# Poly (Methyl Methacrylate)/Biphasic Calcium Phosphate/Nano Graphene Bone Cement for Orthopedic Application

## Abstract

**Background:** The aim of this study was to make a bioactive bone cement based on poly (methyl methacrylate) (PMMA) with suitable mechanical properties. **Methods:** PMMA has been modified by fabricating a composite consisting of biphasic calcium phosphate (BCP) 68 wt%, PMMA 31 wt% and graphene (Gr) 1 wt% (PMMA/BCP/Gr), 32 wt% of PMMA, and 68 wt% of BCP (PMMA/BCP) and pure PMMA by milling, mixing with monomer liquid, and casting. The modified cements were evaluated regarding mechanical properties, bioactivity, degradation rate, and biocompatibility. **Results:** The scanning electron microscopy (SEM) images of hydroxyapatite (HA) formed on samples surface after 28 days of immersion in simulated body fluid (SBF) demonstrated that bioactivity was obtained due to the addition of BCP, and the degradation rate of the cement was enhanced as well. Investigations of mechanical properties revealed that BCP increased the elastic modulus of PMMA more than 1.5 times, but predictably decreased elongation. The addition of 1 wt% Gr increased elongation and yield strength from 16.39% ± 1.02% and 61.67 ± 1.52 Mpa for PMMA/BCP to 35.18% ± 2.42% and 78.40 ± 2.06 Mpa for PMMA/BCP/Gr, respectively. MG63 cells survival and proliferation improved from 127.55% ± 7.03% for PMMA to 201.41% ± 10.7% for PMMA/BCP/Gr on Day 4 of culture. **Conclusion:** According to the obtained results of mechanical and biological tests, it seems that new PMMA/BCP/Gr bone cement has a potentiality for usage in orthopedic applications.

**Keywords:** *Bioactivity, biphasic calcium phosphate, bone cement, graphene, poly (methyl methacrylate)*

**Farnoosh Pahlevanzadeh[1], Mehdi Ebrahimian-Hosseinabadi[2]**

[1]Department of Tissue Engineering, Najafabad Branch, Islamic Azad University, Najafabad, Iran, [2]Department of Biomedical Engineering, Faculty of Engineering, University of Isfahan, Isfahan, Iran

## Introduction

Bone can self-repair in partial injuries, but in order to treatment of Wider damages,[1] bone cements and implants can help surgeons. Bone cements is used for fixing implants, and leads to a uniform distribution of stress between the bones and implants.[2] Moreover, tumor surgery and percutaneous vertebroplasty[3] are other fields in which bone cements can be utilized. For instance, it can strengthen the compression fractures of vertebrae.[4] One of the common bone cements is poly (methyl methacrylate) (PMMA)[5] which is used due to its favorable properties such as nontoxicity and ease of functionalization.[6] One of the most important problems of the implants is loosening in the long-term using in the physiological environment of the human body,[2,7] which can be due to fatigue[8] and nonbioactivity of PMMA. PMMA as an artificial material leads to form a fibrouse layer is formed on PMMA as an bio-inert material. This may leads to micro movements and failure of implants as a consequence.[9] The other obvious disadvantages of PMMA are lack of osseointegration,[10] sharp rise in temperature,[2] release of methyl methacrylate monomers and subsequent necrosis of the surrounding tissue.[11] In addition, PMMA is not able to induce osteogenesis.[12] To overcome these shortages, adding bioactive ceramics to the PMMA-based cement is a suitable method to improve the bone cement properties. Chen *et al.*[13] incorporated calcium silicate-based bioceramic (Akermanite) to PMMA for making bioactive bone cement with suitable mechanical properties and fabricated a bioactive cement with 100 MPa compressive strength. In general, it has been previously examined and documented that bioactive ceramics such as calcium phosphates,[9] hydroxyapatite,

**Address for correspondence:**
*Dr. Mehdi Ebrahimian-Hosseinabadi, Department of Biomedical Engineering, Faculty of Engineering, University of Isfahan, Azadi Sq., Isfahan 81746-73441, Iran. E-mail: m.ebrahimian@eng. ui.ac.ir*

