Preparation and Characterization of Chitosan/PEG/Gelatin Composites for Tissue Engineering

Hua Hong, Changsheng Liu, Wenjing Wu

State Key Laboratory of Bioreactor Engineering, Ultrafine Materials, and Engineering Research Center for Biomedical Materials of Ministry of Education, East China University of Science and Technology, Shanghai 200237, People's Republic of China

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ABSTRACT: Chitosan scaffolds have gained much attention in tissue engineering. However, brittleness and low biodegradability limit scaffolds application, especially in use as guided tissue regeneration membranes (GTRm) in surgical operations. The first objective of this work is to improve the brittleness of the chitosan membrane, which is not desired for use via adding polyethylene glycol (PEG) to chitosan, and the second objective is to accelerate the degradation rate by blending gelatin with the binary chitosan-PEG mixture. The addition of PEG softened the blend membrane in vision and in touch. The tensile compliant increased from 7.87×10^{-3} (MPa^{-1}) for chitosan membrane to 3.63×10^{-1} (MPa^{-1}) for chitosan-PEG-gelatin (CPG) membrane. Degradation results *in vitro* indicated that CPG membrane degraded faster and weight loss increased more significantly than chitosan membrane and the lowest tensile strength of CPG membrane could meet the requirement of the application. CPG membrane showed significant improvement in degradation and flexibility in comparison with the chitosan membrane. Cell adhesion, viability, and proliferation onto the external surface of CPG membrane with C2C12 cell had been evaluated *in vitro* and quantified by a methyl thiazolyl tetrazolium (MTT) reduction assay. © 2009 Wiley Periodicals, Inc. J Appl Polym Sci 114: 1220–1225, 2009

Key words: chitosan; blends; brittle; membrane; degradation

INTRODUCTION

Owning to abundant sources, friendliness to the environment, natural polymers like chitosan have been widely investigated for biomedical application. Chitosan has been demonstrated to be a nontoxic, nonantigenic, and biocompatible polymer with bioadhesive, wound healing, and antimicrobial properties. Chitosan and some of its modified types have been reported for use in biomedical applications, such as artificial skin and sutures, drug carriers etc. Although chitosan seems to have excellent properties as a biomaterial, for applications as biodegradable implant for use in the human body, concerns have been raised regarding its low degradation rate, which has important implications for implantable systems. Chitosan is a crystalline polysaccharide and is normally soluble in aqueous solution at pH $\leq$ 7. Highly deacetylated chitosan exhibits low degradation rate in aqueous media and may last several months and thus results in great limits in the development of inexpensive and versatile membrane systems.[1] Various chemical modification techniques including graft copolymerization are conducted to accelerate the degradation rate of the chitosan membrane. However, chemical modifications change the fundamental chemistry structure of chitosan; there are still some considerations concerning the reductions or loss of the advantageous properties of chitosan.[2–4]

Gelatin is a soluble protein derived from partially denatured collagen. Attractive properties of gelatin, such as low immunogenicity, plasticity, adhesiveness, promotion of cell adhesion and growth, and low cost, make it ideally suitable as a biomaterial for tissue engineering.[5] Gelatin contains free carboxyl groups on its backbone and has the potential to blend with chitosan to form a network by hydrogen bonding.[6–8]

For the chitosan application as guided tissue regeneration membrane (GTRm), brittleness would lower the function of GTRm and is not convenient for clinical operation. One example is the barrier membrane used in the human periodontium by GTR. The brittleness of the membrane is unable to provide sufficient space adjacent to the defect to allow for the regeneration of the desirable tissue

Correspondence to: C. Liu (liucs@ecust.edu.cn).
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(such as periodontal ligament and bone) into the space by precluding epithelial cells and gingival connective tissue cells which are believed to propagate at a greater rate. So the improvement of membrane brittleness is highly desired for the function and ultimate clinical application of GTRm.[9,10]

In this study, we aimed to improve the brittleness of the membrane by a simple method blending a popular softener like polyethylene glycol (PEG) with chitosan and also aimed to develop new composite scaffolds that combine the advantages of the three types of biomaterial.[11,12]

Blending two or more polymers is an approach to develop new biomaterials exhibiting combinations of properties that could not be obtained by individual polymers.[13] The macroscopic properties of multiphase systems generally cannot be deduced solely from the properties of each phase, because the morphology of polymer blends is often influenced by the interaction between the components of the blends that in turn affect the properties of the blends.[14,15] The blending membranes deserve attention because its process is simple and mild. Chitosan, blended with various polymers, has been widely investigated to improve the mechanical properties of chitosan.[16] However, no results have been reported concerning the effect of blending polymers on either the improvement of the degradation rate or the pliability of chitosan membrane.

