CHAPTER 5

Fumarate-based Macromers as Scaffolds for Tissue Engineering Applications

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Summary

Our laboratory has developed a number of novel synthetic scaffold materials based on fumaric acid for an assortment of tissue engineering applications. These biodegradable materials include poly(propylene fumarate) (PPF), poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)), and oligo(poly(ethylene glycol) fumarate) (OPF), each of which can be applied as an injectable liquid and crosslinked in situ to form a polymer network. Although each fumarate-based macromer presents a unique set of physical and mechanical properties, the materials can be tailored for particular applications, ranging from cell encapsulation to gene delivery. The injectability and biodegradability of fumarate-based polymers, coupled with the ease with which they can be modified, uniquely situate fumarate-based macromers as excellent scaffolds for tissue engineering.

Keywords: polymer, injectable, biodegradable, scaffold, controlled release

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List of Abbreviations

AA: ascorbic acid
APS: ammonium persulfate
β-TCP: β-tricalcium phosphate
BAPO: bis(2,4,6-trimethylbenzoyl) phenylphosphine oxide
BP: benzoyl peroxide
MSCs: marrow stromal cells
NVP: N-vinyl pyrrolidone
OPF: oligo(poly(ethylene glycol) fumarate)
OPN: osteopontin (extracellular matrix protein)
PEG: poly(ethylene glycol)
PEG-DA: poly(ethylene glycol) diacrylate
PLGA: poly(D,L-lactic-co-glycolic acid)
PNVP: poly(N-vinyl pyrrolidone)
PPF: poly(propylene fumarate)
P(PF-co-EG): poly(propylene fumarate-co-ethylene glycol)
PF-DA: propylene fumarate-diacrylate
RGD: Arg-Gly-Asp peptide sequence
TEA: triethylamine
TEMED: *N*,*N*,*N*',*N*'-tetramethylethylenediamine
TGF-β1: transforming growth factor-β1
UV: ultraviolet

Introduction

Over the past fourteen years, our laboratory has developed expertise in the fabrication of synthetic scaffolds with tailored chemistries for a variety of tissue engineering applications. These novel materials are based on fumaric acid, a natural product found in mammalian cell metabolism [1-3]. We have demonstrated these materials to be non-toxic to surrounding cells and tissues [4-8] and, therefore, well-tolerated *in vivo* [5]. Additionally, as these materials are based on naturally-occurring compounds, they degrade over time in the body to natural products which can be safely and efficaciously excreted [5]. Thus, after implantation of fumarate-based scaffolds, no further surgery would be needed for removal of the material, thereby reducing discomfort to the patient and cost of the procedure [9]. Additionally, these materials can crosslink *in-situ*, thus providing the possibility of injecting the scaffolding directly into the defect site. In this case, the material is initially a liquid that, after exposure to light or other initiating agents, cures into a solid in less than 15 minutes [2, 6, 10, 11]. Scaffold injectability offers a great advantage in that no invasive surgeries would be needed for either application or removal of the material [9].

Our laboratory has focused on developing three types of fumarate-based macromers: poly(propylene fumarate) (PPF), poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)),

and oligo(poly(ethylene glycol) fumarate) (OPF). Each of these materials has distinct physical and mechanical properties, and each can be tailored to promote repair in response to the specific requirements of the damaged tissue. This chapter will summarize the synthesis and crosslinking of each of these macromers, as well as provide examples of modifications for specific tissue engineering applications.

Poly(propylene fumarate) (PPF)

Synthesis and Purification

While a variety of synthetic techniques for this polymer have been utilized [12-14], currently our laboratory follows a two-step procedure involving bis(hydroxypropyl) fumarate as an intermediate [15]. In the first step, diethyl fumarate is combined with 1,2 propanediol in a 1:3 molar ratio. The reaction is run for approximately eight hours in an inert atmosphere in the presence of the catalyst $ZnCl_2$ (0.01 mol), with a slow increase in temperature from 100 to 160°C. This reaction produces the intermediate bis(hydroxypropyl) fumarate as well as ethanol, and the reaction is halted when 80-100% of the theoretical ethanol has been collected in a receiving flask.

