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DENDRIMER FUNCTIONALIZED RADIATION GRAFTED HYDROGELS FOR TISSUE ENGINEERING APPLICATIONS

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ABSTRACT

Low density polyethylene (LDPE) films were modified by γ-ray radiation grafting of 2-hydroxyethylmethacrylate (HEMA). The covalent immobilization of polyglycerol dendrimer (PGLD) on LDPE-G-HEMA surface was performed by using dicyclohexyl carbodiimide (DCC)/N,N-dimethylaminopyridine (DMAP) method. The occurrence of grafting polymerization of HEMA and further immobilization of PGLD was quantitatively confirmed by photoelectron spectroscopy (XPS) and fluorescence, respectively. The LDPE-G-HEMA surface topography after PGLD coupling was studied by atomic force microscopy (AFM). The hydrophilicity of the LDPE-G-HEMA film was remarkably improved compared to that of the ungrafted LDPE. The core level XPS ESCA spectrum of HEMA-grafted LDPE showed two strong peaks at ~286.6 eV (from hydroxyl groups and ester groups) and ~289.1 eV (from ester groups) due to HEMA brushes grafted onto LDPE surfaces. The results from the cell adhesion studies shows that MCT3-E1 cells tended to spread more slowly on LDPE-G-HEMA than on LDPE-G-HEMA-i-PGLD.

1. INTRODUCTION

The twentieth century was a watershed for the development of lifesaving surgical procedures and techniques that also vastly improved the quality of human life. The clinical surgeries were informed by the extraordinary explosion in knowledge of human anatomy, physiology, and biology. All the knowledge converged to a complex array of pre-existing lines of work from the interdisciplinary between the clinical medicine, engineering, and science. Actually, an intense research has been devoted to the development of scaffolds in the area of medicine that has come to known as tissue engineering.

The success of polymeric scaffolds is determined by the response it elicits from the surrounding biological environment. This response is governed, to a large extent, by the surface properties of the scaffold. Multiple approaches have been developed to provide micrometer to nanometer scale alterations in polymer surface to enable improved protein and cell interactions. Chemical modification of polymeric scaffold surfaces by radiation grafting is one of the upcoming approaches that have been employed successfully to providing cell adhesion on polymeric scaffolds [1-2].

The achievements regarding cell culture on hydrogels surfaces prepared by radiation-grafting can be described as promising techniques to prepare scaffolds to tissue engineering due to their biocompatibility and a vast literature has been published here by Hoffmann and co-workers [3-8]. However, the cellular response to radiation-grafted polymer surfaces including adhesion and growth of cells cannot be controlled or modified through changes in the graft-polymer structure. Graft copolymers do not possess functional groups, other than end groups, that permit chemical modification to change their properties, thereby limiting the applications of these materials in tissue engineering.

There is a need the development of polymer materials which include a sufficient concentration of derivatizable groups to permit the chemical modification of the polymer for different biomedical applications. This strategy is important to providing a better control of the process of cell adhesion and growth on synthetic surfaces.

Macromolecules with dendritic structure appear to be promising alternatives because of their high periphery functionality. Polymers with a dendritic (multi-level) branched architecture have been investigated extensively over the past twenty years. These fascinating macromolecules have tree-like structures, with “branches” consisting in repeating units divergently emerging from a central core [9-10].

The functional groups on dendrimer periphery make it possible the covalent or ionic attachment of a biological molecule to the graft copolymer. The dendrimer-functionalized graft copolymers can be used in a wide range of biomedical applications including tissue engineering and drug delivery.

The present work was dedicated to the design and development of new biomaterials suitable for hard tissue implants. The study aims the synthesis of 2-hydroxyethyl methacrylate (HEMA) grafted onto low density polyethylene (LDPE-G-HEMA) based polymer matrix functionalized with polyglycerol dendrimer (PGLD). The chemical strategy of this research was structured on two parts: 1) the synthesis of the radiation graft copolymer LDPE-G-HEMA and 2) the immobilization of the PGLD onto the graft copolymer.

