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ORIGINAL PAPER

Magnetic poly(glycerol dimethacrylate) latex particles: synthesis, characterization and cellular interactions

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Abstract Magnetic poly(glycerol dimethacrylate) (m-poly(GDMA)) latex particles were synthesized by precipitation polymerization in the presence of iron oxide nanoparticles obtained by co-precipitation of Fe^{2+} and Fe^{3+} salts. The mean size of iron oxide nanoparticles and m-poly(GDMA) latex particles was determined by dynamic light scattering as 57 and ~800 nm, respectively. Vibrating sample magnetometer results showed that iron oxide nanoparticles and m-poly(GDMA) latex particles are superparamagnetic, and their saturation magnetizations are 74.37 and 3.85 emu/g, respectively. MC3T3-E1 preosteoblasts were seeded in the presence of m-poly(GDMA) latex particles. Through the magnetic particles, magnetic field was applied onto the cells by an appropriate magnet (4500 G). Cell–particle interactions were observed by optical microscope and scanning electron microscope. Mitochondrial activities of cells was measured by 3-[4,5-dimethylthiazol-2-y1]-2,5-diphenyl tetrazolium bromide test. The results show that the presence of m-poly(GDMA) particles in the culture medium did not affect the proliferation behaviour of MC3T3 cells and no toxicity against to L929 fibroblastic cell proliferation was observed when the particle concentration is 100 pg per cell.

Keywords Iron oxide nanoparticles · Magnetic latex particles · Cytotoxicity · Cellular interaction · Poly(GDMA)

Introduction

Nano-size magnetic polymeric particles have been used and also investigated in biology and medicine for cell selection [1, 2], magnetic resonance imaging (MRI) [3],

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hyperthermia [4], targeted drug delivery [5] and bone tissue engineering [6]. There are variety of methods to produce magnetic polymeric particles in desired properties such as colloidal stability, uniform size or narrow size distribution, high and uniform magnetite content, superparamagnetic behaviour and enough surface functional groups for coupling of active bioligands [7]. Magnetite (iron oxide, Fe_3O_4) is commonly used for the construction of magnetic particles since it is known to exist in numerous parts of human body [8]. In addition, polymeric coating ensures stability of magnetic particles in physiological medium providing nontoxicity by avoiding leakage of iron and enables chemical modification for attachment of biologically active compounds [9]. Therefore, it is possible to use such those particles in a number of cell-based applications including living cells, such as in tissue engineering that is a rapidly evolving field in medicine to repair damaged tissue by using cell preparations of different origin [10].

To understand the possibilities of magnetic nanoparticles' usage for cell-based applications the interactions of particles with cells have been investigated [11]. The factors that are size, surface properties, cell type and endocytotic pathways influence the cellular adherence and cellular uptake of these particles [12]. While the cellular uptake is specific for nanoparticles in the range of 50–200 nm, bigger particles up to micron-size have been used by attaching to the cells. In addition, interactions between cells and particles should be altered by using monomers in different functionalities and wettabilities. It is possible to prepare biodegradable or nondegradable nanoparticles by using a wide variety of monomers or polymers, such as styrene, acrylic acid, methyl methacrylate, isoprene, butylcyano acrylate, poly(caprolactone), poly(L-lactide), poly(D,L-lactide-*co*-glycolide), dextran, etc. [13–15]. Miniemulsion or inverse miniemulsion processes have been extensively used for the production of magnetic nanoparticles.

Our previous work has highlighted the potential use of poly(glycerol dimethacrylate)-based, poly(GDMA), latex particles in cell cultures. The monosize latex particles were prepared by a precipitation polymerization method developed by Saraçoğlu et al. [16]. L929 mouse fibroblasts were cultured on the poly(GDMA)-based latex particle coated tissue culture polystyrene (TCPS) surfaces [17]. The similarity of molecular structure and swelling ability of poly(GDMA) particles to the highly biocompatible material, poly(2-hydroxyethyl methacrylate), make them promising material for various biotechnological and biomedical applications [18]. However, magnetic form of poly(GDMA) particles has not been synthesized and investigated in cell cultures until now. In this study, magnetic poly(glycerol dimethacrylate) (m-poly(GDMA)) latex particles were synthesized by precipitation polymerization in the presence of iron oxide nanoparticles and their potential effect on mammalian cell behaviour was investigated in L929 cell cultures.

