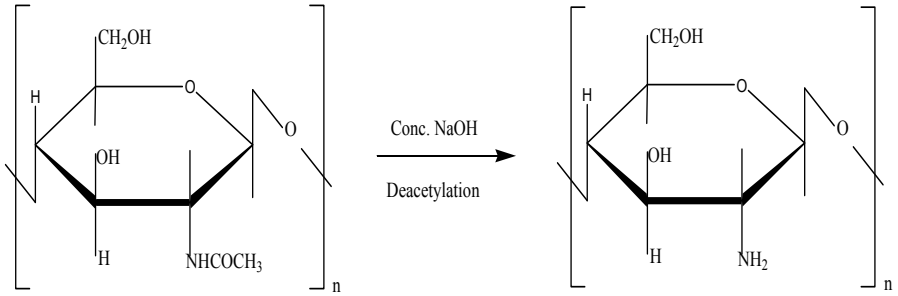
***Keywords :*** Chitosan; scaffold; hydrochloric acid; supercritical $CO_2$; hydrogel

### 1. Introduction :

Tissue engineering, a technique to create new tissue from cultured cells, has now been considered as a potential alternative to organ or tissue transplantation [1]. The scaffolds provides a necessary template and physical support to guide the differentiation and proliferation of cells into targeted functional tissues or organs [2]. A tissue engineering scaffold should be biocompatible and biodegradable, and the degradation product should be non-toxic, and the degradation rate must be consistent with the growth rate of new tissue. The scaffold must be of large enough surface area for cell adhesion and be porous because space is required for cell

seeding, growth and production of extracellular matrix [3]. To allow a high density of seeded cells and to promote neovascularization when being implanted *in vivo*, the scaffolds should have high porosity, large surface area, suitable pore size, and highly interconnected pore structure, in addition to biocompatibility and biodegradability [4-7].

The polysaccharide chitosan is being explored as a substrate for biological reactions. Chitosan, the deacetylated derivative of chitin and the second most abundant natural polysaccharide that is found in insect and crustacean exoskeletons and the cell walls of fungi. Due to its good biocompatibility, biodegradability, low toxicity and low cost, chitosan has already been used for drug delivery, sutures and skin repair [8]. Chitosan is a "green material" because of its biodegradability and biocompatibility. Recently, the use of chitosan has been focusing on medical applications.




Chitin Chitosan

Figure (1) : Structure of chitin and chitosan.

The preparation of porous structures in chitosan may provide a wide variety of applications ranging from filters for separations, scaffolds for cell and tissue growth to controlled substance release etc. In particular, porous scaffolds are one of the promising material for tissue engineering applications. Various processing techniques have been and are being developed to fabricate these scaffolds such as solvent-casting, particulate or porogen leaching, membrane lamination, fiber bonding, phase separation/inversion, high pressure gas foaming, freeze-drying, melt-based technologies, and the solid free foam fabrication (3D-printing) [9-11]. Lyophilization is a bio-clean method. During the whole process, there are no poisonous substances involved and no organic solvent remaining [8]. The use of supercritical carbon dioxide (sc.CO2) to prepare these scaffolds is also now being considered as a recent technique.

Supercritical carbon dioxide ($sc.CO_2$) is nontoxic, nonflammable and naturally abundant, and has low critical temperature and pressure [12]. It has been used as a solvent [13] or a foaming agent [14-18] to prepare microcellular structures for both biodegradable and nonbiodegradable polymers, and thus is a promising process medium for fabrication of tissue-engineering scaffolds.

The current investigation focuses on preparation of chitosan based porous scaffolds from chitin by supercritical carbon dioxide method instead of lyophilization. The advantage of supercritical carbon dioxide process is that it is solvent-free technique, the porous structures fabricated from this process have excellent mechanical properties, and the pore size, shape and porosity are highly tunable [12].

## 2. EXPERIMENTAL :

### 2.1. *Chemicals*

Chitin and Chitosan (79% degree of deacetylation) was supplied by Central Institute of Fisheries Technology (CIFT), Cochein, India and used without further purification. Sodium hydroxide, Sodium carbonate, and hydrochloric acid were of analytical grade.