EXPERIMENTAL

Materials and methods

Preparation of chitosan-PEG matrices and chitosan-PEG-gelatin composite matrices

A mixture of chitosan/PEG (50/50, w/w) was prepared by dissolving chitosan powder (medical grade with molecular weight of 30 KD and 90% deacetylated, purchased from Shanghai Kabo Industrial Trade Company, China) and polyglycol 6000 (analytical grade) in acetic acid (2%, s %) aqueous solution. This blend is hereby named as solution CP. The solution G was the gelatin aqueous solution with 2% (wt %) concentration. The blended solution CPG (chitosan/PEG/gelatin = 35/35/30) was ternary mixture of the solution CP with G. All reagents were used without further purification. Homogeneous mixture solutions CP and CPG were obtained via ultrasonic blend for 30 s and then filtered with sand core funnel in vacuum. The mixture was cast onto teflon dishes, 20 cm in diameter, and spread slowly to form an even liquid film. The liquid film was prevaporized in an oven at 50°C for 8 h to form the membrane. The subsequent membrane was immersed in a sodium hydroxide solution (4%, wt %) for 10 min. The obtained membrane was washed repeatedly with double-distilled water and prefrozen at −20°C for 4 h and then freeze dried using Boyikang FD-3 freeze drier at −45°C for 2 h.

Degradation investigation

The biodegradation media were a physiological media prepared by dissolving sodium chloride in distilled water to form 0.9% (wt %) concentration. The degradation studies were performed at 37°C at 240 rpm with constant stirring to mimic the physiologic conditions.

Blend membranes were cut into different sizes as the test demand. Dried membrane samples were put into cuvettes and 10 mL of saline solution was added, respectively. Then all of the samples were placed into a culture box at 37°C with stable rotations at 240 rpm. The culture media were changed with fresh physiologic media under frost state every 2 days. Measurements were conducted weekly from 1 to 5 weeks. Samples were rinsed five times with distilled water and vacuum dried for 24 h before weight loss was analyzed. The following was the relative evaluation.

Scanning electron microscopy

The surface and cross-sectional morphologies of the membrane and pore distributions, sizes, and interconnectivity were observed with scanning electron microscopy (SEM, JEOL model JSM-840). Segments of the outer surface and the interior of the specimen were prepared by the fracture of membrane in liquid nitrogen. Samples were carefully fixed on SEM sample holders using conductive tape. The samples were then sputter coated with gold at a thickness of 20–50 nm using JFC-1011 fine AUTO sputter–coater with a current of 20 mA for 80 s. The coated membranes were analyzed by SEM with an acceleration voltage of 15 kV.

Weight loss

In vitro degradation behavior was evaluated by recording weight loss over time under dynamic culture condition. The test specimens were cut into samples sized of 1 × 1 cm. Weight loss was defined as the difference between the initial weight of the sample and the residual weight after culture for a certain period of time (1, 2, 3, 4, and 5 weeks, respectively). The percentage of weight loss (wt %) was calculated by comparing the dry weight (W_t) remaining at a given degradation time t with the initial weight (W_i) according to the equation:

$$\text{Weight loss} = \left[(W_i - W_t) \Big/ W_i \right] \times 100\%$$

Where W_i (g) was initial dry weight of the sample. W_t (g) was the residual weight after a certain time t of immersion. Each experiment was repeated three times and the average value was taken as the weight loss.

