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Citation: SCIENCE CHINA Materials ; doi: 10.1007/s40843-016-5104-9

View online: http://engine.scichina.com/doi/10.1007/s40843-016-5104-9

Published by the Science China Press

ARTICLES SCIENCE CHINA Materials

mater.scichina.com link.springer.com Published online 11 November 2016 | doi: 10.1007/s40843-016-5104-9

# Growth enhancing effect of LBL-assembled magnetic nanoparticles on primary bone marrow cells

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**ABSTRACT** **Magnetic field has been considered to have positive effect on growth of bone. Because a magnetic nanoparticle can be regarded as one magnetic dipole, the macroscopic assemblies of magnetic nanoparticles may exhibit magnetic effect on local objects. This paper fabricated macroscopic film of γ-$Fe_2O_3$ nanoparticles by layer-by-layer (LBL) assembly on poly-D,L-lactic acid (PLA) scaffold, and studied the magnetic effect of the assembled γ-$Fe_2O_3$ nanoparticles film on primary bone marrow cells. The primary bone marrow cells were extracted from a mouse and cultured on the PLA substrate decorated by the film of γ-$Fe_2O_3$ nanoparticles after purification. Quantitative PCR (q-PCR) was used to show the cellular effect quantitatively. A just-found magnetosensing protein was employed to verify the magnetic effect of assembled film of nanoparticles on primary cells. It was exhibited that the decoration of nanoparticles enhanced the mechanical property of the interface. By acting as the adhesion sites, the LBL-assembled film of nanoparticles seemed beneficial to the cellular growth and differentiation. The expression of magnetosensing protein indicated that there was magnetic effect on the cells which resulted from the assembly of magnetic nanoparticles, implying its potential as a promising interface on scaffold which can integrate the physical effect with good biocompatibility to enhance the growth and differentiation of stem cells. The LBL-assembled film of magnetic nanoparticles may boost the development of novel scaffold which can introduce the physical stimulus into local tissue *in vivo*.**

**Keywords:** magnetic nanoparticles, layer-by-layer assembly, tissue engineering, bioelectronics, bone cells

## INTRODUCTION

Regenerative medicine has been considered to change the therapeutic paradigm essentially [1,2]. In this area, a critical issue is the regulation effect of materials upon cellular growth and tissue repair. Here, scaffold, seed cell and bioactive interface are three essential factors [3,4]. Recently, stem cell is extensively regarded as the favorable source of seed cell due to the capability of pluripotent differentiation and good immune compatibility [5,6]. Thus, the scaffold should be suitable for the stem-cellular growth and differentiation. Certainly, it should also be of good biocompatibility and appropriate mechanical property, especially for the repair of bone tissue. Owing to such advantages as mentioned, poly-lactic-acid (PLA) and poly-D,L-lactic-co-glycolic-acid (PLGA) are the commonly used scaffold materials in clinic which have been approved by FDA. However, the polymer-based scaffold remains to be improved, such as the alleviation of inflammation [7], the efficiency for cellular adhesion [8] and the promotion for differentiation [9]. Fortunately, there is a bioactive interface between the scaffold and the cells that can be ingeniously designed to harmonize the scaffold and the cells. There have been many important discoveries about the effect of interface. For example, surface topography was discovered to have significant influence on the cellular behavior and fate [10,11]. This effect will turn more important when the topographical size reaches the nanoscale [12], which inspires us that the nanoparticles-decorated surface may play an unexpected role in the interaction between scaffold and cells. Furthermore, the presence of nanoparticles can possibly bring about some physicochemical influences, such as redox, mechanical

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stress and electromagnetic effect. These factors have been proved to play important roles in the cellular growth and tissue repair.

Magnetism has been considered positive to the repair of bone tissue which results from the influence on bioelectricity [13–15]. However, it is inconvenient and of low efficiency to load a magnet on body. Thus, it occurs to us that the local magnetic effect can be provided by making a magnetic interface on the scaffold around tissue defect. Iron oxide nanoparticles currently attract increasing interests from biomedical researchers because of their unique magnetism and good biocompatibility [16]. One magnetic nanoparticle can be regarded as a magnetic dipole. Due to the very small size, thermal energy can even make the magnetic moment randomly orient to generate the so-called superparamagnetism. However, we discovered previously that the collective magnetism of nanoparticles had a transition from the superparamagnetism into the weak ferromagnetism after the assembly [17]. The aggregation-induced coupling of magnetic dipolar moments can account for this phenomenon, which reinforced the magnetic energy of system to overcome the perturbance of thermal energy. Thus, it is hypothesized that the assembled film of magnetic nanoparticles is possibly able to exert the magnetic action on the vicinal cells. Thus, it is potentially beneficial for the tissue repair to coat the traditional scaffold with a layer of magnetic nanoparticles.

