Colloid Polym Sci (2004) 282: 1316–1322
DOI 10.1007/s00396-004-1066-7

ORIGINAL CONTRIBUTION

R. Murugan
S. Ramakrishna

Ce(IV) ion initiated graft polymerization of glycidylmethacrylate onto a demineralized bone matrix: effect of reaction parameters

Received: 3 November 2003
Accepted: 7 January 2004
Published online: 6 May 2004

R. Murugan (✉) · S. Ramakrishna
Surface Modification & Nanobioengineering Laboratory, Division of Bioengineering, NUS Nanoscience & Nanotechnology Initiative, National University of Singapore, 9 Engineering Drive 1, 117576, Singapore, Singapore
E-mail: engmr@nus.edu.sg
Tel.: +65-6874-6593
Fax: +65-6874-2162

Abstract Studies on chemical modification of demineralized bone matrix (DBM) have opened new arenas in the field of clinical orthopedics owing to its potential osteoinductivity with desired chemical functionality. To widen its usage to biomolecular delivery, graft polymerization of glycidylmethacrylate onto DBM was carried out by a free-radical initiating process using ceric ammonium nitrate as an initiator. The evidence of the grafting reaction was examined by chemical analysis using Fourier transform IR spectroscopy. The grafting condition was standardized by regulating the reaction parameters such as the concentrations of the backbone, the monomer and the initiator, the polymerization temperature and time. The optimum polymerization temperature and time to have the maximum grafting yield were 40 °C and 3 h, respectively. The percentage of grafting and the percentage of grafting efficiency were determined as a function of the reaction parameters, and both were found to increasing initially and thereafter decrease in most of the cases. The grafting results are discussed in a detailed fashion and a reaction mechanism is proposed.

Keywords Demineralized bone matrix · Glycidylmethacrylate · Ceric ammonium nitrate · Grafting · Optimum condition

Introduction

The last few decades have witnessed a significant augmentation of new bone graft substitutes for the treatment of bone defects arising from trauma, tumors or bone diseases. Demineralized bone matrix (DBM) is a natural macromolecular biomaterial habitually used in clinical orthopedics owing to its extensive osteogenesis [1, 2, 3]. The rationale for using DBM is based on the ability to generate bone in heterotopic and orthotopic regions through a process of bone induction. It is known to be biocompatible, biodegradable, osteoinductive, cost-effective, readily available from approved tissue banks and suitable for biomedical applications as a graft extender, a graft enhancer and a graft substitute [4, 5, 6]. It appears to stimulate new bone formation through an osteoconductive mechanism as well [7]. DBM-related biomaterials have been in clinical use for the past 10 years as the demineralization process destroys the antigenic substances associated with bone tissues and is less immunogenic than calcified allograft [8, 9]. Experimental evidence also supports its bone-healing characteristics with biological tissues and hence it is used as a restorative agent in craniofacial defects, mandibular defects and other osseous reconstructive applications [10, 11, 12]. Besides, it is believed to be even used as a carrier for biomolecular delivery. A wide variety of biomolecules can be delivered using functionalized DBM. The term biomolecules as used herein refers to growth factors, for example, bone morphogenetic

proteins and therapeutic molecules, such as gentamicin. For this, it needs specific chemical functionality to carry the respective molecules to the targeted sites. Therefore, modifying the DBM by functionalizing pertinent reactive groups through a chemical route would definitely make them suitable for the delivery of biomolecules. In this study, the DBM was functionalized with epoxy groups with the view that it may be utilized for the delivery of biomolecules through chemical bonding between amino and epoxy groups.

