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Hydrogel-mediated DNA delivery confers estrogenic response in non-responsive osteoblast cells

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Abstract

Oligo (polyethylene glycol) fumarate (OPF) hydrogel has been employed in musculoskeletal tissue engineering for photo-encapsulation of chondrocytes and as a matrix for marrow stromal cells differentiation. In this study, we have studied the application of OPF hydrogel for co-encapsulation of DNA and bone cells and examined whether co-encapsulation can enhance gene transfer by maintaining the DNA within the cellular microenvironment. Our results showed that plasmid DNA encoding green fluorescence protein (GFP), co-encapsulated with bone tumor cells, was capable of transfecting the cells and the transfected tumor cells continuously expressed GFP protein over the time course of study (21 days). Furthermore, we have examined the co-encapsulation of estrogen receptor (ER) encoding plasmid DNA and human fetal osteoblast cells (hFOB) that lack endogenous ER. Our results show that the transfected cells responded to estrogen as alkaline phosphatase (ALP), and estrogen response element (ERE)-directed luciferase enzyme activities increased with estrogen-treatment. Taken together, these studies show that OPF hydrogel could be further explored for targeted gene delivery in bone and other tissues encapsulated within the hydrogels.

Keywords

Bone tissue engineering; DNA delivery, Hydrogel; Osteoblast, Estrogen receptor

INTRODUCTION

Polymer-based matrices, microspheres and nanospheres have been employed in the past by various research groups for controlled release systems for proteins, such as hormones, cytokines and growth factors.¹⁻³ Among them, photocrosslinkable synthetic hydrogels are receiving increased attention for cell and protein delivery vehicles in tissue engineering due to spatial and temporal control over crosslinking.⁴ Recently, Nakayama et al reported the release of adenovirus-associated DNA from vascular stents coated with photocrosslinkable hydrogels.⁵ Other investigators demonstrated that photo-polymerization was compatible with DNA encapsulation, and through the use of protection agents, the damaging effect of photo-initiated radicals could be reduced.⁴

Estrogen exerts its physiological actions mainly through two known estrogen receptors, ER α and ER β .^{6,7} The classical action of estrogen involves transcriptional induction, which involves binding of ER to DNA and is known as genomic effect. However, it has been demonstrated that ER mediates transcriptions that are not dependent on ER binding to DNA, but require the presence of other transcription factors, and also that ER can directly bind to

functional proteins and trigger regulatory events.⁸ Estrogen also elicits anti-apoptotic effects, stimulation of nitric oxide and prolactin synthesis, which do not directly involve transcriptional regulation by ER. These are known as non-genomic effects. The evaluation of estrogenic status and the mechanism of action of estrogen are important in many pathophysiological conditions.

Current strategies to enhance nonviral gene delivery involve the complexation of DNA with cationic polymers or lipids. Cationic polymers or lipids can self-assemble with DNA to form particles that are capable of being endocytosed by cells.⁹⁻¹⁰ In general, DNA delivery from biomaterials can be categorized into two fundamental approaches: sustained release and immobilization. Sustained-release systems are designed to maintain elevated concentrations locally by supplying DNA to balance the loss by degradation or clearance. Alternatively, DNA can be immobilized within a biomaterial scaffold. Synthetic systems based on the immobilization of nonviral DNA complexes have a guiding principle that the substrate must be designed to maintain the DNA locally, yet allow for cellular internalization.

We have previously reported that oligo(polyethylene glycol) fumarate (OPF) could be crosslinked using long wavelength UV light and mechanical property, and swelling ratio of the hydrogels could be controlled by the change in crosslinking levels.¹¹ We have also shown that photocrosslinkable OPF hydrogel can be successfully used for encapsulation of the chondrocytes and as a matrix for osteoblastic differentiation of marrow stromal cells.¹¹⁻¹² In our present work, we have used OPF for co-encapsulation of DNA and bone cells. We hypothesized that co-encapsulation of DNA complexes with cells can enhance gene transfer by maintaining the DNA within the cellular microenvironment. We have employed photocrosslinkable OPF hydrogels as matrix to deliver ER DNA and induce estrogenic response in human fetal osteoblast (hFOB) cells, which lack endogenous ER.

