Rapid hard tissue embedding method for embedding graphene nanomaterials - a multilayered graphene hydrogel membrane

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ABSTRACT

Graphene has attracted great interest for its uses in tissue regeneration. The multilayered graphene hydrogel (MGH) membrane shows great potential as a barrier membrane in guided bone regeneration. However, when using the conventional methyl methacrylate embedding process, the MGH membrane swelled and lost its natural form, which seriously limited the observation of bone regeneration. We developed a rapid embedding method by adding the cross-linker Tetra (ethylene glycol) diacrylate and increasing the polymerization temperature (45 °C) and initiator concentration (64% BPO in penetration liquid). By the rapid embedding method, the shape and properties of the MGH membrane can be well preserved, and the bone tissue can also be well embedded. The resulting blocks have uniform hardness, which makes it possible to obtain thin sections using a cutting and grinding procedure. The rapid embedding method has no significant effect on the conventional methods of bone regeneration detection. The interface between the MGH membrane and new bone tissue can be clearly observed. This new hard tissue embedding method can lead to better observation

of the interaction between bone and graphene, providing an optimized experimental technology for the study of hard tissue regeneration with graphene in the future.

Keywords: graphene, hydrogel, hard tissue embedding, bone regeneration

1. Introduction

The hard tissue embedding and slicing technique has been mainly applied to mineralized tissues, including bone and teeth. This procedure entails several special processing steps: fixation, osmotic dehydration, embedding and precise slicing to obtain thin sections. In terms of the quality and quantity of regenerated bone tissue, the hard tissue embedding technique can maintain the integrity of calcification deposition and original bone morphology^[1]. It can also avoid the longtime traditional method of decalcification. These advantages are conducive to the quantitative study of new bone, the differentiation of mature bone and immature bone, and the fluorescence labeling study of bone dynamics. Due to the advantages of the hard tissue embedding technique, it is widely used in the research and development of innovative biomaterials for bone regeneration.

Graphene has a unique planar aromatic network and has been used to construct functional biological systems through integration with nucleic acids, proteins, peptides, and cells. It has attracted great interest in the field of biomedicine including drug delivery, biosensors, tissue regeneration^[2,3,4,5,6]. In recent years, graphene has been reported to promote the growth of osteoblasts and the osteogenic differentiation of mesenchymal stem cells (MSCs) through pre-enrichment of osteogenic inducers, inherent mechanical strength, and wrinkled nano morphology, which enhances the attraction of graphene to bone regeneration^[7]. At present, graphene nanomaterials have been extensively added to bone implants, hydrogels, and scaffold materials to fabricate implant coating and composite materials. By improving properties such as mechanical behaviors, electrical conductivity, and bone inductivity, the graphene composites can enhance bone regeneration^[8,9].

Graphene nanomaterials are a kind of special materials with slow degradation^[10]. After 3-4 months of bone reconstruction period, graphene still exists around the new bone tissue, confirmed by macroscopic observation^[11]. At present, in the studies of bone regeneration by graphene nanomaterials, the samples of new bone tissue often need to be embedded in paraffin after decalcification. Due to the decalcification process, a lot of information about bone regeneration is missed by this method. Therefore, an appropriate embedding method, which can effectively incorporate both bone regeneration tissues and graphene nanomaterials simultaneously for further detection, is exactly important for the studies on bone regeneration with graphene. Is the conventional methyl methacrylate (MMA) hard tissue embedding technology suitable for embedding graphene, and can the interface between bone regeneration tissues and graphene nanomaterials be clearly observed? Until now, no specific research has been done.

In our previous study, we used a multilayered graphene hydrogel (MGH) membrane to explore the application of graphene as a biological barrier membrane in guiding bone regeneration^[11]. The MGH membrane could mimic natural bone collagen fiber and has good mechanical strength and elasticity. The results revealed that it has great potential to be used as a barrier membrane in guided bone regeneration. However, when using conventional MMA hard tissue embedding process (MMA and dibutyl phthalate were used for polymerization), the MGH membrane swelled and lost its natural form, which seriously obstructed the observation of bone regeneration (**Figure 1**). Therefore, it is necessary to explore a suitable hard tissue embedding technology for embedding graphene, such as MGH. Furthermore, the new embedding technology should not influence the common evaluation methods of bone tissue regeneration, such as micro CT, sequential fluorescent labeling, and histological staining.