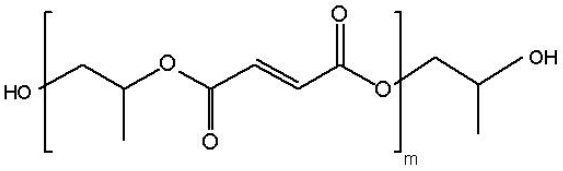
In the second step, the bis(hydroxypropyl) fumarate is transesterified, producing PPF and the by-product 1,2 propanediol. This reaction is run for approximately eight hours under vacuum (<1 mmHg) while the temperature is slowly increased from 90 to 150°C. The number average molecular weight (M_n) of the polymer increases with reaction temperature and time and the reaction is halted when the desired molecular weight is obtained by gel permeation chromatography.

To purify the product, the polymer is dissolved in dichoromethane and then washed with acid (5 wt% HCl in H_2O) to remove the catalyst. The solution is further purified by two washes each of brine and water and then the organic phase is dried using sodium sulfate. After drying, solvents are removed by rotary evaporation, leaving a viscous liquid of purified PPF.

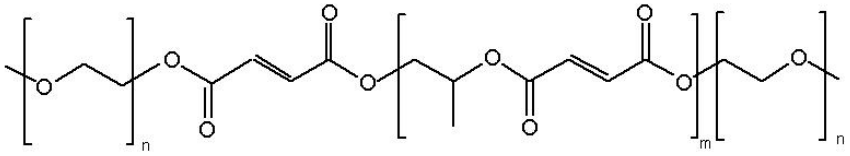
Crosslinking

Because PPF is an unsaturated linear polyester, it can be crosslinked through the double bond occurring in the fumarate unit (see Figures 1 and 2) [12]. Traditional cross-linking agents for this polymer include N-vinyl pyrrolidone (NVP) monomers with benzoyl peroxide (BP) as a radical

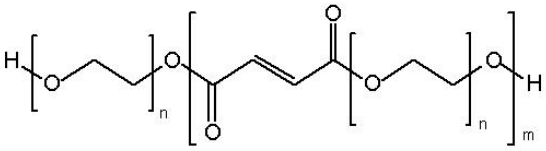
initiator [16, 17]. While the resulting poly(N-vinyl pyrrolidone) (PNVP) crosslinks are not degradable, PNVP is water-soluble and can be excreted through the kidneys [18].



Poly(Propylene Fumarate) (PPF)



Poly(Propylene Fumarate-co-Ethylene Glycol) (P(PF-co-EG))



Oligo(Poly(Ethylene Glycol) Fumarate) (OPF)

Figure 1: Chemical structures of the fumarate-based macromers discussed in the text, including poly(propylene fumarate) (PPF), the methoxy-capped triblock copolymer poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG)), and oligo(poly(ethylene glycol) fumarate) (OPF).

Efforts in our laboratory have also produced a derivative of PPF, propylene fumarate-diacrylate (PF-DA), as a novel crosslinking agent for PPF (see Figure 2). When crosslinked with PPF using BP, the resulting samples showed increasing compressive strength and modulus with higher amounts of the crosslinker, providing a method to tailor mechanical properties to correspond to the tissue to be replaced [19]. In addition, the presence of the fumarate groups in the crosslinker allow for the possibility of biodegradation of the network [20].