Materials and methods

Materials

Ferric chloride ($FeCl_3 \cdot 6H_2O$) and phosphate-buffered saline (PBS, pH 7.4) tablets were purchased from Sigma (USA). Ferrous chloride ($FeCl_2 \cdot 4H_2O$) and the

monomer, glycerol dimethacrylate (GDMA, 85 %, mixture of isomers), were supplied from Aldrich Chemical Co. (USA) and used without further purification. Ammonia solution (25 %), acetone and hydrochloric acid (HCl, 37 %) were purchased from Merck (Germany). Ethanol (96 %) was obtained from Aklar Kimya (Turkey). Methanol was received from BİRPA® (Turkey). Toluene was obtained from Riedel-de Haen (Germany). Acetonitrile and 2,2′-azobisizobutyronitrile (AIBN) were supplied from Aldrich Chemical Co. (USA). All the water used in experiments were ultrapure water which is obtained from Barnsted EasyPURE™ system.

Synthesis of iron oxide nanoparticles

Superparamagnetic iron oxide nanoparticles were synthesized according to Massart's procedure [19]. In brief, an aqueous solution of ferric chloride (40 mL; 4.093 g $FeCl_3 \cdot 6H_2O$) and ferrous chloride (10 mL; 2.595 g $FeCl_2 \cdot 4H_2O$ in 2 M HCl) were added to ammonia solution (500 mL, 0.7 M) at room temperature and the mixture was stirred. The iron oxide precipitate was isolated from the solution by magnetic separation, washed with water 3 times and transferred to the ethanol solution. Then, these nanoparticles were dried at room temperature and pulverized before characterization tests.

Synthesis of magnetic poly(GDMA) latex particles

Poly(GDMA) and m-poly(GDMA) latex particles were synthesized via precipitation polymerization [16]. Polymerization was performed in a sealed reactor placed in a shaking water bath equipped with a temperature control system. The receipt used for the precipitation polymerization of GDMA in the presence or absence of magnetic nanoparticles is given in Table 1. Briefly, 15 mg of iron oxide nanoparticles synthesized before were added into 40 mL polymerization medium containing toluene (30 mL) and acetonitrile (10 mL). The resulting mixture was sonicated for 30 min at 80 % power within an ultrasonic water bath (Sonorex Super 10 P, Bandelin, Germany). Then, 0.16 g of initiator, AIBN, and 1.5 mL of monomer (GDMA) were poured into the sealed cylindrical Pyrex reactor (volume: 100 mL). Then, the sealed reactor was placed in a shaking water bath. The polymerization was allowed to proceed at 70 °C for 24 h (stirring speed: 550 rpm). After completion of polymerization, nonmagnetic particles were removed by supernatant replacement during purification by repeated magnetic separation steps from ethanol.

Table 1 Recipes used for the preparation of poly(GDMA) and m-poly(GDMA) latex particles by precipitation polymerization

Components	Poly(GDMA)	m-Poly(GDMA)
Magnetic nanoparticles		15 mg
GDMA	1.5 mL	1.5 mL
Toluene	30 mL	30 mL
Acetonitrile	l0 mL	10 mL
AIBN	0.16 g	0.16 g

Non-magnetic poly(GDMA) latex particles were also synthesized by using the same procedure but in the absence of iron oxide nanoparticles.

The experimental conditions, such as GDMA concentration, composition of dispersion medium and amount of initiator, were kept constant by taking into account our previous publication [17].