glass ceramics,[14] and titanium dioxide are mixed with PMMA.[15] On the other hand, calcium phosphate-based ceramics can resemble the mineral phase, and as a consequence, these ceramics are bioactive, osteoconductive, and suitable for bone repair.[16] Biphasic calcium phosphate (BCP) ceramics contains a diverse amount of hydroxylapatite (HA), $(Ca_{10}[PO_4]_6[OH]_2)$ and β-tricalcium phosphate (β-TCP $[Ca3[PO4]2]$).[17] HA is a bioactive ceramic which is a poorly soluble material whereas TCP is highly soluble which helps to increase the degradation rate of cements.[18,19] Besides, the results of studies on BCP showed osteoconductivity,[20,21] osteoinductivity, and its extraordinary potentiality for regeneration of bone tissue.[22] In a study by Goncalves *et al.*,[9] HA was added to PMMA-based bone cement, and the results showed that the cements bioactivity remarkably increased and high-cell survival was reported. To give the biodegradability properties to the PMMA-based bone cement, BCP can make cements more favorable for use as biodegradable ceramics in bone cements because of the presence of β-TCP. The study by Schwartz *et al.*[23] indicated new bone formation and osteointegration in the case of using BCP for filling bone defects.

Although extensive studies performed considering the addition of bioactive ceramics into PMMA, to the best of our knowledge, there was not any study focusing on the incorporation of BCP into PMMA. In fact, BCP has been composited with PMMA in the present study to overcome the absence of bioactivity and to enhance the rate of degradation. The inclusion of BCP in PMMA increased the elastic modulus (E value), but decreased elongation by enhancing the cements brittleness and obviously this has led to reducing the cement strength and faster breaking. To solve this problem, graphene (Gr) was utilized due to its high-mechanical properties especially its adequate ductility. Gr is a biocompatible,[24] two-dimensional monolayer of graphite which has been used in many researches to enhance the composites mechanical properties.[25] In addition of favorable mechanical properties, ease of function, high surface area, maintenance of the osteoblasts viability of Gr, and its derivatives led to wide-usage of these nanomaterials in bone cements.[26,27] Finally, it seems that PMMA/BCP/Gr can develop as a potential bioactive bone cement.

## Materials and Methods

### Materials

PMMA powder with molecular weight = 80,000 g/mol and liquid component methyl methacrylate (MMA) containing 20 ppm hydroquinone 2.4% N-N dimethyl-p-toluidine,13.2% butyl methacrylate, and 84.4% monomer MMA (CEMFIX1, Teknimed, France) were used. Gr nanoplates with 4–20-nm thickness (purity >99.5%, layers <30) were purchased from Neutrino (Iran). Diammonium hydrogen phosphate (molecular weight 132.6 g/mol, 1M) was purchased from Merck (Germany).

### Synthesis of biphasic calcium phosphate

BCP was synthesized from intensive areas of tibia bovine bone (a 2-year-old animal) by heating it in two steps. At the first step, the bones were cut into small parts and boiled in hot water for 9 h and dried in an oven at 70°C for 2 h. Afterward, the resultant powder was sintered at 700°C for 60 min. In the next step, the bone was immersed in a combination of diammonium hydrogen phosphate ($[NH_4]_2HPO_4$) with the volume ratio of 1 cm 3 bone: 20 ml $(NH_4)_2HPO_4$, for 24 h and sintered at 700°C for 95 min. Finally, powders were milled at 250 rpm for 3 h to achieve BCP powder. Both heating and cooling rate were selected as 10°C/min.

### Poly (methyl methacrylate)-based cement preparation process

The samples were prepared with various weight percentages of the solid components of PMMA, BCP, and Gr [Table 1]. The powders were milled for 15 min in order to obtain a uniform composition and mixed with liquid component (14.4 ml liquid per 40 gr powder) and casted into molds (10 mm height and 6 mm diameter) according to ASTM F 451-08 and immersed in ringer solution at 37°C for 8 h [Figure 1a]. The presence of bioactive ceramics leads to weaken the elongation and yield strength. To overcome this limitation, Gr with various weight percentages (1 and 3 wt%) was added to the PMMA-based cement. For PMMA/BCP/Gr preparation, the weight percentage of BCP was kept constant and Gr was added to the cement with the weight percentage of 1 wt% pre polymer due to optimized wt% of Gr and it derivatives reported by other researches.[9] Based on the weight percentage of the solid components, the samples were coded into PMMA, PMMA/BCP, and PMAA/BCP/Gr.