Moreover, the iron oxide nanoparticles own the capability of thermogenesis in the presence of alternating magnetic field[18], radicals scavenging [19] and magnetic manipulation [20] so that the assembled film of iron oxide nanoparticles can regulate the cellular behavior by physical stimuli and mitigate the local inflammation. Also, it has been proposed that the nanoparticles can play a protein-like role [21]. Therefore, it is taken for granted that the decoration of nanoparticles on scaffold can enhance the cellular adhesion and growth.

Herein, layer-by-layer (LBL) method was employed to assemble colloidal γ-$Fe_2O_3$ nanoparticles into a uniform, compact and reproducible film on the PLA substrate. This method relies on the electrostatic interaction between nanoparticles and polyelectrolyte so that the film is highly stable even in the complex medium. The primary bone marrow cells were freshly extracted from femur and tibia of a mouse. Then the primary cells were cultured on the assembled film-decorated PLA substrate. It was exhibited that the assembled film of magnetic nanoparticles improved the biocompatibility of the scaffold and promoted cell differentiation. Of more importance, a just-found magnetosensing protein, which can respond to magnetic fields, was used to indicate the role of magnetic effect in these phenomena. The preliminary results support the point that the introduction of magnetism into interface is promising for the development of next-generation scaffold.

## EXPERIMENTAL METHODS

### Synthesis of γ-$Fe_2O_3$ nanoparticles

25% (*w*/*w*) $N(CH_3)_4OH$ was slowly added into the mixture of $Fe^{2+}$ and $Fe^{3+}$ (molar ratio 1:2) until the pH value of the solution reached 13. Then the reaction continued for 1 h to obtain the black colloidal particles ($Fe_3O_4$). Then air was pumped into the reaction system under 95°C water bathing after the pH value was adjusted to 3 by 1 mol $L^{-1}$ HCl. Finally, the reaction system was kept for 3 h to oxidize $Fe_3O_4$ colloidal particles into γ-$Fe_2O_3$ particles. During the whole reaction, vigorous stirring was needed. With this synthesis, the γ-$Fe_2O_3$ nanoparticles were bare, without other molecules. In our experiment, the final colloidal concentration of γ-$Fe_2O_3$ nanoparticles was 3 mg $mL^{-1}$.

### LBL assembly and magnetization

The colloidal suspension of γ-$Fe_2O_3$ nanoparticles was concentrated to 2 mg $mL^{-1}$ for the LBL assembly. The polyelectrolyte solution (PDDA, poly-diallydimethylammonium chloride) was diluted to 2 wt.% with ultra-pure water. A glass slide was firstly treated by the boiling mixture of $H_2SO_4$ and $H_2O_2$ (volume ratio 7:3) for 2 h. After that, the glass slide was washed by the ultra-pure water repeatedly and finally dried by $N_2$ gas stream. Then, the glass slide was dipped into the PDDA solution and the colloidal suspension of γ-$Fe_2O_3$ nanoparticles for 15 and 25 min, respectively. The γ-$Fe_2O_3$ nanoparticles were adsorbed on the glass slide due to the electrostatic interaction which brought about the formation of one bilayer. When the soak in one solution terminated, the glass slide was washed by ultra-pure water repeatedly and finally dried by $N_2$ gas stream. The washing is important. Otherwise, the colloidal suspension will become instable. The steps were duplicated leading to the LBL assembly of nanoparticles.

The magnetization of film was done by putting the fabricated LBL-assembled film into a uniform magnetostatic field for 30 min. The magnetic field was 0.7 T in intensity and parallel to the film.

### Mouse bone marrow cell extraction

Several C57/B6 mice were killed and were dipped in 70% ethanol for tens of seconds. Then the femur and tibia were

cut and put in 5 mL α-MEM medium. The skin and muscle were removed and both ends of bones were cut down. A 25G needle was used to flash the bone marrow out into the α-MEM medium. Then the medium containing the bone marrow was transferred to a 15-mL tube for centrifugal separation with 2000 rpm for 5 min. the pellets were resuspended in the cell culture medium (α-MEM with 15% fetal bovine serum and 1% Penicillin-Streptomyxin) after aspirating the supernatant. Before the culturing, the cells were counted and adjusted to a suitable concentration for cell seeding. The detailed operation can refer to Ref. [22]. This experiment was performed in accordance with the relevant regulations and the experimental qualification was approved by Southeast University and Department of Science and Technology of Jiangsu province.

**Culturing of primary mouse bone marrow cells**

The extracted bone marrow cells were cultured in 24-well plate with 3 wells as one group. The size of one well is just 1.5 cm so that the rounded glass plate with assemblies of nanoparticles can be fitted into the well. The culturing medium was the α-MEM medium containing 15% fetal bovine serum and 1% Penicillin-Streptomyxin. The ascorbic acid-2-phosphate (1 mmol $L^{-1}$) was added into the culturing medium to initiate differentiation. The medium was changed every 3 days. After culturing for about 10 days, multi-cellular fibroblastoid colonies (or CFU-fs) can be observed. These colonies were stained to check expression of alkaline phosphatase by using a reagent kit (bought from Sigma Co.).