Mechanical strength

The mechanical strength was carried out using a Microelectronic Universal testing machine (CMT6104). The test specimens were cut into strips with 5 cm long and 1 cm wide, and the thickness of each strip was measured using vernier calipers. The two ends of each strip were wrapped with adhesive tape before it was clipped between the retaining clips of the testing machine. All specimens were drawn at ambient temperature. The tensile rate was 1 cm/min. At least three sample measurements were performed and the results were quoted as average values. The ultimate tensile strength (δ) and elongation rate ε and tensile compliant D were calculated as follows:

$$\delta_b = \frac{F}{A} \tag{1}$$

$$\varepsilon_b = \frac{l - l_0}{l_0} \times 100\% \tag{2}$$

$$D = 1/E = \delta/\varepsilon \tag{3}$$

where F (g) was the load when the membrane broken, A (cm^2) was the initial cross proportion, l_0 (cm) was the initial length of the membrane, l (cm) was the length between the measurement lines when the membrane was broken. All values were reported as mean ($N \geq 3$).

Cell culture

The CPG samples were sterilized overnight in UV light irradiation and rinsed two times with sterile phosphate-buffered saline and were placed into a 24-well cell culture plate well. C2C12 cells (muscle myoblast, mouse, purchased from ATCC) maintained in Dulbecco's modified eagle medium (DMEM) and supplemented with 10% fetal bovine serum and 100 U of penicillin–streptomycin/mL were seeded at a density of 1×10^5 cells/mL, 100 μL/well onto the samples. The samples were cultured in DMEM medium in a saturated humidified atmosphere of 5% CO_2 at 37°C for 1 week. The culture medium was replaced every 3 days. After cultured for 3 days, an inverted microscope (TE 2000-U, Nikon Co., Japan) was used to detect the growth and morphology of C2C12. A methyl thiazolyl tetrazolium (MTT) reduction assay was performed to quantify the cell viability. The absorbance of the solution was measured at 492 nm using an enzyme-linked immunosorbent assay (ELISA, Biorad Co., USA) plate reader. Experiments were run in triplicate per sample.

RESULTS AND DISCUSSION

Appearance observation and SEM

As a barrier membrane, the implant needs to maintain structural integrity for the supportive function for a certain time period, then degrades completely and be replaced by regeneration tissue.[17] The photographs of samples before degradation and degradation for 3 weeks are presented in Figure 1.

Observed with human eye, the appearance of blend membrane degradation at initial weeks showed no remarkable differences. The shape maintains integrity, which allows it to as a physical supporter.

Figure 1 shows that the chitosan and CPG membrane are all compact membrane [Fig. 1(a,c)] before degradation. After degradation over 3 weeks, more defects and holes occurred on the CPG blend membrane [Fig. 1(d,e)] when compared with the chitosan membrane [Fig. 1(b)]. The holes were connected with each other. At first, degradation medium transferred into the CPG membrane by means of the capillary effect, and the gelatin was the soluble polymer, the dissolving of gelatin form holes in the membrane. Moreover, the resulted holes increased the contact area of CPG with the medium and promoted the degradation further. The biodegraded membrane appeared to be heterogeneously eroded and exhibited an irregular surface with many holes of different sizes at depth of observation [Fig. 1(e)]. Over 2 weeks, a greater extent of biodegradation took place. The results indicated that the addition of gelatin could accelerate chitosan membrane degradation.

Weight loss

Figure 2 indicates the influence of adding gelatin to the chitosan on the weight loss of the composites after immersion in degradation medium for 1–5 weeks. For the chitosan membrane, it not only maintained its physical form but also did so without obvious weight loss only 3% after 1 week of soaking. For the chitosan-PEG-gelatin blend membrane, weight loss reached 22% for 1 week incubation. Chitosan was insoluble in the medium at pH $\leq$ 7 and the glycosidic bond could not degrade easily even in