2. MATERIALS AND METHODS

2.1 Radiation grafting of HEMA onto low density polyethylene films and characterization

The 2-hydroxyethylmethacrylate (HEMA) monomer was purchased by Sigma-Aldrich Co and was distilled in vacuum before using. Commercial LDPE films of 50 μm thickness supplied by Union Carbide were used as a substrate for the radiation graft polymerization. The LDPE films were cut into 2.0x2.0 cm pieces, and ultrasonically cleaned twice in methanol for 5 min, and dried in a vacuum oven at 50 ºC for 24 h. All other chemicals were reagent grades. The graft polymerization reaction was carried out in 100 mL glass ampoule containing a methanol (MeOH) solution of HEMA (30% w/w). The HEMA grafted onto LDPE (LDPE-G-HEMA) was synthesized by placing a LDPE film in a glass ampoule in a solution of HEMA/MeOH (30% w/w). The ampoule was degassed by bubbling nitrogen (N_2) for 15 min., sealed and then irradiated with a ^{60}Co γ-source at an irradiation dose of 0.5-2.0

kGy and a dose rate of 0.061 kGy.h^{-1} to give graft yields on the interval of 5-50% (w/w). The HEMA homopolymer was removed by washing the LDPE-G-HEMA films with 400 mL water:MeOH mixture (30:70) several times (for 1 h each) on the magnetic stirrer at room temperature (25 ºC). The LDPE-G-HEMA films were then extracted with water:MeOH (30:70) in a Soxhlet for 8 h to remove the undesirable residual monomer and HEMA homopolymer, and finally dried under reduced pressure (10^{-3} mbar) at room temperature (25 ºC). The grafting yield was determined by the percentage increase in weight of the LDPE films and the grafting process was confirmed by photoelectron spectroscopy (XPS). The grafting yield was calculated by the equation:

$$Grafting(\%) = \frac{(W_f - mW_i)}{W_i} x100 \qquad (1)$$

where W_f and W_i are the weights of the samples before and after grafting.

The chemical compositions of the nongrafted and functionalized LDPE surfaces were determined by XPS. The XPS measurements were performed on a ESCA-36 McPherson spectrometer with a monochromatic Al K_α X-ray source (1486.71 eV photons) at a constant dwelling time of 100 ms and a pass energy of 40 eV. The samples were mounted on standard sample studs by means of double-sided adhesive tape. The core-level signals were obtained at a photoelectron takeoff angle (measured with respect to the sample surface) of 90º. The X-ray source was run at a reduced power of 225 W (15 kV and 15 mA). The pressure in the analysis chamber was kept at 10^{-7}-10^{-8} Torr during each measurement. All binding energies (BEs) were referenced to the C1s hydrocarbon peak at 284.8 eV. Surface elemental stoichiometries were determined from the spectral area ratios, after correction with the experimentally determined sensitivity factors, and were reliable to within $\underline{+}$10%. The elemental sensitivity factors were calibrated with stable binary compounds of well-established stoichiometries.

For determination of equilibrium water absorbency, the LDPE-G-HEMA graft copolymers were immersed into phosphate buffered saline solution (PBS), pH 7.0 at 37 ºC for different periods of time. It was found that the equilibrium water absorbency is achieved after 2.0 h. In order to determine the equilibrium water absorbency gravimetrically, the surface of the swollen LDPE-G-HEMA film was wiped with filter paper in order to remove the free water and then the samples were weighed. Swelling percentage was determined by the equation:

$$Swelling(\%) = 100[(W_s - W_d)/W_d] \qquad (2)$$

where Ws and W_d are weights of the swollen and initial films, respectively.

The hydrophilicity/hydrophobicity of the LDPE-G-HEMA surfaces was measured by contact angles with deionized water. The contact angles where measured in air by sessile-drop method using a home made optical contact angle goniometer. The LDPE and LDPE-G-HEMA films were conditioned at the equilibrium humidity of the instrument. The water contact angle was measured by putting a sessile drop (3 μL) of bidistilled and deionized water on five different sites of air-side surface of the polymer surfaces (1x1 cm). Three angles were measured on each sample. This was repeated for a total of three samples.

To investigate the surface topography of LDPE modified surfaces, atomic force microscopy was used. Atomic force microscopy was performed on a commercial SPM-9500 J3, Shimadzu using optical beam deflection to monitor the displacement of a microfabricated silicon cantilever having a spring constant of 60 N/m. The AFM was performed with silicon nitride probes mounted on cantilevers in tapping mode to avoid surface damage. The force of interaction was approximately 10^{-9} N. This method significantly improves lateral resolution on soft surfaces and thin films. The AFM images were obtained under ambient laboratory conditions and all the scans were two micron in size.

