Grafting of Glycidyl Methacrylate upon Coralline Hydroxyapatite in Conjugation with Demineralized Bone Matrix Using Redox Initiating System

R. Murugan*[†] and K. Panduranga Rao

Biomaterials Laboratory, Central Leather Research Institute, Chennai-600 020, India

Abstract: Grafting of glycidyl methacrylate (GMA) upon coralline hydroxyapatite in conjugation with demineralized bone matrix (CHA-DBM) using equal molar ratio of potassium persulfate/sodium metabisulfite redox initiating system was investigated in aqueous medium. The optimum reaction condition was standardized by varying the concentrations of backbone, monomer, initiator, temperature and time. The results obtained imply that the percent grafting was found to increase initially and then decrease in most of the cases. The optimum temperature and time were found to be 50 °C and 180 min, respectively, to obtain higher grafting yield. Fourier transform infrared (FT-IR) spectroscopy and X-ray powder diffraction (XRD) method were employed for the proof of grafting. The FT-IR spectrum of grafted CHA-DBM showed epoxy groups at 905 and 853 cm^{-1} and ester carbonyl group at 1731 cm^{-1} of poly(glycidyl methacrylate) (PGMA) in addition to the characteristic absorptions of CHA-DBM, which provides evidence of the grafting. The XRD results clearly indicated that the crystallographic structure of the grafted CHA-DBM has not changed due to the grafting reaction. Further, no phase transformation was detected by the XRD analysis, which suggests that the PGMA is grafted only on the surface of CHA-DBM backbone. The grafted CHA-DBM will have better functionality because of their surface modification and hence they may be more useful in coupling of therapeutic agents through epoxy groups apart from being used as osteogenic material.

Keywords: coralline hydroxyapatite, DBM, GMA, grafting, FT-IR.

Introduction

Owing to physico-chemical and biological properties, hydroxyapatite (HA) seems to be an ideal bone graft substitute in orthopaedic and dental surgery. It is well documented that the HA is highly biocompatible, non-toxic, bioactive and osteoconductive.[1-3] Recently, HA derived from coral species known as coralline hydroxyapatite (CHA) has been attracted as its starting structure is interconnective porous and hence allows faster bone or tissue in-growth upon implantation.[4] The demineralized bone matrix (DBM) is a natural macromolecule being used as a bone graft substitute due to its unique osteoinduction, which means bone formation in exoskeletal sites.[5-7] It has been recognized that in many clinical applications, osteoinductivity serves to enhance the efficacy of the implant.[8] Hence, composite of CHA and DBM, which are the main ingredients of natural bone, have recently attracted as a composite graft substitute to combine the osteoconductive and osteoinductive properties.[9,10]

Glycidyl methacrylate(GMA), ester of methacrylic and 2,3-epoxy porpanol, is a well-known monomer being used to couple with hydroxyl group to form stable covalent bone with biomolecules without any linker.[11] Such grafted macromolecules are more stable during long storage period and relatively resistant against hydrolysis. The graft copolymerization of GMA onto biocomposite backbone is one of the most effective methods to enhance their physico-chemical properties and to impart new functional groups upon it. Recent studies have shown the grafting of GMA onto coralline hydroxyapatite,[12] degelatinized bone,[13] and demineralized bone[14] using ceric ammonium nitrate. In the present communication, we report the results of the grafting of GMA onto CHA in conjugation with DBM in aqueous medium using potassium persulfate/sodium metabisulfite redox initiating system under nitrogen atmosphere.

Experimental

Materials. The CHA and DBM were prepared in our laboratory as reported earlier,[15,16] respectively. GMA (Polysciences,

---

[†]Present address: Biomaterials Lab(E3-05-30), Div. of Bioengineering National University of Singapore, 9 Engineering Drive 1, Singapore-117576.
*e-mail : rmurug@yahoo.com

USA) was purified by distillation under vacuum. Potassium persulfate ($K_2S_2O_8$) and sodium metabisulfite ($Na_2S_2O_5$) were obtained from S.D. Fine Chem Ltd, India. Nitrogen was purified by passing through a freshly prepared alkaline pyrogallol solution. Low conducting H_2O was used for the preparation of solution and in polymerization reaction. All other reagents and chemicals used were of analytical grade.

