Graphene oxide improves chitosan-based biomaterials with applications in bone tissue engineering

SORINA DINESCU[1], SIMONA IGNAT[1], LOREDANA PREDOIU[1], ANCA HERMENEAN[2], MARIANA IONITA[3], MITKO MLADENOV[4],MARIETA COSTACHE[1]*

[1]*University of Bucharest, Department of Biochemistry and Molecular Biology, 050095Bucharest, Romania*

[2]*"Vasile Goldis" Western University of Arad, Department of Histogy, 310414 Arad, Romania*

[3]*University Politehnica of Bucharest,Advanced Polymer Materials Group, 010072 Bucharest, Romania*

[4]*"Ss. Cyril and Methodius" University, Faculty of Natural Sciences and Mathematics, Institute of Biology, 1000 Skopje, Macedonia*

*Address correspondence to: University of Bucharest, Department of Biochemistry and Molecular Biology, 91-95 Spl. Independentei, 050095, Bucharest 5th District, Romania.
Tel.: +4021 3181575; Email: marietacostache@gmail.com

Abstract

Bone tissue engineering (BTE) currently requires modern and efficient approaches for restauration of bone defects. In this context, intelligent biomaterials that are able to induce by their own nature the osteogenic differentiation process are among the best approaches developed for BTE. Chitosan (CH) is a natural biopolymer with good properties for tissue engineering. Graphene and its derivatives, graphene oxides (GO), have been recently discovered to favor cell proliferation, cell adhesion and cell differentiation processes. The excellent mechanical and physical properties that GO displays promotes its use alone or in combinations for bone regeneration applications. The present study proposes a novel biomaterial based on chitosan and GO, CH/GO 1 wt.% as a potential biomaterial for restauration of surface bone defects. In vitro studies suggest that this composite efficiently supports osteogenesis and promotes bone formation.

1. Introduction

Biomaterials designed for bone tissue engineering (BTE) have evolved lately from simple inert filling materials to nature-inspired biodegradable tissue-adapted materials, often reinforced with innovative compounds that allow the material to mimic bone structure and durity. Apart from osteoconductive properties, some of the new materials benefit of a composition that is by itself osteoinductive, thus favoring bone tissue regeneration. These innovative biomaterials can be implanted at the bone lesion site impregnated or not with cells, depending on the strategy adopted for BTE. In the light of these modern approaches developed in bone regeneration, the quality of life in patients with bone defects is considerably improved.

In this context, this study describes a new biomaterial based of natural polymer chitosan (CH), already used in tissue engineering studies (KIM & al. [1]), improved with graphene oxide (GO) component that converts this composition into a biomaterial addressed to bone tissue regeneration. This CH/GO film will be evaluated in this study for BTE applications potential.

Graphene is one of the new generation nanomaterials with proven pro-osteogenic effects (NAYAK & al. [2], GU & al. [3]). One of its derivatives, GO has been tested for various medical application, among which it demonstrated excellent properties for bone regeneration (DEPAN & al. [4], MISRA & al. [5]). Several studies have shown a slight decrease in cell viability when cultured in contact with GO (CAI & al. [6], HONG & al. [7]), but more reports demonstrated that graphene derivatives GO present low cytotoxicity and are biocompatible (SAHU & al. [8], LA & al.[9]), even that it favors cell proliferation (PANDELE & al. [10], PANDELE & al. [11], IONITA & al. [12]).

With respect to cell adhesion, previous reports (SHI & al. [13], DEPAN & al. [14]) have shown that the addition of GO in the composition of the materials favors the interaction between the cells and the substrate. A possible explanation for this is that GO creates a nanotopography on the surface of the material that encourages focal adhesions formation (KIM & al. [15]). Moreover, it's essential for cells to proliferate and adhere to the substrate biomaterial in order to be able to initiate complex differentiation programs such as osteogenesis.