In this paper, a new hard tissue embedding method is reported. In the process of embedding agent polymerization, the shape and properties of graphene can be well preserved without swelling

and deformation. According to the relevant literature, the cross-linker Tetra (ethylene glycol) diacrylate (TTEGDA) features an excellent balance between hydrophilicity and hydrophobicity, which is conducive to the physicochemical compatibility of TTEGDA cross-linked resin with resin-bound polymers^[12]. In this method, we removed the plasticizer dibutyl phthalate, which increased the movement ability of the embedding molecules, and added the cross-linker TTEGDA, which connected the molecular chains to increase the interaction of MMA and form an embedded block. In addition, polymerization temperature and concentration of the polymerization initiator were increased to accelerate the polymerization speed and further avoid the swelling of the MGH membrane. The resulting blocks have uniform hardness, which makes it feasible to obtain thin plates using a cutting grinding procedure. Routine histological staining, microscopic CT, and fluorescence labeling were all successfully conducted. This new hard tissue embedding method is beneficial to more effectively observe the interaction between bone and graphene and directly promote the study on the bone regeneration potential of graphene bulk materials.

2. Materials and Methods

2.1 Bone sample preparation and conventional MMA embedding processing

The preparation procedure of the MGH Membrane has been reported in our previous publication^[11]. In short, chemically converted graphene was synthesized by chemical reduction of a graphene oxide solution obtained by chemical oxidation and exfoliation of natural graphite. A certain amount of chemically converted graphene dispersion (0.37 mg mL^{-1}) was then filtered through a mixed cellulose ester filter membrane ($0.05 \text{ }\mu\text{m}$ pore size; Millipore Co., USA) by vacuum filtration. Once the filtration was accomplished, the MGH membranes were carefully peeled off from the filter membrane using tweezers and stored in water before use. The MGH membranes were then implanted

in eighteen male Fischer rats (body weight 270–300 g) as reported in previous publication^[11]. Briefly, two calvarial defects were created on each animal using a trephine drill with a diameter of 5 mm. Nine rats were covered with the MGH membrane, and nine were left blank. The experimental protocol was formally approved by the Institutional Animal Care and Use Committee (IACUC) of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (Animal Welfare Ethics acceptance number: No.DWLL2020-0339).

Polyfluorochrome sequential labeling was carried out to assess dynamic bone mineralization including the speed of bone formation and mineralization. The rats received tetracycline hydrochloride (25 mg kg⁻¹) (Sigma, Chemical Co, St. Louis, MO, USA) subcutaneously 2 weeks postoperatively, and alizarin red s (30 mg kg⁻¹) (Sigma, Chemical Co, St. Louis, MO, USA) 4 weeks postoperatively, respectively. By 2 weeks before sacrifice, calcein green (20 mg kg⁻¹) (Sigma, Chemical Co, St. Louis, MO, USA) was administrated subcutaneously.

Rats were sacrificed eight weeks after surgery. The defect sites were removed and fixed in 10% neutral formaldehyde solution for 4 days. For conventional MMA embedding, three samples with the MGH membrane and three blank samples were processed as follows:

- (1) 8 hours in 70% ethanol;
- (2) 8 hours in 80% ethanol;
- (3) 8 hours in 90% ethanol;
- (4) 8 hours in 95% ethanol;
- (5) 24 hours in 100% ethanol;
- (6) 8 hours in xylene;

(7) 24 hours in xylene : penetration liquid = 1:1 (volume-to-volume ratio) at 4 °C, penetration liquid is composed of MMA: dibutyl phthalate = 3:1 (v/v);

(8) 24 hours in embedding solution I (1% weight-to-volume ratio benzoyl peroxide (BPO) in penetration liquid) at 4 °C;

(9) 24 hours in embedding solution II (4% (w/v) BPO in penetration liquid) at 4 °C, and;

(10) Replaced the embedding solution II again, vacuumed for 4 hours, and polymerized slowly at 37 °C.