Preparation of Coralline Hydroxyapatite. The CHA was prepared by hydrothermal exchange reaction using coral genus "Goniopora", procured from the Gulf of Mannar, Indian Coast. The coral blocks were dried and cleaned of macroscopic impurities and then heated at 900 °C for 2 h in a muffle furnace (Indfur, India) in order to remove all the organic debris and other trace impurities. The heated coral is washed with de-ionized water and dried overnight. The heat-treatment makes the coral very brittle, which becomes powder even under the slightest pressure. Stoichiometric amount of high purity di-ammonium phosphate, coral powder and water corresponding to HA were ground well in an agate mortar and kept for reaction in a hydrothermal apparatus (High Pressure Reactor HR-1000, Berghof, Germany) for 30 min under high pressure of water vapour. The resultant material (CHA) was washed in de-ionized water and dried at 60 °C overnight.

Preparation of Demineralized Bone Matrix. The DBM was prepared from adult bovine cortical tibia obtained from the local slaughterhouse. In brief, all the soft tissues and bone marrows were removed initially from the cortical bone and reduced to small chips in dimension of 10 mm^3, approximately. The bone chips were washed in distilled water and stored at 4 °C until use. The bone chips were extracted with absolute ethanol, subsequently dehydrated with anhydrous ether, and then air-dried for 12 h. After air-drying the bone chips were fragmented in a liquid-nitrogen impacting mill of particle size of about 100 to 200 μm. The resulting bone powder was demineralized by acid-extraction in 0.5 N HCl (50 mL per gram of bone powder) for 3 days at room temperature. The demineralized bone was washed in distilled water by centrifugation at 10,000 rpm for 10 min at 4 °C until the pH of the supernatant become same as the rinsing water. Finally, demineralized bone was extracted with absolute ethanol as well as ether and then evaporated under chemical hood.

Polymerization Procedure. The polymerization reaction was preformed in a 500 mL three-neck round bottom flask equipped with a mechanical stirrer, reflux condenser and a gas inlet system under nitrogen atmosphere. In a typical reaction, 5 g of CHA-DBM, at a weight ratio of 70:30, was suspended in 100 mL of low conducting water at constant stirring under bubbling of slow stream nitrogen and maintained at required temperature. After 30 min, the freshly prepared 25 mL of initiator solution containing equal molar ratio of potassium persulfate/sodium metabisulfite (1.5× 10^{-3} mol/L) were added followed by the monomer GMA (10

mL). The flask contents were kept under continuous stirring at 300 rpm until the completion of reaction. The grafting reactions were carried out for varying time intervals (1-5 h). After completion of the reaction, the contents were poured in methanol and the precipitated products were filtered and dried. The dried products were extracted with acetone in a Soxhlet apparatus for 3 days to remove unbound PGMA homopolymer. The extracted products were then dried in vacuum to constant weight.

Instruments. The FT-IR spectra were obtained on Nicolet 20-DXB spectrophotometer, USA over the region 4000-400 cm^{-1} for the ungrafted and grafted CHA-DBM in the pellet form mixed with spectroscopic grade potassium bromide. The phase purity and structural changes of ungrafted and grafted CHA-DBM was examined by a high resolution X-ray powder diffractometer (XRD 3000, Sieffert & Co., Germany) in Guinier geometry using Cu Kα radiation at a wavelength of 1.5406 Å with a graphite diffracted beam monochromator. The data were recorded between 20 and 60° at a scanning speed of 1° (2θ) per minute.

Calculation. The percent grafting and grafting efficiency were calculated from the increase in weight of the CHA-DBM after grafting by using the following expressions:

$$\text{Percent grafting (PG)} = (W_g - W_o)/W_o \times 100$$

$$\% \text{ Grafting efficiency (\%GE)} = W_g/(W_g - W_h) \times 100$$

$$\text{Homopolymer (\%)} = W_h/W_m \times 100$$

Where, W_g is weight of grafted CHA-DBM; W_o is the weight of CHA-DBM; W_h is the weight of homopolymer; W_m is the weight of monomer used.
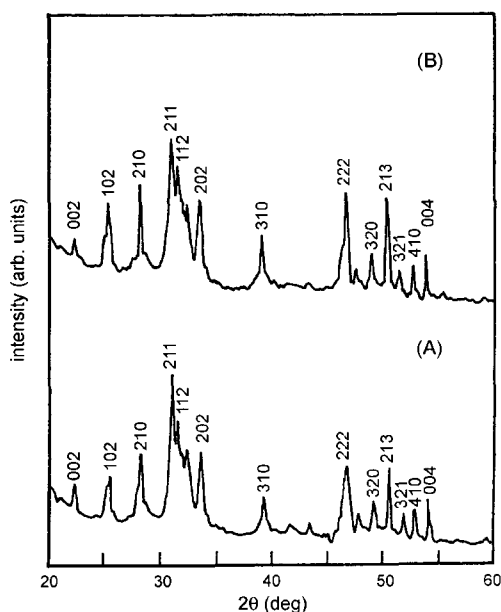
Results and Discussion