Glycidylmethacrylate (GMA), an ester of methacrylic acid and 2,3-epoxy-propanol, bears a reactive epoxide group that can react with amino groups [13] and hydroxyl groups [14, 15] to form a stable covalent bond without a cross-linker. These kinds of grafted molecules are chemically stable during a long storage period and relatively resistant against hydrolysis. As DBM has CH_2OH functional groups, GMA can readily be grafted upon it using a suitable initiator and subsequently biomolecules can also be coupled through epoxy groups. Chemical modification provides an excellent way to make the DBM as an impact biomaterial for broader applications. In our previous studies, the necessity of chemical modification of organic bone grafts [16, 17] and inorganic bone grafts [18, 19] in making new composite bone grafts with a desired functionality was elucidated. The aim of the present investigation was to develop a process for imparting epoxy groups onto DBM macromolecules using GMA as a monomer and ceric ammonium nitrate (CAN) as a redox initiator with the view that the grafted DBM may have a great impact in biomolecular delivery in comparison with ungrafted DBM. For this, the effect of the graft polymerization conditions has to be considered carefully, and this is reported in a detailed fashion in this article.

Experimental

Materials

DBM was isolated from bovine cortical tibia in accordance with the method described earlier with some modifications with an average particle size of 120 μm [20]. GMA was obtained from Polysciences (USA) and was used after distilled purification under a vacuum. CAN, $Ce(NH_4)_2(NO_3)_6$, was purchased from Sigma (USA) and was used as received. Low-conducting water was used for all the experiments. All other chemicals and solvents used were of analytical reagent grade.

Preparation of DBM

The preparation of DBM was as follows. Initially, all the macroscopic impurities of soft tissues and bone marrows were removed from the bovine cortical bone and reduced into small chips of about 10 mm^3. The bone chips were completely washed in distilled water and stored at 4 °C until they were assayed. The bone chips were extracted with absolute ethanol, dehydrated with anhydrous ether and air-dried for 12 h. After air-drying, the bone chips were fragmented in a liquid-nitrogen impact mill to a cocktail particle size ranging between 50 and 200 μm. The resulting bone powder was demineralized by acid-extraction with 0.5 N hydrochloric acid for 3 days at room temperature. The residual acid was removed by continuous washing with 0.1 M sodium chloride solution. The demineralized bone was washed in distilled water by centrifuging at 10,000 rpm for 10 min until the pH of the supernatant became the same as that of the rinsing water. Finally, demineralized bone was extracted with absolute ethanol followed by ether and then evaporated under a chemical hood.

Method of graft polymerization

The grafting of GMA onto DBM was carried out under a nitrogen atmosphere. In brief, DBM (2–6 g) was added to a 250-ml three-neck round-bottom flask equipped with a mechanical stirrer, a reflux condenser and a gas-inlet system. To this, 100 ml low-conducting water was added and the nitrogen gas was purged into the flask to remove oxygen during the reaction. After 15 min, a freshly made 25 ml solution of CAN ($0.5–2.5\times10^{-3}$ mol L^{-1}) dissolved in 0.1 M nitric acid was added to the reaction medium followed by GMA ($1.5–7.5\times10^{-1}$ mol L^{-1}). The reaction flask was placed in a temperature-controlled water bath (30–60 °C) and the grafting reaction was carried out for the desired period (1–5 h) under constant stirring. After completion of the reaction, the grafted product was poured into methanol to induce precipitation, filtered and dried. The resultant product was then Soxhlet-extracted for the removal of unbound polyGMA (PGMA) homopolymer using acetone and dried in a vacuum to constant weight.

Characterization

Determination of grafting yield

The percentage of grafting (%G) and the percentage of grafting efficiency (%GE) were determined as follows:

$$\%G = \frac{\text{weight of grafted polymer}}{\text{weight of backbone}} \times 100,$$

$$\%GE = \frac{\text{weight of grafted polymer}}{\text{weight of grafted polymer} + \text{weight of homopolymer}} \times 100.$$

Chemical analysis

The chemical analysis was carried out to prove the grafting reaction using a Nicolet Fourier transform (FT) IR spectrophotometer (ThermoNicolet Avatar 360, USA) equipped with a deuterated triglyceryl sulfate–KBr window detector using KBr pellets at a ratio of 1 mg powder sample per 300 mg high-purity KBr. The transmissions were recorded between 400 and 4,000 cm^{-1} with 2-cm^{-1} resolution and averaging of 100 scans.