MATERIALS AND METHODS

DNA synthesis

DNA plasmid encoding for green fluorescent protein (GFP) was purchased from Invitrogen (Carlsbad, CA). Plasmid DNAs encoding FLAG-ER and luciferase constructs containing estrogen response element (ERE) were kindly provided by Dr. David Monroe (Mayo Clinic, Rochester MN).¹³ Synthesis and purification of the plasmids were carried out as described (Qiagen Inc., Valencia, CA).

Macromer synthesis

OPF was synthesized using poly(ethylene glycol) (PEG) with the initial molecular weight of 10,000 according to previously published methods.¹⁴ Briefly, 50g PEG was dried by azeotropic distillation from toluene and dissolved in 500 mL distilled methylene chloride. The resulting PEG was placed in an ice bath and purged with nitrogen for 10 minutes, then 0.9 mol triethylamine (TEA; Aldrich, Milwaukee, WI) per mol PEG and 1.8 mol distilled fumaryl chloride (Acros, Pittsburgh, PA) per mol PEG were added dropwise. The reaction vessel was then removed from the ice bath and stirred at room temperature for 48 hrs. For purification, methylene chloride was removed by a rotary evaporator. The resulting OPF was dissolved in ethyl acetate and filtered to remove the salt from the reaction of TEA and chloride. OPF was re-crystallized in ethyl acetate and vacuum dried overnight. The average molecular weight (MW) of OPF was 16246 ± 3710 as determined by gel permeation chromatography (GPC).

Hydrogel formulation

Hydrogels were made by dissolving OPF macromer to a final concentration of 33% (w/w) in deionized water containing 0.05% (w/w) of a photoinitiator (Irgacure 2959, Ciba-Specialty Chemicals, Tarrytown, NY) and 3.3% (w/w) N-vinyl pyrrolidinone (NVP). To produce positively charged hydrogels, 3.3% (w/w) [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC) (75%, Aldrich) was added to the solution. The OPF/MAETAC mixture was pipetted between glass slides with a 1 mm spacer and polymerized using UV light (365 nm) at an intensity of $\sim 8 \text{ mW/cm}^2$ (Blak-Ray Model 100AP) for 10 min.

Attenuated total reflectance Fourier transform infrared microscopy (ATR-FTIR)

The surface of OPF hydrogels with and without modification was characterized using micro ATR-FTIR spectroscopy (Nicolet 8700), coupled to a Continuum microscope (Thermo Electron Corp., Madison, WI). The microscope utilizing an ATR slide on germanium crystal and spectra were collected at the resolution of 4 cm^{-1} for 128 scans with a sampling area of $150 \times 150 \text{ }\mu\text{m}$.

Swelling measurements

After crosslinking, OPF hydrogel were cut into the disks of 10 mm diameter, weighed (W_i) and placed individually into the well of 12-well tissue culture plate containing 2.5 mL PBS. Swollen samples were weighed (W_s) at different time points. The swelling ratio of the hydrogels was calculated using the following equations: Swelling ratio = $(W_s - W_i)/W_i$

DNA encapsulation

OPF (330 mg) was dissolved in 1 mL PBS and 500 μg plasmid DNA encoding green fluorescent protein (GFP) was added to the macromer solution and frozen overnight at -80°C . To produce positively charged hydrogels, 50 μL MAETAC (75%, Aldrich) was added to the solution. The frozen DNA/macromer solutions were then lyophilized for 24 h. The freeze-dried DNA/macromer solutions were rehydrated to a concentration of 33% with PBS containing 33 μL N-vinyl pyrrolidinone (NVP) and 0.05% (w/w) Irgacure 2959 (Ciba-Specialty Chemicals). The macromer/DNA mixture was pipetted between glass slides with a 1 mm spacer and polymerized using UV light (365 nm) at the intensity of $\sim 8 \text{ mW/cm}^2$ (Blak-Ray Model 100AP) for 5 min.