Characterization studies

The hydrodynamic sizes of magnetic nanoparticles, poly(GDMA) and m-poly(-GDMA) latex particles were measured in an aqueous medium (pH 7), at room temperature by dynamic light scattering (DLS) (NanoS, Malvern Instruments). DLS was applied with an angle of 170° by using a He–Ne laser (4 mW) operated at 633 nm. The polydispersity index (PDI) values for size distribution in aqueous medium were directly obtained from the software of hydrodynamic size distribution analysis in a NanoS system.

Poly(GDMA) and m-poly(GDMA) latex particles were visualized in dry state by scanning electron microscope (SEM, Zeiss, Evo 50, England). For this purpose, polymer dispersion was dropped onto a copper grid and dried at room temperature. The dried samples were coated under vacuum with a thin layer of gold and then, they were visualized with 2,000× magnification. The diameters of individual beads were measured by a ruler on each photograph printed on an A4 page. All beads in each photo were measured and counted. The number average diameter of latex particles (D_n) was calculated according to Eq. 1 by considering the length of scale bar on each photo, where N_i is the number of beads in a diameter of D_i (in nanometers)

$$D_n = \sum N_i D_i / \sum N_i. \quad (1)$$

Attenuated total reflectance Fourier Transform infrared (ATR-FTIR) spectrum of m-poly(GDMA) particles was recorded by using a Thermo Scientific Nicolet iS10 FTIR spectrophotometer (USA) bearing an ATR attachment with a diamond crystal.

The magnetic properties of the nanoparticles and m-poly(GDMA) latex particles were measured by vibrating sample magnetometer (VSM, PPMS, Quantum Design, USA).

Cell culture studies

Cytotoxicity

The cytotoxicity test (MEM-extract test, MTT test system, 72 h incubation) was conducted according to the ISO 10993/EN 30993 standard [20]. Poly(GDMA) and m-poly(GDMA) latex particles were washed with ethanol (70 %) several times and sterilized by UV light during 15 min. Then, cytotoxicity test was performed towards the growth of L929 mouse fibroblasts. The cells obtained from Animal Cell Culture Collection in the Foot and Mouth Disease Institute, Ankara, Turkey [HUKUK (Cell Culture Collection) 92123004], were seeded into 24-well tissue culture polystyrene

(TCPS) plate with an inoculation density of 5×10^4 cells mL^{-1}. In this study, 150 mg of dry particles were incubated at 37 °C for 24 h in 30 mL medium. The medium consists of Dulbecco's Modified Eagle's Medium (DMEM) (Sigma, Germany) with 10 % v/v fetal bovine serum (FBS). After 24 h of incubation, particles were removed from extract by using 0.22 μm filter. The L929 culture medium was replaced with various dilution of extracts (25, 50 and 100 %). During 3 days, mitochondrial activity of cells was assessed with MTT (3-[4,5-dimethylthiazol-2-yl]-diphenyltetrazolium bromide) analysis.

MC3T3 cells and cell seeding

The MC3T3-E1 preosteoblasts which are anchorage-dependent and grown in monolayer cultures were obtained from Riken Cell Bank (Japan) for cell culture studies. MC3T3-E1 cell line derived from mouse calvarium tissue is an immortalized cell-line. The cells were subcultured in culture flasks (Nunc™, Germany) using α-MEM (Sigma, USA) supplemented with 10 % (v/v) FBS and 1 % (v/v) antibiotic solution (penicillin–streptomycin, Sigma, USA). The cells, maintained at 37 °C in a humidified CO_2 (5 %) atmosphere (Heraus Instruments, Germany) were dissociated with 0.25 % trypsin–EDTA, centrifuged and resuspended in medium prior to cell seeding. All the cell culture studies were carried out in a Laminar Flow Cabinet (Bioair, Type II Laminar Flow Cabinet, Italy). Specific growth rate and doubling time of MC3T3-E1 preosteoblasts were found as 0.019 h^{-1} and 36 h, respectively.