Results and discussion

Proof of grafting

The existence of PGMA grafting was confirmed by chemical analysis by comparing the FTIR spectrum of ungrafted DBM with that of grafted DBM (Fig. 1). The results of ungrafted DBM (Fig. 1, spectrum a) show the absorption peaks at 1,669 and 1,541 cm^{-1} due to the presence of an amide group and the merging of N–H and adsorbed O–H groups was noticed at 3,410 cm^{-1}. The grafted DBM (Fig. 1, spectrum b) exhibits new functional groups apart from the characteristic absorptions of DBM. The observed new peaks at 906 and 845 cm^{-1} are assigned to epoxide groups of grafted PGMA. Another two peaks observed at 1,731 and 1,260 cm^{-1} are associated with carbonyl and C–O functional groups of grafted PGMA, respectively. These results confirmed the grafting of PGMA onto the DBM backbone.

Effect of backbone

The grafting dependency on the DBM backbone was studied by changing its amount in the range between 2 and 6 g by keeping the other parameters, including the monomer concentration (6×10^{-1} mol L^{-1}), the initiator concentration (1.5×10^{-3} mol L^{-1}), the polymerization temperature (40 °C), time (3 h) and the total volume (100 ml), constant. The %G and %GE increased initially with increasing DBM up to 5 g and beyond this quantity the %G and %GE were considerably reduced with further increase of the DBM concentration (Fig. 2). This behavior is attributed to the availability of more grafting sites for the initiation of graft polymerization at a higher concentration of DBM. The observed decreasing trend in %G and %GE with a higher amount of DBM over a critical value (5 g) may be attributed to deactivation of the radical activity of the DBM backbone soon after its formation owing to termination of grafting chains between the backbones. Further, the viscosity of the reaction medium may also obstruct the movement of the DBM macroradicals and hence both the %G and %GE are reduced.

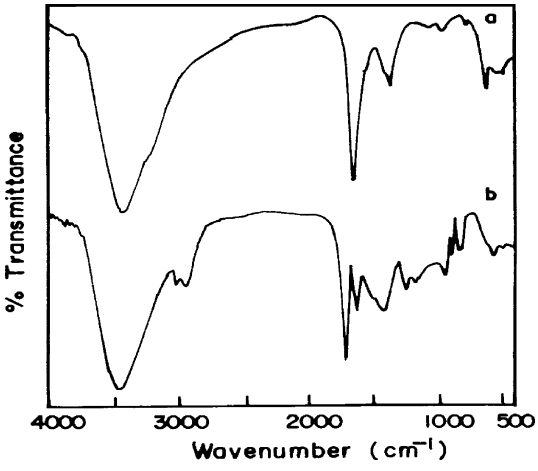


Fig. 1 Fourier transform R spectra of ungrafted demineralized bone matrix (*DBM*) (*a*) and grafted DBM (*b*), showing epoxide groups at 906 and 845 cm^{-1}, a carbonyl group at 1,731 cm^{-1} and C–O bonding at 1,260 cm^{-1} for the proof of grafting

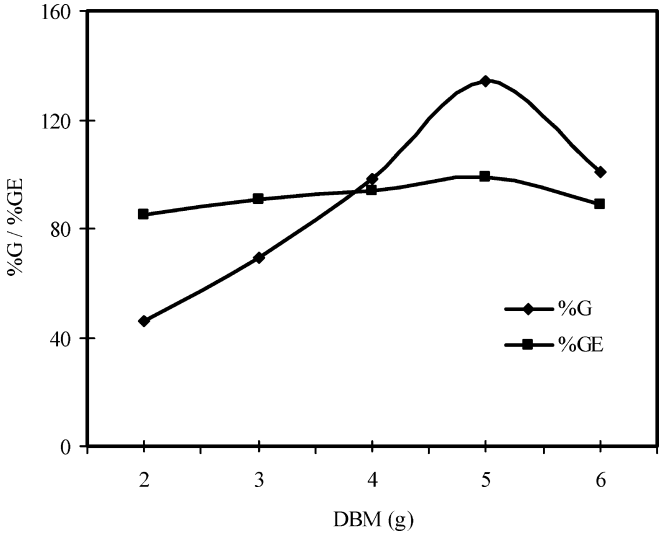


Fig. 2 Effect of backbone on the grafting reaction: glycidylmethacrylate (*GMA*) 6.0×10^{-1} mol L^{-1}; ceric ammonium nitrate (*CAN*) 1.5×10^{-3} mol L^{-1}; temperature 40 °C; time 3 h; total volume 100 ml