**Characterization of materials and cells**

The elastic module of γ-$Fe_2O_3$ granular film was tested by NanoTest™ Vantage (Micro Materials Corp. Ltd., UK). The tip used in the measurement is Berkovich indenter tip. Stiffness data can be extracted from the contact rigidity and contact depth.

Cellular viability was measured by CCK8 assay and flow cytometry after culturing for 7 days, respectively. CCK8 kit was purchased from Beyotime Co. Ltd. (Shanghai, China). Every well of 24-well plate was added with 50 μL CCK8 medium. After incubation for 2 h in 37°C, the 24-well plate was measured with an ELISA microplate reader, by which the optical density (OD) value of each well under 450-nm wavelength was recorded. Then the cellular viability can be calculated as the following formula:

$$\text{viability}\,(\%) = \frac{\text{OD}_\text{i} - \text{OD}_\text{blank}}{\text{OD}_\text{cells} - \text{OD}_\text{blank}} \times 100\%,$$

where $OD_i$ is the OD value of each well, $OD_{blank}$ is the OD value of the well with the culturing medium but without the cells and $OD_{cells}$ is the OD value of the well with cells cultured in the absence of nanomaterials.

Before the flow cytometry measurement, the cells in 24-well plate were washed by phosphate buffer saline (PBS) buffer. Then the cells were trypsinized and the cells from one group (3 wells) were gathered into one vial, which were furthermore treated twice by centrifugation and washing with PBS. Finally, the volume of cellular suspension was 1 mL. Finally, the cells were stained with propidium iodide and analyzed by the flow cytometry (FACSCalibur™, BD Biosciences, USA) using a wavelength of 488 nm.

## RESULTS AND DISCUSSION

The disk-like PLA substrate was fabricated by spin coating of polymer solution on a glass plate. The cross-section of PLA substrate was shown in Fig. 1a. Compared with the naturally-dried PLA film on glass substrate, the PLA film made by spin coating shows more level (Supplementary information, Fig. S1a). The topography of polymer substrate is important because the subsequent assembly depends on the property of surface. Mechanical property of PLA substrate was characterized by Nanoindentor (Fig. S1b). It can be seen that the elastic modulus of sample (12.1230 GPa) is higher than that of naturally-dried sample (7.0942 GPa). This means that the surface homogeneity indeed influences the mechanical property on interface although the bulk material may be little affected. The elastic modulus of the spin-coated PLA substrate is comparable to that of bone (about 10–30 GPa), so it is suitable for culture of bone cells.

To assemble the colloidal nanoparticles on PLA substrate, plasma was employed to treat the surface for hydrophilization. Contact angle *versus* time was used to check the performance of plasma treatment (as shown in Fig. S2). Based on the data, the sample with 15-min treatment and contact angle of 47.43° was chosen for the next step. Then the PLA plate was marinated into the PDDA solution and γ-$Fe_2O_3$ colloidal suspension alternatively. The γ-$Fe_2O_3$ nanoparticles are approximated 13 nm in size and formed aggregates of about 100 nm in suspension (Figs S3a and b). With the increase of assembled layers, the film becomes dense and uniform (Fig. S4). After assembly of 5 bilayers, the film completely covered the surface of substrate. The morphological observation for 5-layers-assembled film is shown in Figs 1b and c. Compared with the naturally-dried film, the LBL-assembled film was more uniform macroscopically and poriferous microscopically (Fig. S5). Thus, it is more suitable as the interface between scaffold and cells. The elastic modulus was also measured