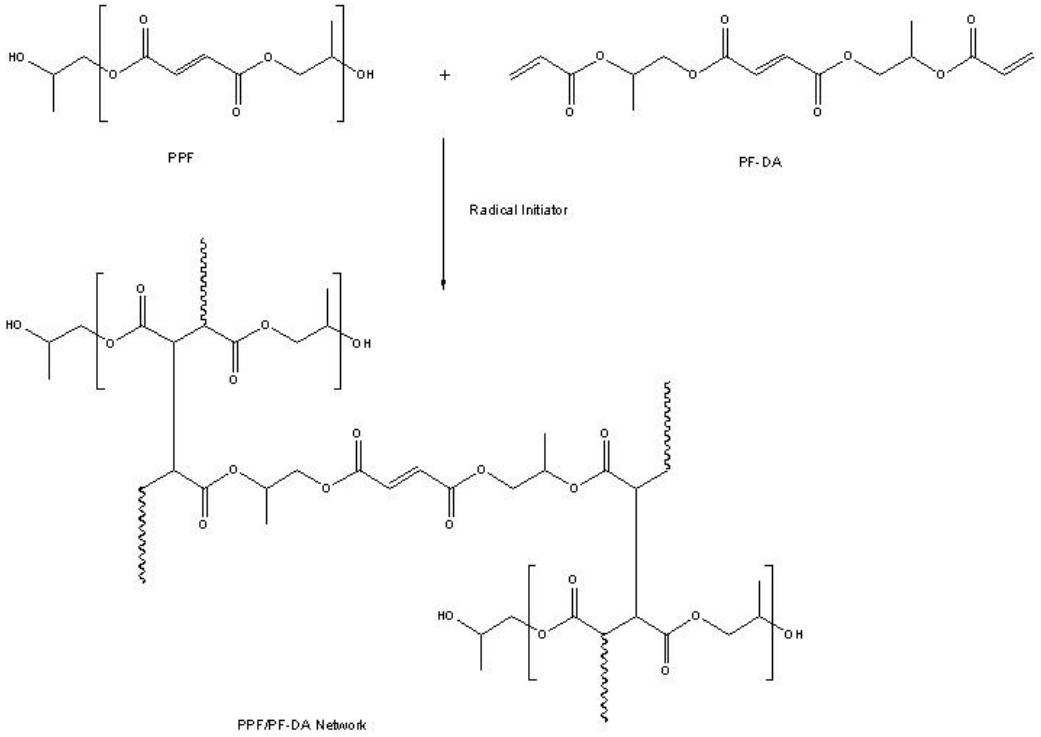


Figure 2: Crosslinking of poly(propylene fumarate) (PPF) with the crosslinker propylene fumarate-diacrylate (PF-DA) in the presence of thermal or photo-based radical initiators. PPF can also be crosslinked without crosslinking agents using photoinitiators. Water-soluble thermal initiators have been employed to crosslink poly(propylene fumarate-co-ethylene glycol) (P(PF-co-EG) and oligo(poly(ethylene glycol) fumarate) (OPF) via similar reaction schemes.

Instead of addition of a crosslinking molecule, PPF chains can be covalently bonded to themselves by addition polymerization. It has been shown in our laboratory that this is possible by the addition of the photoinitiator bis(2,4,6-trimethylbenzoyl) phenylphosphine oxide (BAPO) and exposure to ultraviolet (UV) light [15, 21, 22]. BAPO has also been used as an initiator for networks of PPF and PF-DA [23], or PPF and diethyl fumarate [24].

Modification for Specific Applications

In studies using the material as a scaffold to encourage bone ingrowth, PPF was combined with ceramic particles such as β-tricalcium phosphate (β-TCP) [16, 25, 26]. These PPF/β-TCP composites were implanted in rat tibiae for up to five weeks. At this time, the material was observed to be gradually replaced by bone from the perimeter inward [17].

To investigate their use for delivery of bioactive factors to promote bone formation, crosslinked PPF foams were placed in rabbit cranial defects with or without pre-adsorption of transforming growth factor-β1 (TGF-β1) [7, 27]. At eight weeks, a mild inflammatory response was seen for all scaffolds and significantly more bone ingrowth was evident for samples coated with TGF-β1. Additionally, PPF has been used to form composite scaffolds incorporating poly(D,L-lactic-co-glycolic acid) (PLGA) microspheres to control delivery of osteogenic factors in a rabbit radial defect model [28-30]. Guided bone formation was observed to bridge up to 80% of the segmental defect after 12 weeks of implantation of PPF scaffolds releasing the osteogenic thrombin peptide TP508 [31] (see Figure 3).