### *2.2. Instruments and equipment*

Qunatitative and qualitative Fourier transform infrared spectra were obtained from Perkin Elmer Infrared spectrophotometer. Spectra were taken with a resolution of 2 $cm^{-1}$ and were averaged over 16 scans, in the range 4000-500 $cm^{-1}$. The morphology was studied by using a JEOL JEM-1230 transmission electron microscope (TEM) and a JEOL JSM-5200 scanning electron microscope (SEM) at 80 and 15 kV, respectively. The porosity measurement was done by using the micromeritics porosimeter: ASAP2010 model.

### *2.3. Procedures*

#### *2.3.1. Preparation of chitin whiskers*

Chitin was treated in hydrochloric acid and stirred for 2 h to obtain the colloidal solution. The residues were collected after centrifugation and treated with hydrochloric acid for two times. Finally, the residues were dialyzed in distilled water until they were neutral. The prepared chitin was then subjected to solvent-exchange into acetone and ethyl alcohol prior to sc.$CO_2$ treatment.

#### *2.3.2. Supercritical fluid (SCF) drying*

The solvent-exchange products were placed inside a sealed chamber of the SCF reactor (Thorr Co, USA). The pressure were raised to 200 bar. The reaction was left for 2 h and a flow of $CO_2$ was then applied through the sample in order to replace all the organic solvent with $CO_2$. The pressure was then released slowly to the atmosphere.

#### *2.3.3. Preparation of chitosan scaffold*

The supercritical carbon dioxide treated chitin was treated with NaOH and $Na_2CO_3$ respectively and then neutralize. .....

## 3. Results and Discussion :

Tissue engineering has emerged as a promising alternative to treat the loss or malfunction of a tissue. In this paper we have described procedures for fabricating tissue scaffolds from chitosan, an enzymatically degradable polysaccharide with broad potential by a greener technique. The polymer's hydroxyl and amino groups provide several possibilities for derivatization or grafting of desirable bioactive groups, and chitosan's pH-dependent solubility allows use of relatively mild processing methods. This feature is particularly important if incorporation of bioactive species is desired prior to forming a three dimensional microstructure. Formation of dense chitosan membranes and extruded fibers has been extensively described and characterized elsewhere [19, 20], and provide alternate approaches for biomedical implant fabrication.

Chitin (precursor of chitosan) which is the major structural molecule in arthropod cuticles. Interactions with matrix proteins and connective tissue components are numerous and undoubtedly intimate in that environment. Chitosan, the deacetylated derivative of chitin which creates the potential for a variety of interactions in the mammalian implant environment. However, collagen is the exceptional case, interactions with mammalian proteins have not been extensively characterized. However, many interesting interactions with mammalian tissues have been reported. The cells involved ranged from osteoblasts and fibroblasts to macrophages and keratinocytes [21-27]. In many cases the cellular interactions have been positive from the tissue repair and regeneration standpoint.

### *3.1. Chemical structure analysis*

The FTIR spectra of chitosan, Sc.$CO_2$ chitin and Sc.$CO_2$ chitosan are shown in Fig. 2. The IR spectrum of chitin (Fig. 2(c)) shows characteristic peaks at 1628.7 $cm^{-1}$ and 1560.1 $cm^{-1}$ for amide I and amide II. Fig 2 (a) and (b) shows the spectra of chitosan and Sc.$CO_2$ chitosan. From the chitosan spectrum, it can be found that the distinctive absorption bands at 1662 cm-1 (amide I), 1598 cm-1 (-NH2 bending) and 1380 cm-1 (amide III). The absorption bands at 1156 cm-1 (antisymmetric stretching of C-O-C bridge), 1075 and 1033 cm-1 (skeletal vibration involving the C-O stretching are characteristics of its saccharide structure. As compared to chitosan, Sc.$CO_2$ chitosan shows a new peak at 1559.6 cm-1 represents the free primary amino group (-$NH_2$) at $C_2$ position of glucosamine.