Based on several *in vitro* osteogenesis studies, it has been concluded that there is a possible correlation between the proportion of GO in the biomaterial and the degree of osteogenic differentiation of the cells cultivated in contact to it (LEE & al. [16]). In particular, GO was proved to accelerate osteogenic differentiation of mesenchymal stem cells (MSCs) (NAYAK & al. [2]).

All these being considered, the aim of this study was to evaluate a novel film biomaterial based on CH and improved with 1 wt.% GO (CH/GO 1 wt.%) for its ability to support osteogenic differentiation of murine preosteoblasts, by comparison with a pure CH biomaterial.

2. Materials and Methods

2.1. Culture system achievement

Chitosan/graphene oxide (CH/GO 1 wt.%) composite films and control chitosan films were prepared and tested for biocompatibility as previously described [art Preparation and *in vitro*, bulk, and surface investigation of chitosan/graphene oxide composite films]. Cells from MC-3T3E1 cell line were seeded with a density of 40000 cells/cm2 on the surface of the materials and the resulting cell-material hybrid was maintained for 72 hours in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Steinheim, Germany), supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Foster City, CA) and 1% antibiotic (Sigma-Aldrich, Steinheim, Germany) and standard *in vitro* culture conditions (37°C, 5% CO2, humidity).

Once the system equilibrated with the media and culture conditions, the normal complete DMEM media was removed and replaced with osteogenic differentiation cocktail (Life Technologies, Foster City, CA). The osteogenic inducing media was changed every two-three days, up to 21 days of differentiation.

2.2. qPCR assessment of osteogenic markers

Osteogenic markers osteopontin (*opn*) and osteocalcin (*ocn*) gene expression was evaluated after 7 and 21 days of induced *in vitro* differentiation in order to determine if the degree of differentiation was influenced by the composition of the material and, in particular, by GO.

Briefly, total RNA was isolated using Trizol and following manufacturer's instructions (Life Technologies, Foster City, CA, USA). Isolated RNA samples from T0 (the moment of osteogenic induction) and 21 days of differentiation were tested for concentration and purity on NanoDrop (ThermoFischer Scientific, Waltham, MA, USA) and for RNA integrity (RIN) on the Agilent BioAnalyzer 2100 system (Agilent Technologies, Waldbronn, Germany). Total cellular RNA was reverse transcribed to corresponding cDNA using iScript cDNA Synthesis kit (BioRad, Hercules, CA, USA). *Opn* and *ocn* specific primers were designed using Primer3 software and were optimized for the best annealing temperature by gradient PCR. The reaction was performed on a Corbett thermocycler in 50-60°C range of temperatures using standard PCR components (Promega, Madison, WI, USA). *Opn* and *ocn* primer sequences are presented in table 1.

Table 1. Mouse *opn* and *ocn* primer sequences used for qPCR gene expression evaluation.

Mouse osteopontin	5'- CTGGCAGCTCAGAGGAGAAG -3'	103 bp
	5'- TTCTGTGGCGCAAGGAGATT -3'	
	5'-CCCGGTTCATGGTCTGGTTG -3'	
Mouse osteocalcin	5'-GGTAGTGAACAGACTCCGGC -3'	134 bp
	5'-AGGCGGTCTTCAAGCCATAC -3'	

Once the conditions were optimized, qPCR for *opn* and *ocn* pattern of expression was performed on a LightCycler 2.0 carrousel-based system using LightCycler Fast Start DNA Master SYBR Green I Kit (Roche, Mannheim, Germany). *Opn* and *ocn* gene expression was normalized to both glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and TATAA binding protein (TBP) reference genes, as well as to the basal levels of these genes in normal mouse bone tissue.