Evidence of Grafting by FT-IR. Figure 1 shows the IR spectra of ungrafted and grafted CHA-DBM samples. The ungrafted CHA-DBM shows the characteristic peaks of both the CHA and DBM phases as OH-3420 cm^{-1}, PO_4^{3-} (v_3)-1050 cm^{-1}, PO_4^{3-} (v_4)-620 and 570 cm^{-1}, CO_3^{2-} - 1450, 871 cm^{-1} pertaining to CHA, and amide absorption peaks at 1634 and 1541 cm^{-1} pertaining to DBM. The grafted CHA-DBM shows the presence of epoxy group of PGMA at 905 and 853 cm^{-1} and ester carbonyl group of PGMA at 1738 cm^{-1} in addition to the absorption peaks of CHA-DBM. These results obviously show that the PGMA has grafted onto CHA-DBM.

X-ray Diffraction Study. The XRD patterns of ungrafted and grafted CHA-DBM were shown in Figure 2(A) and (B), respectively. The results indicate that both the diffracted peaks are almost identical and expose mainly the apatite phase due to the presence of higher amount of CHA than DBM. All the patterns show uniformly broad peaks around the characteristic peak regions 26, 28, 29, 30-35, 39, 46, 49,
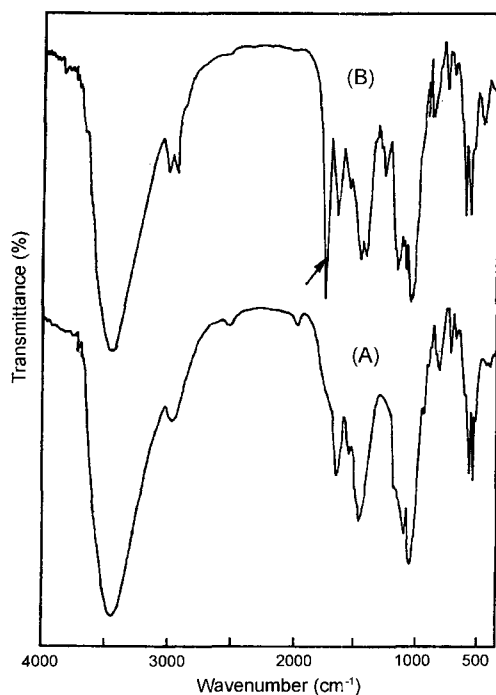
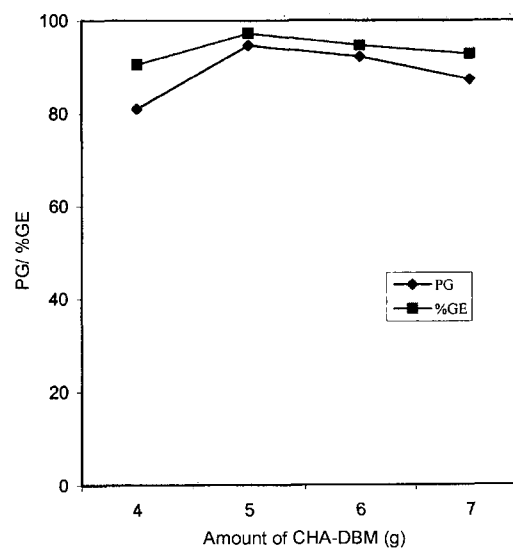**Figure 1.** FT-IR spectra of (A) ungrafted CHA-DBM and (B) grafted CHA-DBM.

Table I. List of Lattice Cell Parameters Calculated from the XRD Data

| Samples	Lattice Constants		
	$a_0 = b_0$ (Å)	c_0 (Å)	c_0/a_0 (Å)
Ungrafted (CHA-DBM)	9.4273	6.8869	0.7305
Grafted (CHA-DBM)	9.4296	6.8857	0.7302
JCPDS 9-432*	9.4180	6.8840	0.7309

*Joint committee on powder diffraction data for HA.