### Characterization of biphasic calcium phosphate powder

The phase composition of the BCP powders was assessed by X Ray diffraction (XRD) was used for investigation of cements compositions (xrd, Philips, the Netherlands) performed with Cu-Kα radiation (λ = 0.154 nm, 40 kV, 40 mA). The weight percentages of β-TCP and HA forms were calculated by Reynaud's equations.[28]

The average particle size in a supernatant fluid was evaluated by a dynamic light scattering (DLS) method (Vasco, Cordouan technologies, France) at 25°C and 657 nm wavelength. Laser power was 35.6%. Before the test, the samples were dispersed in distilled water with

| Table 1: Various polymethyl methacrylate-based cements (weight %) | | | |
|---|---|---|---|
| Samples | PMMA (%) | BCP (%) | Gr (%) |
| PMMA | 100 | | - |
| PMMA/BCP | 32 | 68 | - |
| PMMA/BCP/Gr | 31 | 68 | 1% |

Gr – Graphene; PMMA – Poly (methyl methacrylate); BCP – Biphasic calcium phosphate
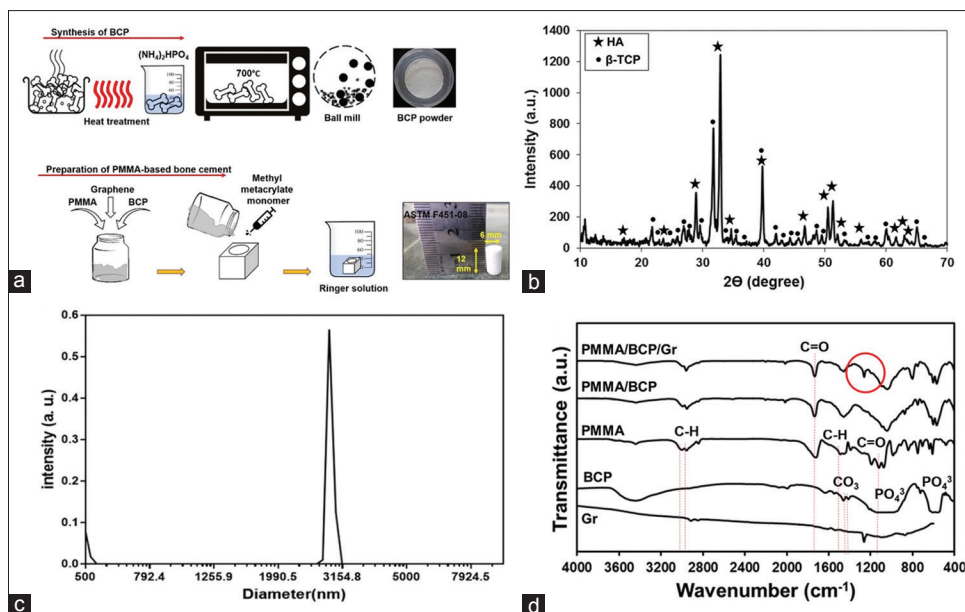
Figure 1: Schematic representation of biphasic calcium phosphate synthesis and fabrication of samples according to ASTM F451-08 standard for mechanical tests (a), X-ray diffraction pattern of biphasic calcium phosphate powders (b), particle size pattern of biphasic calcium phosphate by dynamic light-scattering method (c), and Fourier transform infrared spectroscopy spectrum of raw materials and obtained cements (d)

the concentration of 1 wt% and sonicated for 20 min. The results of BCP particles are an average of three measurements.

## Characterization of poly (methyl methacrylate)-based cements

For observing the effect of adding Gr and BCP on the cements, surfaces and investigating HA formation on the samples surfaces scanning electron microscopy (SEM) were used. The samples were placed in an oven for 2 h at 60°C to remove the residual moisture before the test. The discs were coated by gold for 150 s and observed by SEM (SEM, Philips XL30 at an operating voltage of 20 kV and the electrical current of 10 mA).

Fourier-transform infrared spectroscopy (FTIR, Bruker tensor) was used to evaluate the chemical composition of BCP and the composites over a range of 4000-400 cm$^{-1}$ and a resolution of 2 cm$^{-1}$.

To evaluate the bioactivity of the cements, the samples with the same shape and the same surfaces were exposed to ultraviolet radiation for 30 min and then placed in falcons containing 10 ml of simulated body fluid (SBF)[29] and were incubated at 37.0°C for 28 days. Three discs were examined for each PMMA-based cement type.

*In vitro* degradation of the PMMA-based cements specimens immersion in phosphate buffer saline (PBS) for 28 day was used for investigation of cements. after drying and weighing bone cements, the loss of samples weight was determined.

Although bone is under pressure made by different forces such as bending, stretching, and pressing, the endurance of

pressure due to a person's weight is very significant. The compressive test was performed in order to mechanical properties evaluation. To this, the prepared samples (*n* = 5) were poured into cylindered shape molds. The samples were dried after 8 h of immersing in the Ringer solution and the test speed was 5 mm/min.