To explore the idea of cell delivery with PPF, other research has examined the addition of osteoblast-type cells to an injectable PPF construct [31-33]. In our laboratory, a method for encapsulation of cells has been developed for short-term protection from unfavorable crosslinking conditions, such as the presence of radical species and potential temperature rise. With this technique, cells are encapsulated in gelatin microspheres crosslinked on the surface to prevent premature dissolution [31]. It has been shown that marrow stromal cells (MSCs), when encapsulated in this manner and added to crosslinking PPF, exhibit significantly higher initial viability than non-encapsulated cells [32].

Further studies have demonstrated that PPF scaffolds can be mechanically reinforced through the incorporation of nanoparticles and nanotubes to form composites. The inclusion of surface-modified carboxylate alumoxane nanoparticles (1 wt %) dispersed within PPF/PF-DA has been shown to result in a greater than 3-fold increase in flexural modulus compared to the PPF/PF-DA material alone [34]. Further, increasing degrees of nanoparticle loading into the material did not result in significant loss of flexural or compressive strength [34]. Similar results have been observed upon the incorporation of functionalized single-walled carbon nanotubes within PPF scaffolds [35]. Specifically, nanocomposites formed with as low as 0.1 wt % loading of functionalized single-walled carbon nanotubes exhibited a 3-fold increase in both compressive modulus and flexural modulus and a 2-fold increase in both compressive offset yield strength and flexural strength in relation to PPF scaffolds alone [35].

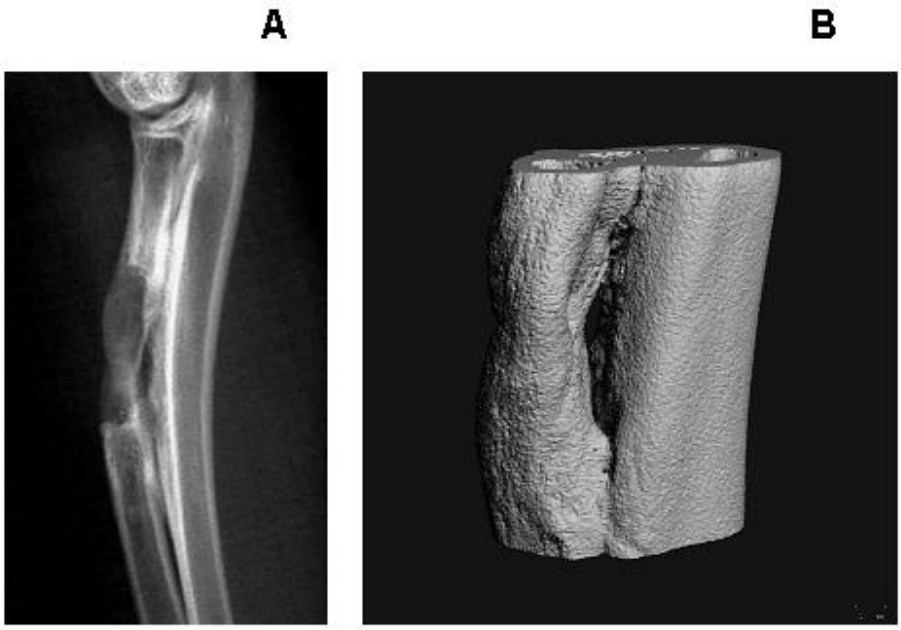


Figure 3: Radiograph (A) and micro-computed tomography image (B) of bone regeneration in a radial segmental defect in a rabbit model upon release of the osteogenic peptide TP508 from PPF scaffolds (adapted from [29-30]) (note: images not to same scale).