Figure 3. Effect of backbone on grafting reaction: GMA, 6.0 × 10^{-1} mol/L; $K_2S_2O_8$/$Na_2S_2O_5$, 1.5 × 10^{-3} mol/L each; temperature, 50 °C; time, 180 min; total volume, 100 mL.

Figure 2. XRD pattern of (A) ungrafted CHA-DBM and (B) grafted CHA-DBM.

and 50° (2θ), which indicates that the materials are microcrystalline in nature. The lattice cell parameters of the ungrafted and grafted CHA-DBM were calculated by refining the XRD data by least square fitting method using 'CELN' programme. The calculated cell values of grafted CHA-DBM

are comparable to ungrafted CHA-DBM as well as reported values as listed in Table I. The crystalline structure of grafted CHA-DBM does not undergo any changes and there is no secondary phase transformation detected due to grafting, which suggest that the grafted PGMA chains have not distorted the crystal lattice of CHA-DBM and hence it is grafted only on the surface of CHA-DBM backbone.

Effect of Backbone Concentration. The dependence of the grafting reaction on the concentration of CHA-DBM was studied in the range of 4-7 g by weight (Figure 3). The percent grafting and grafting efficiency increased initially with increase of CHA-DBM concentration up to 5 g and thereafter decreased with further increase in the CHA-DBM concentration. The increase of grafting yield at the initial stage of the reaction may be due to the availability of more number of reactive sites, which increased with increase in the concentration of CHA-DBM. The decrease in percent grafting and grafting efficiency is due to the production of more CHA-DBM macroradicals and hence increasing the chance of interaction with each other to terminate the grafting.

**Effect of Monomer Concentration.** The results of percent
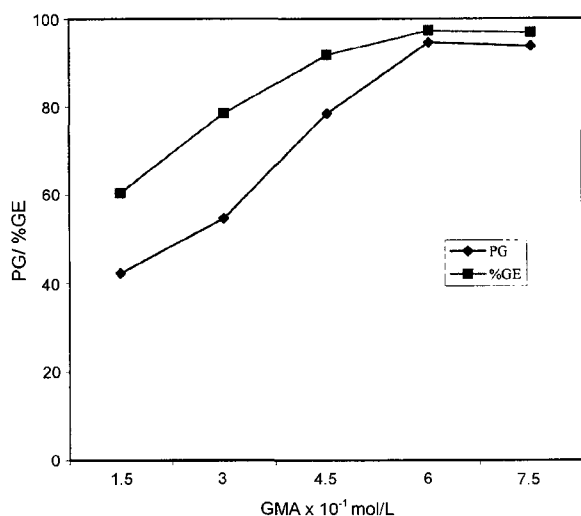
Figure 4. Effect of monomer on grafting reaction: CHA-DBM, 5.0 g; $K_2S_2O_8/Na_2S_2O_5$, 1.5×10^{-3} mol/L each; temperature, 50 °C; time, 180 min; total volume, 100 mL.
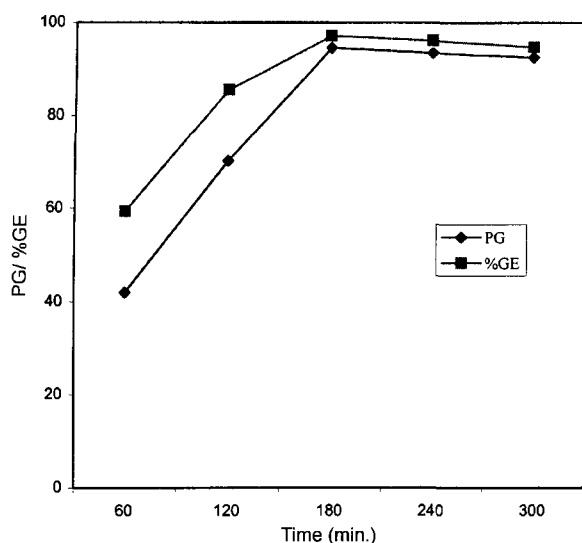

Figure 5. Effect of initiators on grafting reaction: CHA-DBM, 5.0 g; GMA, 6.0×10^{-1} mol/L; temperature, 50 °C; time, 180 min; total volume, 100 mL.

grafting and grafting efficiency over the range of monomer concentration 1.5-7.5 $\times$ 10^{-1} mol/L were shown in Figure 4. The results suggest that upon increasing GMA in the reaction medium, both the percent grafting and grafting efficiency increased and reached maximum at a monomer concentration of 6.0×10^{-1} mol/L. The increase in rate of grafting observed upon increase in the monomer concentration may be due to the greater availability of monomer to the grafting sites. The monomer concentration at above 6.0 $\times$ 10^{-1} mol/L, the percent grafting and grafting efficiency remained steady.