2.2 Polyglycerol dendrimer synthesis and characterization

PGLD with generation 3 (G-3) was synthesized by step-by-step allylation and dihydroxylation reactions in according to Haag et al [11]. A PGLD dendrimer with a number average molecular weight of 1,7 kDa with an average of 26 hydroxyls per molecule was synthesized in a step-growth process denominated divergent synthesis. The successful synthesis of PGLD G-3 (88% yield) was confirmed by ^{1}H-NMR: (300 MHz, CD_3OD). Shift (δ, ppm): 4.8 (OH), 4.04 (OCH_2-CH-CH_2), 3.95-3,40 (CH_2-O-CH_2). ^{13}C-NMR (75 MHz, CD_3OD). Shift (δ, ppm): 62.9 (CH_2-OH, terminal unity), 82.0-81.5, 80.6-79.8, 74.7-73.9, 73.8-72.2, 71.8-70.7, 64.7, 63.1 (CH_2-O-CH_2, polyether backbone).

A Bruker MALDI-MS with time-of-flight (TOF) mass analyzer and microchannel plate detector (MCP) was used for the determination of large molecular weight PGLD dendrimer molecules. The energy source was a nitrogen laser operating at a wavelength of 337 nm and pulse width of 3 ns. The matrix used was indoleacrylic acid which was prepared by forming a saturated solution in acetone. Then a target plate was spotted with 5 µL of matrix and followed by 5 µL of dilute sample. The sample was allowed to completely dry before use. Standards were used to calibrate the instrument. The real mass of the PGLD G-3 determined from MALDI mass spectra were found to be 1721.8 (+Na) (Figure 1). This value was consistent with the theoretical molecular weight of PGLD G-3 (1689) reported by literature [11].

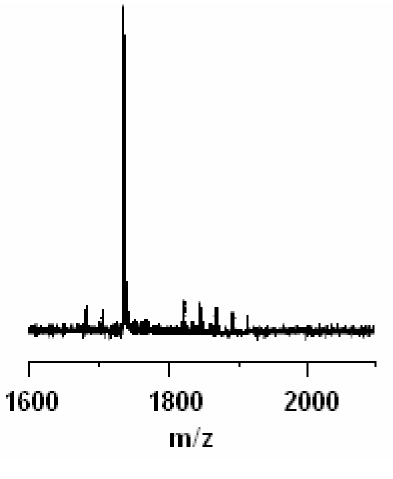

Figure 1- MALDI-TOF mass spectra of polyglycerol G3.0 dendrimer.

2.3 PGLD immobilization onto HEMA hydrogels

The PGLD was immobilized onto LDPE-G-HEMA fims using the PGLD was coupled to LDPE-G-HEMA hydrogels using dicyclohexyl carbodiimide (DCC)/N,Ndimethylaminopyridine (DMAP) as coupling esterification methodology [12]. The LDPE-G-HEMA was immersed in a 1:4 mixture of pyridine and CH_2Cl_2 containing 30 mol% of DMAP and PGLD at a concentration of 0.5 M. After stirring at room temperature (25ºC) for 6h, the LDPE-G-HEMA containing the coupled PGLD (LDPE-G-HEMA-i-PGLD) was transferred to a solution of water/pyridine (1:1 v/v) and maintained for 24 h. The LDPE-G-HEMA-i-PGLD was then washed with $NaHSO_4$ (1M), then (Na_2CO_3 (10% w/w) and finally, with saturated NaCl solution to remove pyridine and DMAP. The esterification reaction between PE-g-HEMA and PGLD was accomplished by fluorescence measurements (Varian Cary Elipse). Previously to the immobilization onto LDPE-G- HEMA chains, PGLD was labeled with rhodamine B by reacting the PGLD G-3.0 with 8 equiv of rhodamine B isothiocyanate (6.0×10^{-5} mol) [13]. The labeled PGLD was purified by dialysis until no traces of free dye was detected by HPLC. Figure 2 illustrates the reaction scheme of the HEMA radiation grafting onto LDPE and PGLD immobilization onto LDPE-G-HEMA surface.