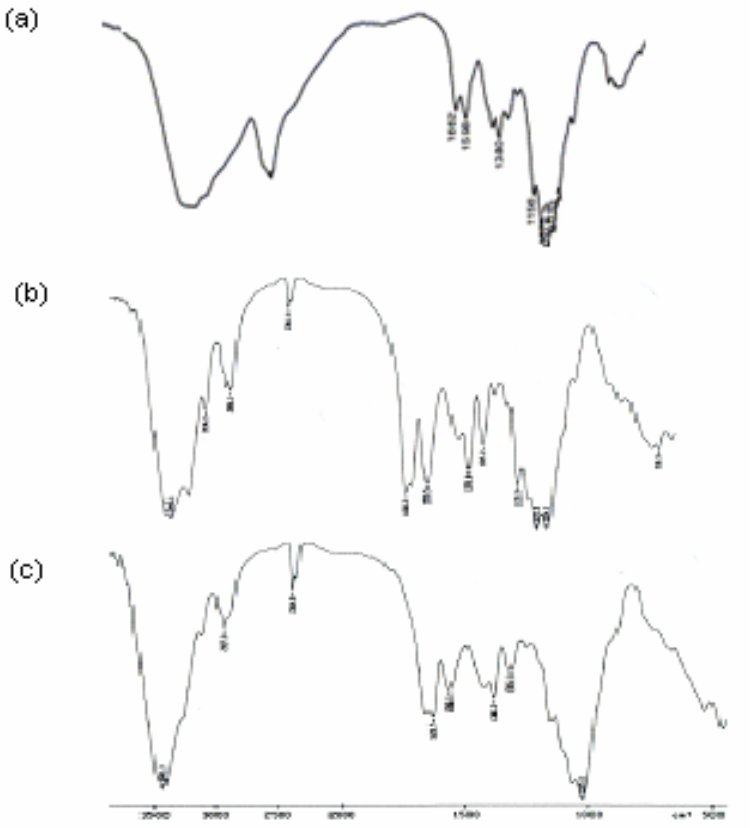



Figure (2) : FTIR spectra of (a) chitosan, (b) Sc. $CO_2$ chitosan, (c) Sc. $CO_2$ chitin.

### *3.2. Morphological studies :*

The scanning electron micrographs (SEMs) of the chitin and supercritical carbon dioxide chitin are shown in Figure 3(a) & 3(b), (c), (d) respectively. It exhibited a nonporous, smooth membranous phases consisting of dome shaped orifices, microfibrils and crystallites. It also exhibited flat lamellar phases on which a large number of protruding microfibrils are evident.

The electron micrographs of chitosan and supercritical carbon dioxide chitosan are shown in Figure 3(e), & 3(f), (g), (h) respectively. The SEMs of chitosan derivative exhibited a polyphasic microporous structure. The pore dimensions are non-uniform with thin walls.