Effect of monomer

The influence of the monomer concentration on the grafting reaction was studied at various amounts of GMA between 1.5 and 7.5×10^{-1} mol L^{-1} while other reaction parameters were kept constant as stated previously in addition to 5 g DBM. As can be seen from theresults (Fig. 3), the %G and %GE increase with increasing monomer concentration up to 6.0×10^{-1} mol L^{-1}. However, beyond this value, the monomer concentration has a small effect on the %G and becomes a decreasing tendency. Thus, the GMA concentration of 6.0×10^{-1} mol L^{-1} was recognized as an optimum value. With a further increase of monomer, the %G remains relatively stable though there is a slight deviation with an optimum level. The enhancement of the rate of grafting upon increasing the monomer concentration to an optimum value could be ascribed to the greater availability of monomer to the grafting sites. The slight decreasing trend in %G may be due to the higher affinity of the monomer for its homopolymer over DBM macroradicals. On the other hand, with further increase of monomer concentration the total volume of the reaction medium increases, which leads to a reduction in the concentration of initiator and hence %G was reduced accordingly.

Effect of initiator

The effect of the initiator concentration was examined by keeping the amount of DBM at 5 g, the concentration of GMA at 6.0×10^{-1} mol L^{-1}, the polymerization time at 3 h and the temperature at 40 °C. The result obtained by changing the initiator concentration from 0.5×10^{-3} to 2.5×10^{-3} mol L^{-1} is shown in Fig. 4. Both the %G and %GE have reached a maximum value at the critical concentration of CAN (1.5×10^{-3} mol L^{-1}). A further increase in CAN concentration was accompanied by a slight decrease in grafting yield. Increasing the initiator concentration induces the dissociation of CAN. The rapid dissociation of CAN might account for the higher grafting yield in the initial stage of the reaction, because less Ce(IV) would have been available for graft initiation [21]. The dissociation of CAN increases the free-radical concentration in the polymerization medium and the free radicals actively involved in many graft polymerizations [22]. They can directly interact with the DBM backbone to form active sites and may also initiate less homopolymer formation. The homopolymer chains may undergone chain-transfer reactions with the DBM backbone and thus created additional active sites upon it, which in turn led to higher %G. A further increase in the amount of CAN over 1.5×10^{-3} mol L^{-1} results in the enormous primary radicals (DBM radicals) and growing macroradicals of the side chains, which may interact with each other, resulting in a termination of reactive sites and hence a reduction in the %G and %GE.

Effect of temperature

Determining an optimum temperature is one of the significant factors to standardize the grafting reaction. To study the effect of polymerization temperature on the

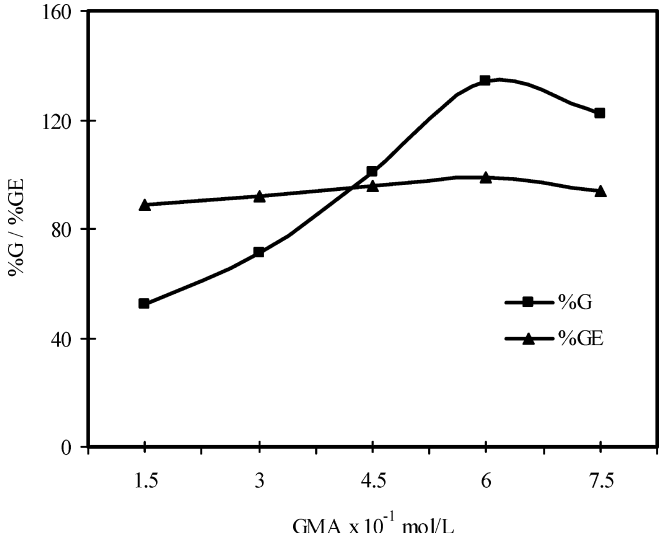


Fig. 3 Effect of monomer on the grafting reaction: DBM 5.0 g; CAN 1.5×10^{-3} mol L^{-1}; temperature 40°C; time 3 h; total volume 100 ml