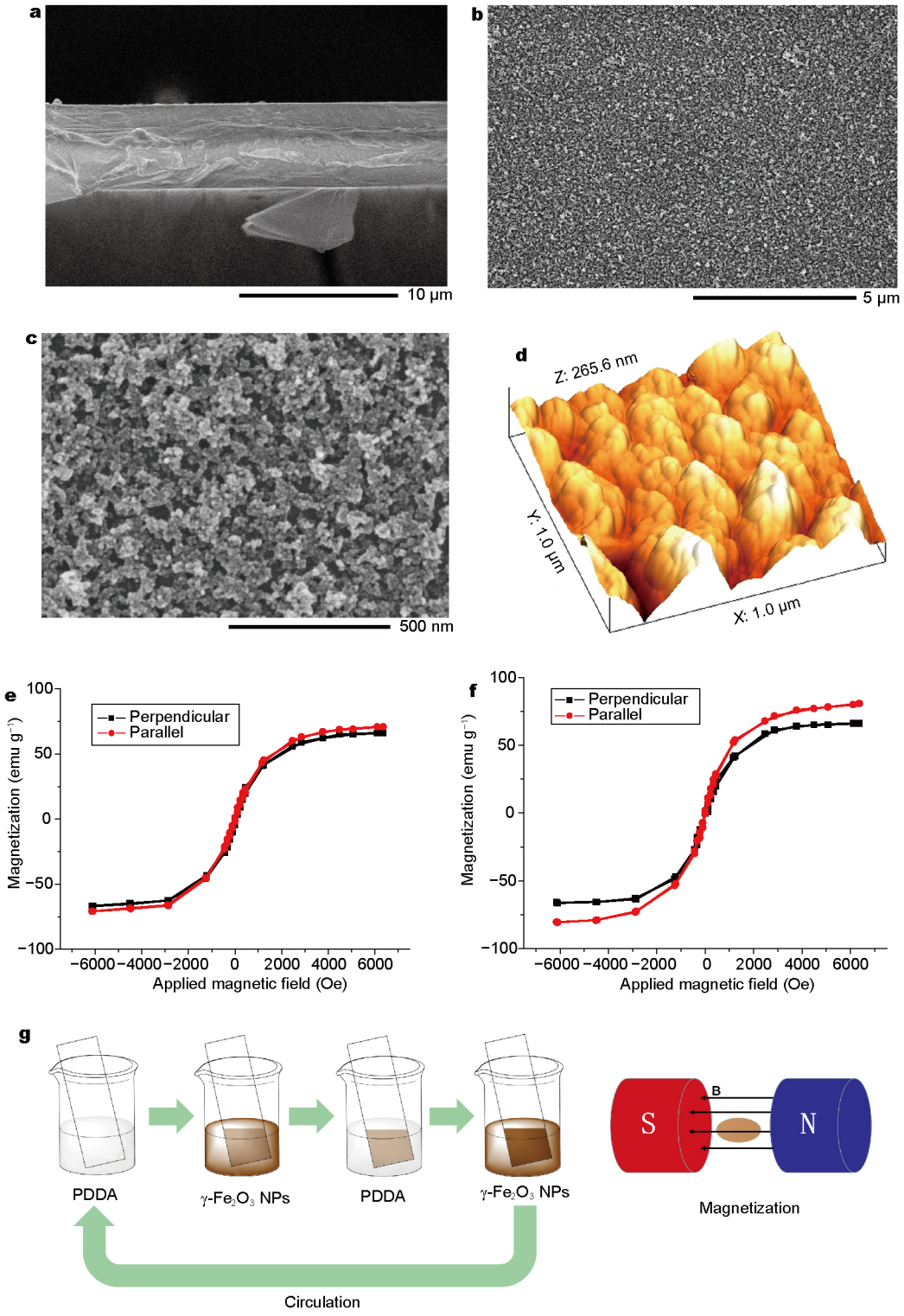


**Figure 1** Morphological observation of LBL-assembled film. (a) Cross-section of PLA substrate fabricated by spin coating; (b) LBL-assembled γ-$Fe_2O_3$ nanoparticles on PLA substrate; (c) local magnification of b; (d) 3D AFM image of the granular film. (e and f) Magnetic hysteresis loops of LBL-assembled film of γ-$Fe_2O_3$ nanoparticles before and after magnetization. (g) Schematic drawing of the fabrication process of LBL-assembled film and the magnetization.

by the nanoindentor. The result was 95.5620 and 94.9036 GPa for LBL-assembled and naturally-dried film, respectively (Fig. S6). The thickness and the three-dimensional (3D) morphology of granular film were characterized by atomic force microscopy (AFM), which is shown in Fig. 1d. From the 3D-reconstructed image, the thickness is about 265.6 nm. The aggregation of nanoparticles played an important role so that the 6-layers assembly can result in such a thickness. It is worth noting that the presence of granular film can lead to a nearly 8-fold increase of elasticity. We measured the elastic modulus of another PLA sample fabricated by the freeze-drying method. Although the absolute values were much less than those fabricated by the spin coating method without and with the LBL-assembled film of nanoparticles, the increasing multiples were approximate. The scanning electron microscopy images of the granular film before and after nanoindentation experiment are shown in Fig. S7. Actually, the indentation depth in our experiments is just several micrometers since the thickness more than 200 nm is possible to influence the experimental results. The magnetic hysteresis curves for the assembled film of γ-$Fe_2O_3$ nanoparticles before and after magnetization were measured by a vibrating sample magnetometer (Figs 1e and f). It is obviously seen that the magnetic interaction along the magnetization direction turns stronger. It was considered the magnetic moments should be aligned into the identical orientation to yield local magnetic ordering even if the magnetic field was removed. It should be mentioned that although the nanoparticles were immobilized, the magnetic moments were rotatable. The 13 nm size made the magnetic moments of γ-$Fe_2O_3$ nanoparticles rotate freely even without the reversal of nanoparticles themselves. This is the common thermogenic mechanism for magnetic nanoparticles, i.e., the Neel's relaxation. The experimental process of LBL assembly is schematically shown in Fig. 1g.