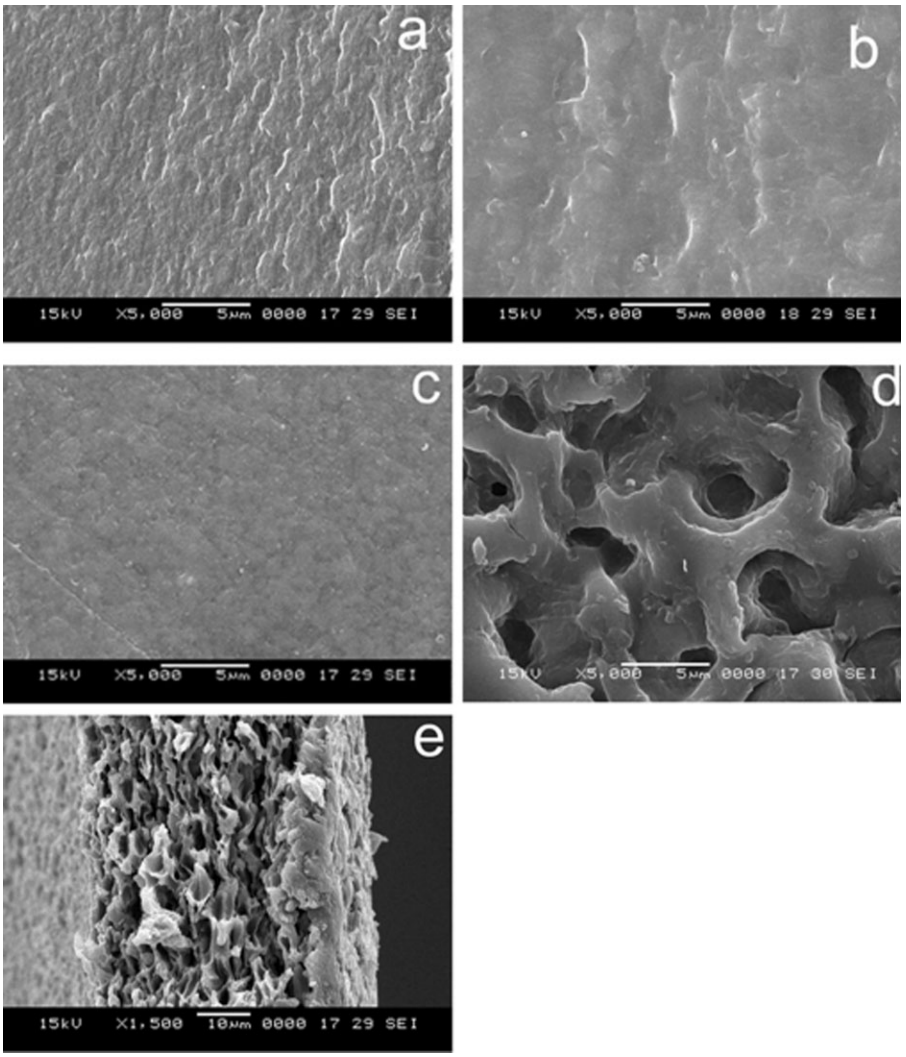

Figure 1 SEM micrographs of chitosan-based blend membrane; (a) chitosan membrane before degradation, (b) chitosan membrane degradation for 3 weeks, (c) CPG membrane before degradation, (d) CPG membrane degradation for 3 weeks, and (e) CPG membrane cross section after degradation 3 weeks.

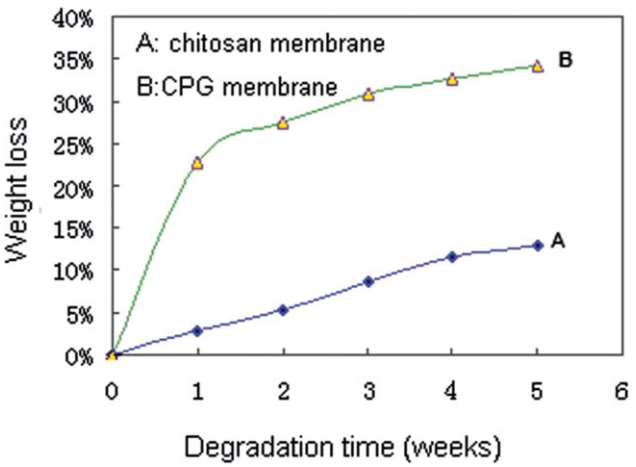

Figure 2 Curves of weight loss of chitosan and CPG membrane for different degradation times. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the presence of lysozyme. Because of the gelatin dissolving and the synergetic effect as declared before, CPG composites showed an increase in weight loss. Weight loss was dependent mainly on the content of gelatin. So the addition of gelatin accelerating the weight loss and the degradation rate of the chitosan composites.

Mechanical strength

Playing a role as a supporting barrier, the chitosan-based blend membrane required adequate mechanical strength. In the mean time, the flexibility and pliability were expected for clinical application.

