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Effect of Swelling Ratio of Injectable Hydrogel Composites on Chondrogenic Differentiation of Encapsulated Rabbit Marrow Mesenchymal Stem Cells *In Vitro*

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Abstract

An injectable, biodegradable hydrogel composite of oligo(poly(ethylene glycol) fumarate) (OPF) and gelatin microparticles (MPs) has been investigated as a cell and growth factor carrier for cartilage tissue engineering applications. In this study, hydrogel composites with different swelling ratios were prepared by crosslinking OPF macromers with poly(ethylene glycol) (PEG) repeating units of varying molecular weights from 1,000 ~ 35,000. Rabbit marrow mesenchymal stem cells (MSCs) and MPs loaded with transforming growth factor-β1 (TGF-β1) were encapsulated in the hydrogel composites in order to examine the effect of the swelling ratio of the hydrogel composites on the chondrogenic differentiation of encapsulated rabbit marrow MSCs both in the presence and absence of TGF-β1. The swelling ratio of the hydrogel composites increased as the PEG molecular weight in the OPF macromers increased. Chondrocyte-specific genes were expressed at higher levels in groups containing TGF-β1-loaded MPs and varied with the swelling ratio of the hydrogel composites. OPF hydrogel composites with PEG repeating units of molecular weight 35,000 and 10,000 with TGF-β1-loaded MPs exhibited a 159 ± 95 and a 89 ± 31 fold increase in type II collagen gene expression at day 28, respectively, while OPF hydrogel composites with PEG repeating units of molecular weight 3,000 and 1,000 with TGF-β1-loaded MPs showed a 27 ± 10 and a 17 ± 7 fold increase in type II collagen gene expression, respectively, as compared to the composites with blank MPs at day 0. The results indicate that chondrogenic differentiation of encapsulated rabbit marrow MSCs within OPF hydrogel composites could be affected by their swelling ratio, thus suggesting the potential of OPF composite hydrogels as part of a novel strategy for controlling the differentiation of stem cells.

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Keywords

injectable hydrogels; crosslinking; marrow mesenchymal stem cells; gelatin microparticles; TGF-β1; chondrogenic differentiation; cartilage tissue engineering

Introduction

Articular cartilage tissue often lacks the ability of self-repair and is difficult to fully regenerate with currently available treatments [1]. Therefore, tissue engineering strategies have been investigated as an alternative to treat articular cartilage defects [1, 2]. The success of a tissue engineering approach relies on the proper selection of cells, bioactive molecules, and scaffolding materials [1]. One attractive candidate for cells in this approach is mesenchymal stem cells (MSCs), since MSCs may be easily isolated from the bone marrow and expanded without losing their capacity to differentiate into cells of various mesenchymal lineages, including chondrocytes [3]. Numerous studies have demonstrated the successful transplantation of autologous MSCs for cartilage and bone tissue engineering applications [4–6].

Recently, various hydrogel materials have been developed for use as delivery vehicles of bioactive molecules and cells in tissue engineering [7–10]. Hydrogels are physically or chemically crosslinked three-dimensional polymers swollen in water that allow the controlled release of bioactive molecules [11]. Among those, injectable hydrogel materials, which can gel under physiological conditions, have held great potential. Cells and bioactive factors can be easily incorporated in the system and injected to the defect site, which allows minimally invasive procedures for tissue repair [12, 13]. Moreover, depending on the selection of crosslinking molecules, the mechanical properties of hydrogels can be easily tailored [14].

A novel, degradable fumarate-based macromer, oligo(poly(ethylene glycol) fumarate) (OPF), has been developed in our laboratory as an injectable hydrogel carrier for growth factors for orthopaedic tissue engineering. Previous studies have demonstrated the potential of degradable OPF hydrogels for *in vitro* osteogenesis by rat marrow mesenchymal stem cells (MSCs). OPF hydrogels with two different OPF formulations of varying poly(ethylene glycol) (PEG) molecular weight were prepared for encapsulation of rat marrow mesenchymal stem cells and cultured for 4 weeks both with and without osteogenic supplements [15]. That study demonstrated that the molecular weight of the PEG repeating unit in the macromer affected the hydrogel swelling ratio and that changes in the swelling ratio of OPF hydrogels resulted in different amounts of calcium deposition by encapsulated rat marrow MSCs. In addition, OPF hydrogels have been recently investigated as a cell and growth factor delivery system for cartilage tissue engineering [16]. In that study, rabbit marrow MSCs encapsulated with TGF-β1-loaded MPs in OPF hydrogels showed an increase in gene expression of type II collagen and aggrecan, demonstrating that the incorporation of gelatin microparticles into a hydrogel matrix is a promising strategy for localized and sustained release of a growth factor. Additional studies have demonstrated the degradation of OPF hydrogel composites with encapsulated MPs both *in vitro* and *in vivo* [17, 18].

The present study asked whether the swelling ratio of OPF hydrogel composites affected the proliferation and chondrogenic differentiation of encapsulated rabbit marrow MSCs. OPF hydrogel composites of different swelling ratios encapsulating rabbit marrow MSCs and gelatin MPs loaded with or without TGF-β1 were prepared and cultured for 4 weeks to investigate the effect of hydrogel swelling on the chondrogenic differentiation of encapsulated rabbit marrow MSCs as measured by gene expression of two chondrogenic markers, collagen type II and aggrecan.

Materials and Methods

OPF synthesis and characterization

OPF was synthesized from fumaryl chloride and PEG according to a previously established method [19]. PEG of four different nominal molecular weights (35,000 g/mol, 10,000 g/mol, 3,300 g/mol and 1,000 g/mol) was used to prepare OPF macromers of four different repeating units (namely OPF 35K, OPF 10K, OPF 3K and OPF 1K). Macromer molecular weight was determined by gel permeation chromatography (GPC; Model 410; Waters, Milford, PA) using a refractive index detector (n=3). The purified macromer was stored at −20°C and sterilized prior to use by exposure to ethylene oxide for 14 h.