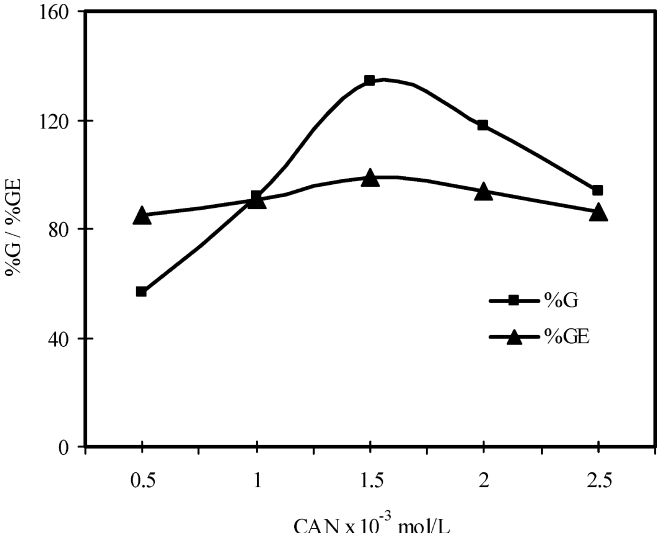


Fig. 4 Effect of initiators on the grafting reaction: DBM 5.0 g; GMA 6.0×10^{-1} mol L^{-1}; temperature 40°C; time 3 h; total volume 100 ml

reaction parameters, the grafting of GMA onto DBM was carried out at different temperatures ranging from 30 to 60 °C. The results are depicted in Fig. 5. The %G and %GE increased by increasing the polymerization temperature from 30 to 40 °C and then slightly decreased with further increase of temperature to 50 °C. The maximum %G was observed at 40 °C, which was presumably an ideal condition for the grafting reaction. Beyond this temperature, the %G decreased. This can be ascribed to the fact that with an increase in temperature, more radicals are formed that might have enhanced the grafting reaction. However, the %G was found to decrease at 50 °C, which may be due to the denaturation of DBM macromolecules at about 50 °C [23], causing a radical termination, and thus %G and %GE are reduced.

Effect of time

The influence of the polymerization period on the %G and %GE is shown in Fig. 6. With an increase in the polymerization time, the grafting yield increased rapidly up to 3 h after which it leveled off with further increase in the polymerization period. The increase in the %G and %GE with respect to time is accounted for by an increase in the number of grafting sites on the DBM backbone in the initial stage of reaction. A longer reaction period has little effect on the %G since the number of active sites remains almost stable upon increasing the grafting time and hence there is no further change in the %G observed. Thus, the optimum polymerization time is proposed as 3 h.

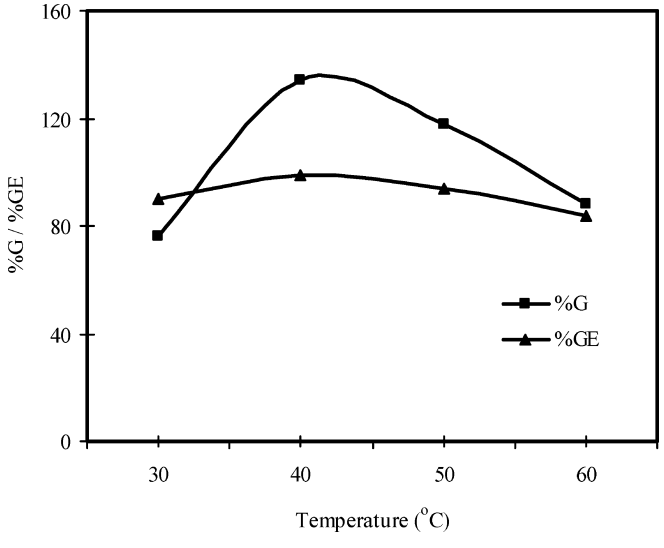


Fig. 5 Effect of temperature on the grafting reaction: DBM 5.0 g; GMA 6.0×10^{-1} mol L^{-1}; CAN 1.5×10^{-3} mol L^{-1}; time 3 h; total volume 100 ml