Poly(propylene fumarate -co-ethylene glycol) (P(PF-co-EG))

Synthesis and Purification

P(PF-co-EG) is a block copolymer based on PPF, but, unlike PPF, due to the addition of the poly(ethylene glycol) (PEG) chains, many formulations of this copolymer are water-soluble (see Figure 1) [36]. In the current scheme used in our laboratory, a triblock copolymer with methoxy-capped endgroups is synthesized in a three-step procedure [3]. The first two steps are identical to those described above for PPF. The resulting polymer is further transesterified with methoxy poly(ethylene glycol) under vacuum (<1 mmHg) at 160°C until evidence of copolymer formation is observed via GPC.

For purification, the product is dissolved in dichloromethane, filtered and precipitated in ethyl ether to remove the catalyst. The ethyl ether is removed by rotary evaporation if necessary and then the product is dried under vacuum. The purified copolymer is either a waxy solid or a powder at room temperature.

Crosslinking

Like PPF, P(PF-co-EG) also contains many unsaturated double bonds through which crosslinking can occur (Figure 1) [3]. To date, our laboratory has explored crosslinking only via thermal radical initiation. The two most commonly employed systems are the NVP/BP combination described above [36] or a bimolecular initiation system of ascorbic acid (AA) and ammonium persulfate (APS) used with a poly(ethylene glycol) diacrylate (PEG-DA) crosslinker [37]. The advantage to the later system is that a pore-forming agent such as sodium bicarbonate can be added to the mixture during crosslinking. The interaction with ascorbic acid produces carbon dioxide, allowing for highly porous hydrogel materials to be produced while maintaining the injectability of the system [10].

Modification for Specific Applications

When implanted in a cage implant model in rodents, this copolymer exhibited a normal wound healing response over 21 days [5]. In order to more specifically control cellular response to this material, our laboratory has focused on modification techniques to covalently attach a variety of cell adhesion ligands to P(PF-co-EG). In one set of experiments, scaffolds derivatized with the Arg-Gly-Asp (RGD) peptide sequence, known to interact with integrin receptors on the cell surface, increased MSC attachment and spreading on P(PF-co-EG) in a specific and dose-dependent manner [38]. Ligand density and copolymer composition also affected migration ability of MSCs on the scaffold surface [38]. Furthermore, after attachment, the MSCs retained their ability to differentiate *in vitro* to produce bone-like, mineralized tissue [39]. These types of scaffolds may be most directly applicable to guided tissue regeneration for dental defects, where proliferation and migration of specific cell types is desired.

Another approach involves modification of P(PF-co-EG) to direct protein adsorption at the surface, which will then impact the global response to the material, including everything from blood coagulation to cell adhesion [40, 41]. Our laboratory has developed a technique to include positively-charged agmatine moieties in P(PF-co-EG) hydrogels, which may affect the type and amount of adsorbed proteins [41]. In the presence of serum proteins, the adhesion of smooth muscle cells was significantly greater with increased incorporation of positive charge [41].

Additionally, polymer systems based upon P(PF-co-EG) have been shown to be thermoreversible, with solutions of the polymer being liquid below 25^{o}C and gels above 35^{o}C [42] (see Figure 4). These thermoreversible hydrogels can be applied toward the encapsulation and delivery of cells and/or bioactive factors for tissue engineering. Bovine articular chondrocytes encapsulated in P(PF-co-EG) hydrogels remained viable and synthesized proteoglycans throughout culture *in vitro* [42].

Oligo(Poly(ethylene glycol) fumarate) (OPF)

Synthesis and Purification

OPF, like P(PF-co-EG), is a water-soluble macromer, however, it is comprised of alternating PEG and single fumarate moieties (see Figure 1), which results in greater hydrophilicity than for P(PF-co-EG), with OPF hydrogels swelling up to 17-fold [43]. Synthesis has traditionally proceeded through reaction of PEG, fumaryl chloride and triethylamine (molar ratios 1:0.9:0.9) in a single step [2]. After azeotropic distillation, PEG is dissolved in dichloromethane and fumaryl chloride and triethylamine (TEA) are added drop-wise over several hours while the reaction flask is held near 0°C. The reaction is then left stirring for 1-2 days at room temperature to assure maximal conversion.