Gelatin microparticle preparation

Gelatin microparticles were fabricated from acidic gelatin (Nitta Gelatin Inc., Osaka, Japan) following established procedures [20]. Briefly, a gelatin solution was prepared by dissolving 5 g of gelatin in 45 ml of distilled, deionized water (ddH_2O) at 60°C. Then, this solution was added dropwise to 250 ml of chilled olive oil while stirring at 500 rpm. After 30 min, 100 ml of chilled acetone (4°C) was added to the emulsion. After an additional 60 min, the microspheres were collected by filtration and washed with acetone. These microparticles were then crosslinked in 0.1 wt % Tween 80 (Sigma, St. Louis, MO) solution with 10 mM glutaraldehyde (GA) (Sigma, St. Louis, MO) while stirring at 500 rpm at 15°C. After 15 h, crosslinked microparticles were collected by filtration, washed with ddH_2O, and then agitated in 25 mM glycine solution for 1 h to inactivate any unreacted GA. These microparticles were collected by filtration, washed with ddH_2O, and then lyophilized overnight. Finally, dried microparticles were sieved to obtain particles of 50–100 μm in diameter and sterilized with ethylene oxide for 14 h.

OPF hydrogel composite swelling and degradation experiment

For swelling studies, OPF 35K, OPF 10K, OPF 3K and OPF 1K hydrogel composites encapsulating gelatin microparticles were prepared in the same manner as would be used for cell encapsulation. Briefly, 0.1 g of OPF and 0.05 g of the crosslinking agent poly(ethylene glycol) diacrylate (PEG-DA; nominal MW 3400, Nektar Therapeutics, Huntsville, AL) and 0.0219 g of microparticles were combined in 578 μl of phosphate buffer saline (PBS). Equal volumes (46.8 μl) of the thermal radical initiators, 25 mM ammonium persulfate (APS) and 25 mM N,N,N′,N′-tetramethylethylenediamine (TEMED) in PBS, were then added. After gentle mixing, the suspension was quickly injected into Teflon molds (6 mm diameter, 1 mm thickness) followed by incubation at 37°C for 8 min. Hydrogel composites were transferred to PBS and cultured statically at 37°C for 4 weeks. At day 1, 7, 14, 21 and 28, the swelling ratio and sol fraction of OPF hydrogel composites were then determined by the following equations.

$$\text{Swelling ratio} = \frac{W_s - W_d}{W_d}$$
$$\text{Sol fraction} = \frac{W_i - W_d}{W_i}$$

Here, W_i, W_s, and W_d represent the weight of dried hydrogel composites after crosslinking, the weight of hydrogel composites after swelling in PBS, and the weight of dried hydrogel composites after swelling, respectively. The swelling ratio is defined as the fractional increase in the weight of the hydrogel due to water absorption. The sol fraction represents the fraction of the polymer following a crosslinking reaction that is not part of a crosslinked network. A decrease in sol fraction over time reflects polymer loss and characterizes the extent of hydrogel degradation.

Rabbit marrow MSC isolation and pre-culture

Rabbit marrow MSCs were isolated from the tibias of 4 month old New Zealand white rabbits as previously described [16]. Briefly, after anesthesia, rabbit bone marrow was collected into a 10 ml syringe containing 5000 U of heparin. The bone marrow was then filtered through a cell strainer (40 μm) and cultured in Dulbecco's modified Eagle's medium − low glucose (DMEM-LG) supplemented medium containing 10% v/v fetal bovine serum (Gemini, Calabasas, CA), 250 μg/l fungizone, 100 mg/l ampicillin, and 50 mg/l gentamicin (Invitrogen) for 2 weeks. In an effort to reduce any interanimal variation, a pool of rabbit marrow MSCs from a total of 6 rabbits was collected together, placed in medium containing 20% FBS and 10% dimethyl sulfoxide (DMSO), and cryopreserved in liquid nitrogen prior to use. For experiments, cryopreserved cells were thawed at 37°C, seeded in T-75 flasks, and expanded for 14 days of culture in DMEM supplemented medium containing 10% v/v fetal bovine serum (Gemini), 250 μg/l fungizone, 100 mg/l ampicillin, and 50 mg/l gentamicin. Cells from one preparation were used in all experiments and were cultured up to passage three before the encapsulation process.

Microparticle and rabbit marrow MSC encapsulation

Before encapsulation, OPF 35K, OPF 10K, OPF 3K and OPF 1K were combined with PEG-DA at a 2:1 ratio of OPF to PEG-DA by weight. These polymer mixtures and gelatin microparticles were sterilized with ethylene oxide for 14h. Then, sterilized MPs were loaded with TGF-β1 (R&D Systems, Minneapolis, MN) by immersing them in aqueous TGF-β1 solution at pH 7.4 and incubating them at 4°C for 15h according to established methods [21]. At this pH, there is ionic complexation of gelatin microparticles and TGF-β1 due to the negative charge of the acidic gelatin (IEP of 5.0) and positive charge of TGF-β1 (IEP of 9.5) [22]. The total TGF-β1 loading of the microparticles in each gel was 25 ng, which resulted in a concentration of 10 ng TGF-β1/ml relative to the medium (2.5 ml) used for culturing each gel. TGF-β1-free MPs were also prepared for comparison.

For encapsulation of isolated rabbit marrow MSCs and gelatin microparticles in OPF hydrogel composites, a combination of OPF and PEG-DA were dissolved in 300 μl PBS and mixed with the 110 μl microparticle swelling solution. Equal volumes (46.8 μl) of the thermal radical initiators, 25 mM ammonium persulfate (APS) and 25 mM N,N,N′,N′-tetramethylethylenediamine (TEMED) in PBS, were then added. After this mixture was vortexed, a 168 μl PBS suspension containing 6.7 million cells was added to achieve a cell concentration of 10 million cells/ml in the final suspension. After gentle mixing, the suspension was quickly injected into Teflon molds (6 mm diameter, 1 mm thickness) followed by incubation at 37°C for 8 min. Final gel constructs were transferred into 12 well tissue culture plates. Each well contained one gel construct and 2.5 ml chondrogenic medium, which was DMEM supplemented with ITS+ Premix (6.25 μg/ml insulin, 6.25 μg/ml transferrin, 6.25 μg/ml selenous acid, 5.35 μg/ml linoleic acid and 1.25 μg/ml bovine serum albumin) (BD Biosciences, Franklin Lakes, NJ), 1 mM sodium pyruvate, 50 μg/ml ascorbate 2-phosphate (Sigma-Aldrich), 10^{-7} M dexamethasone (Sigma-Aldrich), 250 mg/l fungizone, 100 mg/l ampicillin and 50 mg/l gentamicin. The medium was changed every 3 days. For the comparison with TGF-β1 loaded MPs groups (noted as +), the same OPF cell-hydrogel composites with blank MPs (noted as −) were also prepared and cultured exactly as described above. At day 7, 14, and 28, samples were collected from each group for biochemical assays (n=4) and quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) (n=4). Samples at day 0 were collected immediately after the encapsulation process, including four samples from each OPF formulation (OPF 35K, OPF 10K, OPF 3K and OPF 1K) for biochemical assays and a total of four samples from TGF-β1-free groups used as a control for all groups for RT-PCR analysis. In addition, cell-free hydrogel composites (n=3) were also prepared following the same methods. These cell-free hydrogel composites were analyzed with samples to establish