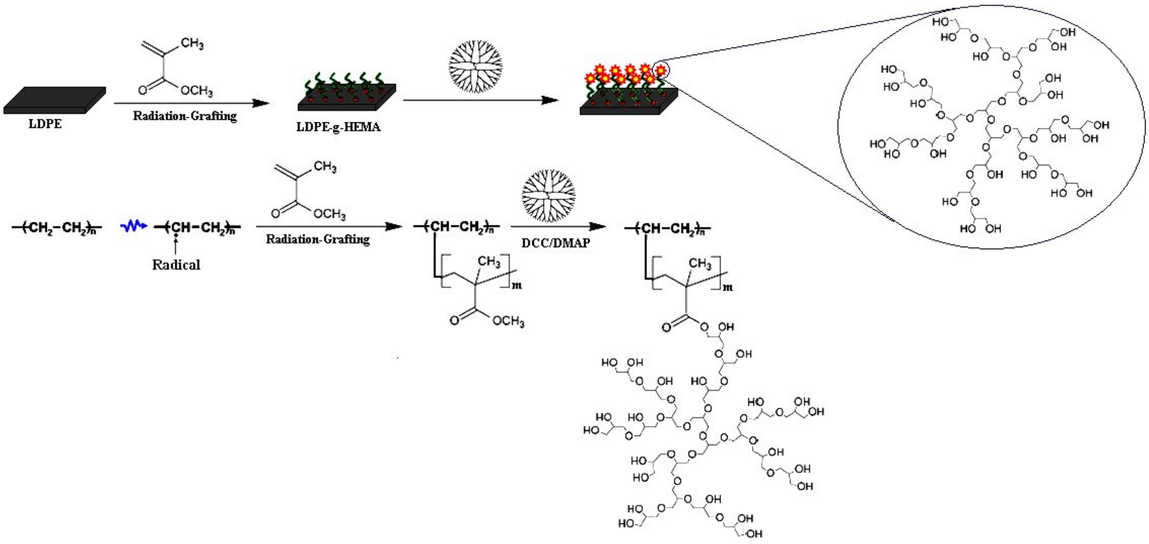

Figure 2- Illustration of the radiation grafting of the LDPE-G-HEMA-i-PGLD synthesis.

2.4 Cell adhesion

MC3T-E1 osteoblast cell adhesion on LDPE-G-HEMA and LDPE-G-HEMA-i-PGLD were analyzed by neutral red assay after 2, 4, 6 and 24 h. MC3T3-E1 cells, an osteoblastic cell line established from normal mouse calvaria, were grown in α-modified minimum essential medium (α-MEM; 1.8 mM Ca^{2+}, 0.81 mM Mg^{2+}, 1.0 mM H_2PO_4, 50 μg/mL ascorbic acid) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (GIBCO-BRL) in 5% CO_2 at 37°C. The medium was changed twice weekly. After 7 day culture, the MC3T3-E1 cells were removed from the culture dish using trypsin solution (0.25% trypsin, 0.05% EDTA, 0.1% glucose in PBS), centrifuged and resuspended in DMEM medium to adjust the cell density to 2.0×10^5 cells/mL. A volume of 100 μL of the cell suspensions were placed on LDPE-G-HEMA and LDPE-G-HEMA-i-PGLD. Tissue culture polystyrene commercial 24-well cell culture plates were used as control. To acess the number of viable adherent cells on LDPE-G-HEMA and LDPE-G-HEMA-i-PGLD substrates neutral red assay (NR) was performed. At the end of cell incubation time at 37 ºC the medium was removed and replaced with 200 μL/well of NR solution (0.4% w/v in water). After the reaction time, the medium was discarded and the cultures wshed twice with PBS. The cells were lysed with 1% (w/v) acetic acid in 50% ethanol solution for 10 min to release the dye. The lysates were collected and the optical density was measured (540 nm).

3. RESULTS AND DISCUSSION

In order to verify the presence of HEMA on LDPE surfaces, XPS analysis was carried out. LDPE and LDPE-G-HEMA surfaces were characterized by XPS. The high resolution C1s and O1s core levels were taken on LDPE-G-HEMA surface. After the HEMA grafting on LDPE, the oxygen atomic percent of LDPE-G-HEMA increased at the expense of a decrease in the carbon atomic percent. Using the peak fit software available with the XPS instrument; these two peaks can be analyzed. As can be seen in Figure 3 the values of the ratios CO/CH peaks of different grafting yields were calculated and plotted against the degree of grafting. It can be seen that ratios of the CO/CH peaks increased with the grafting yield and level off at a 50% (w/w) grafting yield. This means that the distribution of grafted chain in the surface increased with increasing grafting yield. At saturated level of CO/CH ratio, the distribution of HEMA grafted chain may be interpreted as becoming homogeneous on the surface of the polymeric matrices.

Figure 4 showed the influence of grafting yield on the water contact angle and water uptake of the final grafted LDPE. The water contact angle decreased sharply from 92º to 42º at grafting yield of 30% (w/w) and then decreased slowly to a constant value of 32º at grafting yield of 50% (w/w). The water uptake increased correspondingly. The reduction of water contact angle is certainly caused by the introduction of HEMA. Therefore, it can reveal relatively the amount of HEMA on LDPE surface. Surfaces with contact angles in the range of 20–60° are generally considered to be hydrophilic and show more resistance to protein adsorption [14].