For cell culture studies, ethanol (70 %) containing m-poly(GDMA) latex particles (net particle concentration, 100 pg/cell) was placed in 24-well TCPS dishes. Ethanol evaporated and particles stuck onto the surface of culture dish. Sterilization was achieved by UV light (15 min) before cell seeding. A cylindrical magnet (4500 G, surface area of magnet was same with culture area of plate) was then placed at the reverse side of the cell culture plate to provide magnetic force vertical to the plate during the cell culture studies. MC3T3-E1 preosteoblasts were trypsinized from the flasks and cell suspension was obtained by the addition of fresh differentiation medium similar to the culture medium stated above, but supplemented with 2 mM L-glutamine (Sigma, USA), 10 mM β-glycerol phosphate and 50 μg mL^{-1} L-ascorbic acid. Cell suspension (1 mL) containing 5×10^4 cells was seeded onto m-poly(GDMA) latex particles in 24-well dishes. Cells were cultivated in 5 % CO_2 at 37 °C in 95 % relative atmospheric humidity for 5 days. Half of differentiation medium was replenished every 3 days. In this study, TCPS surfaces without particles were used as positive control.

MTT assay

Mitochondrial activity of cells proliferated in particle-containing TCPS dishes was measured by MTT test during 4 days. In each well of cell culture dishes, culture medium was replaced with 600 μL serum-free medium and 60 μL MTT solution (2.5 mg mL^{-1} in PBS).The dishes were incubated in a humidified atmosphere, with 5 % CO_2 at 37 °C during 3 h and then, the medium was decanted. MTT was

converted into an insoluble purple formazan dye by mitochondrial dehydrogenase enzymes. Only active mitochondrial dehydrogenases of living cells converted MTT into the insoluble purple formazan dye. After addition of 400 μL absolute isopropanol, cells are lysed and the precipitated formazan is dissolved. Formazan concentrations were quantitatively determined by measuring the optical density (OD) at 570 nm with background correction of the OD at 690 nm using a spectrophotometer (ASYS Hitech UVM 340 Plate Reader, Eugendorf, Austria). Results were compared with control groups.

Observation of cell–particle interactions

Cell–particle interactions were observed by optical microscope and SEM. Optical observations were carried out with inverted microscope (Olympus IX71, Japan). Photographs were taken with digital camera which is connected to the microscope (Olympus C-4000, Japan).

For SEM procedure, the samples were firstly washed 2 times with PBS after removing medium. The cell samples were fixed with 2.5 % glutaraldehyde for 30 min. After washing with PBS (pH 7.4) 3 times, the cell samples were dehydrated through a series of ethanol concentrations (30 %, 50 %, 70 %, 90 % and dry ethanol). Following, hexamethyldisilazane was dropped onto the cell samples and 5 min later it was removed from the samples. Once the samples were air-dried, they were then sputtered under vacuum with a thin layer of gold before viewing under SEM (Zeiss, Evo 50, England) in different magnifications.

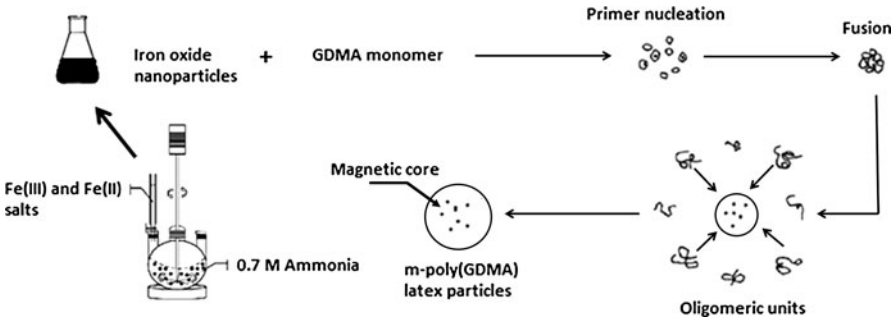
Statistical analysis

All data are expressed as means ± standard deviations of a representative of three similar experiments carried out in triplicate. Statistical analysis was performed by one-way analysis of variance (ANOVA) in conjunction with Tukey's post hoc test for multiple comparisons using SPSS Version 9.0 Software.