any background contribution from the hydrogel to fluorescence and absorbance measurements in the biochemical assays.

Biochemical assays for cell proliferation and GAG production

At each time point, samples and cell-free hydrogels were removed from medium, rinsed in 2 ml PBS, homogenized with a pellet grinder (Fisher Scientific) and digested in 500 μl of a proteinase K solution (1 mg/ml proteinase K (Sigma-Aldrich), 10 μg/ml pepstatin A (Sigma-Aldrich), and 185 μg/ml iodoacetamide (Sigma-Aldrich) in tris-EDTA solution (6.055 mg/ml Tris(hydroxymethyl aminomethane) (Sigma-Aldrich), 0.372 mg/ml EDTA (Sigma-Aldrich), pH 7.6 adjusted by HCl)) at 60°C for 16 h. After collection and digestion of all samples and cell-free hydrogels, specimens underwent three repetitions of a freeze/thaw/sonication cycle (30 min at −80°C, 30 min at room temperature, 30 min of sonication) for complete extraction of DNA from the cell cytoplasm. DNA and glycosaminoglycan (GAG) assays were then run for samples at each time point (n=4).

DNA content was calculated by measuring double-stranded DNA content using the PicoGreen assay (Molecular Probes, Eugene, OR) according to the manufacturer's instructions. The PicoGreen dye binds to double-stranded DNA (dsDNA) and results in fluorescence corresponding to the concentration of dsDNA, which was measured by a plate reader (FL x800, Bio-Tek Instrument, Winooski, VT) at a wavelength of 490 nm. The fluorescence of negative, cell-free hydrogels was subtracted from the fluorescence values of experimental groups to account for fluorescence of the material alone.

Similarly, glycosaminoglycan content was also determined using a biochemical assay, the dimethylmethylene blue dye (DMMB) assay (Sigma-Aldrich), as previously described [23]. Upon DMMB binding to GAG, a pink color is produced, allowing for quantification of GAG by measuring absorbance at 520 nm. GAG content in hydrogels was calculated by comparison to a curve generated from standards of known amounts of porcine chondroitin sulfate B (Sigma-Aldrich). A microplate reader (BIO-TEK Instrument, Winooski, VT) was utilized for the absorbance measurements.

Real time PCR

Total RNA was extracted from hydrogel composites at each time point via the RNeasy Mini Kit (Qiagen, Valencia, CA). Briefly, hydrogels were transferred into RNA lysis buffer solution and homogenized by gentle pipetting. The homogenized solution was purified using a Qiagen shredder column and total RNA was extracted with the RNeasy Mini Kit. RNA samples were then reverse-transcribed to cDNA using Oligo dT primers and superscript III transcriptase. The final cDNA transcripts were then subjected to real time PCR (7300 Real-Time PCR System, Applied Biosystems, Foster City, CA) to determine the expression of genes for type II collagen and aggrecan. Gene expression data were analyzed using the $2^{-\Delta\Delta Ct}$ method as reported previously [4, 24]. Briefly, all gene expression data were normalized to the expression of the housekeeping gene, glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) and expressed as the fold ratio as compared with those of a control group. For this study, the control group contained rabbit marrow MSCs embedded in OPF with blank MPs (n=4, one sample from each OPF formulation) that were analyzed immediately after encapsulation (day 0). The sequence of primers for GAPDH, type II collagen, and aggrecan were as follows [16]: GAPDH: 5′-TCACCATCTTCCAGGAGCGA-3′, 5′-CACAATGCCGAAGTGGTCGT-3′; type II collagen gene: 5′-AACACTGCCAACGTCCAGAT-3′, 5′-CTGCAGCACGGTATAGGTGA-3′; Aggrecan: 5′-GCTACGGAGACAAGGATGAGTTC-3′, 5′-CGTAAAAGACCTCACCCTCCAT-3′.

Statistical analysis

Swelling ratio, sol fraction, DNA, GAG, and gene expression were reported as means ± standard deviation. Repetitive ANOVA and Tukey's multiple comparison test were used to determine possible significant differences ($p < 0.05$) in the DNA, GAG, and gene expression between groups.

Results

OPF characterization

The number average molecular weight (M_n) and weight average molecular weight (M_w) of OPF macromers and the parent PEG for the synthesis were determined by GPC and are shown in Table 1.

OPF hydrogel composite characterization

Swelling studies indicated that equilibrium swelling of the hydrogel composites had been reached after being immersed in PBS overnight. Swelling ratios of the hydrogels remained the same afterwards for the 28 day culture period (data not shown). The equilibrium fold swelling of OPF hydrogel composites is shown in Figure 1 (A). OPF 35K had a swelling ratio of 15.1 ± 0.3, which was statistically higher than that of all the other OPF formulations. In addition, OPF 10K hydrogel composites had a statistically higher swelling ratio (13.9 ± 0.2) than either OPF 3K (13.0 ± 0.4) or OPF 1K (12.7 ± 0.3). There was no statistical difference between OPF 3K and 1K. The results indicated that swelling ratio increased as the PEG molecular weight in the OPF formulation increased. Sol fraction of OPF hydrogel composites over time is shown in Figure 1 (B). For each OPF formulation, there was little change in sol fraction over time. However, OPF 10K had a significantly lower sol fraction than OPF 35K, OPF 3K and OPF 1K at each time point.