DNA release

Polymerized gels were cut into 7 mm diameter disks, and placed in 2.5 ml PBS at 37°C on an orbital shaker. Buffer was collected every 3-4 days, and DNA concentration was determined by the PicoGreen assay according to the manufacturer's guideline. Parallel gels without DNA were used to eliminate the effect of hydrogel degradation products on the PicoGreen assay.

Cell culture

MG-63 cells (human osteosarcoma cell line, ATCC) were grown to confluence in standard culture flasks in a 1:1 mixture of Dulbecco's Modified Eagle Medium (DMEM) (Gibco BRL, Rockville, NY) supplemented with 10% Fetal Bovine Serum (Invitrogen), 2mM l-glutamine and 1.5 g/L sodium bicarbonate and incubated in a humid environment with 5% CO_2 . Culture medium was changed every 2 days. The cells were then trypsinized and used in transfection experiments.

Cell transfection by plasmid DNA

Transfection experiments in MG63 cells were performed to check the biological functionality of plasmid DNA. DNA was first complexed with Jet-PEI (Qbiogene, Irvine, CA) as transfection agent at 3 μ L Jet-PEI/ μ g DNA for 20 min. One mL of 33% OPF macromer was added to the solution of DNA complex and frozen overnight at -80°C , followed by lyophilizing for 24 hrs. The freeze-dried DNA/macromer solutions were rehydrated with PBS containing NVP and initiator to the volume of 1 mL, as described above, and mixed with MG63 cell (25×10^6) pellets. The cell mixture was transferred to the glass mold with spacer and polymerized using 365 nm light at the intensity of $\sim 8 \text{ mW/cm}^2$ for 10 min. The same number of cells without incorporation of plasmid DNA was encapsulated into the hydrogel as control. The resulting hydrogel-cell constructs (7 mm in diameter and 1 mm in thickness) were placed into the 12-well plates with 2.5 mL culture media. The constructs were harvested after 14 and 21 days and transfection of the encapsulated cells with entrapped plasmid encoding GFP was assessed by confocal laser microscopy using a FITC filter (excitation 480 nm, emission 535 nm). Viability of the encapsulated cells with and without DNA was determined using Live/Dead Kit (Molecular Probes, L3224).

hFOB cell culture

ER-negative human fetal osteoblast cells (hFOB) were kindly provided by Dr. Thomas C. Spelsberg (Mayo Clinic, Rochester, MN) and have been previously described.¹⁵

Estrogen response of transfected hFOB cells

To quantify the functionality of the transfected cells, plasmid DNA encoding FLAG-ER13 was complexed with Jet-PEI and co-encapsulated with 25×10^6 hFOB cells into the hydrogels with different formulation as described above. hFOB cells encapsulated without plasmid DNA were used as control. The hydrogel-cell constructs were placed into the 12-well plates with 2.5 culture media of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco BRL, Rockville, NY, USA) and Ham's F12 medium (Nissui Pharmaceutical, Japan), supplemented with 10% charcoal-stripped Fetal Bovine Serum (Invitrogen), 2 mM L-glutamine and 1.5 g/L sodium bicarbonate and incubated in a humid environment at 34°C with 5% CO_2 . Three days post-transfection, the cells were replaced with fresh medium and treated with 20 nM 17 β -estradiol (Sigma Chemical Co., St. Louis, MO). The cells encapsulated in the polymer were harvested 4 days after estrogen treatment and processed for immunostaining, alkaline phosphatase activity and luciferase assays.

Immunostaining

For immunostaining, cells were washed in phosphate-buffered saline (PBS) and fixed for 20 min at room temperature in 2% Paraformaldehyde in PBS. Cells were rinsed with $1 \times$ TBS, permeabilized with 0.2% tritonx-100 for 10 min. This was followed by a rinse with $1 \times$ TBS and incubation with 10 $\mu\text{g/mL}$ anti-FLAG-FITC antibody (Sigma) for 60 min at room temperature. At the end of the incubation, cells were rinsed with $1 \times$ TBS and visualized under microscope.