2.3. OPN and OCN protein levels of expression

OPN and OCN osteogenic markers protein expression during osteogenic differentiation in contact with CH and CH/GO 1wt.% was assessed by immunohistochemistry techniques and fluorescence microscopy. For this purpose, cell-material hybrids were collected at T0 and 21 days of differentiation and imparafinnated. Paraffin blocks were sectioned in the microtome and obtained sections were exposed to sequential toluene and ethanol baths with the purpose of deparaffination. Deparaffinated sections were gently washed in PBS three times and then exposed to unspecific blocking solution of 2% bovine serum albumin (BSA, Sigma-Aldrich, Steinheim, Germany) for one hour, in room temperature. Fluorescent staining of the sections started with an overnight incubation of the sections with a mix of primary antibodies: rabbit polyclonal anti-osteocalcin (SC-30044, Santa Cruz Biotech., Heidelberg, Germany) and mouse monoclonal anti-osteopontin OPN (SC-7361, Santa Cruz Biotech., Heidelberg, Germany). After a thoroughly wash in PBS, sections were exposed to secondary antibodies solution for one hour: goat anti-rabbit coupled with AF546 (Life Technologies, Foster City, CA, USA) and goat anti-mouse coupled with AF488 (Life Technologies, Foster City, CA, USA). DAPI staining was used to mark cell nuclei in blue and, after a final wash in PBS, the sections were mounted in ImunoHistoMount (SC4086, SantaCruz Biotech, Heidelberg, Germany). Fluorescent staining was visualized using an Olympus IX73 inverted microscope and images processed with Cell F software.

2.4. Statistical analysis

All experiments were performed in triplicate, using three sets of CH and CH/GO 1 wt.% films and the results were expressed as a mean ± standard deviation (SD) using GraphPad Prism Software 6.0 (GraphPad Software Inc., San Diego, CA, USA). Statistical analysis of the qPCR data was performed using one-way ANOVA, followed by Bonferroni correction. Differences between samples were considered statistically significant for $p < 0.05$ and highly significant for $p < 0.001$.

3. Results and Conclusions

3.1. Osteogenic markers pattern of gene expression

In order to evaluate the evolution and the efficiency of the osteogenic differentiation process of murine preosteoblasts in contact with CH and CH/GO 1wt.% during 21 days and to discuss the contribution of GO to the process, quantitative PCR was employed.

Osteopontin (*opn*) is a highly phosphorylated sialoprotein and one of the major components of bone mineralized extracellular matrix (ECM). At bone tissue level, it is involved in mediation and signaling related to adhesion, migration and cell survival (STANDAL & al [17]). *Opn* is secreted by fully differentiated osteoblasts and osteocytes (McKEE and NANCI [18]), as well as by osteoclasts (DODDS & al. [19]). In particular for bone differentiation, *opn*is firstly secreted in the initial stages of osteogenic differentiation, with a maximum of expression when bone matrix mineralization is initiated (SODEK & al [20]).

In our experimental conditions, *opn* gene expression was first detected after 7 days of osteogenic differentiation in both 3D culture systems. Statistical analysis revealed a significant difference between *opn* expression found in cells cultivated in contact with CH/GO 1 wt.% as compared to control (p<0.001). The same significant difference (p<0.001) was registered between 3T3E1/CH/GO 1wt.% and 3T3E1/CH after 21 days of induced *in vitro* osteogenic differentiation process. Interestingly, no difference was observed when comparing *opn* gene expression in the control 3T3E1/CH system at 7 and 21 days, suggesting that no important evolution of the osteogenic process happened in this conditions. On the contrary, after 21 days of induced osteogenesis, a statistically significant increase (p<0.01) in *opn* expression was detected, as related to the levels found after 7 days of differentiation. Overall, the early activation of *opn* found after only 7 days of differentiation confirms that the osteogenic process was initiated in these 3D cultures. Moreover, the composition of the scaffolds could trigger an influence on osteogenic process evolution, since the presence of GO resulted in an increased *opn* expression both after 7 and 21 days of experimental conditions.
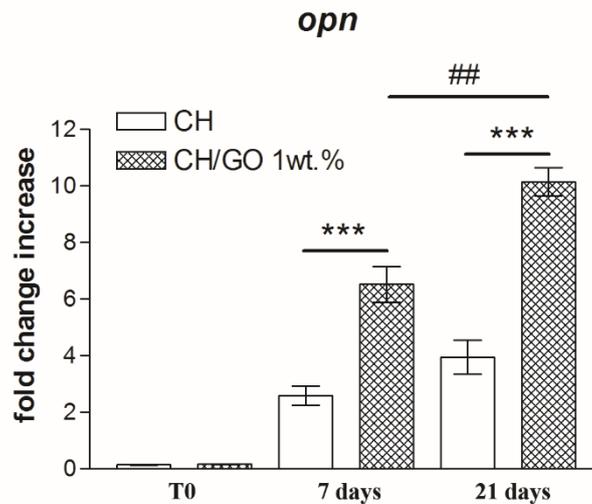
Fig.1 – *opn* gene expression profile in 3T3E1/CH and 3T3E1/CH/GO 1 wt.% systems during 21 days of induced *in vitro* osteogenesis, as revealed by qPCR. [*] refers to comparison between samples at the same time point and [#] refers to comparison between time points for the same sample.