Biochemical assays

DNA content at each time point is depicted in Figure 2. All groups showed a trend of decreasing DNA content over the culture period. A significant decrease in DNA content was seen for all the treatments at day 28 as compared to day 0. Differences were also found between groups with different swelling ratios at the same time point. More specifically, when blank MPs were encapsulated, a statistically higher DNA content was observed in both OPF 35K− and OPF 10K− hydrogel composites compared with OPF 3K− or OPF 1K− hydrogel composites at day 7, 14 and 28. While in groups with TGF-β1-loaded MPs, significantly higher DNA content was observed in samples for OPF 35K+ at day 28 as compared to those for OPF 3K+ or OPF 1K+.

As shown in Figure 3, for all of the eight treatments, there was a higher GAG/DNA content at later time points (day 7, 14 and 28) as compared to day 0. Especially in the TGF-β1 treated groups, a significant increase in GAG/DNA content was observed in OPF 35K+ samples at day 14 and day 28, OPF 10K+ samples at day 7, OPF 1K+ samples at day 28 as compared to day 0. No significant difference was found in the groups loaded with blank MPs during the culture period except for OPF 10K− at day 28. Additionally, swelling ratio was determined to have an influence on GAG/DNA content, as evidenced by the significantly higher GAG/DNA values for OPF 35K+ hydrogel composites than both OPF 3K+ and OPF 1K+ hydrogel composites at day 14.

Real time PCR

Results of collagen type II gene expression over time are presented in Figure 4. Statistical analysis revealed significantly higher collagen type II gene expression levels for the OPF 35K

+ and OPF 10K+ samples (158.8 ± 95.4 and 89.1 ± 31.5 fold increase, respectively) at day 28 than their corresponding TGF-β1-free groups OPF 35K− and OPF 10K− (62.2 ± 34.4 and 29.9 ± 11.3 fold increase, respectively). However, there was no significant change in collagen type II gene expression level for the OPF 3K and 1K samples over the culture period regardless of the presence of TGF-β1. At day 28, the OPF 35K+ samples were determined to have a significantly higher level of collagen type II gene expression than other treatments. OPF 10K + samples had a significantly higher collage type II gene expression level than OPF 3K+ and OPF 1K+ samples at day 28.

Similar to collagen type II gene expression, aggrecan gene expression for all the treatments showed an increasing trend during the culture period, as shown in Figure 5. The results demonstrated a significantly higher aggrecan gene expression level for the OPF 35K+ (23.7 ± 12.0 fold increase), OPF 10K+ (14.2 ± 7.4 fold increase) and OPF 35K− (12.8 ± 6.4 fold increase) samples at day 28 compared with day 0. Additionally, significant differences were also seen between OPF 35K+ samples and OPF 3K+ or OPF 1K+ samples in the presence of TGF-β1-loaded MPs at day 28, while there was no significant change between the groups with blank MPs.

Discussion

The objective of this study was to examine the effect of the swelling ratio of OPF hydrogel composites with gelatin MPs on the chondrogenic differentiation of encapsulated rabbit marrow MSCs. This study was designed to assess how OPF hydrogel composites with gelatin MPs of four different swelling ratios (OPF 35K, OPF 10K, OPF 3K, and OPF 1K) affected chondrocyte-specific gene expression of encapsulated rabbit marrow MSCs. For each OPF formulation, composites encapsulating MSCs and TGF-β1-loaded MPs (noted as '+') and composites encapsulating MSCs and blank MPs (noted as '−') were included.

The synthesis of OPF from PEG was characterized by GPC. The results indicated a decrease in the number of PEG chains incorporated in the macromer as the PEG molecular weight increased, which was probably due to steric hindrance of large PEG molecules thus affecting the addition of a fumarate unit to the end of the chain [19].

Although many factors, such as molecular weight of macromer, concentration of macromer, crosslinker and initiator as well as crosslinking extent, can influence the swelling ratio of a crosslinked composite, in the current study the swelling ratio of OPF hydrogel composites was adjusted by changing the initial PEG molecular weight for OPF synthesis based on previous reports [15, 25]. Theoretically, as the PEG molecular weight increases, the spacing between the crosslinks becomes larger and therefore the resulting hydrogel will have a larger mesh size, exhibiting as a higher swelling ratio. A previous study has proved that OPF 10K hydrogels had a significantly larger swelling ratio (17.5 ± 0.2) than OPF 3K hydrogels (13.4 ± 0.4) [25]. The results of this study, as expected, demonstrated a significant difference in composite swelling ratio between OPF formulations even though MPs were present. Specifically, OPF 35K and OPF 10K hydrogel composites were shown to have a statistically higher swelling ratio than that of OPF 3K and OPF 1K composites.

Sol fraction of the hydrogel composites represents the fraction of the polymer that is not involved in the crosslinked network. The sol fraction of OPF hydrogel composites was measured over time to evaluate the degradation of hydrogel composites and its potential influence on cell behavior. The results revealed no significant change in sol fraction over a 28-day culture period for each OPF formulation, suggesting little hydrogel degradation occurred. The results further indicated that hydrogel degradation may not be a factor that would affect cell behavior in the present study. OPF 10K hydrogel composites had a lower sol fraction as

compared to that of other OPF formulations at all the time points, which was probably due to the varied molar ratios of fumarate double bonds to acrylate double bonds for the OPF formulations examined because the same weight ratios of OPF to PEG-DA were used. However, this result did not present any concern since the goal of this study was to examine hydrogel composites with different swelling ratios.

Cell encapsulation was performed using a thermal radical initiation system (APS/TEMED), which enables crosslinking *in situ*. Compared to photoinitiation, a thermal initiation system would be beneficial in areas where there is limited light penetration. Although it is known that the radical initiators may elicit some toxicity to the cells, the initiator concentration used in the present study has been previously shown to be cytocompatible for MSCs in OPF hydrogel [15].