Alkaline phosphatase activity

Alkaline phosphatase (ALP) activity of encapsulated cells was measured as described¹⁶ after homogenizing the specimens with tissue grinder. Twenty-four hrs post-transfection, the cells were treated with 17 β -estradiol and harvested after 72 hrs of treatment. ALP activity was measured and normalized to that of untransfected cells.

Luciferase assay

Transfection of Luciferase constructs and the Luciferase assays were performed as described. 17 hFOB cells were co-transfected with FLAG-ER and ERE-Luciferase constructs using Jet-PEI as described above. All transfections performed had an internal transfection control plasmid containing Renilla Luciferase (pRL-CMV-Luciferase) (Promega), which allowed the normalization of Luciferase units. Twenty-four hours post transfection, the cells were treated with 20 nM 17 β -estradiol. The cells were harvested after 48 hrs of treatment using 300 μ L of passive lysis buffer provided in a Luciferase Assay Kit (Promega). Luciferase assays were performed using the dual Luciferase reporter assay system according to the manufacturer's protocol (Promega) and read on a TD-20E luminometer (Turner, Sunnyvale, CA).

Statistical analysis

All data are reported as mean $\pm$ standard deviation (SD) for $n = 3$. Single-factor analysis of variance (ANOVA) was performed with StatView version 5.0.1.0 (SAS Institute, Inc, Cary, NC) to assess the statistical significance of the results. Bonferroni's method was employed for multiple comparison tests at significance levels of at least 95%.

RESULTS

Hydrogel structure and characterization

Figure 1 reveals chemical structure of OPF macromer and MAETAC monomer. OPF was crosslinked in the presence of initiator and NVP as an accelerator and co-monomer for photo-crosslinking. To produce positively charged hydrogel, MAETAC, which is a bifunctional molecule containing both a pH-independent cationic head (quaternary ammonium) and a reactive methacryl group, was copolymerized with OPF hydrogel. ATR-FTIR spectrum of OPF hydrogel after crosslinking is shown in Fig. 2a. Bands at 1650 and 1086 cm^{-1} are assigned to carbonyl and carbon oxygen bonds (C-O-C) of OPF, respectively. After copolymerization with MAETAC, a new peak emerged at 1725 cm^{-1} that is characteristic of methacryl carbonyl from MAETAC (Fig. 2b).

Hydrogel swelling

The swelling ratio of the OPF hydrogels was calculated over the course of the release study (Fig. 3). All swelling ratio profiles remained unchanged during the release for 21 days. In general, the equilibrium swelling of positively charged hydrogels was significantly greater than hydrogels without charge ($p < 0.05$).

DNA release

Figure 4 reveals DNA release profile from hydrogels with and without charge. An initial release of about 30% was observed from neutral hydrogel after 3 days. This trend continued over 10 days followed by a sustained release over 28 days. Unlike neutral hydrogels, there is almost no release of DNA from positively charged hydrogels over three weeks of study. After 28 days, only small amount of DNA was released from this hydrogel formulation. This can be attributed to complexation of DNA with positively charged monomer.

In vitro cell transfection

Figure 5 reveals MG63 osteosarcoma cells with and without GFP encoding plasmid DNA encapsulated within neutral hydrogel. It appears that majority of the encapsulated cells remained viable after 21 days in culture, and incorporation of plasma DNA did not affect viability of cells. Transfection of MG63 cells by co-encapsulated plasmid DNA encoding GFP after 14 and 21 days are shown in Fig. 6a and 6b, respectively. The encapsulated cells

continuously expressed GFP at 14 and 21 days as complexed DNA was delivered to the bone tumor cells.

Figure 7 shows that OPF hydrogel-mediated transfection of FLAG ER plasmid leads to the expression of ER α protein in ER-negative hFOB cells after 4 days. To compare the effect of hydrogel formulations on cell transfection, ALP and luciferase enzyme activities of the encapsulated hFOB cells were measured in the presence and absence of estrogen. In ER-transfected cells, estrogen treatment resulted in a 1.8-fold increase in ALP activity compared to the control (Fig. 8). Our results show that estrogen increased ALP activity in the presence of positively-charged hydrogel as opposed to uncharged hydrogels (Fig. 8).