Osteocalcin (*ocn*), a marker characteristic for the last steps of the osteogenic process, was activated quite late in cells exposed to our differentiation conditions, since the first statistically significant differences between samples were identified after 21 days of osteogenesis. There was a very small *ocn* level of gene expression found at 7 days of differentiation, suggesting the late activation of this gene. In this case, an increasing trend of *ocn* gene expression was found both in 3T3E1/CH and 3T3E1/CH/GO 1wt.% systems, with higher differences for the system containing GO (p<0.001). This observation suggests that osteogenesis takes place in CH 3D scaffolds when cells are exposed to osteogenic inducers, but the process of differentiation appears to be more efficient in the presence of GO. This conclusion is supported by *ocn* gene expression quantified after 21 days of differentiation, when a statistically significant (p<0.01) increase in *ocn* levels was registered for 3T3E1/CH/GO 1 wt.%, as compared to control.
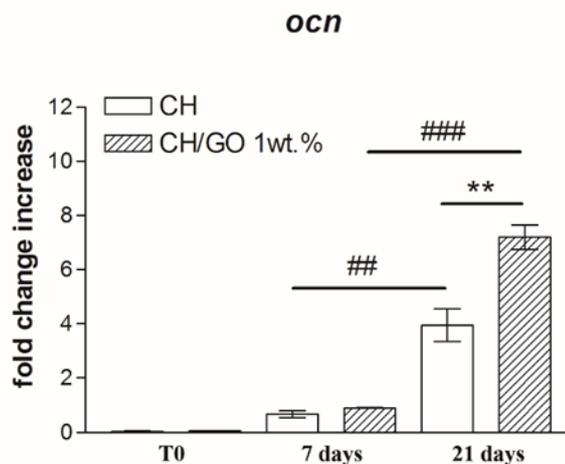
Fig.2 – *ocn* gene expression profile in 3T3E1/CH and 3T3E1/CH/GO 1 wt.% systems during 21 days of induced *in vitro* osteogenesis, as revealed by qPCR. [*] refers to comparison between samples at the same time point and [#] refers to comparison between time points for the same sample.