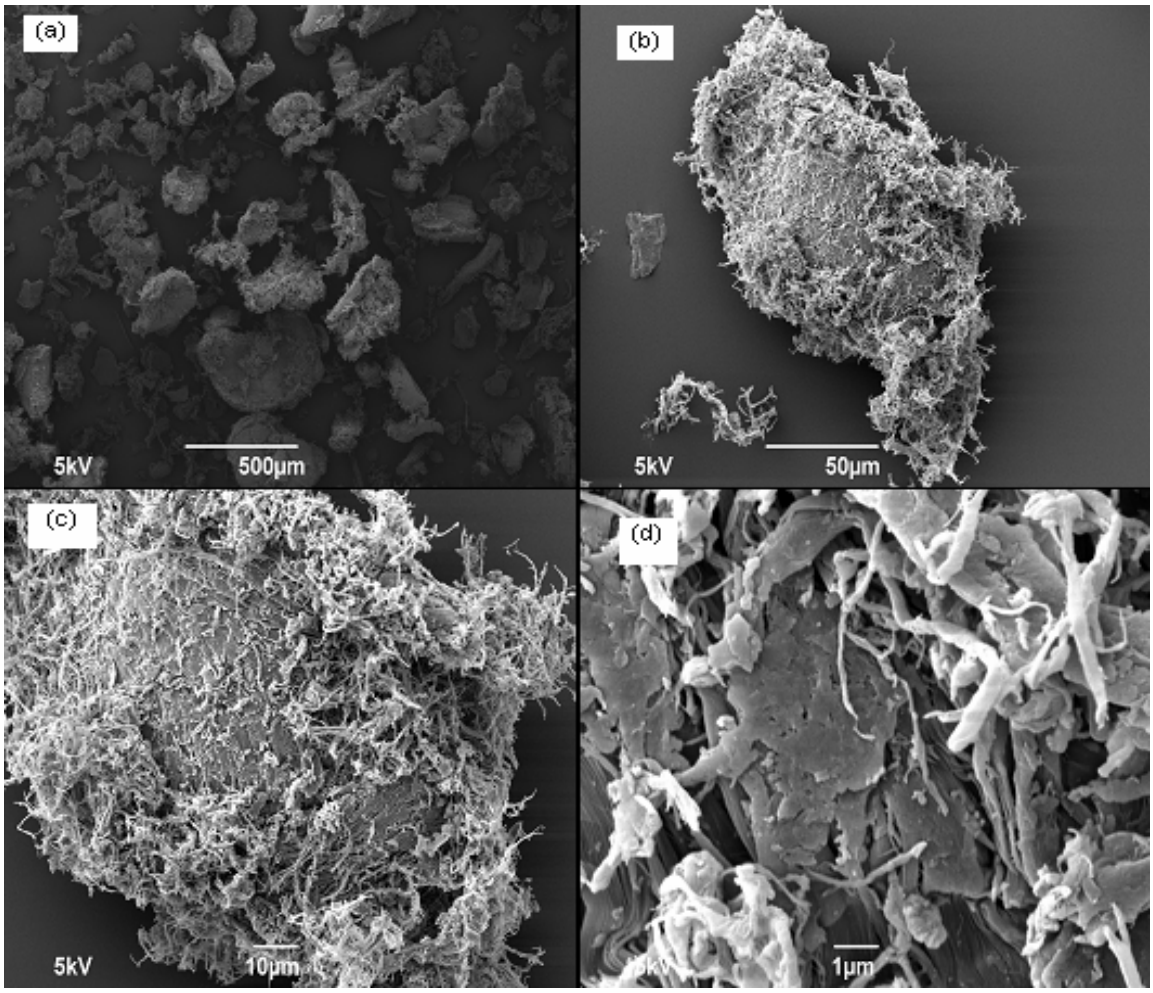



Figure (3) : Continued on Next Page .....