Then the primary bone marrow cells of mouse were cultured on the PLA plates with LBL-assembled film of γ-$Fe_2O_3$ nanoparticles. After culturing for 6 days, the cellular morphology was observed with optical microscopy (Figs 2a–d). Seen from the pictures, the cells show the fairly good growth on the PLA surface with assembled film of nanoparticles. This was also confirmed by the measurement of cellular viability using CCK8 assay and flow cytometry, respectively. Both results show that the PLA sample with assembled film of nanoparticles has much higher viability for the bone marrow cells than the plain PLA sample (Figs 2e and f). This may result from the adhesion sites-like effect of the nanoparticles, which was also found in our other work [23,24]. To confirm the reinforcement of cellular adhesion in the presence of nanoparticles, the expression of integrin and cadherin which are important proteins related to cellular adhesion was measured by q-PCR (Fig. 2g). Both the integrin and the cadherin are obviously up-regulated for the PLA sample with assembled film of γ-$Fe_2O_3$ nanoparticles. More interestingly, it is seen that the magnetic effect can promote the cellular growth. The PLA sample with LBL-assembled film of γ-$Fe_2O_3$ nanoparticles shows even higher cellular viability and the microRNA expression after the magnetization. Considering that there is little morphological variation before and after the magnetization (Fig. S8), the local magnetic ordering is considered to account for this phenomenon. The magnetization may result in the ordered arrangement of magnetic moments to reduce the systematic energy. After the removal of external magnetic field, the thermal energy was incapable of disorganizing the magnetic moments thoroughly. Thus, the remnant magnetic moments of long-range order can affect the cellular growth.

After culturing for 15 days, the differentiation of primary bone marrow cells toward osteoblast was checked by ALP staining. The results shown in Figs 3a–d indicate that the assembled film of γ-$Fe_2O_3$ nanoparticles is beneficial for the cellular differentiation. This is also confirmed by the q-PCR measurement of some proteins related to cellular differentiation, including osteocalcin, osteopontin, runt-related transcription factor 2 (RUNX2) and bone morphogenetic protein 2 (BMP-2) (Fig. 3e). The osteocalcin and the osteopontin are indicators for differentiation toward osteoblast. RUNX2 and BMP-2 are the upstream and the downstream protein for the osteogenic differentiation. Of importance, the magnetization is also found to be capable of further promoting this effect, which hints that the magnetic effect should play a crucial role. Many studies have reported that the magnetic nanoparticles can promote the differentiation of marrow-derived stem cells toward osteoblasts [25–27]. We also utilized the Au nanoparticles to do the same experiments, but it was found that the LBL-assembled colloidal Au film was unbeneficial for the differentiation of cells. To directly prove the point that the assembled film of γ-$Fe_2O_3$ nanoparticles can yield the magnetic effect on cells, the magnetosensing protein was measured by q-PCR, which was just discovered to be capable of responding to the external magnetic field [28]. The result exhibits that the assembled film of γ-$Fe_2O_3$ nanoparticles up-regulated the expression of magnetosensing protein which further rises for the magnetized assembled film of