Figures 3 and 4 show the tensile strength and elongation rate of the chitosan membrane and the CPG membrane. With the addition of PEG both the tensile strength and the elongation rate decreased and the tensile strength decreased more than the elongation rate. The initial tensile strength reached

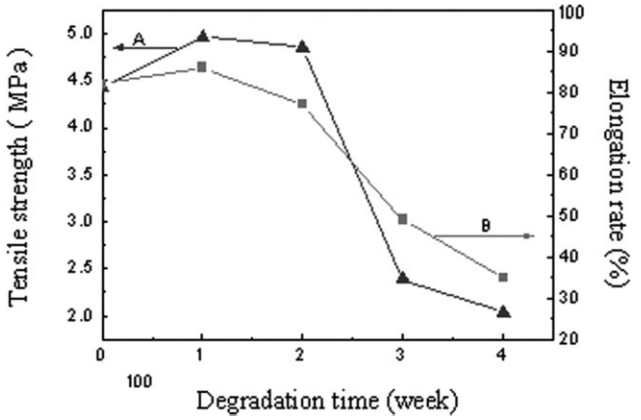

Figure 3 Mechanical properties of chitosan membrane for different degradation times.

4.42 MPa for the chitosan membrane and 1.04 MPa for CPG membrane. At the earliest stage of degradation, hydrolysis first occurred for the gelatin which was soluble. The vacated space was propitious to stretch fully for the chitosan molecule and the regular array, which increased the tensile strength. But along with the degradation, the space vacated by the gelatin dissolving was enough for chitosan molecular to stretch fully and result in the material defect. After approximately 4 weeks of degradation, the tensile strength for the CPG membrane was 0.77 MPa. The maximum tensile strength for the human periodontal ligament was 0.50×10^{-3} MPa as it appeared at the tooth cervix.[18] So the strength of membrane made during this experiment can meet a practical need.

Tensile compliance was used to measure the material pliability. For improving the flexibility and pliability, PEG was added as a softener. The tensile compliance increased from 0.19 MPa^{-1} for the chitosan membrane to 0.36 MPa^{-1} for the CPG membrane.

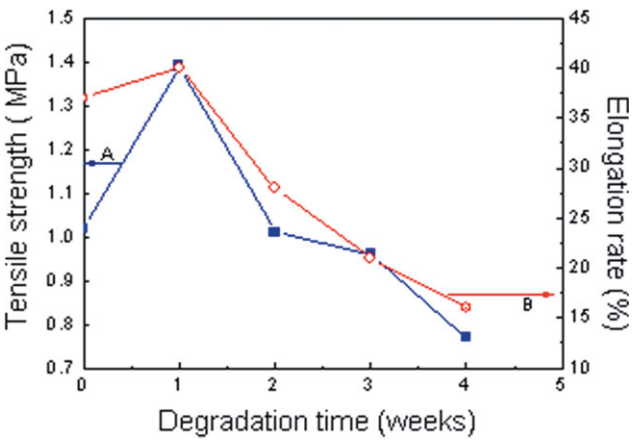

Figure 4 Mechanical properties of CPG membrane for different degradation times. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