Results and discussion

Synthesis of magnetic nanoparticles

In this study, for the synthesis of magnetic nanoparticles the method including co-precipitation of Fe^{2+} and Fe^{3+} salts in aqueous medium was selected. Chemical co-precipitation has been widely used to produce magnetite nanoparticles due to its ease and large volume capability. Conventionally, iron oxide nanoparticles were prepared by adding a base to an aqueous mixture of Fe^{2+} and Fe^{3+} chloride at a 1:2 molar ratio (Scheme 1). Ammonia was chosen as a base for co-precipitation, because it leads to small particles [21]. Average size of the iron oxide nanoparticles was measured as 57 nm by DLS. The magnetic properties of the iron oxide nanoparticles were determined by VSM. The saturation magnetization of the



Scheme 1 Preparation of m-poly(GDMA) latex particles

nanoparticles was 74.37 emu/g and these particles show a typical superparamagnetic behaviour at room temperature without any hysteresis loop.

Synthesis of magnetic poly(GDMA) particles

Because of their hydroxyl functionality poly(GDMA) particles can be easily derivatized and hence biochemical ligands can be covalently attached onto the particles. In addition, the water swellability, and the similarity of its molecular structure to a highly biocompatible material, poly(HEMA), poly(2-hydroxyethyl methacrylate), make poly(GDMA) particles as suitable materials in many biotechnological and medical applications.

In this study, we used single-stage precipitation polymerization method developed recently by Saraçoğlu et al. [16] for the synthesis of monodisperse, crosslinked hydrophilic poly(GDMA) particles. Thermally initiated polymerization was performed in a medium containing acetonitrile and toluene without using a stabilizer. A rather hydrophilic dimethacrylate monomer, GDMA also provided crosslinking due to the presence of bifunctional methacrylate groups in its structure. After the addition of monomer GDMA and initiator AIBN into toluene–acetonitrile mixture, free radicals were formed by the decomposition of AIBN. Primary nucleus particles were generated by aggregation of oligomers. Continuous capture of oligomers from solution suggested that the particle surface is swollen with solvent. This transient surface gel layer stabilized the poly(GDMA) latex particles during their growth stage, by a process termed auto-steric stabilization [22].

Magnetic nanoparticles produced before were added into polymerization medium to prepare m-poly(GDMA) particles. Scheme 1 illustrates the preparation method. It is believed that if a magnetic compound is dispersed in the reaction medium, it adsorbs components of the system including monomer, stabilizer and radicals coming from the initiator [23].

Characterization studies

Figure 1 shows the general view of poly(GDMA) latex particles. They have an average size of 791 nm without coagula and PDI was calculated as 1.03, indicating

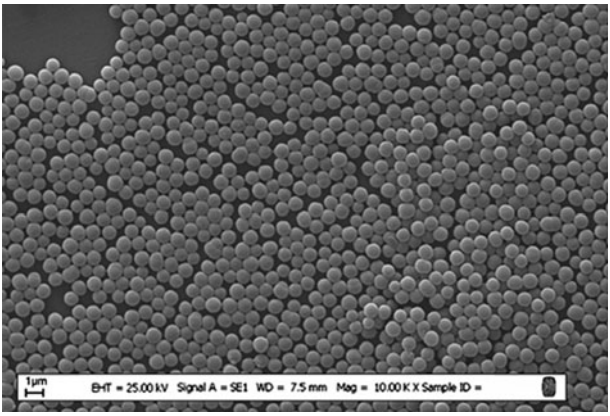


Fig. 1 SEM images of poly(GDMA) latex particles

that the microspheres were monodispersed (a PDI of less than 1.05 is usually considered as being monodispersed [24]). Magnetic-poly(GDMA) latex particles had an average size of 821 nm and PDI was calculated as 1.02. However, they may form clusters consisting more than ten particles.