3.2. Osteogenic markers OPN and OCN protein expression

Bone matrix proteins Opn and Ocn expression was monitored up to 21 days of differentiation in our conditions and revealed by fluorescence microscopy and immunostaining. The general profile of both Opn and Ocn expression is correlated to the gene expression profile, suggesting the functionality of differentiated osteoblasts and their ability to produce bone matrix.
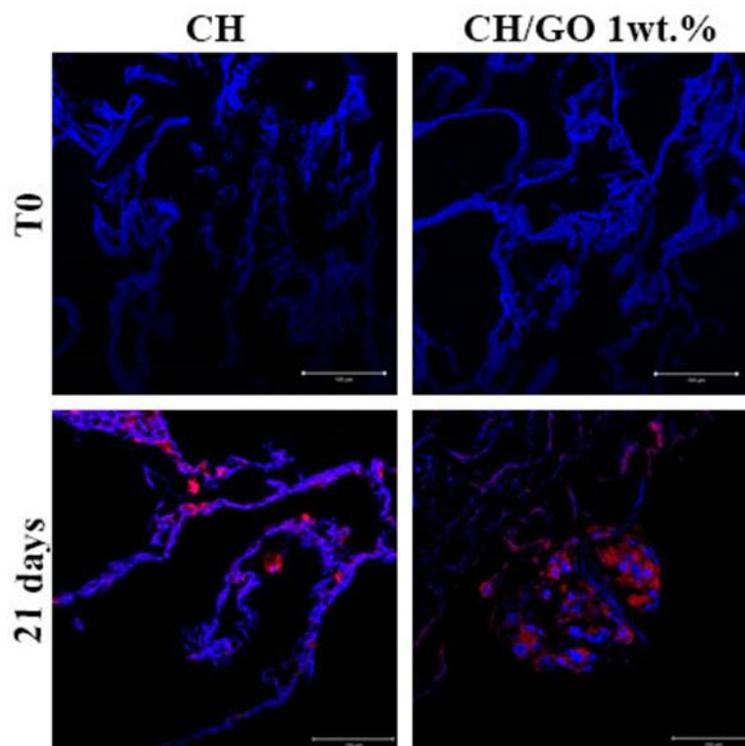

Fig. 3- Fluorescence microscopy micrographs displaying Opn protein expression before (T0) and after 21 days of osteogenic induction in CH and CH/GO 1 wt.% systems. Opn is labeled in red due to the secondary antibody coupled to TRITC that was used for immunostaining. Nuclei are stained in blue (DAPI).

Opn was found to be expressed in both CH and CH/GO 1 wt.% systems after 21 days of induced osteogenesis (fig. 3), as compared to the moment of induction (T0). Opn was expressed in low levels in dispersed cells distributed within CH material, while entire groups of differentiating cells expressing higher levels of Opn were revealed by fluorescence microscopy in CH/GO 1 wt.% system.

In parallel, Ocn protein expression was detected after 21 days of differentiation, but to a lower level than Opn (fig.4). FITC-labeled cells expressing Ocn were identified by fluorescence microscopy in CH/GO 1 wt.% system and, to a very low extent, in the control CH system, proving that osteogenesis took place faster and with higher efficiency in the

presence of 1 wt.% GO. These observations correlate with previous findings and with the fact that Ocn is a late-expressed protein secreted by bone ECM.
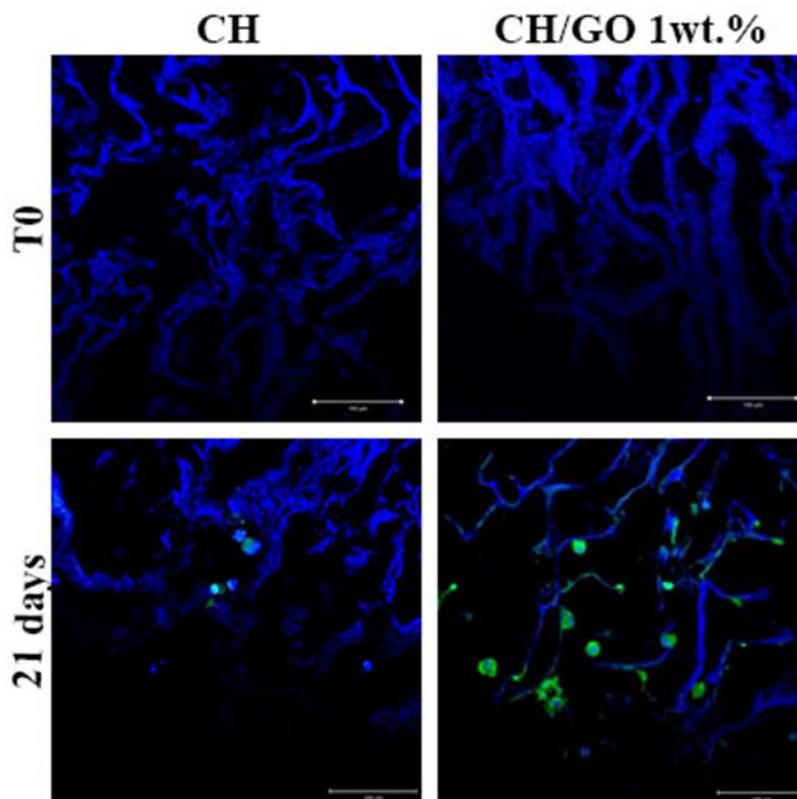