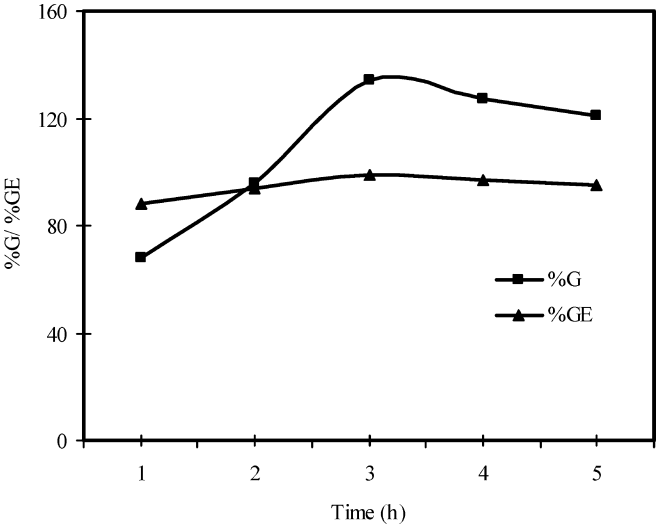


Fig. 6 Effect of time on the grafting reaction: DBM 5.0 g; GMA 6.0×10^{-1} mol L^{-1}; CAN 1.5×10^{-3} mol L^{-1}; temperature 40 °C; total volume 100 ml

Optimum condition for grafting

The optimum conditions for the graft polymerization of GMA onto DBM are listed in Table 1. The results were determined by studying the influential reaction parameters, including the concentrations of the backbone, the monomer and the initiator, the temperature and time. The optimum conditions given in Table 1 are associated with the maximum %G. However, the %G and %GE can be manipulated corresponding to clinical applications, because less polymer grafting is preferred for some cases to avoid overdosage of toxicity with surrounding tissues upon implantation in our body.

Reaction mechanism

The introduction of active groups onto the DBM backbone was achieved by the mechanism described in the following.

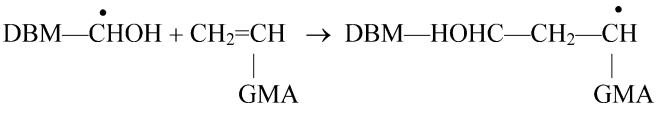
Radical formation

The reactive vicinal where the grafting initiated on the DBM is CH_2OH. The overall reaction mechanism is that the ceric ions attack the DBM backbone and a DBM–ceric complex is formed. The ceric(IV) ions in the complex are reduced to ceric(III) ions by oxidizing a hydrogen atom and thus creating a free radical on the DBM backbone (Scheme).

$$\text{DBM—CH}_2\text{OH} + \text{Ce(IV)} \rightarrow \text{DBM—}\dot{\text{C}}\text{HOH} + \text{Ce(III)} + \text{H}^+$$

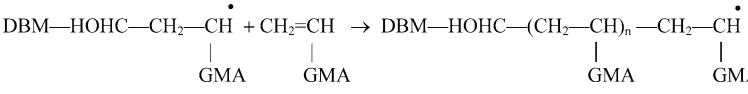
Graft initiation

The grafting of GMA onto DBM is initiated by the free radical reacting with a monomer (Scheme).