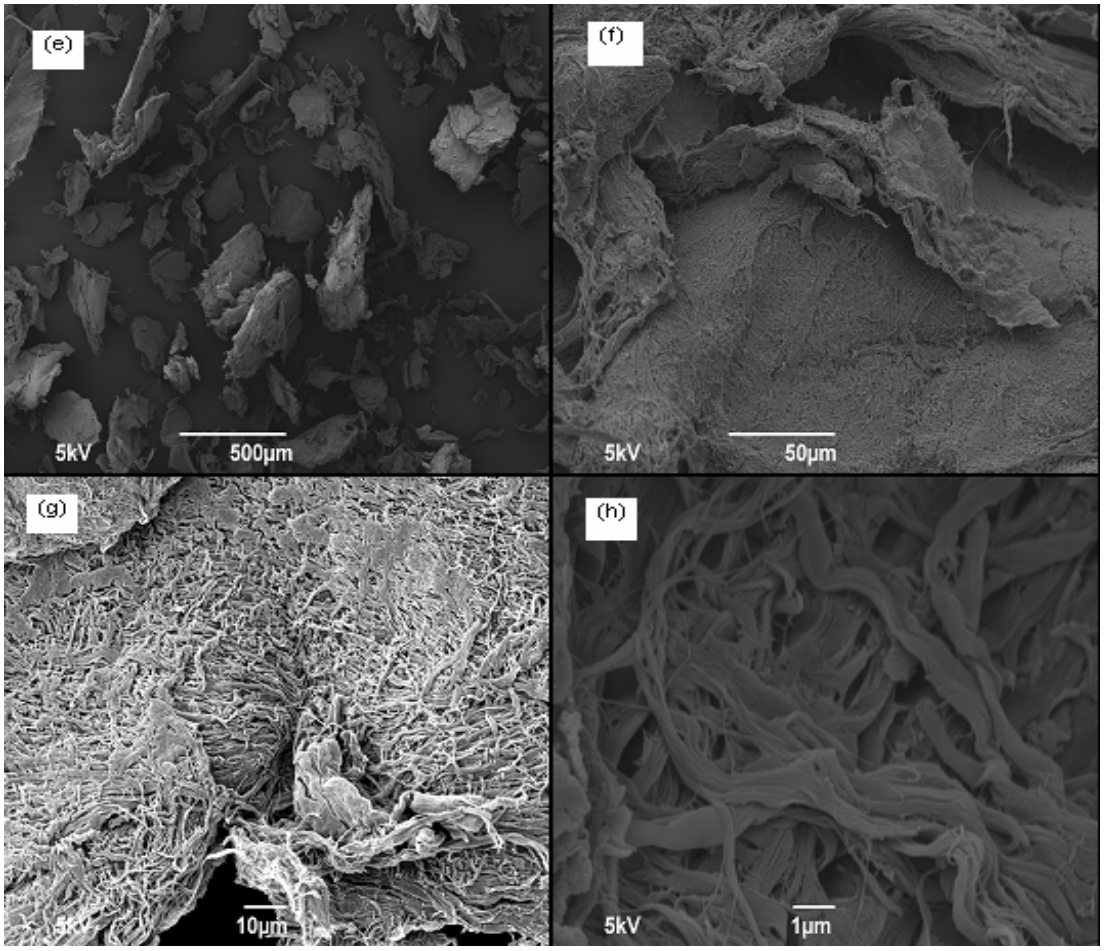



Figure (3) : Scanning electron micrographs of (a): chitin (b), (c), (d) : sc.$CO_2$ treated chitin (e) : native chitosan (f), (g), & (h) : sc.$CO_2$ treated chitosan.

The transmission electron micrographs of sc.$CO_2$ chitin and chitosan are shown in Fig. 4(a) and 4 (b).

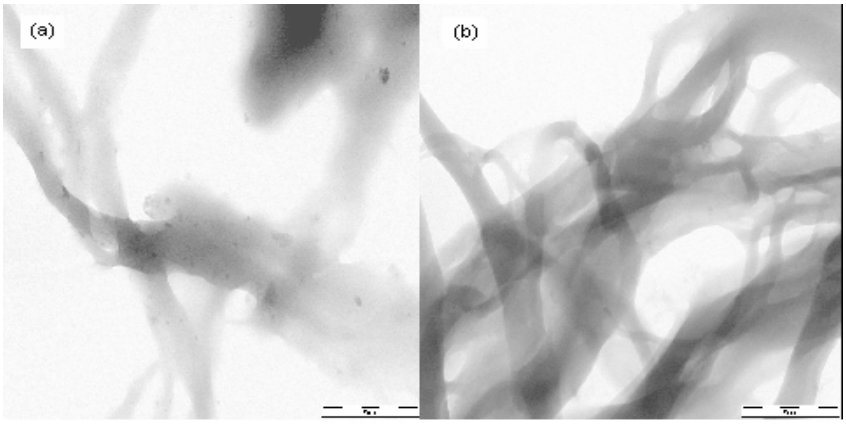



Fig. 4. Transmission electron micrographs of supercritical carbon dioxide treated (a) chitin, (b) chitosan.

### *3.3. Porosity of scaffold*

Control of scaffold pore morphology is critical for controlling cellular colonization rates and organization within an engineered tissue. Furthermore, angiogenesis is a requirement for some scaffold application scenarios and can be grossly affected by material porosity. Pore morphology can also be expected to significantly affect scaffold degradation kinetics and the mechanical properties of the developing tissue. While controlled rate freezing is limited by the magnitude and directionality of thermal gradients, it does provide a simple, straightforward and reproducible way of introducing directional pores into a polymer structure. The methods detailed here allow optimization of pore morphology over a physiologically relevant range and serve as an additional method of tailoring scaffold properties for particular tissue requirements. BET measurement was applied to evaluate the scaffold for its porosity and surface area. The summary report of the porosimetry is described below.

**BET Surface Area Report of supercritical carbon dioxide treated chitin :**

Single point BET surface area: 181.96 $m^2/g$
BET surface area: 194.09 $m^2/g$
Relative pressure range: 0.050 to 0.200
Monolayer volume: 44.5930 cc/g
C: 71.6353
Slope: 0.022112
Intercept: 0.000313
Correlation coefficient: 0.99991
Total surface area: 8.947 $m^2$
BET Surface Area Report of sc.$CO_2$ treated chitosan:
BET surface area (sc.$CO_2$ chitin treated in NaOH) : 207 $m^2/g$
BET surface area (sc.$CO_2$ chitin treated in $Na_2CO_3$): 194 $m^2/g$

## 4. Conclusions :

It is demonstrated in the present work that porous supercritical chitosan scaffolds can be prepared by the proposed supercritical carbon dioxide method. Compared with the lyophilization method, the presented method is more time and energy efficient. Chitosan scaffolds have been proved to be a potential tissue engineering matrix with porous structure, suitable pore size. Porous structures could be easily fabricated with control over pore morphology. The moderate surface area is expected for better proliferation for biological study indicating the potential applicability to tissue engineering of the scaffolds prepared by the proposed method.