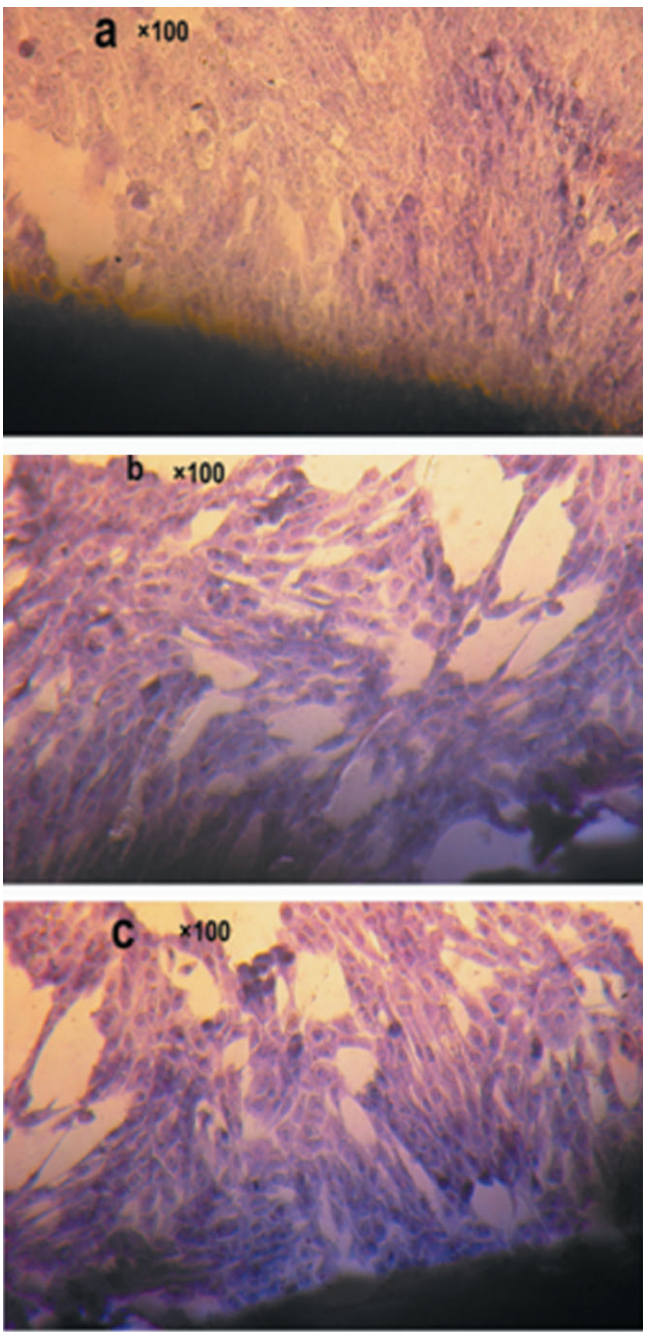

Figure 5 The morphologies of cells growth on chitosan blend membrane (a) control group, (b) chitosan membrane, and (c) CPG membrane. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The addition of PEG improved the tensile compliance of chitosan membrane. This enhancement of the tensile compliance value may be ascribed to the addition of gelatin weakened the intermolecular interaction of chitosan.

In vitro evaluation by cell culture

Figure 5 presents the results of the morphology of C2C12 cultured on the chitosan membrane and the

CPG membrane under a 5% CO_2 atmosphere at 37°C for 3 days. It indicates that there were no significant differences among chitosan membrane, CPG membrane, and control group. Cell grew and proliferated rapidly on the membrane to become confluent at some areas when observed 48 h later. The samples not only ensured the C2C12 cells grow normally but also promoted the proliferation, which means the CPG membrane had both the biocompatibility and bioactivity.[19–21] One possible reason is the function of chitosan is similar to the glycosaminoglycans based on the similar structure. Glycosaminoglycans, main protein component of the extracellular matrixes, played an important role during the process of adhesion, proliferation, and shaping of the cell, so the chitosan had the activity of reconstructing, inducing, and stimulating the connective tissue. Gelatin contains Arg-Gly-Asp (RGD)-like sequence that promotes cell adhesion and migration.[22] The results also show there had no cytotoxicity for CPG membrane.

The cytotoxicity test was evaluated by the value of OD_{490} (optical density, OD) of various specimens. From the statistical analysis, it seems that there were no significant differences among the CPG membrane ($OD_{490} = 1.480$), CP membrane ($OD_{490} = 1.478$), and chitosan membrane ($OD_{490} = 1.482$). As chitosan is a well-known biomaterial without toxicity to cells, the near OD_{490} value of CPG and chitosan suggested that the CPG was biocompatible with the cell.

CONCLUSIONS

The chitosan and CPG blend membranes were prepared by a solution casting and freeze-dry method. It was demonstrated that it was possible to accelerate the degradation rate and increase the compliance in the extension of chitosan membranes by blending chitosan with PEG and gelatin. PEG served as an effective softener for the blended membranes and gelatin improved the degradation rate greatly. The mechanical properties of the membranes after 4 weeks of degradation confirmed that the lowest tensile strength for CPG membrane could meet the demand of clinical application, especially, since the cell culture results showed that the CPG scaffold was biocompatible. The method provided in this study was simple and low cost and the results were satisfied. This is a satisfactory, promising, feasible method for fabricating GTRm.

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