DNA assay results showed a trend of decreasing DNA content in all groups during the culture period. As we previously reported, the cell loss may be attributed to the encapsulation process which resulted in some cell death, especially over the first few days of cell culture [15, 16]. Additionally, the decrease in DNA content may also be due to the culture of cellular constructs in chondrogenic medium, which is chemically defined and serum free. The absence of serum in the medium was shown to be beneficial for chondrogenic differentiation of mesenchymal cells but not for cell attachment and growth [26, 27]. In fact, a previous study involving encapsulation of bovine chondrocytes in similar OPF/MP composites showed a significant increase in DNA content after culture in a serum-containing medium for 14 days [20]. OPF swelling ratio was determined to have a significant influence on DNA content in the hydrogel composites. At day 7, 14 and 28, a significant difference in DNA content was observed between higher swelling ratio composites (OPF 35K− and 10K−) and lower swelling ratio composites (OPF 3K− and 1K−). In addition, in the presence of TGF-β1-loaded MPs, OPF 35K hydrogel composites (OPF 35K+) also had a higher DNA content than OPF 3K as well as OPF 1K hydrogel composites (OPF 3K+ and 1K+) at day 28. OPF 35K and 10K had comparatively higher swelling ratios, indicative of larger mesh sizes, which possibly facilitated nutrient and growth factor diffusion and resulted in higher DNA content.

GAG is a marker of chondrocytic phenotype, and therefore GAG deposition in the hydrogel composites was measured to evaluate MSC chondrogenic differentiation. The results revealed that GAG/DNA content increased after day 0 and remained similar after day 7 for all the formulations (Figure 3), which provided evidence of MSC chondrogenic differentiation in the hydrogel composites. Significant increase in GAG production as compared to day 0 was seen more frequently in the groups with TGF-β1 (OPF 35K+ at day 14 and 28, OPF 10K+ at day 7 and OPF 1K+ at day 28) than those without TGF-β1 (only OPF 10K− at day 28), suggesting the stimulative effect of TGF-β1 on chondrogenic differentiation. However, a generally low GAG production by rabbit marrow MSCs encapsulated in a hydrogel composite was seen and this may be explained by the tight polymer network surrounding cells, thus limiting the deposition of GAGs only in the pericellular space. Alternatively, rabbit marrow MSCs may need additional signaling to sustain GAG production following chondrogenic differentiation. Further study is needed to elucidate the relationship between encapsulated rabbit marrow MSC differentiation and extracellular matrix production.

To further characterize the *in vitro* chondrogenesis by rabbit marrow MSCs as a function of the swelling ratio of hydrogel composites, gene expressions of two chondrogenic-specific markers, collagen type II and aggrecan, were measured by quantitative RT-PCR [4, 24].

The RT-PCR results showed that the presence of TGF-β1 in the hydrogel composites significantly promoted collagen type II gene expression. For each OPF formulation, samples cultured with TGF-β1-loaded MPs expressed more collagen type II than those with blank MPs.

Additionally, significant increases were found in OPF 35K+ and OPF 10K+ samples compared with OPF 3K+ or OPF 1K+ samples at day 28, indicating that chondrogenic differentiation was accelerated in hydrogel composites with higher swelling ratios. This result may be due to better diffusion of signaling molecules and nutrients in hydrogel samples with a higher swelling ratio. However, it could also be due to the different local concentrations of TGF- β1 in the hydrogel phase after its release from the gelatin MPs. A previous study investigating the release kinetics of TGF-β1 from OPF 10K and OPF 3K hydrogel composites prepared with different crosslinking agents in the absence of encapsulated cells showed that both composites exhibited a burst release after 3 days followed by a sustained release for a period of 25 days. However, the relative amount of TGF- β1 released from each hydrogel composite was different, suggesting a different local concentration of TGF- β1 inside the corresponding hydrogel [17].

Aggrecan gene expression demonstrated a similar trend as that for collagen type II. Upregulation of the gene expression was seen over time for all the treatments, especially when TGF-β1 was present. Samples in OPF 35K+, with high swelling ratio, resulted in a significantly higher level of aggrecan gene expression at day 28 than all the other formulations. Interestingly, some extent of chondrogenic differentiation was also observed in OPF 35K− hydrogel composites since both collagen type II and aggrecan gene expression were upregulated at day 28. This could be due to the chondrogenic potential of chemically defined medium. Previous literature reports indicated that chondrogenic differentiation was achieved in only 25% of the samples cultured in chondrogenic medium without addition of TGF-β1, while in the presence of TGF-β1 chondrogenic differentiation was induced for all the samples [26].

Both collagen type II and aggrecan gene expressions suggested that hydrogel composites of higher swelling ratio, including OPF 35K and OPF 10K hydrogel composites, promoted chondrogenic differentiation of encapsulated rabbit marrow MSCs. This result was consistent with the findings of a previous study concerning the osteogenic differentiation of rat marrow MSCs encapsulated in OPF hydrogels. OPF 10K hydrogels promoted osteopontin production and calcium deposition over OPF 3K hydrogels in the presence of dexamethasone [15]. Both studies suggested that the swelling ratio of hydrogel composites, which is related to the hydrogel mesh size, may affect nutrient transport and drug delivery throughout the hydrogels and thus influence the proliferation and differentiation of encapsulated cells. Although hydrogel degradation may also affect MSC differentiation, this was not the case in the present study since equilibrium swelling of the hydrogel composites was reached within one day and little change in swelling ratio or degradation was seen during the culture period.

Conclusion

Rabbit marrow MSCs were encapsulated in hydrogel composites of crosslinked OPF and gelatin microparticles of different swelling ratios and cultured over a 28-day culture period. Real time RT-PCR demonstrated chondrocyte-specific gene expression in hydrogel composites containing TGF-β1-loaded microparticles. Additionally, enhanced upregulation of chondrocyte-specific genes such as collagen type II and aggrecan was observed in OPF hydrogel composites with higher swelling ratios, which is indicative of larger mesh sizes. The results suggest that chondrogenic differentiation in this system was affected by the swelling ratio (or mesh size) of surrounding hydrogels. Taken together, these results indicate that OPF hydrogel composites of tailored swelling characteristics can be used as part of a novel strategy for controlling the differentiation of MSCs.