Figure 2 shows ATR-FTIR spectrum of m-poly(GDMA) latex particles. The peak at 3,500 cm^{-1} was assigned to –OH groups in poly(GDMA). The presence of ester carbonyl groups was confirmed by peak at 1732.3 cm^{-1}. The peak at 1,635 cm^{-1} represented C=C bands. These results indicated that poly(GDMA) was polymerized on the magnetic nanoparticles. Thus, we can conclude that magnetic nanoparticles were located inside the latex particles.

Figure 3 shows the magnetization curve of the m-poly(GDMA) latex particles as recorded by VSM. The saturation magnetization of the m-poly(GDMA) latex particles was 3.85 emu/g and these particles show a typical superparamagnetic behaviour at room temperature without any hysteresis loop. That means in the absence of magnetic field, the magnetization vectors of the m-poly(GDMA) latex

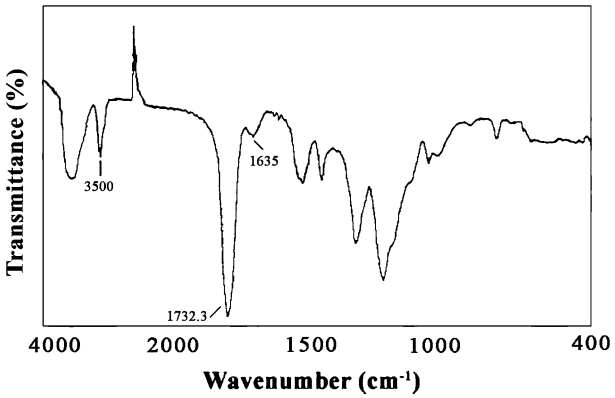


Fig. 2 ATR-FTIR spectrum of m-poly(GDMA) latex particles

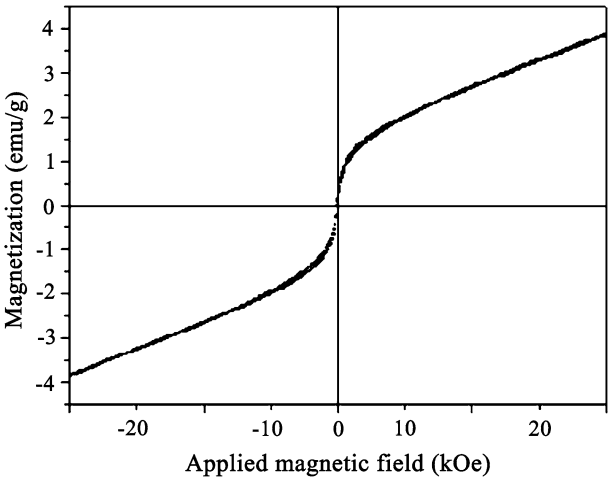


Fig. 3 Magnetization curve of m-poly(GDMA) latex particles obtained by VSM at room temperature

particles are orderless because of thermal movement, and do not exhibit any overall magnetism. Linear interpolation between saturation magnetization of iron oxide nanoparticles and magnetic latex particles can be used to calculate the content of iron oxides in the microspheres and there was a good agreement between the estimated results obtained by VSM and the results obtained by atomic absorption spectrophotometry [9]. When we applied this calculation to our results, it was found that m-poly(GDMA) latex particles have 5.17 wt% of iron oxide nanoparticles since iron oxide nanoparticles have saturation magnetization of 74.37 emu/g.

Cell culture studies

Cytotoxicity test

Cytotoxicity of m-poly(GDMA) latex particles were tested by quantitative analysis using MTT test. L929 mouse fibroblasts grown on TCPS dishes were exposed to the different dilutions of extracts (25, 50 and 100 %) of m-poly(GDMA) particles at the end of 24th hour and proliferation of cells was measured by MTT assay during 3 days. As a control, no extract added cell cultures were used. The extracts of the particles showed no inhibition of the cell metabolism compared to control group. From quantitative scores, it was concluded that the extracts of the particles demonstrated no cytotoxic reactivity in this test as seen in Fig. 4.