To determine whether ER-negative cells respond to estrogen-treatment after hydrogel-mediated ER DNA delivery, we have studied the effect of estrogen after co-transfecting ER plasmids along with ERE-luciferase constructs. Estrogen treatment resulted in a 2.5-fold increase in luciferase activity compared to the control (untransfected cells) (Fig. 9). Our results show that luciferase activity of the cells encapsulated within positively-charged hydrogel significantly increased in the presence of estrogen compared to the cells encapsulated within the uncharged hydrogels (Fig. 9).

DISCUSSION

For bone and cartilage tissue engineering, methods have previously been developed for encapsulation of marrow stromal cells or osteoblasts, demonstrating excellent cell viability and an ability to generate cartilaginous and bone like matrix.^{11, 18, 19} The addition of DNA to this system may provide additional tools to alter cell behavior and enhance tissue formation. In this study, we have examined if co-encapsulation of the DNA and bone cells enhances gene transfer by increasing the DNA concentration in cellular microenvironment. Initially, we demonstrated that MG63 cells remain viable after encapsulation into the hydrogel for 21 days. It appears that cells encapsulated within OPF hydrogel are able to exchange nutrients and waste over 21 days of culture due to hydrogel permeability. The photoencapsulated plasmid DNA appeared to maintain the ability to produce the encoded protein in transfected bone cells. Encapsulated bone tumor cells continuously expressed GFP protein after transfection with plasmid DNA. Similar observation has been previously reported by Quick et al that photoencapsulated DNA preserved its active and supercoiled conformation within the PEG hydrogel.²⁰ It has been further shown that photoencapsulated DNA within their degradable hydrogel was capable of transfecting the chondrocytes.

Previous study by Segura et al has shown that cationic polymers modified with biotin resulted in efficient internalization of complexed DNA and further transgene expression.²¹ In another work, these authors have reported that DNA immobilization enables spatial patterning of gene transfer by transfecting cells that attach and orient along a topographical pattern on a hyaluronic acid-collagen hydrogel.²² These authors have stated that enzymatic degradation of the cationic polymers is not necessary for transfection and the duration of transgene expression is extended for surface mediated delivery related to bolus delivery. Our data also showed that DNA release from positively charged hydrogel was negligible during the first two weeks and only 10% of the loaded DNA was released from the hydrogel after 28 days. It appears that positively charged groups in MAETAC interact with DNA through electrostatic interaction and induces condensation of the DNA within the hydrogel network. Given these data, it appears that with the addition of a positive charge to the polymer matrix, DNA can be immobilized within the hydrogel by electrostatic interaction as well. This provides an elevated concentration of DNA for cell internalization. It has been reported that increasing the concentration of the DNA in the cellular microenvironment by adsorbing DNA to silica particles that settle on the surface increased transfection.²³

To investigate the functionality of transfected cells, hFOB cells lacking endogenous ER were transfected with FLAG-ER plasmid DNA. Immunostaining confirmed expression of FLAG ER after 4 days of culture. The transfection efficiency of the cells was further investigated in different hydrogel environment with incorporation of MAETAC. ATR-FTIR data confirmed that positively charged monomer (MAETAC) is covalently incorporated into the hydrogel. Our results also show that incorporation of the positively charged monomer increased swelling ratio of the hydrogel. Furthermore, we demonstrated that entrapment of hFOB cells with FLAG ER encoding plasmid DNA complexes into the positively charged hydrogel promotes cellular internalization and further gene expression. Our results show that ER DNA delivery leads to increases in alkaline phosphatase and estrogen-dependent luciferase activities. ER is essential for estrogen-dependent regulation of alkaline phosphatase activity and ERE-driven reporter gene expressions. Previous results show that ER-negative hFOB cells do not respond to estrogen treatment and ER expression is required for estrogen-mediated increases in ALP activities.¹⁶ Thus, our present study demonstrates that hydrogel-dependent ER DNA delivery confers estrogen response in hFOB cells.