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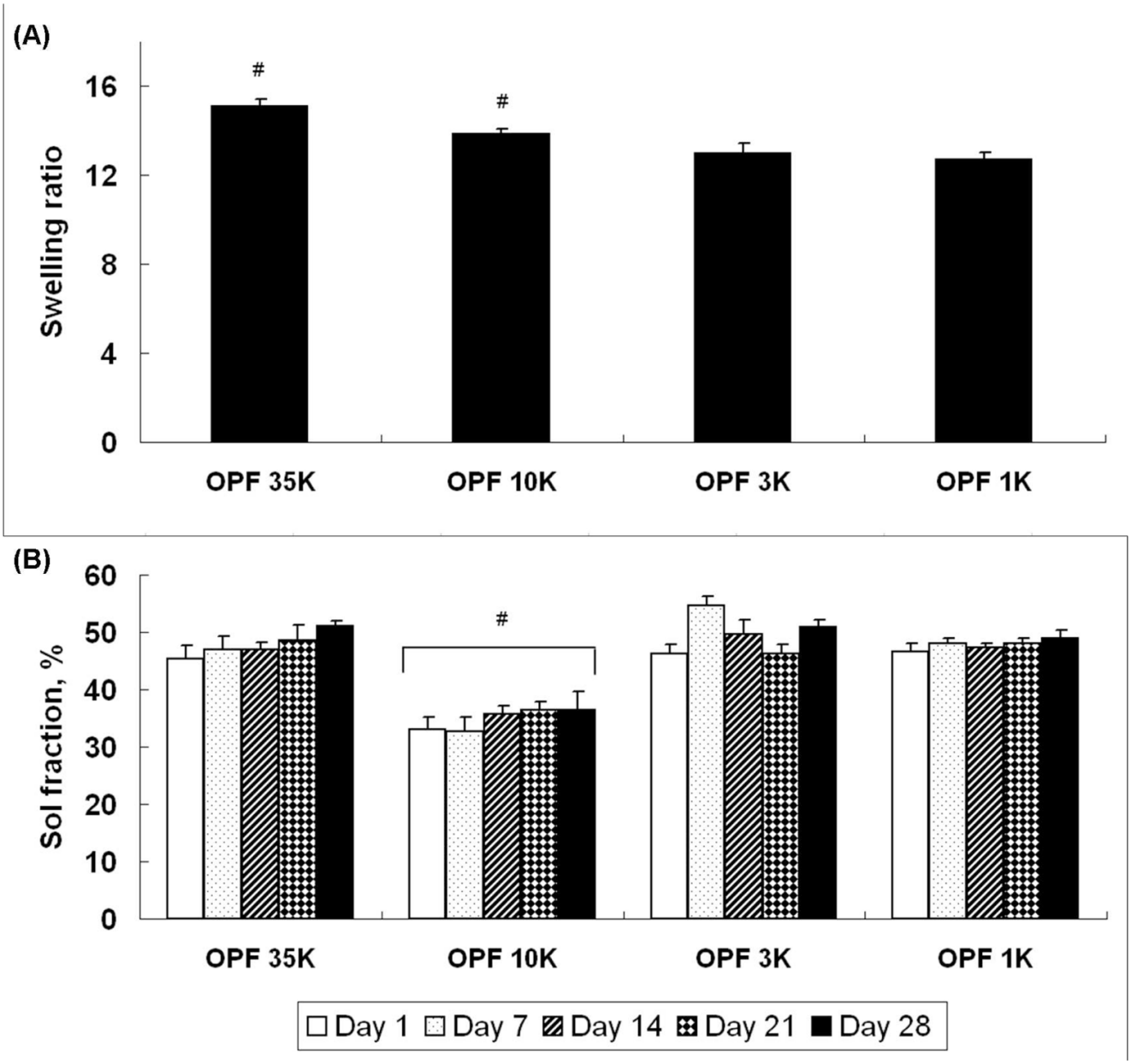

Figure 1.

Swelling ratio (A) of OPF 35K, 10K, 3K and 1K hydrogel composites after swelling in PBS overnight and sol fraction (B) of OPF hydrogel composites after immersion in PBS for up to 28 days. The symbol (#) indicates a significant difference as compared to other OPF formulations ($p<0.05$). Error bars represent means ± standard deviation for n=4.