Fig. 4- Fluorescence microscopy micrographs displaying Ocn protein expression before (T0) and after 21 days of osteogenic induction in CH and CH/GO 1 wt.% systems. Opn is labeled in green due to the secondary antibody coupled to FITC that was used for immunostaining. Nuclei are stained in blue (DAPI).

Gene expression profile correlated well with protein expression for Opn and Ocn in the case of CH and CH/GO 1wt.% composites. This correlation revealed the activation of osteogenic differentiation program in murine preosteoblasts and the ability of these materials to support bone differentiation during *in vitro* studies of 21 days. As observed with Opn expression, the simple formation of cell groups during osteogenesis in contact with CH/GO 1 wt.% proves the beneficial properties of this material for BTE, since the initiation of bone formation requires the agglomeration and signaling of cells in small groups. In addition, the fact that both *opn* and *ocn* genes activated after 7 days of differentiation in CH/GO 1wt.% system supports the idea that this material improved with GO has osteoinductive and osteoconductive properties required for successful BTE. In our experimental conditions, the contribution of GO added in the material's composition resulted in a more efficient osteogenesis process.

4. Conclusions

Overall, a novel biomaterial based on natural compound chitosan and with additional 1 wt.% GO content proved to generate better results after *in vitro* osteogenesis than the control pure CH system. Thus, CH/GO 1 wt. % would represent a better candidate for BTE

applications than the commonly used CH biomaterials. Graphene and its derivatives, GO, reveal excellent properties that enhance bone development, thus encouraging the use of these compounds for further BTE applications.