Chemical pure grade MMA (purity $\geq 98.0\%$) and BPO (purity $\geq 98.0\%$) were purchased from Sinopharm Chemical Reagent (Shanghai, China). The other reagents such as ethanol (purity $\geq 99.7\%$), xylene (purity $\geq 99.0\%$), and dibutyl phthalate (purity $\geq 99.5\%$) are analytical grade and were supplied by Sinopharm Chemical Reagent Co., Ltd., Shanghai, China.

2.2 Rapid embedding processing

The composition of penetration liquid changes from MMA : dibutyl phthalate = 3:1 (v/v) to MMA : TTEGDA = 10:1 (v/v). By adding the cross-linker TTEGDA (Sigma, Chemical Co, St.Louis, MO, USA) and testing different polymerization temperatures, polymerization initiator BPO concentrations in embedding solution II, and embedding time, we aimed to find the best combination for rapid embedding of both the MGH membrane and bone tissue. Testing polymerization temperatures included 37 °C, 40 °C, 45 °C, 50 °C, and 60 °C, while initiator BPO concentration in embedding solution II is arranged from 2% to 128% (w/v) multiply. The other steps remained unmodified. The whole test time is three days. Then the other twelve samples were embedded by the best combination process and polymerized.

2.3 Microcomputed Tomography (micro-CT) Scanning

Each specimen was subjected to micro-CT scanning using a desktop micro-CT device (eXplore Locus, GE HealthCare, USA). The micro-CT scanner was set at a voltage of 80 kV and a current of 80 μ A. The micro-CT scanner's auxiliary software (Version ABA 2.2, GE Healthcare, USA) was used to make a 3D reconstruction from sequential scans. A cylindrical region of a diameter of 5 mm and a height that covered the entire thickness of the calvarial bone was chosen as the region of interest (ROI). The bone volume fraction (BVF) was evaluated by the volume ratio of the mineralized tissue in ROI. Bone mineral density (BMD) was calculated as the major index to evaluate the quality and strength of the regenerated bone.

2.4 Cutting and grinding processing

The specimens were cut and ground from the center of the defects perpendicular to the sagittal suture with the Exact Cutting and Grinding Equipment (Exact Apparatebau, Norderstedt, Germany) to a thickness of 80 μ m, according to the technique described by Donath & Breuner.

2.5 Sequential fluorescent labeling observation

Fluorescent labeling was observed under confocal laser scanning microscope (LSM710, CarlZeiss, Germany) as reported. Briefly, five photographs were taken in the same area for each sample. Three used fluorescence microscopy images to analyze the fluorochromes tetracycline, alizarin red s, and calcein green. One merging image of three fluorescent labeling was used to reflect

the mineralization of calvarial defects, and one regular image under transmission light microscopy combined with the former merged image to show the newly formed bone in an overlapping manner.

2.6 Histological observation

Afterward, each section was further ground to a thickness of 40 μm . The undecalcified sections were further stained with Van Gieson's picric acid-fuchsin. The newly formed bone within the defect was evaluated using the Olympus light microscope (Olympus BX51, Tokyo, Japan).

2.7 Statistical analysis

The data were shown as mean values $\pm$ standard deviation. The Shapiro–Wilk test was used to test the normality of the distribution. As all the measurements were normally distributed, independent t test was applied to compare the differences between MGH group and blank group. All statistical analyses were performed using SPSS (SPSS 19.0, Chicago, IL, USA) statistical software packages. A two-tailed p value < 0.05 between the testing groups was considered to indicate statistical significance.

3. Results

3.1 Conventional MMA embedding processing

After the processes of dehydration and penetration, bone tissue and the MGH membrane retained their original structure. Nevertheless, when embedded in conventional MMA mixture for 1 day at 37 $^{\circ}\text{C}$, the MGH membrane began to dissolve and finally exhibited a mist trait when the MMA matrix

coagulated to an embedded block after 7 days (**Figure 1a**). The MGH film swelling severely influenced the histological and histomorphologic observation of calcified tissues and the interface between the MGH membrane and newly formed bone (**Figure 1b**).

3.2 Rapid embedding processing

The ideal hard tissue embedding method requires short time consumption, no destruction of biomaterials, no influence on the activity of proteins in the tissue, and a clear and transparent embedding matrix for easy observation. Polymerization temperature and polymerization initiator BPO concentration are closely related to the above properties. Thus, 35 combinations of different polymerization temperatures and BPO concentrations of rapid embedding processing were set up for testing (**Figure 2**).