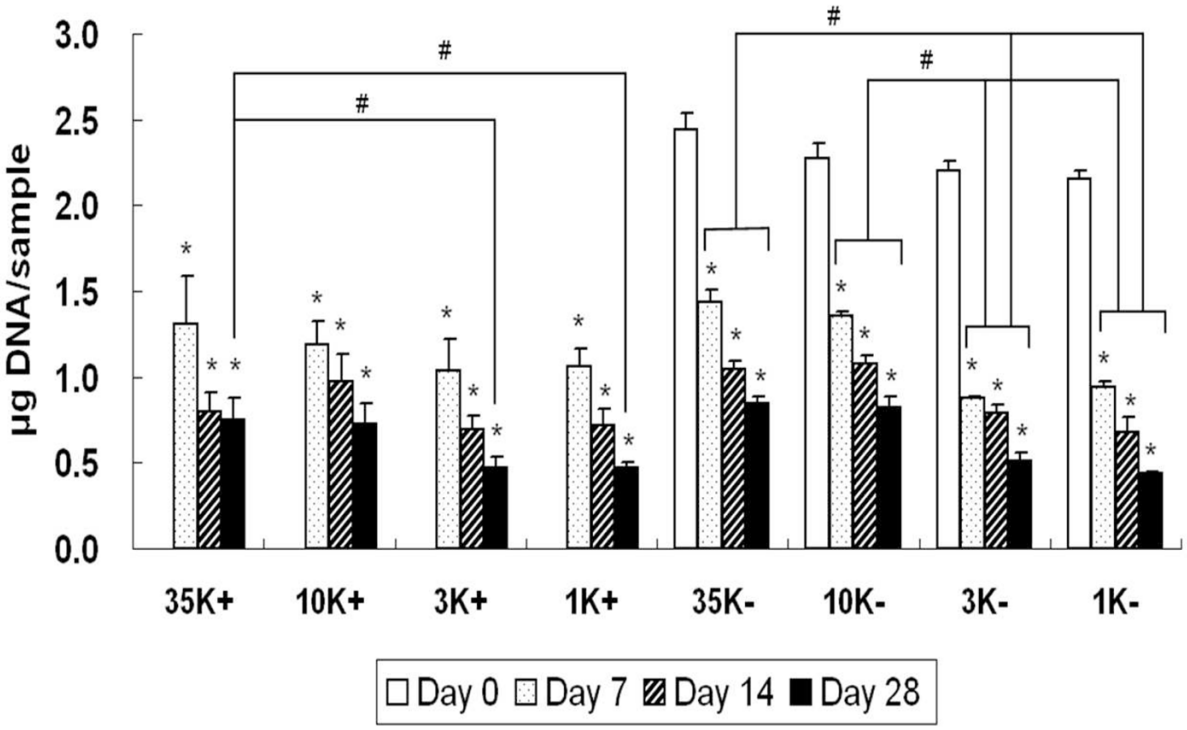

Figure 2.

DNA content of OPF 35K, 10K, 3K and 1K hydrogel composites encapsulating rabbit marrow MSCs with TGF-β1 loaded MPs (+) or blank MPs (−) over a 28-day culture period. Samples marked by (*) exhibited significant DNA decrease compared to samples at day 0 ($p<0.05$). The day 0 samples from each blank formulation served as the controls for all samples (blank and TGF-β1-loaded) associated with the respective OPF (OPF 35K, 10K, 3K or 1K). The symbol (#) indicates a significant difference in DNA content as compared to other OPF formulations at the same time point ($p<0.05$). Error bars represent means ± standard deviation for n = 4.

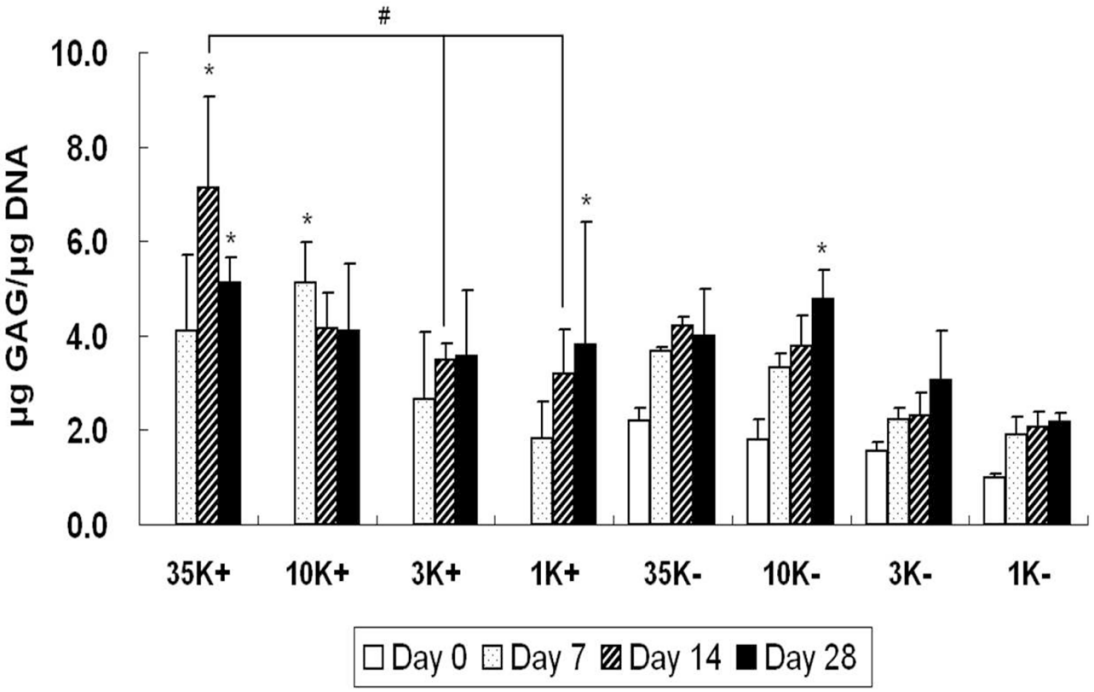

Figure 3.

GAG/DNA content of OPF 35K, 10K, 3K and 1K hydrogel composites encapsulating rabbit marrow MSCs with TGF-β1 loaded MPs (+) or blank MPs (−) over a 28-day culture period. Samples marked by (*) exhibited significantly higher GAG/DNA compared to samples at day 0 ($p<0.05$). The day 0 samples from each blank formulation served as the controls for all samples (blank and TGF-β1-loaded) associated with the respective OPF (OPF 35K, 10K, 3K or 1K). The symbol (#) indicates a significant difference in GAG/DNA content as compared to other OPF formulations at the same time point ($p<0.05$). Error bars represent means ± standard deviation for n = 4.