Effect of Initiator Concentration. The effect of initiator concentration was studied by keeping the concentration of GMA at 6.0×10^{-1} mol/L and the polymerization time at 3 h. Figure 5 shows the result obtained by changing the initiator concentration from 0.5×10^{-3} to 2.5×10^{-3} mol/L. The percent grafting and grafting efficiency increased up to a critical initiator concentration of 1.5×10^{-3} mol/L and then slightly decreased at above this concentration. The increase in the initiator concentration increases the dissociation rate and therefore increases the free radical concentration in the polymerization medium. The free radicals take place in many reactions in the graft copolymerization.[17] They can directly interact with the CHA-DBM backbone to form active sites and may also be possible to initiate the homopolymerization of GMA. The active homopolymer chains may give chain-transfer reactions with the CHA-DBM backbone and create additional active sites upon it due to which the percent grafting will be higher. Further increase in the concentration over 1.5×10^{-3} mol/L results in the enormous primary radicals (CHA-DBM radicals) and growing macroradicals of side chains, which may interact with each other resulting in termination of reactive sites and grafting efficiency.
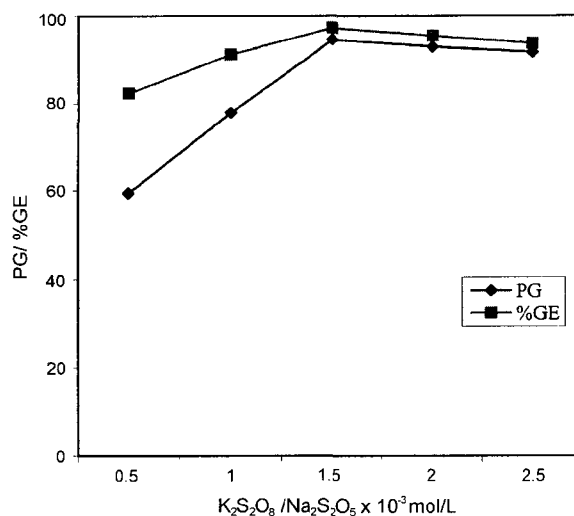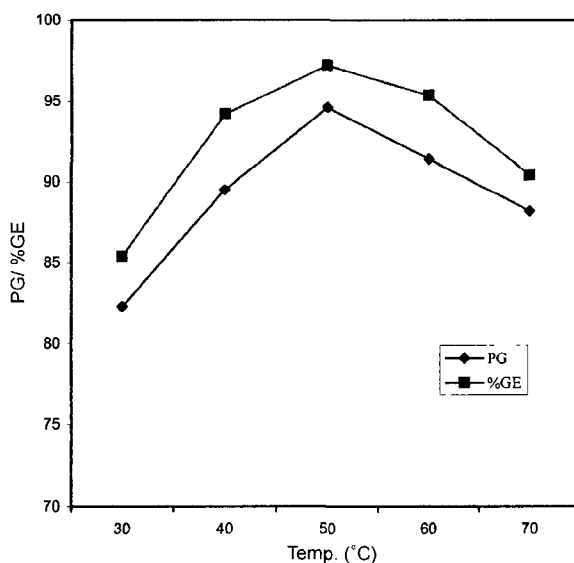
Figure 6. Effect of temperature on grafting reaction: CHA-DBM, 5.0 g; GMA, 6.0×10^{-1} mol/L; $K_2S_2O_8/Na_2S_2O_5$, 1.5×10^{-3} mol/L each; time, 180 min; total volume, 100 mL.