To evaluate the *in vitro* biocompatibility and cytotoxicity of the PMMA-based cement, the 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) assay and cell attachment test were performed. The MG63 cell line was purchased from Pasteur Institute of Iran and cultured in Dulbecco's modified Eagle medium (DMEM, Bioidea, Iran), low-glucose supplemented with 10% (vol/vol), fetal bovine serum (FBS, Bioidea, Iran), and 1% (vol/vol) penicillin/streptomycin (pen/strep, Bioidea, Iran). The culture condition contained humidity and 5% CO$_2$ at 37°C. The medium was replaced every other day. After the cells covered 70%–80% of the flask, they were separated by 0.25% trypsin-EDTA solution (Bioidea, Iran). The cells were counted by Trypan blue procedure. After enumeration, they were seeded on the cement samples (*n* = 3), which had already been placed into 24-well plates, in the same density with the control well (10$^4$ cell/well). The cells were incubated for 7 days at 37°C under 5% CO$_2$.

To evaluate the cements for maintenance ability of MG63 cells viability, MTT (Sigma-Aldrich, Germany) assay was performed. The cement discs were washed with PBS for 3 times, and then, the samples were sterilized by 70% (vol/vol) ethanol for 2 h and exposed to UV light for 2 h. The discs were plunged into DMEM medium containing 10% (vol/vol) FBS and 1% (vol/vol) penicillin/streptomycin

overnight. Colorimetric assay was used for cell relative viability evaluation. After 1, 4, and 7 days of incubation, the culture medium was discarded and washing with PBS was performed. The samples were incubated in 0.5 mg/mL MTT in the PBS solution for 4 h to form formazan crystals. Dimethyl sulfoxide (DMSO) was added to insoluble formazan crystals in order to change the solubility properties and placed into a shaker at 37°C for 1 h. After that, the obtained solution from each sample and blank (DMSO, wavelength = 540 nm) was placed into a 96-well plate and their optical density (OD) was measured using microplate reader (Bio-Rad, Model 680). The cell relative viability was calculated with the obtained parameter. The MG63 cells viability was calculated by Eq.1.[30]

$$\text{Relative cell viability}\,(\%) = \frac{A_{\text{sample}} - A_{\text{b}}}{A_{\text{c}} - A_{\text{b}}} \times 100 \qquad (1)$$

Where $A_{\text{sample}}$ is the absorbance of the sample, $A_{\text{b}}$ and $A_{\text{c}}$ are the blank and control (tissue culture plate) absorbance, respectively.

### Statistical analysis

The results were reported as the mean ± standard deviation (SD) and analyzed using Tukey's post-hoc test using GraphPad Prism Software (V.6) with the $P < 0.05$ to reveal the significant difference between all data.

## Results and Discussion

### Biphasic calcium phosphate powder analysis

By quantitative description of the XRD pattern in Figure 1b, the BCP powders contained 32 wt% β-TCP and 68 wt% HA. As demonstrated in another research, BCP properties can be optimized by changing the percentage of β-TCP and HA.[31] Increasing the percentage of β-TCP can enhance the solubility of ceramic and improve degradable rate subsequently. In this research, the percentage of each of the formed phases was very similar to the appropriate amount reported for the bone defects repair in other studies.[32,33]

The particle size of BCP measured by DLS test was between 500 nm and 10 μm [Figure 1c] and the most common particle size was 3 μm. The particle size was important due to its effectiveness on mechanical properties. As Chaiyabutr et al.[34] reported in their study, with the decrease in the particle size, mechanical properties significantly improved.

### Bone cements analysis

For further characterization of HA and β-TCP, PMMA, and the reaction between these materials FTIR spectroscopy was used. According to Figure 1d, the FTIR spectra of calcined BCP revealed the characteristic peaks of the OH bands in HA at 3439 cm⁻¹ in the calcined sample.

Moreover, $CO_3$ groups at 1412 and 1456 cm⁻¹ were found in the spectra of BCP related to the formation of HA crystals after calcination. The broad bands at 999–1129 and 580 cm⁻¹ are related to the $PO_4^3$ groups. Similar results demonstrated in other researches.[35,36] Furthermore, FTIR spectra of PMMA revealed the characteristics peaks at 2996 and 2954 cm⁻¹ related to axial deformation of the CH bonds of aliphatic carbons, at 1460 cm⁻¹ assigned to the angular deformation of CH bond. The bands related to axial and angular deformation of carbonyl group (C = O) were revealed at 1718 and 1191 cm⁻¹, respectively which were assessed in a similar study.[37] FTIR spectra of the Gr containing cement revealed that the shift of the peak from 1262 cm⁻¹[38] to 1258 cm⁻¹ indicates the hydrogen bonding between PMMA chains and Gr, which might be due to the effect of Gr, which can add some functional group in the crosslinking process of PMMA. Furthermore, after adding BCP to the polymer and Gr matrices, a peak at 1550 cm⁻¹ decreased, and at 1370 cm⁻¹ the peak got stronger which reveals that the components of the cement chemically interact with each other.