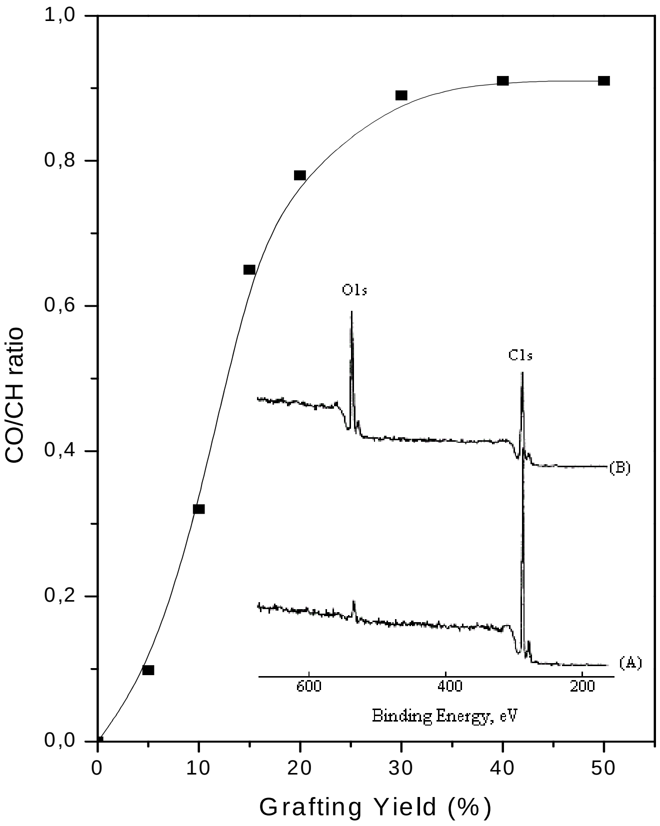

Figure 3- CO/CH ratio for LDPE-G-HEMA obtained from XPS spectra (inside) of LDPE (A) and LDPE-G-HEMA (B). Grafting yield: 50% (w/w).

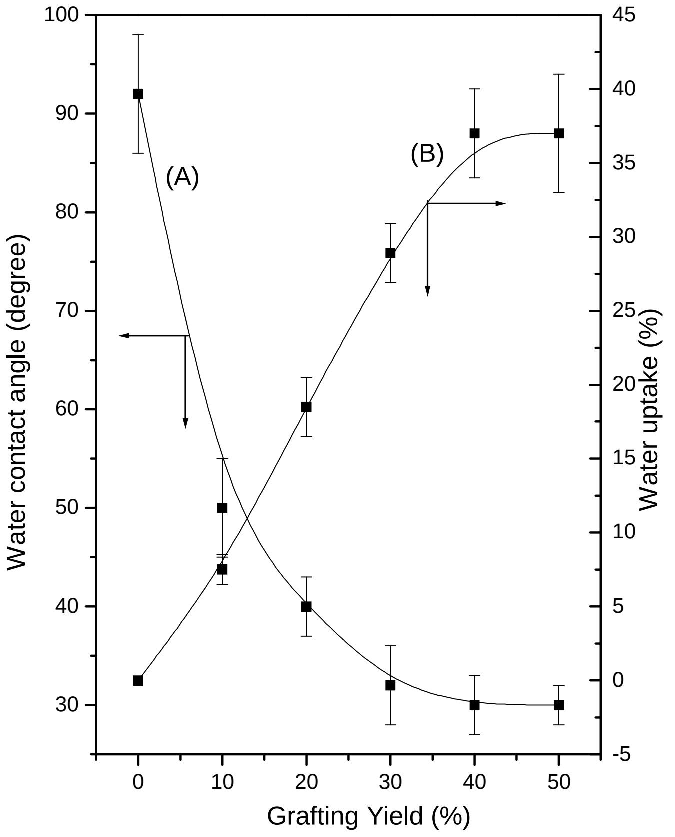

Figure 4- Water contact angle measurement (A) and water uptake (B) of the grafted LDPE films as a function of grafting yield (%).