To purify the oligomer, the solvent is removed by rotary evaporation, followed by dissolution of the product in ethyl acetate. The solution is filtered to remove the salt produced by the reaction of the chloride ions and TEA, and the oligomer is recrystalized twice in ethyl acetate. Finally, the purified OPF is precipitated in ethyl ether and dried under vacuum (<1 mmHg) to produce a powder.

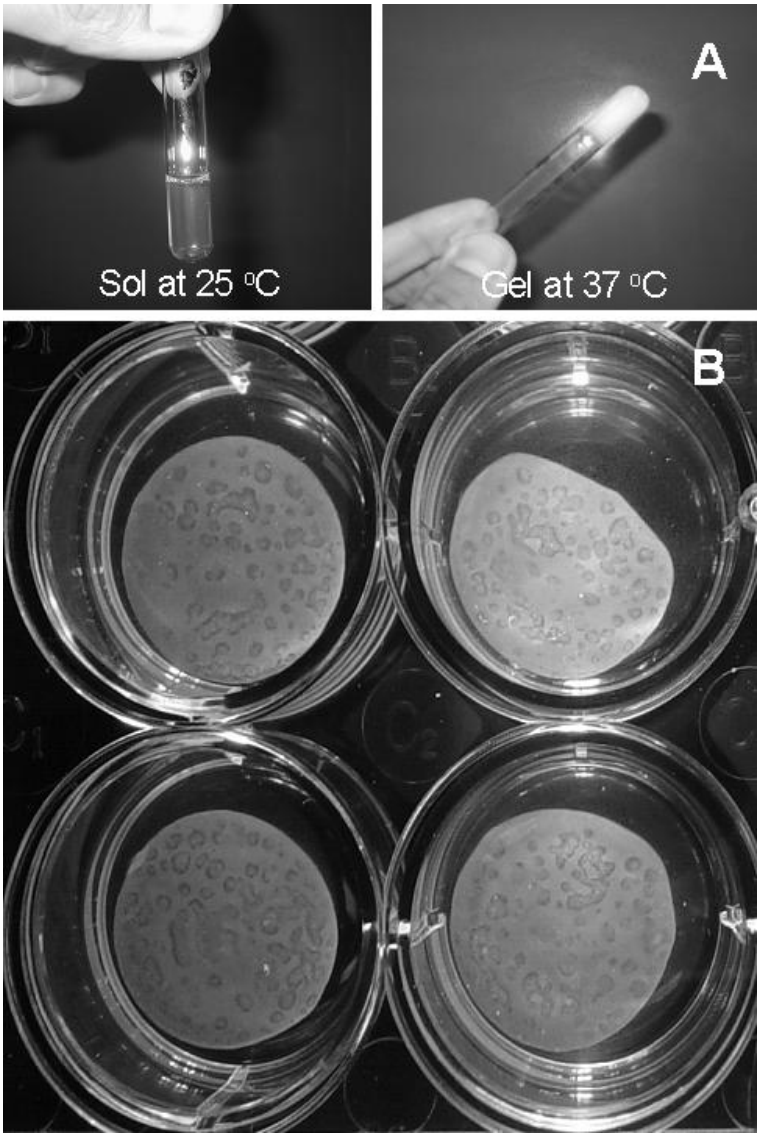


Figure 4: Thermoreversible solutions of P(PF-co-EG) demonstrated below (25°C) and above (37°C)the lower critical solution temperature (A). Clusters of viable chondrocytes are visible encapsulated with P(PF-co-EG) hydrogels following 8 days of culture (B) [3, 42].

Crosslinking

Although OPF contains fewer double bonds by weight than the polymers previously discussed, crosslinking still readily occurs though the fumarate units (Figure 1). Crosslinking is achieved using AA/APS, the thermal radical initiators discussed previously [11], or another radical-forming pair, APS and *N*,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED) [43]. The advantage to the later combination is that, when combined in equimolar amounts, the APS/TEMED system remains neutral [43], while the APS/AA system is strongly acidic [44]. Both PEG-DA [11, 43]

and *N*,*N*'-methylene bisacrylamide [45] have been used in conjunction with these radical initiators as crosslinking agents for OPF.