### Investigation of bioactivity

The PMMA-based cements surfaces presented in Figure 2 show that the BCP and Gr were distributed homogeneously. The presence of bioactive ceramics in a composite leads to form bone and strong bone-cement bonding.[13,39] Hence, BCP was added to PMMA-based cements as a bioactive material to turn the bio-inert cement to a bioactive type. The surface of bioactive bone cement [Figure 2] indicates that 68 wt% BCP powder was adequate to avoid entrapping within the cement and enough area of surface was allocated to bioactive substance for exposure to body environment and be able to create osteoconductivity for bone cement, as confirmed by Kokubo and Takadama.[40] In this research, it was revealed that the bioactive material should be used with more than 60 wt% so that it can be considered as an osteoconductive material. The bioactivity test results showed that the inclusion of BCP into the cement led to the formation of hydroxyapatite on the samples surfaces, resulting in bioactivation of the cement. The SEM images of the samples surfaces after 28 days of immersion in SBF indicated that HA was not formed on cement surface in the absence of BCP [Figure 2], but on the surface of the samples with BCP, HA could be clearly observed in a spherical-like morphology [Figure 2].

The degradation curve was drawn by samples weight measured on day 1, 10, 20, and 28. According to Figure 3, PMMA/BCP/Gr cement had the maximum rate of degradation which can be due to the degradation of BCP and releasing of Gr, although with less power and importance whereas the pure PMMA indicated poor degradation. It should be mentioned that if the cement shows bioactivity and degradability at the same time the degradation to its components could take place gradually and therefore the
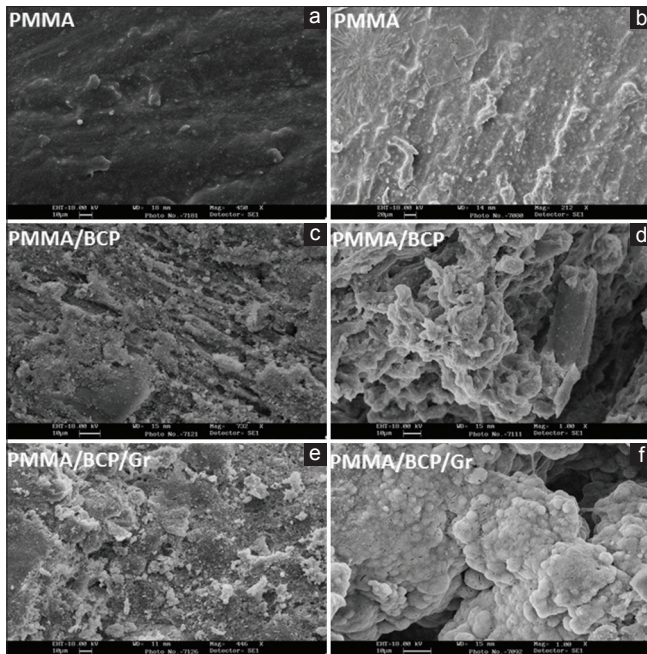
Figure 2: Scanning electron microscopy images of poly (methyl methacrylate) (a), poly (methyl methacrylate)/biphasic calcium phosphate (c), poly (methyl methacrylate)/biphasic calcium phosphate/graphene (e) before and poly (methyl methacrylate) (b), poly (methyl methacrylate)/biphasic calcium phosphate (d), poly (methyl methacrylate)/biphasic calcium phosphate/graphene (f) after 28 days immersion in simulated body fluid



Figure 3: Degradation curve of poly (methyl methacrylate)-based bone cements

bone cells have enough time for reproducing. Such events result in synthetic materials elimination and normal tissue formation in the patient's body.[41]