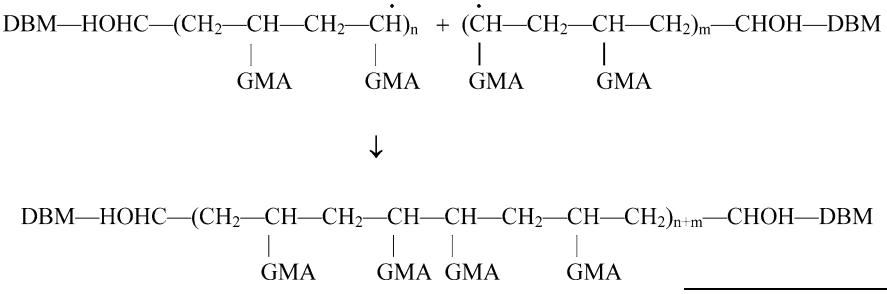
Graft propagation

In the presence of GMA, the DBM radical is chemically coupled to a monomer unit, resulting in a covalent bond between GMA and DBM to create a chain reaction for propagation (Scheme).



Graft termination

Finally, the termination of the grafting reaction is achieved through a combination of two radicals (Scheme).



Conclusions

The graft polymerization of GMA onto DBM was effectively initiated with CAN under an inert atmosphere. The chemical analysis proved the grafting reaction by the existence of epoxy groups of polymer chains on the grafted DBM. The optimum reaction condition for the maximum yield of grafting was standardized by regulating the reaction parameters. Both the %G and the %GE were dependent on the concentrations of the reaction ingredients. This study suggests that it is possible to custom-make the DBM with a specific property-balancing for a given function by the grafting technique. The grafted DBM may be used as a carrier for biomolecular delivery by coupling the biomolecules with epoxy groups.

Table 1 Optimum conditions for graft polymerization

Reaction factors	Measurements
Backbone	5 g
Monomer	6×10^{-1} mol/L
Initiator	1.5×10^{-3} mol/L
Temperature	40 °C
Time	3 h
Total volume	100 mL

Acknowledgement Financial support of the National University of Singapore Research Project Grant is gratefully acknowledged.

References

1. Urist MR (1965) Science 150:893
2. Hagen JW (1992) J Biomed Mater Res 26:897
3. Niederwanger M, Urist MR (1996) J Oral Implant 12:210
4. Cook SD, Dalton JE, Prewett AB, Whitecloud TS (1995) Spine 20:877
5. Edwards JT, Diegmann MH, Scarborough NL (1998) Clin Orthop 357:219
6. Helm GA, Sheehan JM, Sheehan JP, Jane JA, DiPierro CG, Simmons NE, Gillies GT, Kallmes DF, Sweeney TM (1997) J Neurosurg 86:93
7. Martin G, Boden SD, Morone MA, Titus L (1999) Spine 24:637
8. Sandhu H (2000) Clin Orthop 371:56
9. Guizzardi S, Di Silvestre M, Scandroglio R, Ruggeri A, Savini R (1992) Spine 17:701
10. Urist MR, Silverman BF, Buring K, Dubuc FL, Rosenberg JM (1967) Clin Orthop 53:243

11. Tiedeman JJ, Garvin KL, Kile TA, Connolly JF (1995) Orthopedics 18:1153
12. Urist MR, DeLange RJ, Finerman GAM (1983) Science 220:680
13. Svec F, Jehlickova A (1981) Angew Makromol Chem 99:11
14. Eckert AW, Grobe D, Rothe U (2000) Biomaterials 21:441
15. Murugan R, Panduranga Rao K (2003) J Biomater Sci Polym Ed 14:457
16. Murugan R, Panduranga Rao K (2003) Polym Bull 49:395
17. Murugan R, Panduranga Rao K (2003) Macromol Res 11:14
18. Murugan R, Panduranga Rao K (2002) Trends Biomater Artif Organs 16:43
19. Murugan R, Ramakrishna S Biomaterials (2004) Biomaterials 25:3073
20. Mizuno S, Glowaki J (1996) Biomaterials 17:1819
21. Kantouch A, Hebeish A, El-Refie MH (1970) Eur Polym J 6:1575
22. Ikada Y, Nishizaki Y, Sakurada I (1994) J Polym Sci Part A Polym Chem 12:1829
23. Von Hippel PH (1967) Treatise on collagen. Academic, New York