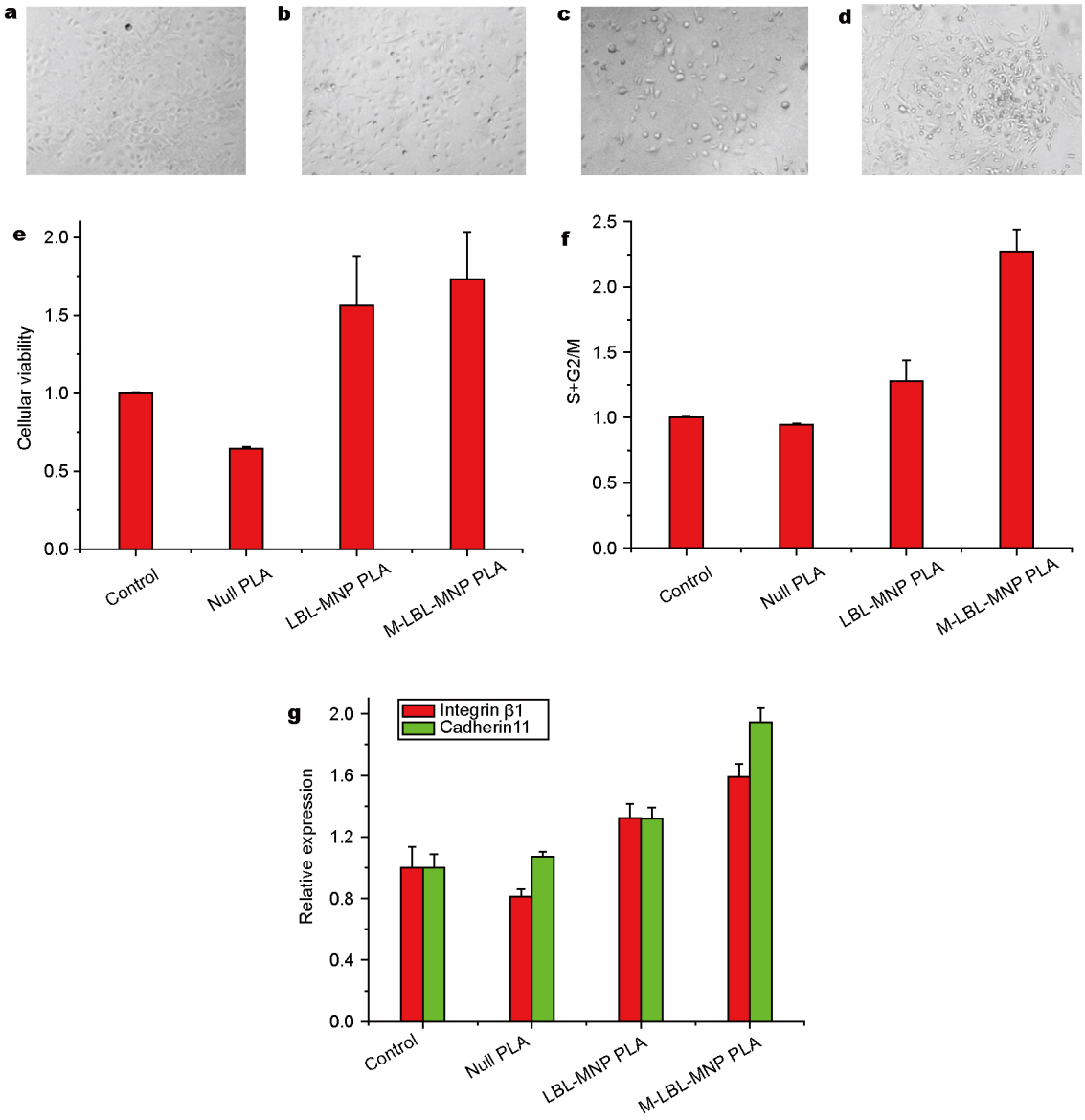


**Figure 2** Characterization of cellular growth. (a–d) Optical observation of cells growing on cellular culturing plate (control), blank PLA film (null PLA), PLA film with LBL-assembled magnetic nanoparticles (LBL-MNP PLA) and PLA film with LBL-assembled magnetic nanoparticles after magnetization (M-LBL-MNP PLA); (e, f) proliferation measurement of cells cultured on the four surfaces using CCK8 assay and flow cytometry, respectively; (g) q-PCR measurement of integrin and cadherin for cells growing on the four surfaces.

nanoparticles (Fig. 3f). Here, one magnetic nanoparticle or aggregate of nanoparticles was regarded as a magnetic moment. The adjacent magnetic moments will interact to form ordered arrangement to reduce the systematic energy. Certainly, this ordered arrangement should be highly localized [29,30]. If the ordered arrangement of magnetic moments is regarded as a small magnet, a cell is actually subjected to several small magnets. This is why the magnetosensing protein was also up-regulated for the LBL-assembled film without magnetization. After the film was magnetized, just as mentioned above, the magnetic moments of long-range ordering obviously enhanced the strength of collective magnetic effect upon the cells so that the cellular effect became more significant.

The influence from $Fe^{3+}$ release was excluded by measurement of Fe concentration (Fig. S9), since the results show there is no significant variance for the different samples. The surface capping of PDDA molecules on nanoparticles can account for the low Fe concentration in the cellular culturing medium. The surface capping by polymer