Interactions with MC3T3 preosteoblasts

New strategies for bone tissue engineering are based on the use of stimulatory cues, e.g. electrical stimulations, magnetic stimulation, electromagnetic field stimulation, etc., to provide localized and guided regeneration. The results of early studies have indicated that mechanical forces that applied to osteoblasts induce ECM production,

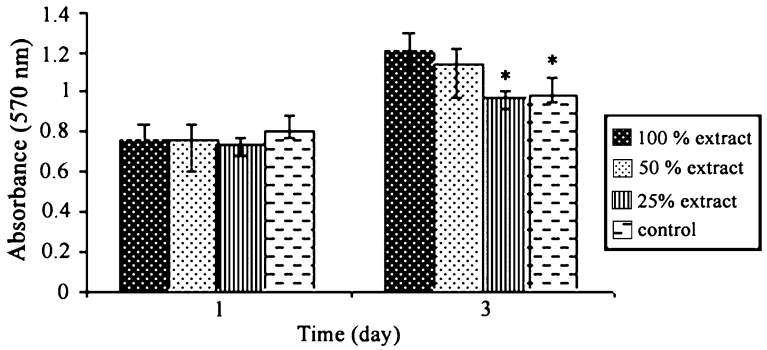


Fig. 4 MTT results for cytotoxicity test of m-poly(GDMA) latex particles during 3 days with L929 cells. No extract added wells were used as control group (statistically significant difference, $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$)

upregulate bone-related gene production and increase osteo-related protein production [25]. This is why, a number of techniques based on compressive or tensile loading have been developed to mechanically stimulate cell seeded scaffolds. Because of the some drawbacks of such those applications, new approaches that apply mechanical strains directly to the cell by means of magnetic particles have been investigated. In those approaches, a magnetic core is usually coated with a polymeric layer or protein layer (collagen type I, RGD, fibronectin, BSA, etc.) that minimize hydrophobic interactions, enhancing colloid dispersion and biocompatibility to allow adherence to the cell membrane and then, magnetic field is applied [26, 27].

Mitochondrial activities of MC3T3-E1 preosteoblasts seeded in the presence of m-poly(GDMA) latex particles (100 pg of particle per cell) was compared with control group (no magnet, no particle) to investigate the direct effect of m-poly(GDMA) latex particles on MC3T3-E1 preosteoblasts proliferation (Fig. 5). The m-poly(GDMA) latex particles at this concentration and the presence of magnetic effect did not enhance or inhibit the growth of cells ($p > 0.05$) during the 5-day culture period. Long-term culture studies (3–4 weeks) have indicated that integrins, when subjected to mechanical stresses, lead to rearrangements in the internal cytoskeleton (CSK), and these changes alter cellular biochemistry [26].

Optical microscope photograph in Fig. 6a shows the fibroblastic morphology of crystal violet dyed MC3T3-E1 preosteoblasts. The interactions between MC3T3-E1 cells and m-poly(GDMA) latex particles were shown by SEM photograph (Fig. 6b) and optical photographs (Fig. 6c, d). SEM image clearly shows that the m-poly((GDMA) particles attached to the MC3T3 cells. Optical views indicate that the particles have not been internalized by the cells, they remained on the outside of the cells during the cultivation period. It was reported that, internalized particles have the potential to influence a number of mechanotransduction signalling pathways [27]. However, in our study, mechanic stress applied by a magnet caused alignment of F-actin stress fibres in adherent cells, although the noninternalization of magnetic particles. Optical photographs in Fig. 6c and d also show that MC3T3 cells responded to mechanic stress by increasing their area of contact compared to control group (Fig. 6a).