Within three days of embedding, with the increase of polymerization temperature and concentration of polymerization initiator, the polymerization speed was accelerated, groups 7, 13, 14, 20, 21, 25, 26, 27, 28, 31, 32, 33, 34, and 35 were able to coagulate completely in nearly 10 hours (**Table. 1**). Groups 7, 14, 21, and 28 had the highest concentration of polymerization initiator (128% w/v BPO), and the embedding matrix was slightly muddy, which could adversely affect the observation of hard tissue sections, so these groups were excluded. In groups 13, 25, 31, 32, and 33, the problems of membrane enlargement, bending, and roughness caused them to be excluded. In the remaining five groups, the polymerization temperature of 50 °C and 60 °C accounted for two groups respectively, and 45 °C accounted for one group. In these groups, the embedded blocks were all solidified and transparent, and the MGH membrane had no obvious deformation. Considering the detrimental effects of excessive polymerization temperatures on protein activity in bone tissue, we

selected group 20 (with 64% w/v BOP at 45 °C) as the most appropriate group for further observation of bone and the MGH membrane (**Figure 2**).

3.3 Micro-CT Scanning

The MGH membrane was invisible as in previous micro CT images^[11]. In the three-dimensional reconstruction and the observation of the sagittal and coronal planes of the micro CT images, it could be clearly seen in the MGH membrane group that the bone defect was almost covered by new bone. In the blank group, only a small amount of new bone was found at the edge of the defect (**Figure 3**). The BVF of the MGH group was $65.21 \pm 5.98\%$, significantly higher than that of the blank group ($19.34 \pm 8.454\%$). The BMD of the MGH group was $423.1 \pm 35.75 \text{ mg/cm}^3$, which was also significantly higher than that of the blank group ($131.8 \pm 48.94 \text{ mg/cm}^3$). It was suggested that the fast embedding method had no effect on the observation and measurement of micro CT.

3.4 Cutting and grounding processing

By using Exact Cutting and Grinding Equipment, the fast embedding specimens could be easily cut and ground to a thickness of 80 μm . The resin sections obtained were complete and transparent. There was no significant difference between the fast embedding and conventional MMA embedding.

3.5 Sequential fluorescent labeling observation

Under the confocal laser scanning microscope, the trichromatic sequential fluorescent labeling was clearly observed on the sections. The MGH group showed excellent new bone formation and mineralization in the whole observation period (2 weeks, 4 weeks, and 6 weeks). In the MGH group,

the new bone tissue was full of the defect, while in the blank group, only a small amount of bone remodeling and osteogenesis were found at the edge of the defect area (**Figure 4**).

3.6 Histological observation

Van Gieson's staining of histological sections obtained by the fast embedding method showed that bone tissue was stained red, and collagen fiber was stained light blue. In the MGH group, almost all defect spaces were filled with new bone, and the structure of bone tissue was clear. The three-layered bone structure was readily visible, corresponding to the lamellar bone containing diploic bone surrounded by external and internal cortical bone with flattened lining osteoblasts. In the diploic bone, the structure of small blood vessels and the space of osteocytic lacunae containing osteocytes could be observed. This three-layer structure was very similar to the original bone tissue (**Figure 5**).

4. Discussion

The MGH membrane is composed of pure graphene and has a simple multilayer structure. The graphene sheets in the membrane are connected by partial $\pi - \pi$ stacking. Due to the repulsive force caused by hydration, the graphene sheets still keep a large separation, and the resulting high porosity is conducive to penetration and transmission of nutrients. The membrane is good for cell adhesion and matrix deposition and has excellent osteoinductive and osteoconductive ability. It can promote bone formation and mineralization in the early stage of cranial defect in rats, revealing that it has great potential to be used as a barrier membrane in guided bone regeneration^[11].