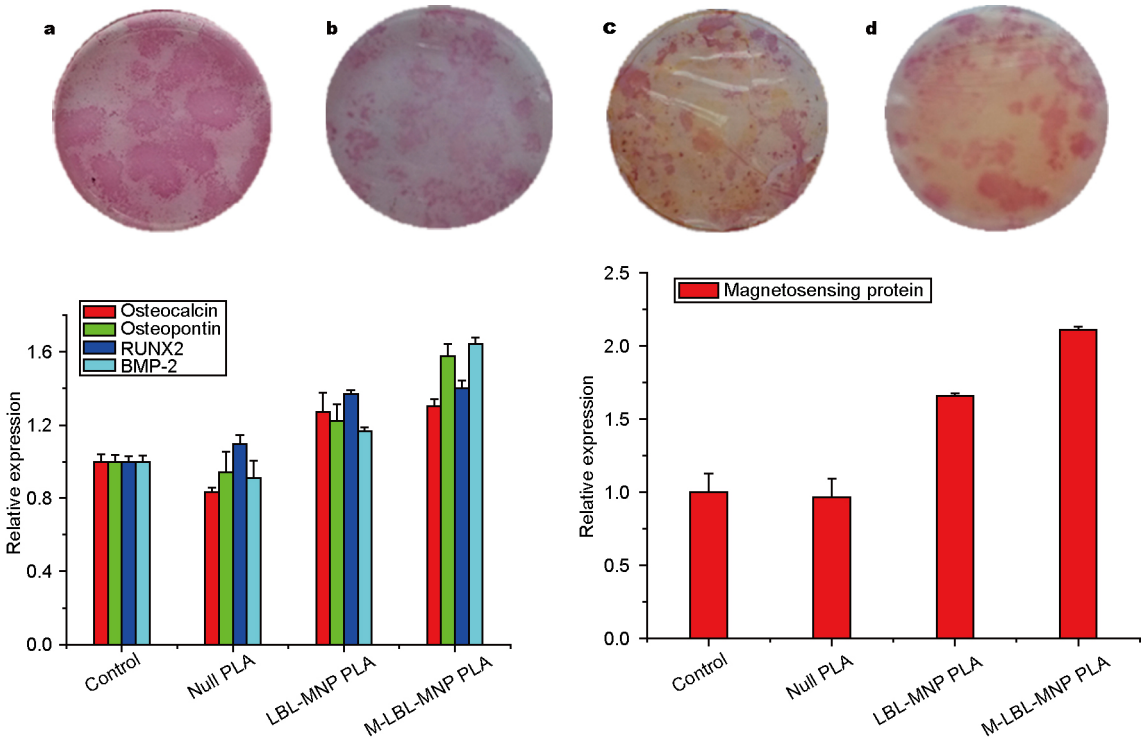


**Figure 3** Characterization of cellular differentiation. (a–d) ALP staining of cells growing on cellular culturing plate (control), blank PLA film (null PLA), PLA film with LBL-assembled magnetic nanoparticles (LBL-MNP PLA) and PLA film with LBL-assembled magnetic nanoparticles after magnetization (M-LBL-MNP PLA); (e) mRNA measurement of osteocalcin, osteopontin, RUNX2 and BMP-2 proteins for cells cultured on the four surfaces with q-PCR method; (f) mRNA measurement of magnetosensing protein for cells growing on the four surfaces with q-PCR method.

prevented the nanoparticles from contacting with the aqueous medium so that the reaction activity was greatly reduced. Thus, the enhancement effect on cellular growth and differentiation of the LBL-assembled film of $\gamma$-$Fe_2O_3$ nanoparticles should root in the magnetism which resulted from the assembly of magnetic nanoparticles, forming the small domains of magnetic ordering. It is the magnetic ordering that affect the cellular growth. Although we are incapable of characterizing the microstructures directly, the hypothesis can explain the experimental phenomena reasonably. This concept is schematically illustrated in Fig. 4. It means that the nanoparticles play the role by magnetism rather than by entering into the cells, which also indicates the assembled film of nanoparticles on scaffold may have higher safety in biomedical applications than the free-floating nanoparticles.

The growth enhancing effect of the LBL-assembled film of magnetic nanoparticles can be explained by the influence of magnetism upon cellular membrane potentials, which trigger cascaded signal transductions [31]. The cellular membrane potential is extremely critical to control the physiological functions of cells, which get dynamically maintained by the difference between ion concentrations inside and outside the cell membrane. Thus, the cellular membrane potential should be capable of being affected by the magnetic field because the moving charges are subjected to the Lorentz force under a magnetostatic field. As mentioned above, the ordered alignment of magnetic moments can yield a localized magnetic field, which can further influence the transmembrane streaming of some ions, especially $Ca^{2+}$ [32,33]. $Ca^{2+}$ is an important physiological transmitter and Ca element is also directly relative with formation of bone so that the presence of magnetism or magnetic materials can enhance the growth of osteoblasts (including the viability of cells). However, the detailed biological process of signal transductions of magnetism on osteoblasts remains under exploration. Seen from the above analysis, the growth enhancing effect of the LBL-assembled film of magnetic nanoparticles roots in the magnetism rather than the amount of magnetic materials. If the magnetic moments of nanoparticles are randomly arranged, the adding number of layers should be of little improvement on the effect. This is because only the magnetic nanoparticles adjacent to cells play the role. Otherwise, the adding number of layers may be good to the effect. Actually, only one layer of magnetized magnetic