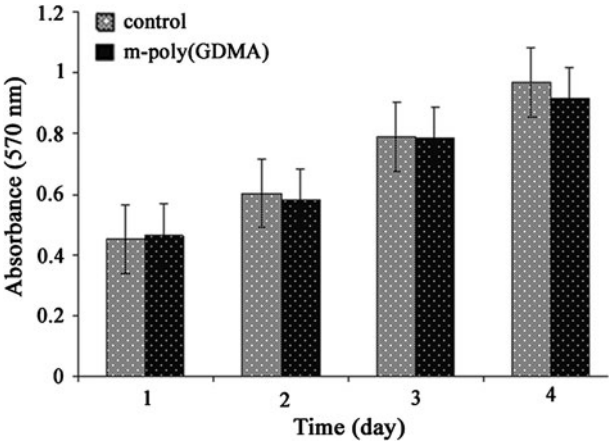


Fig. 5 Mitochondrial activities of MC3T3-E1 cells treated with m-poly(GDMA) latex particles on TCPS surface (statistically significant difference, $n = 3$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, TCPS without latex particles is control group)

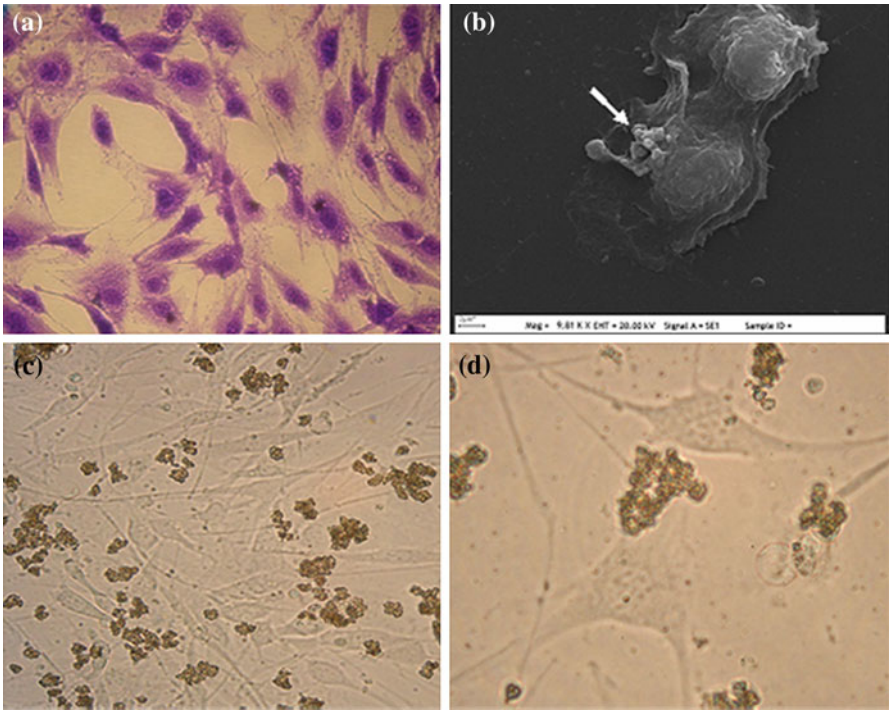


Fig. 6 **a** Optical microscope photograph of crystal violet dyed MC3T3-E1 preosteoblasts. **b** SEM photograph showing the interaction between MC3T3-E1 cells and m-poly(GDMA) latex particles. The *arrow* indicates m-poly(GDMA) latex particles (*bar* 1 μm). **c**, **d** Optical photographs showing the proliferated cells and magnetic particles at the 5th day of culture in magnifications (×20) and (×40), respectively

Conclusion

In this study, magnetic latex particles, m-poly(GDMA), containing magnetite and hydrophilic monomer (glycerol dimethacrylare) were prepared by precipitation polymerization in the diameter of ≈ 800 nm and in monodispersed form. Short-term (up to 1 week) studies in cell cultures suggest that m-poly(GDMA) particles are reasonably well tolerated by L929 mouse fibroblasts and MC3T3 preosteoblasts, with no obvious signs of cell toxicity observed. Although these results are preliminary, m-poly(GDMA) particles appear to have an influence on osteoblastic activity.

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