Dendrimer macromolecules macromolecules are open and soft material rather than rigid and due to this reason they tend to deform on surfaces. The electrostatic interactions between the brushes of grafted HEMA on LDPE surfaces and the surface groups of PGLD may produce deformations on immobilized dendritic macromolecule (PGLD). The changes in the topography of the LDPE-G-HEMA surfaces after PGLD immobilization were studied by AFM. Representative AFM images of the LDPE-G-HEMA and LDPE-G-HEMA-i-PGLD are shown in Figure 5. The surface roughness of the LDPE-G-HEMA increased significatively after PGLD immobilization indicating that the hydroxyl groups of dendrimer macromolecule were activated and efficiently immobilized onto the brushes of grafted HEMA chains. In the AFM images of Figure 5, one can see many separate and randomly globular structures on the LDPE-G-HEMA-i-PGLD surface. In each image, the particles appear to be substantially uniform in size; i.e., they appear to be essentially monodisperse. The driving force for dendrimer molecules to aggregate into globular structures can be related to their dewetting properties on the LDPE-G-HEMA surface. There are a few large irregular clusters in Fig. 5, perhaps caused possibly by dendrimer aggregation due to the HEMA brushes interaction.

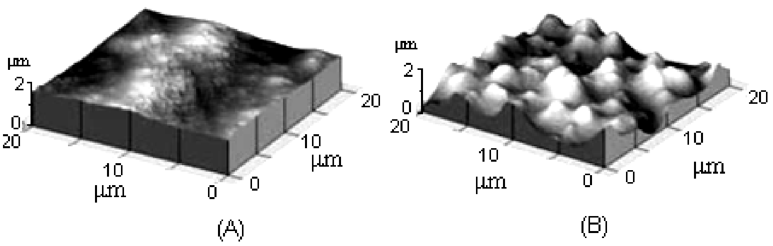

Figure 5- AFM images of LDPE-G-HEMA (A) and LDPE-G-HEMA-i-PGLD (B) surfaces.

The results from the MC3T3-E1 osteoblast cell adhesion tests are shown in Figure 6. After cultivation on LDPE-G-HEMA and LDPE-G-HEMA-i-PGLD for 1 h, only 20% of MCT3T3-E1 cells were judged as spread on the polymer surfaces. During the next few hours, however, the percentage of spread cells increased rapidly from 20 to 80%, and all cells finished spreading within 24 h (Fig. 6). Cells tended to spread more slowly on LDPE-G-HEMA than on LDPE-G-HEMA-i-PGLD (Fig. 6). A further interesting possibility for the LDPE-G-HEMA-i-PGLD would be to immobilizing on dendrimer surface certain growth factors (osteogenic protein-1, BMP-2 or IGF-1/TGF-b1), which are known to stimulate bone formation could be tied to the biomaterial.

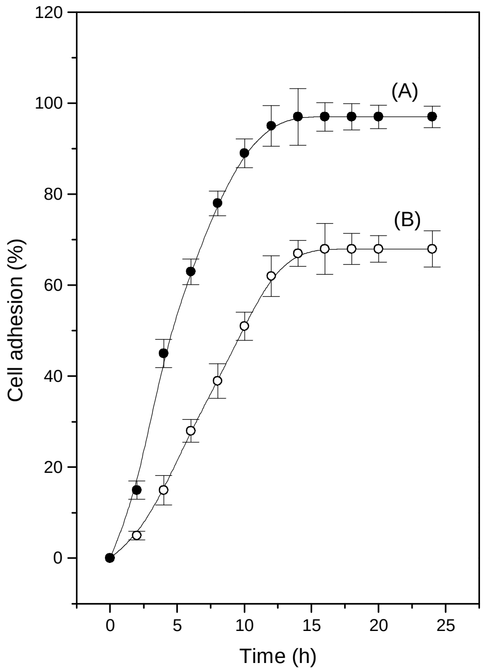

Figure 6- MC3T3 osteoblast cell adhesion assays on LDPE-G-HEMA (A) and LDPE-G-HEMA-i-PGLD (B).

CONCLUSIONS

The results of this study clearly show that osteoblasts are able to grow onto LDPE-G-HEMA-i-PGLD. The favorable cell attachment properties show promise for use of LDPE-G-HEMA-i-PGLD in bone tissue engineering. Since LDPE-G-HEMA-i-PGLD possesses multiple hydroxyl groups that are easily functionalized, it can be tailored for specific purposes. This newly developed LDPE-G-HEMA-i-PGLD might reduce the need for autologous bone and is therefore an interesting alternative to biomaterials field. The next step will be to test the LDPE-G-HEMA-i-PGLD in *in vivo* animal experimentation. The results of this *in vitro* study are quite promising; therefore there is a high chance that the animal experiments will be also successful.

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