Modification for Specific Applications

OPF has been modified using techniques similar to those employed for the P(PF-co-EG) hydrogels to include cell attachment ligands such as RGD [8] and a 15 amino acid sequence derived from the extracellular matrix protein osteopontin (OPN) [46, 47]. Both the non-modified and RGD-modified hydrogels exhibited no deleterious inflammatory response after subcutaneous implantation in rodents [48]. In *in vitro* experiments, it was found that MSCs migrate longer distances than fibroblasts on hydrogels containing the OPN-derived peptide and that MSCs migrated farther on OPN- than RGD-modified gels [46]. In addition, MSCs retained their ability to differentiate and produce mineralized extracellular matrix when cultured *in vitro* on the OPN-derivatized OPF hydrogels [47]. As with P(PF-co-EG), these results may provide a means to control cell behavior for guided tissue regeneration applications.

OPF may also have uses in replacement of non-load-bearing bone defects. Recent work in our laboratory has developed a crosslinking technique that is mild enough to include MSCs in the precursor solution and then crosslink the hydrogel around the cells for injectable cell delivery applications [43]. Results have demonstrated that the encapsulated MSCs retain their ability to differentiate and produce calcified matrix over 28 days *in vitro*, and that hydrogels with greater swelling further promote this differentiation [49]. Further studies have demonstrated the capability to deliver both cells and growth factors with OPF hydrogels [50]. Specifically, bovine chondrocytes embedded in composite hydrogels co-encapsulating gelatin microparticles loaded with TGF-β1 proliferated and produced glycosaminoglycans to a greater extent than in constructs without TGF-β1 [50].

Further, OPF hydrogels have been investigated as delivery systems for plasmid DNA. Specifically, plasmid DNA has been physically entrapped within OPF hydrogels during crosslinking and subsequently released, with the release kinetics depending upon tunable physical properties of the OPF oligomer [51] (see Figure 5). Additionally, hydrogel composites of OPF and cationized gelatin microspheres have been shown to allow for prolonged controlled release of therapeutic plasmid DNA both *in vitro* [52] and *in vivo* [53, 54], with a maintenance of the structural integrity of the DNA [51].