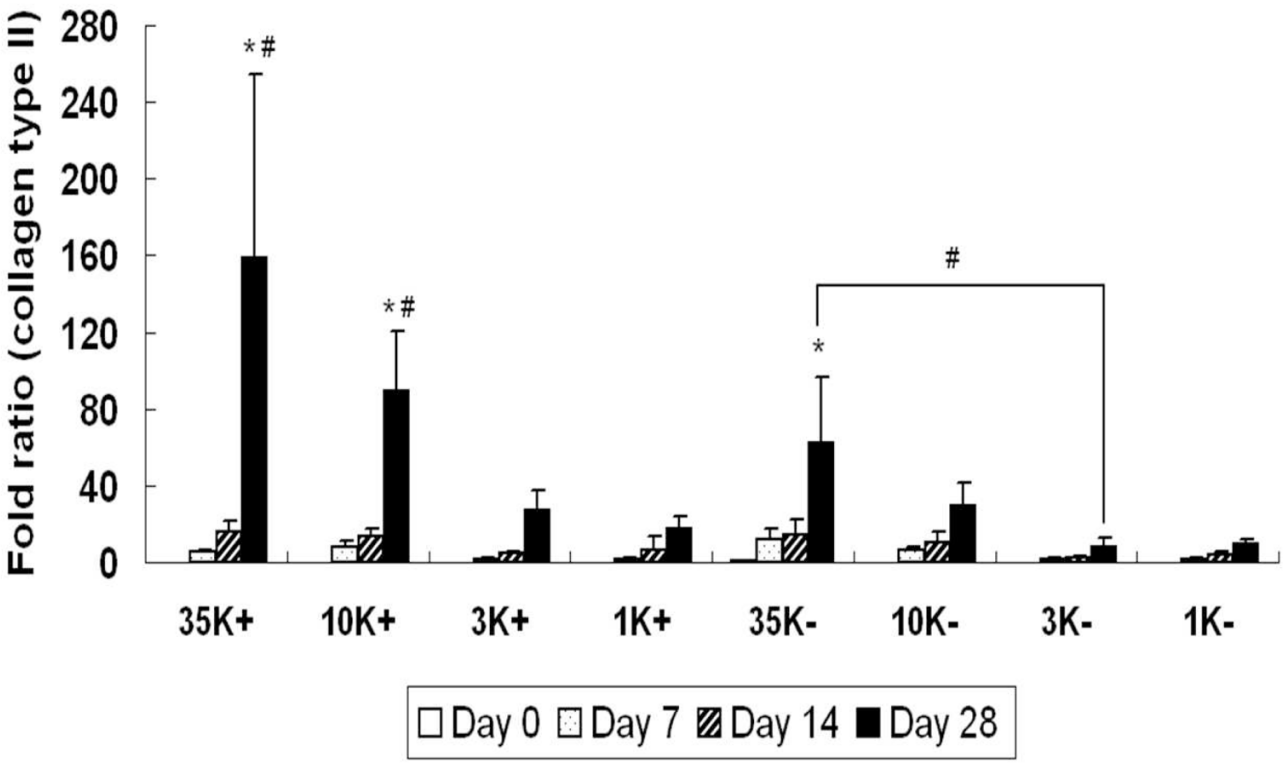

Figure 4.

Quantitative collagen type II gene expression for OPF 35K, 10K, 3K and 1K hydrogel composites encapsulating rabbit marrow MSCs and TGF-β1-loaded MPs (+), or rabbit marrow MSCs and blank MPs (−). Data are presented as a fold ratio after being normalized to GAPDH values. The average expression level of controls (Day 0) is represented as one and is shown with the OPF 35K− group. Within a given hydrogel formulation, significantly higher ($p<0.05$) gene expression than the day 0 value (control) is noted with (*). Samples indicated with (#) had significantly higher gene expression than other OPF formulations at the same time point ($p<0.05$). Error bars represent means ± standard deviation for n = 4.

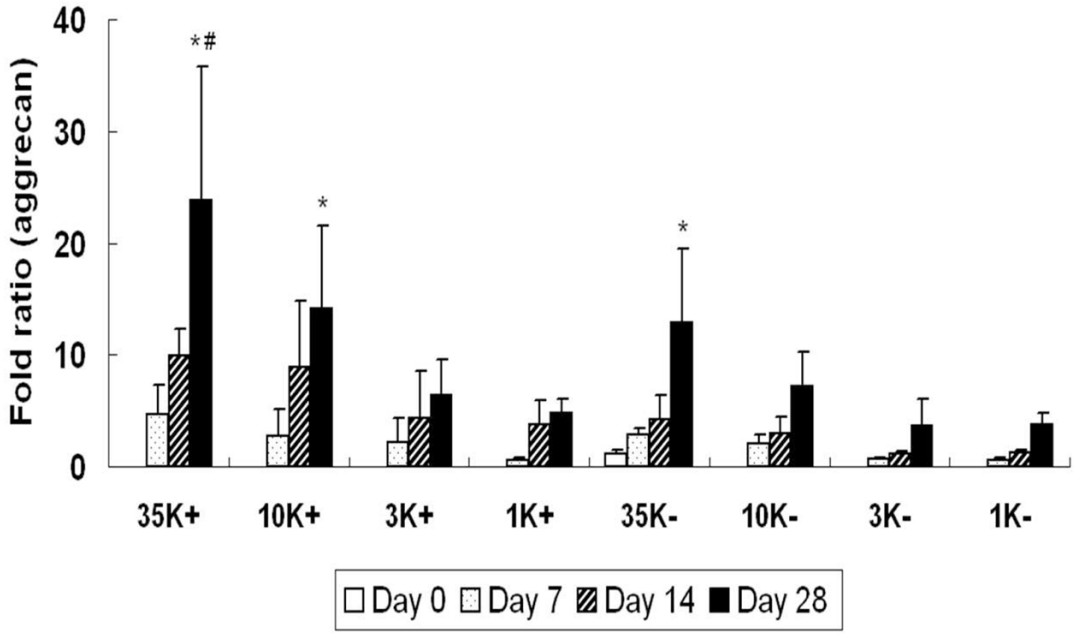

Figure 5.

Quantitative aggrecan gene expression for OPF 35K, 10K, 3K and 1K hydrogel composites encapsulating rabbit marrow MSCs and TGF-β1-loaded MPs (+), or rabbit marrow MSCs and blank MPs (−). Data are presented as a fold ratio after being normalized to GAPDH values. The average expression level of controls (Day 0) is represented as one and is shown with the OPF 35K− group. Within a given hydrogel formulation, significantly higher ($p<0.05$) gene expression than the day 0 value (control) is noted with (*). Samples indicated with (#) had significantly higher gene expression than other OPF formulations at the same time point ($p<0.05$). Error bars represent means ± standard deviation for n = 4.

Table 1

Number (M_n) and weight (M_w) average molecular weights of PEG and OPF as determined by GPC.

	M_n	M_w		M_n	M_w
PEG 35K	38,380 ± 1,310	62,170 ± 2,110	**OPF 35K**	41,520 ± 1,570	125,580 ± 4,750
PEG 10K	8,870 ± 280	11,570 ± 360	**OPF 10K**	9,230 ± 300	64,970 ± 2,110
PEG 3K	2,900 ± 90	3,390 ± 100	**OPF 3K**	4,290 ± 140	39,540 ± 1,250
PEG 1K	860 ± 30	1,000 ± 30	**OPF 1K**	2,930 ± 90	15,560 ± 490