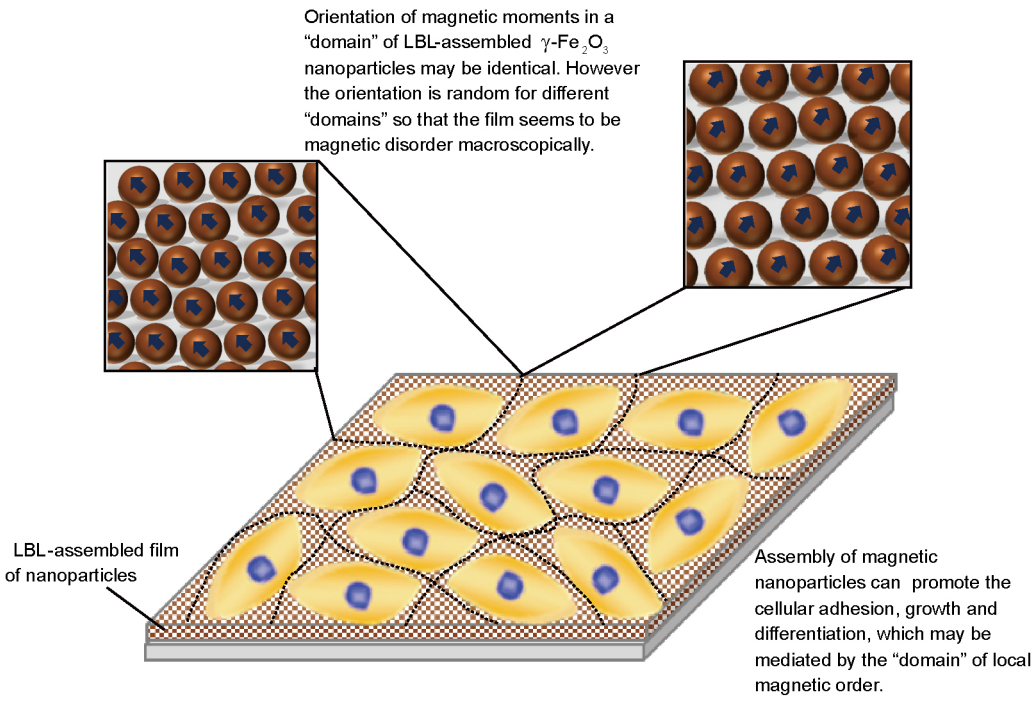


**Figure 4** Schematic showing of the mechanism for effect of the assembled film of magnetic nanoparticles on the cells. The key point lies in the local magnetic order. The magnetization augments the magnetic order and thus can enhance the cellular effect as the above-mentioned phenomena.

nanoparticles has the stronger growth enhancing effect than the multilayers of magnetic nanoparticles with LBL assembly [17].

## CONCLUSION

A uniform and contact film of nanoparticles was fabricated by the LBL-assembly of γ-$Fe_2O_3$ nanoparticles, which can be used as a bioactive interface of PLA scaffold. By coating with film of nanoparticles, the mechanical property on interface was enhanced. The experiments with primary bone marrow cells show that the LBL-assembled film of γ-$Fe_2O_3$ nanoparticles can promote the growth and differentiation of cells. It is proved that the assembly-mediated magnetic effect plays an important role here. We believe this novel interface between scaffold and stem cells will boost the development of next-generation scaffolds and their application in tissue engineering, organ repair and regenerative medicine.

**Received 18 July 2016; accepted 28 September 2016; published online 11 November 2016**

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**Acknowledgments** This work was supported by the National Basic Research Program of China (2013CB733801) and the National Natural Science Foundation of China (21273002 and 61601227). Sun J is also thankful to the supports from the special fund for the top doctoral thesis of Chinese Education Ministry (201174). Lou Z and Li Y thank the Natural Science Foundation of Jiangsu Province (BK20160939 and BK20130608). All authors are thankful to the supports from Collaborative Innovation Center of Suzhou Nano Science and Technology.

**Author contributions** Sun J conceived and designed the experiments, analyzed the results and wrote the manuscript. Liu X did all the cell experiments with help of Yang Y and Li Y. Zhang J prepared the samples and did the characterization with Tang S. Lou Z carried out the AFM characterization. Liu X and Wang P involved in the paper writing. Gu N supervised the project. All authors contributed to the general discussion.

**Conflict of interest** The authors declare that they have no conflict of interest.

**Supplementary information** Supplementary data are available in the online version of the paper.

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## 磁性纳米颗粒层层自组装膜对原代小鼠骨髓细胞的生长促进作用

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**摘要** 磁场一直以来都被认为对骨生长具有促进作用. 磁性纳米颗粒可以被看作是一个磁偶极子, 因此宏观的磁性纳米颗粒组装膜也可能对附近的物体具有磁效应. 本文通过层层自组装方法在聚乳酸支架表面制备了宏观γ-$Fe_2O_3$纳米颗粒组装膜, 研究了γ-$Fe_2O_3$纳米颗粒组装膜对原代小鼠骨髓细胞的磁作用. 原代小鼠骨髓细胞从小鼠体内新鲜提取, 并在前述生物材料表面培养. 定量PCR用来定量表征细胞效应, 磁场的影响通过检测一种刚刚发现的磁感应蛋白来指示. 结果表明, 表面纳米颗粒组装可以显著增强聚合物支架的力学性质, 促进细胞生长和分化. 磁感应蛋白检测结果表明这是由于磁性纳米颗粒组装导致的磁效应引起的. 本文用磁感应蛋白证明了磁性纳米颗粒层层自组装膜可以通过对细胞的磁效应促进干细胞的生长和分化, 该磁性纳米颗粒组装膜将会促进新一代组织工程支架的研发, 有可能将物理刺激效应引入到体内局部组织修复中.