## Mechanical properties

Because the PMMA-based bone cement is used to replace the hip joint which is under a high load, its mechanical properties, particularly the compressive strength, must be favorable. As can be seen in the stress-strain curves [Figure 4a], the inclusion of BCP in PMMA increased the elastic modulus (E value), but decreased elongation by enhancing the cements brittleness and obviously this has led to reducing the cement strength and faster breaking. In fact, by addition of BCP, the comparatively weak interfacial PMMA/BCP interactions may result in reducing elongation. In addition, the shrinkage amount is not equal for the PMMA and BCP particles, so the BCP particles squeezing in the PMMA matrix leads to compressive stress emerged in the matrix of PMMA close to the BCP particles. This hoop stress leads to loose bonding between the matrix of polymer and BCP particles and the PMMA matrix. Similarly, Renteria-Zamarron et al.[42] investigated the effect of wollastonite addition to mechanical properties of PMMA cements and their result showed that compressive strength of PMMA cement decrease after wollastonite addition. In addition, similar results reported by Serbetci et al. study which reported that the addition of HA up to 14.3 wt% led tp reduction of compressive strength.[43] To enhance the weakness of cement strength, Gr was utilized
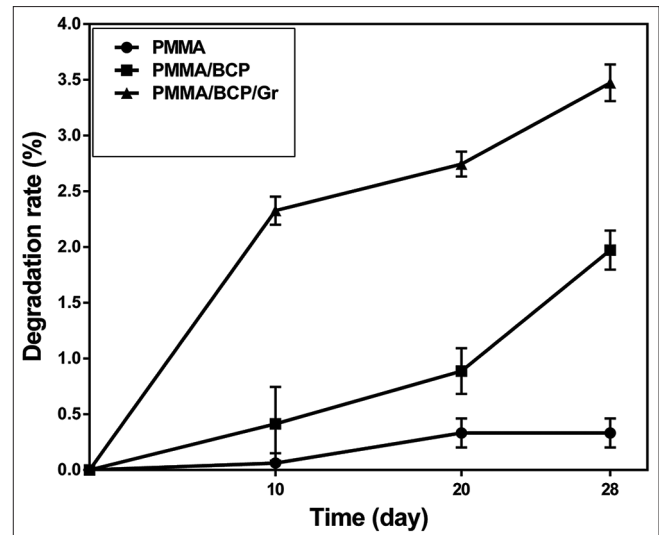
due to its high mechanical properties especially its adequate ductility. Although there is no significant difference between PMMA/BCP/Gr and PMMA/BCP in elastic modulus as can be seen in Figure 4b ($P > 0.05$), elongation was predictably improved due to Gr ductility feature. Addition of BCP ceramic raised the E value as confirmed by the obtained results of previous studies in the case of mixing ceramics with PMMA. However, in this study, the rate of increase is higher and this could be due to the use of 68 wt% of ceramic in the composites versus 50 wt% of the utilized ceramic in the research done by Chen et al. and differences in ceramic type as well.[13] The elastic modulus increased from 789.16 ± 12.87 Mpa for PMMA to 1218.46 ± 11.32 Mpa for PMMA/BCP cement, but it indicated minimum elongation approximately 16.39% ± 1.02% versus PMMA elongation 56.25% ± 1.72%. Elongation went up from 16.39% ± 1.02% to 35.18% ± 2.42% by inclusion of 1% Gr into PMMA/BCP. In addition, this amount of Gr led to increase PMMA/BCP yield strength from 61.67 ± 1.52 Mpa to 78.40 ± 2.06 Mpa for PMMA/BCP/Gr. So adding Gr to PMMA/BCP played an important role in improving the yield strength and elongation value, which were both in the allowed range of the bone cements for each parameter [Figure 4c and d].

Normally, the compressive strength in the yield area for commercial PMMA is in the range of 70–120 Mpa,[13] and this value for PMMA was 96.67 ± 1.52 Mpa in our study, whereas this property of bone is close to 170 Mpa. Mechanical investigation revealed that although PMMA still has the highest yield strength among the samples, the elastic modulus improved from 789.16 ± 12.87 for PMMA to 1185.68 ± 10.18 for PMMA/BCP/Gr.