5. Acknowledgements

References

1. KIM IY, SEO SJ, MOON HS, YOO MK, PARK IY, KIM BC, CHO CS.Chitosan and its derivatives for tissue engineering applications.Biotechnol Adv.;26(1):1-21 (2008).
2. NAYAK T.R., ANDERSEN H., MAKAM V.S., KHAW C., BAE S., XU X., EE P.L., AHN J.H., HONG B.H., PASTORIN G., ÖZYILMAZ B. Graphene for controlled and accelerated osteogenic differentiation of human mesenchymal stem cells. ACS Nano;**5**:4670–4678 (2011).
3. GU M., LIU Y., CHEN T., DU F., ZHAO X., XIONG C., ZHOU Y. Is graphene a promising nano-material for promoting surface modification of implants or scaffold materials in bone tissue engineering? Tissue Eng Part B Rev.;**20**:477–491 (2014).
4. DEPAN D., GIRASE B., SHAH J.S., MISRA R.D. Structure-process-property relationship of the polar graphene oxide-mediated cellular response and stimulated growth of osteoblasts on hybrid chitosan network structure nanocomposite scaffolds. ActaBiomater. ;**7**:3432 (2011).
5. MISRA R.D., CHAUDHARI P.M. Cellular interactions and stimulated biological functions mediated by nanostructured carbon for tissue reconstruction and tracheal tubes and sutures. J Biomed Mater Res A. ;**101**(528) (2013)
6. CAI X., TAN S., YU A., ZHANG J., LIU J., MAI W. Sodium 1-naphthalen esulfonate-functionalized reduced graphene oxide stabilizes silver nanoparticles with lower cytotoxicity and long-term antibacterial activity. Chem Asian J. ;**7**:1664 (2012).
7. HONG B.J., COMPTON O.C., AN Z., ERYAZICI I., NGUYEN S.T. Successful stabilization of graphene oxide in electrolyte solutions: enhancement of biofunctionalization and cellular uptake. ACS Nano. ;**6**(63), (2012)
8. SAHU A., CHOI W.I., TAE G.A stimuli-sensitive injectable graphene oxide composite hydrogel. ChemCommun.;**48**:5280.(2012)
9. LA WG, PARK S, YOON HH, JEONG GJ, LEE TJ, BHANG SH, HAN JY, CHAR K, KIM BS . Delivery of a therapeutic protein for bone regeneration from a substrate coated with graphene oxide. Small. ;**9**:4051-4060. (2013).
10. PANDELE AM., DINESCU S., COSTACHE M., VASILE E., OBREJA C., IOVU H. AND IONITA M . Preparation and *in vitro*, bulk, and surface investigation of chitosan/graphene oxide composite films. Polymer Composites. ;**34**(12):2116-2124.(2013)
11. PANDELE AM., IONITA M., CRICA L., DINESCU S., COSTACHE M., IOVU H.Synthesis, characterization, and *in vitro* studies of graphene oxide/chitosan-polyvinyl alcohol films. Carbohydrate Polymers. ;**102**:813-820.(2014)
12. IONITA M., VASILE E., CRICA L.E., VOICU S.I., PANDELE A.M., DINESCU S., PREDOIU L., GALATEANU B., HERMENEAN A., COSTACHE M. Synthesis, characterization and *in vitro* studies of polysulfone /graphene oxide composite membranes. Composites: Part B. ;**72**:108-115. (2015)
13. SHI X.T., CHANG H.X., CHEN S., LAI C., KHADEMHOSSEINI A., WU H.K. Regulating cellular behavior on few-layer reduced graphene oxide films with well-controlled reduction states. AdvFunct Mater. ;**22**:751(2012)
14. DEPAN D., MISRA R.D. The interplay between nanostructured carbon-grafted chitosan scaffolds and protein adsorption on the cellular response of osteoblasts: structure function property relationship. . ActaBiomater;**9**(6084). (2013)
15. KIM J., KIM Y.R., KIM Y., LIM K.T., SEONWOO H., PARK S. Graphene-incorporated chitosan substrata for adhesion and differentiation of human mesenchymal stem cells. J Mater Chem B. ;**1**:933. (2013)
16. LEE W.C., LIM C.H., SHI H., TANG L.A., WANG Y., LIM C.T., LOH K.P . Origin of enhanced stem cell growth and differentiation on graphene and graphene oxide. ACS Nano. ;**5**:7334–7341. (2011)

17. STANDAL T., BORSET M., SUNDAN A., Role of osteopontin in adhesion, migration, cell survivaland bone remodeling. ExpOncol. 26, 179-184(2004).
18. MCKEE M. D., NANCI A., Osteopontin and the bone remodeling sequence. Colloidalgoldimmunocytochemistry of an interfacial extracellular matrix protein. Ann NY Acad Sci. 760, 177-189(1995).
19. DODDS N. B., CONNOR I. R., JAMES I. E., RYKACZEWSKI E. L., APPELBAUM E., DUL E., Human osteoclasts, not osteoblasts deposit osteopontin onto resorption surfaces: an *in vitro* and*in vivo* study of remodeling bone. J Bone Miner Res. 10, 1666-1680(1995).
20. SODEK J., GANSS B., MCKEE M. D.,Osteopontin. Crit Rev Oral Biol Med. 11(3), 279-303(2000).