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**References :**


[1] Langer R, Vacanti JP. Science 1993;260:920.
[2] Ma L, Gao C, Mao Z, Zhou J, Shen J, Hu X, Han C. Biomaterials 2003;24:4833.
[3] Wang A-J, Cao W-L , Ao Q, Gong K, Kong L-J, Gong Y-D, Zhang X-F. Asian Chitin Journal 2006;2:69.
[4] Mooney DJ, Park S, Kaufmann PM, Sano K, Mcnamara K, Vacanti JP, Langer R. J Biomed Mater Res 1995; 29:959.
[5] Mikos AG, Sarakinos G, Lyman MD, Ingber DE, Vacanti JP, Langer R. Biotech Bioeng 1993; 42: 716.
[6] Mooney DJ, Kaufmann PM, Sano K, Mcnamara KM, Vacanti JP, Langer R. Transplant Proc 1994;26:3425.
[7] Schugens CH, Grandfils CH, Jerome R, Teyssie P, Delree P, Martin D, Malgrange B, Moonen G. J Biomed Mater Res 1995;29:1349.
[8] Wang A-J, Cao W-L, Gong K, Ao Q, J-L Kong, He C-Z, Gong Y-D, Zhang X-F. Asian Chitin Journal 2006; 2:53.
[9] Larry VM. WTEC Panel on Tissue Engineering Research Final Report, Academic Press: Boston, 2003;pp. 1-22.
[10] Hollander AP, Hatton PV. Methods in Molecular Biology, Biopolymers Methods in Tissue Engineering, 2004:Vol. 238, Humana Press Inc.: Totowa NJ.
[11] Gomes ME, Ribeiro AS, Malafaya PB, Reis RL, Cunha AM. Biomaterials 2001;22:883.
[12] Hu X, Lessery AJ. J Cellular Plastics 2006;42:517.
[13] Shi C, Huang Z, Kilic S, Xu J, Enick RM, Beckman EJ, Carrm AJ, Melendez RE, Hamilton A D. Science 1999;286:1540.
[14] Goel SK, Beckman EJ. Polymer Engineering and Science 1994;34:1148.
[15] Arora KA, Lesser AJ, McCarthy TJ. Macromolecules 1999;32:2562.
[16] Arora KA, Lesser AJ, McCarthy TJ. Macromolecules 1998;31:614.
[17] Mooney DJ, Baldwin DF, Suh NP, Vacanti JP, Langer R. Biomaterials 1996;17:1417.
[18] Sheridan MH, Shea LD, Peters MC, Mooney DJ. J Controlled Release 2000;64:91.
[19] Rathke TD, Hudson SM. Rev Macromol Chem Phys 1994;C34:375.
[20] East GC, Qin Y. J Appl Polym Sci 1993;50:1773.
[21] Muzzarelli R, Biagini G, Pugnaloni A, Filippini O, Baldassarre V, Castaldini C, Rizzoli C. Biomaterials 1989;10:598.
[22] Muzzarelli R, Baldassarre V, Conti F, Ferrara P, Biagini G, Gazzanelli G, Vasi V. Biomaterials 1988;9:247.
[23] Klokkevold PR, Vandemark L, Kenney EB, Bernard GW. J Periodontol 1996;67:1170.
[24] Muzzarelli RA, Mattioli-Belmonte M, Tietz C, Biagini R, Ferioli G, Brunelli MA, Fini M, Giardino R, Ilari P, Biagini G. Biomaterials 1994;15:1075.
[25] Peluso G, Petillo O, Ranieri M, Matteo S, Ambrosio L, Calabro D, Avallone B, Balsamo G. Biomaterials 1994;15:1215.
[26] Lee KY, Ha WS, Park WH. Biomaterials 1995;16:1211.
[27] Shahabeddin L, Berthod F, Damour O, Collombel C. Skin Pharmacol 1990;3:107.