The conventional MMA embedding method can observe the combination of hydroxyapatite, titanium implant, and other materials with bone tissue, and evaluate

the function of the materials. However, we found that this conventional MMA embedding method was not suitable for our MGH membrane. In the conventional MMA embedding, graphene nanosheets were dispersed and deformed, resulting in interface pollution, which makes it impossible to observe the clear graphene / bone tissue interface. The conventional MMA embedding time is long, with partial polymerization commencing after 7 days of embedding, and the complete polymerization usually requiring more than one month. During the polymerization process, dibutyl phthalate serves as a plasticizer that can increase the mobility of MMA monomers^[13]. Dibutyl phthalate may also act as electron acceptors that can generate π - π electron-donor-acceptor interactions with the graphene sheets (π donors)^[14]. The possible reason for the deformation of the MGH membrane is that the MMA matrix penetrated into the MGH membrane with the help of dibutyl phthalate and polymerized in the long polymerization time, which destroyed the π - π interaction between the graphene nanosheets, resulting in an increase in interlayer spacing and volume and the subsequent loss of the original assembly of graphene nanomaterials (**Figure 6**). A small number of scholars have tried to use improved embedding methods by reducing polymerization temperature and changing the embedding system to optimize the observation of biomaterial/bone interface, but they were not popularized due to the complex and time-consuming process involved^[15,16]. Therefore, it is necessary to find a suitable and convenient embedding method for the MGH membrane.

In the new embedding method, the cross-linker TTEGDA was added. TTEGDA has two acrylate groups, so it can connect two MMA molecular chains to make them chemically cross-linked (**Figure 6**)^[17,18]. After crosslinking, the viscosity of the embedded matrix increased, and the molecular diffusion ability decreased, so it is difficult for the long chain TTEGDA to enter into the MGH membrane. In addition, by increasing the polymerization temperature and initiator concentration, the polymerization speed was further accelerated, and the embedded matrix around the material polymerized within 9 hours. The volume of polymerized embedded block was fixed, avoiding swelling of the MGH membrane^[19]. After that, MMA in the MGH membrane polymerized gradually. Based on the results of embedding, we found the lowest temperature, which allows rapid polymerization without affecting the MGH membrane. That was group 20 with 64% BOP concentration at 45 °C. Under this condition, we can get about 9 hours of rapid embedding. When embedding is accelerated, the embedding temperature will rise. At this time, we need to thaw the embedding block on ice to prevent explosive agglomeration.

Therefore, based on the results of the experiment, we decided to use the following rapid embedding method: (1) 8 hours in 70% ethanol; (2) 8 hours in 80% ethanol; (3) 8 hours in 90% ethanol; (4) 8 hours in 95% ethanol; (5) 24 hours in 100% ethanol; (6) 8 hours in xylene; (7) 24 hours in xylene : penetration liquid = 1:1 (v/v) at 4°C, penetration liquid is composed of MMA : TTEGDA = 10:1 (v/v); (8) 24 hours in embedding solution I (1% (w/v) BPO in penetration liquid) at 4°C; (9) 24 hours in embedding solution II (64% (w/v) BPO in penetration liquid) at 4°C; (10) replaced the

embedding solution II again, vacuumed for 4 hours, and polymerized at 45°C for about 9 hours of rapid embedding.

By the rapid embedding method, the shape and properties of the MGH membrane can be well preserved, and the bone tissue can also be well embedded. The resulting blocks have uniform hardness, which makes it possible to obtain thin sections using a cutting and grinding procedure. The rapid embedding method has no significant negative effect on bone regeneration, detected using conventional methods, such as histological staining, micro CT, and fluorescence labeling. The interface between the MGH membrane and new bone tissue can be clearly observed by histological staining, micro CT, and fluorescent labeling. The new bone tissue is full of the defect area covered by the MGH membrane, suggesting that graphene has good bone regeneration potential. By avoiding the destruction of the π - π stacking interaction in graphene materials, the rapid embedding method is conducive to structure maintenance of graphene nanomaterials and better observation of the interaction between bone and graphene. This new hard tissue embedding method provides technical support for embedding graphene and bone tissue simultaneously, which will directly promote the research on bone regeneration of graphene bulk materials.

5. Conclusion

In conclusion, we developed an easy and reliable embedding method to effectively preserve and embed both the MGH membrane and bone tissue. Based on

this technique, the interface between the MGH membrane and new bone tissue can be clearly observed by bone evaluation methods, including histological staining, micro CT, and fluorescent labeling. This rapid embedding method provides a feasible experimental technique to promote the application of the graphene membrane as a barrier membrane for guided bone regeneration, facilitating the study of hard tissue regeneration by graphene nanomaterials in the future.