It appears that both hydrogel formulations (neutral and charged) effectively delivers the DNA to the co-encapsulated cells by maintaining an elevated concentration of DNA within the cellular microenvironment, however positively charged hydrogel also enhances functionality of the delivered DNA. This might be due to the electrostatic interaction between the DNA and MAETAC within the hydrogel network that induces condensation of the DNA. DNA condensation induced by cationic polymer has been investigated previously using atomic force microscopy and demonstrated that DNA condensation by positively charged polymer retarded DNA mobility in electrophoresis and inhibited enzyme cleavage.²⁴

Taken together, these findings suggest that the co-encapsulation of the DNA complexes into the OPF hydrogel and further addition of positively charged group to OPF hydrogel had significant effect on transfection of cells. However, further work is necessary to understand the effects of charge and its variations in cellular internalization and transgenic expression of encapsulated cells.

CONCLUSIONS

In this study, we have demonstrated that photocrosslinkable OPF hydrogels have the potential for sustained delivery of DNA complexes. The DNA encapsulated into the OPF hydrogels is capable of transfecting co-encapsulated cells. Thus, our findings suggest that the delivery of DNA from biomaterials and complexation with cationic polymers may enhance gene transfer by maintaining an elevated concentration of DNA within the cellular microenvironment.

Acknowledgments

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Fig. 1. Chemical formula of oligo-(polyethylene glycol) fumarate (OPF) (a), and [2-(methacryloyloxy) ethyl]-trimethylammonium chloride (MAETAC) (b) used for fabrication of hydrogels. OPF macromer and MAETAC were crosslinked in the presence of the photoinitiator Irgacure 2959 and ultraviolet light to form positively charged hydrogel.

Fig. 2. Micro-ATR-FTIR of hydrogels after crosslinking and lyophilization: neutral hydrogel (a), positively charged hydrogel with incorporation of 200 mM MAETAC (b).

Fig. 3.
Equilibrium swelling ratio of neutral and positively charged OPF hydrogels.

Fig. 4. Cumulative DNA release from hydrogels of different formulations. The release of DNA was measured in PBS buffer at 37°C.

Fig. 5. Viability of MG63 osteosarcoma cells encapsulated within OPF hydrogel with GFP plasmid DNA after 14 days (a), and 21 days (b); without GFP plasmid DNA after 14 days (c), and 21 days (d). Live cells are stained green and dead cells are stained red. All magnifications are $\times 40$. Scale bar=20 μm .

Fig. 6.
Detection of GFP protein expression in MG63 osteosarcoma cells.
OPF hydrogels co-encapsulated with GFP-encoding plasmid DNA and MG 63 cells were visualized under confocal laser microscope. Untransfected cells (a); transfected cells after 14 days (b), and 21 days (c) in culture.

Fig. 7.

Detection of ER-Flag protein expression. ER-Flag transfected cells (a); ER-Flag untransfected cells (b).

OPF hydrogels co-encapsulated with ER-Flag encoding plasmid DNA and MG 63 cells were stained with anti-FLAG-FITC antibody (Sigma) and visualized under microscope after 4 days.

Fig. 8.

Estrogen-dependent alkaline phosphatase activity in ER-negative cells OPF hydrogels coencapsulated with ER-Flag encoding plasmid DNA and hFOB cells were treated with 17β -estradiol (20 nM) for 72 hrs, and the alkaline phosphatase activities were measured. Values are the mean $\pm$ SE. (n=3 replicate cultures). * $P < 0.05$ (compared to no estrogen control)

Fig. 9. Estrogen-dependent Luciferase activity in ER-negative cells. OPF hydrogels were coencapsulated with ER-Flag encoding plasmid DNA, ERE-luciferase plasmid DNA and hFOB cells. Transfected cells were treated with 17β -estradiol (20nM) for 48 hrs and the luciferase activities were measured. Values are the mean $\pm$ SE. (n=3 replicate cultures). * $P < 0.05$ (compared to no estrogen control)