Effect of Reaction Temperature. Figure 6 shows the effect of temperature on the percent grafting of PGMA onto CHA-DBM for a given period of reaction time (3 h). The grafting reaction has been studied at five different temperatures i.e. 30, 40, 50, 60, and 70 °C. From the results it is clear that the percent grafting and grafting efficiency have been found to increase with increase in the temperature of reaction medium from 30 to 50 °C and thereafter decreases with further increase of temperature to 60 °C. The optimum temperature

Figure 7. Effect of time on grafting reaction: CHA-DBM, 5.0 g; GMA, 6.0× 10⁻¹ mol/L; $K_2S_2O_8$/$Na_2S_2O_5$, 1.5× 10⁻³ mol/L each; temperature, 50°C; total volume, 100 mL.

required for maximum grafting is 50°C. The percent grafting was found to decrease at above the optimum reaction temperature of 50°C. The observed decreasing trend may be due to the denaturation of DBM macromolecules at above 50°C,[18] which lead to decrease of percent grafting.

Effect of Reaction Time. The change of percent grafting and grafting efficiency with time was shown in Figure 7. From the result, the percent grafting was found to increase up to 180 min, and then leveled-off over the time period (180-300 min). The gradual increase in percent grafting with time may be due to the increase in the number of grafting sites on CHA-DBM at the initial stage of the graft copolymerization. Over the reaction time (180 min), there was no significant change in the percent grafting, which may be due to the number of active sites that remain almost constant and hence no significant improvement is obtained in the percent grafting.

Conclusions

In this study, it was shown that the redox-initiating system could effectively initiate the grafting of GMA upon CHA-DBM backbone in aqueous medium. The optimum reaction condition for the grafting was standardized. The optimum temperature and time are found to be 50°C and 180 min, respectively, to yield higher percent grafting. The percent

grafting and grafting efficiency can be controlled by varying the reaction parameters. The FT-IR results proved the existence of epoxy group of PGMA on the grafted CHA-DBM. The XRD analysis shows that the crystallographic structure of grafted CHA-DBM does not undergo any changes due to the grafting, which implies that the PGMA chains are grafted only on the surface of CHA-DBM. These findings suggest that it is possible to tailor-make the CHA-DBM having specific property balance for a given function by this grafting technique. The therapeutic macromolecules can be coupled to the grafted chains through epoxy group and can be used in bone drug delivery systems apart from being used as osteogenic and bone filling material.

References

(1) K. Ono, T. Yammamura, T. Nakammura, and T. Kokubo, *Biomater.*, **11**, 265 (1990).
(2) M. Jarcho, *Clin. Orthop. Rel. Res.*, **157**, 259 (1981).
(3) K. De Groot, *Biomater.*, **1**, 47 (1980).
(4) C. Muller-Mai, C. Voigt, S. R. De Almeida Reis, H. Herbst, and U. M. Gross, *J. Mater. Sci. Mater. Med.*, **7**, 479 (1996).
(5) J. Glowaki and J. B. Mulliken, *Clin. Plast. Surg.*, **12**, 233 (1985).
(6) S. Mizuno and J. Glowacki, *Biomater.*, **17**, 1819 (1996).
(7) M. R. Urist, *Science*, **150**, 893 (1965).
(8) T. K. Sampath and A. H. Reddi, *Proc. Natl. Acad. Sci. USA*, **80**, 6591 (1983).
(9) T. Sato, M. Kavamura, K. Sato, and T. Miura, *Clin. Orthop.*, **263**, 254 (1991).
(10) S. Kotani, T. Yammamura, T. Nakamura, T. Kitsugi, Y. Furita, K. Kawanable, and T. Kokubo, *Clin. Orthop.*, **278**, 226 (1992).
(11) A. W. Eckert, D. Grobe, and U. Rothe, *Biomater.*, **21**, 441 (2000).
(12) R. Murugan and K. Panduranga Rao, *J. Appl. Polym. Sci.*, (2002, under revision).
(13) T. Sathian, T. P. Sastry, Y. L. Narayana, and G. R. Krishnan, *Proceedings of XXV IULTCS Congress*, **CTO-4**, 576 (1999).
(14) K. Panduranga Rao, *J. Biomat. Sci. Polym. Ed.*, **7**, 623 (1995).
(15) M. Sivakumar, T. S. Sampathkumar, K. L. Shantha, and K. Panduranga Rao, *Biomater.*, **17**, 1709 (1996).
(16) S. Mizuno and J. Glowaki, *Biomater.*, **17**, 1819 (1996).
(17) Y. Ikada, Y. Nishizaki, and I. Sakurada, *J. Polym. Sci., Polym. Chem. Ed.*, **12**, 1829 (1974).
(18) P. H. Von Hippel, *Treatise on Collagen*, G.N. Ramachandran, Ed., Academic Press, New York, 1967, Vol.1, pp 253.