Acknowledgments

Chengqi Lyu and Chunyan Li contributed equally and should be considered as joint first authors.

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Conflict of interest

The authors declared that they have no conflicts of interest to this work.

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Figure 1. General pictures of conventional MMA embedding of the MGH membranes (a). The left bottle showed the MGH membrane was just embedded in conventional MMA matrix, while the left bottle showed the MGH membrane swelled and lost its natural form after 7 days of embedding in conventional MMA matrix. General pictures of the conventional MMA embedding of the MGH membrane and bone tissue (b).

Figure 2. General pictures of the rapid embedding of MGH membranes of 35 groups.

Figure 3. Micromorphometric analysis of treated calvarial defects by rapid embedding including superficial, coronal, and sagittal section views of micro-CT images taken at the eighth week after surgery.

Figure 4. Fluorochrome labeling histomorphometric change analysis showed three-colored fluorescence in the defects rendering the newly formed bone.

Figure 5. Van Gieson's staining of calvarial undecalcified sections by the rapid embedding after 8 weeks of implantation. Scale bars, 250 μm

Figure 6. Diagrams of polymerization process of conventional MMA hard tissue embedding method and polymerization and crosslink process of new rapid hard tissue embedding method. Structural formulas of the compounds are shown in stick representation. (PMMA is presented in both short and long-chain structural diagrams.)

TABLE 1. The rapid embedding of MGH membranes with testing polymerization temperatures included 37 °C, 40 °C, 45 °C, 50 °C and 60 °C, while initiator BPO concentration was arranged from 2% to 128% (w/v) multiplier.

Temperature	No.	BOP concentration	Coagulated	Most coagulated	Not coagulated	Clear	Muddy	Membrane undeformed	Membrane deformed	Take out time (most coagulated)	Problem
37°C	1	2%			√	√		√		≥3d	
	2	4%			√	√		√		≥3d	
	3	8%			√	√		√		≥3d	
	4	16%			√	√			Curved	≥3d	
	5	32%			√	√			Thickening and coarse	≥3d	
	6	64%		√		√			Large, coarse and gauze	≥3d	
	7	128%	√				Slightly muddy	√		12h	
40°C	8	2%			√	√		√		≥3d	
	9	4%			√	√			Slightly curved	≥3d	
	10	8%			√	√		√		≥3d	
	11	16%			√	√			Curved and coarse	≥3d	
	12	32%		√		√			huge, coarse and curved	70h	
	13	64%	√			√			Curved and coarse	46h	
	14	128%	√			√	Slightly muddy	√		9.5h	
45°C	15	2%			√	√		√		≥3d	
	16	4%			√	√			Deformed and coarse	≥3d	
	17	8%			√	√			Large and coarse	≥3d	
	18	16%		√		√			Large and coarse	70h	
	19	32%		√		√			Large and coarse	24h	
	20	64%	√			√		√		9h	Most suitable
	21	128%	√				muddy and precipit	√		6h	

							ated				
50°C	22	2%			√	√			Coarse		
	23	4%			√	√			Large and coarse	≥3d	
	24	8%		√		√			Slightly curved	72h	
	25	16%	√			√			Large and coarse	22h	
	26	32%	√			√		√		8.5h	The temperature is slightly high?
	27	64%	√			√		√		4h	The temperature is slightly high?
	28	128%	√				Slightly muddy	√		2h	
60°C	29	2%			√	√			Large and curved	≥3d	
	30	4%		√		√			Curved and coarse	64h	
	31	8%	√			√			Shrunk and curved	7h	
	32	16%	√			√			Curved and crumpled	3h	
	33	32%	√			√			Slightly curved	2h	
	34	64%	√			√		√		1.75h	The temperature is slightly high?
	35	128%	√			√		√		1.25h	The temperature is slightly high? BPO concentration too high?

Table of Contents

The use of the graphene hydrogel shows great potential in bone regeneration. In this study, the authors describe a novel approach to effectively preserve and embed both the graphene hydrogel and bone tissue. Based on this technique, the multilayered graphene hydrogel did not swell as it did when using a conventional embedding method, while retaining successful application of the conventional methods of bone regeneration detection.

Keywords: graphene for bone regeneration

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