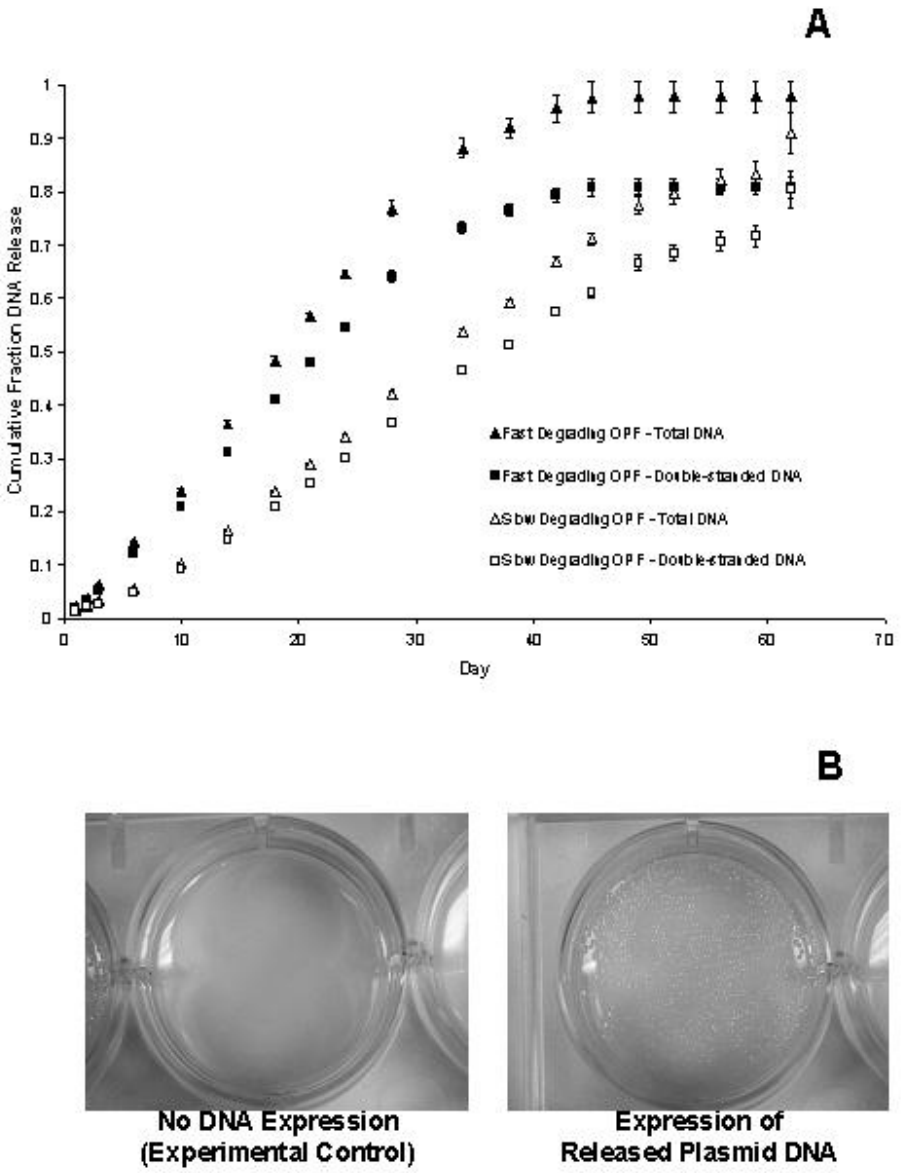


Figure 5: Profiles of plasmid DNA release from OPF hydrogels *in vitro* (n =5) (A). Error bars represent ± standard deviation (adapted from [51]). Plasmid DNA retained bioactivity over the course of release as indicated by surviving *E. coli* colonies expressing the antibiotic resistance gene of the released plasmid relative to the experimental control when cultured on antibiotic selective culture plates (B) [51].

OPF has also been explored for use a delivery vehicle for bioactive factors to promote cartilage regeneration [45, 50, 55-58]. An advantage to the OPF system is that it has the ability to form laminated structures if a second layer is added before the first has completely crosslinked [11]. This provides the opportunity to effect regeneration of complex defects involving more

than one tissue type, such as osteochondral defects in the knee. A bilayered system including gelatin microspheres containing TGF-β1 was well-tolerated and showed excellent repair of osteochondral defects in rabbits at 14 weeks [55]. In other experiments, the gelatin microspheres were loaded with more than one growth factor to obtain both spatial and temporal control of release profiles [56, 57]. In this case, since complexation occurs between the charged bioactive molecules and oppositely-charged gelatin, a main mechanism of release is degradation of the gelatin by enzymes secreted by surrounding cells, allowing localized delivery as needed during the repair process [58]. Complete degradation of the OPF constructs was observed in osteochondral defects in rabbits at 12 weeks post-surgery, with greater osteochondral repair associated with OPF scaffolds releasing the growth factor IGF-1 when compared to untreated defects [56].

Conclusion

Given the diversity in structure and function of human tissues, biomaterials that can be tailored for specific applications are important to the success of repair strategies involving tissue engineering. This chapter has reviewed three fumarate-based macromers, each of which has distinct physical and mechanical properties. In time, each polymer may find its own unique application. The advantage to all of these materials, however, is that they may be used as part of an injectable, biodegradable system. Further developments of these fumarate macromers could provide a significant step towards the ideal of minimally-invasive tissue engineering for a variety of tissue types.

Acknowledgements
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