Finally, it is evident that PMMA/BCP/Gr indicated desirable mechanical properties and its elastic modulus enhanced dramatically in comparison to uniform PMMA. Besides, the strength of this bioactive cement was in the
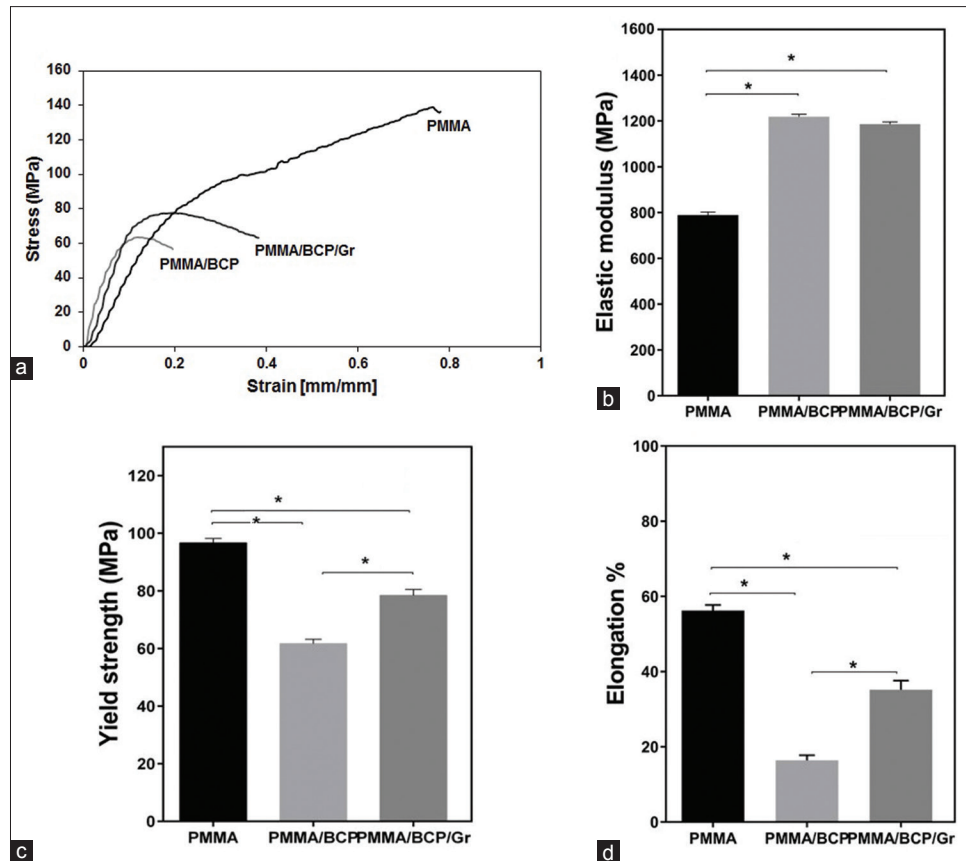
Figure 4: Stress-strain curves of poly (methyl methacrylate)-based bone cements (a), elastic modulus (b), yield strength (c), and elongation (d)

standard range of compressive strength for bone cements based on PMMA >70 MPa.[13]

## Cell viability

It was investigated and proved that the substrate stiffness may have an essential effect on cell structure, but it should be noted that the cell type also plays an important role.[44] Considering the ascending trend of charts [Figure 5] for all specimens from day 1 to day 7, cell viability increased which demonstrated there is no toxicity. According to Figure 5, it is evident that osteoblasts were survived and reproduced better on all modified bone cements than on common bone cement PMMA ($P < 0.05$). The MG63 cells viability were affected by the presence of BCP and Gr. BCP, which may be due to the formation of apatite-like layers. In another study, in which calcium-silicate, based ceramic was added to PMMA, it was illustrated that in addition to apatite formation ability, releasing calcium ions could be another reason for cell viability enhancement.[13] Besides, in the previous *in vivo* studies, it had been reported that a layer of calcium phosphate stimulated the growth of bone cells.[45] In comparison to PMMA, PMMA/BCP was associated with 139.85% and 122.37% proliferation enhancement on day 4 and 7 of culture, respectively.

PMMA/BCP/Gr indicated that the highest cell viability among the modified cements. Actually, the incorporation

of Gr and BCP into PMMA increased the cell viability from 127.55% ± 7.03% for PMMA to 201.41% ± 10.7% for PMMA/BCP/Gr on day 4 of culture, which may due to taking advantage of both additives simultaneously and enhancement of the elastic modulus of the substrate, which can affect the cell viability of the MG63 cells. Similar results have been demonstrated in another study in which a substrate with suitable stiffness could improve cell proliferation and even mesenchymal stem cell differentiation to osteoblast.[45]

To evaluate the effects of BCP and Gr on the cell response, the MG63 cells were cultured on the cement and the cell attachments and morphology were investigated. Gr affects the cell morphology as can be seen in the SEM images of cultured MG63 cells presented in Figure 6. It seems that MG63 cells morphology did not affect by existence of BCP. In addition, osteoblast extended well on PMMA/BCP cements surfaces and it can be due to excellent biocompatibility of BCP. However, the presence of Gr in the substrate caused the spherical morphology of osteoblasts, reduction of their length and their tendency to grow separately and unlike cell aggregation. Zhang *et al*.[46] investigated the effect of another carbon-based material (carbon nanotubes) on osteoblasts proliferation and differentiation and reported that osteoblasts morphology was affected and became round with less length similar to the present study.
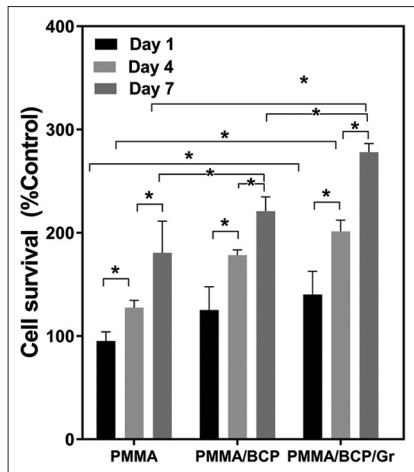
Figure 5: Cell viability of MG63 cells cultured on poly (methyl methacrylate)-based cements on day 1, 4, and 7 of culture

Simultaneous consideration of biological features, mechanical properties, and cell behavior results reveals that PMMA/BCP/Gr can be a promising bone cement for orthopedic applications. It is clear that *in vivo* investigation must be performed to examine PMMA/BCP/Gr osteointegration.

## Conclusion

Modified bone cement was successfully prepared by casting according to ASTM F451-08. Bioactivity, the increase of elastic modulus more than 1.5 times and improvement of the cell viability and proliferation occurred by adding BCP to PMMA, but it decreased elongation through increasing the cement brittleness. Incorporation of Gr at 1 wt% to the obtained PMMA/BCP cement led to increase ductility, and subsequently decreased brittleness and elongation enhancement from 16.39% ± 1.02% for PMMA/BCP to 35.18% ± 2.42% for PMMA/BCP/Gr. The MTT assay demonstrated that PMAA/BCP/Gr cement was cell-friendly and indicated highest cell viability among samples. According to the obtained results of mechanical and biological tests, it seems that new PMMA/BCP/Gr bone cement has a potentiality for usage in clinical applications.

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### Conflicts of interest

There are no conflicts of interest.

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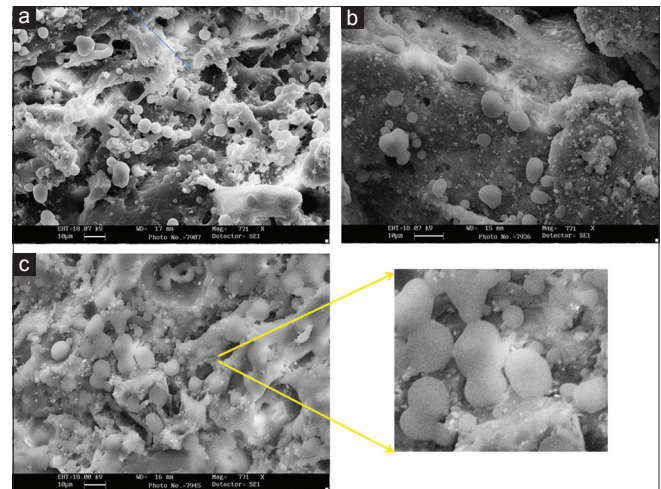
Figure 6: Scanning electron microscopy images of MG63 cells morphology cultured on poly (methyl methacrylate) (a), poly (methyl methacrylate)/biphasic calcium phosphate (b) and poly (methyl methacrylate)/biphasic calcium phosphate/graphene (c)

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# BIOGRAPHIES

**Farnoosh Pahlevanzadeh** received the M. Sc. degree in tissue engineering from Azad Islamic university, Najafabad branch. Now, she is a Ph.D. student of biomedical engineering at Isfahan University of technology. She is interested in biomaterials, tissue engineering, improving the properties of ceramics and polymers in order to use for biomedical applications, and also, the use of carbon compounds such as Carbon nano tubes, graphene and graphene oxide in order to enhance biomaterials properties.

**Email:** farnoosh.pahlevanzadeh@gmail.com

**Dr. Mehdi Ebrahimian-Hosseinabadi** is currently an assistant professor in the department of biomedical engineering at the University of Isfahan. His research interests include developing micro or nanoscale biomaterials for tissue engineering. Furthermore, He is working on design, synthesis and characterization of next-generation of biomaterials for a variety of applications, including but not limited to, the wound healing and fabrication of biomedical devices.

**Email:** m.ebrahimian@eng.ui.ac.ir