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A Heparin-Mimicking Polymer Conjugate Stabilizes Basic Fibroblast Growth Factor (bFGF)

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Abstract

Basic fibroblast growth factor (bFGF) plays a crucial role in diverse cellular functions from wound healing to bone regeneration. However, a major obstacle to the widespread application of bFGF is its inherent instability during storage and delivery. Herein, we describe stabilization of bFGF by covalent conjugation of a heparin-mimicking polymer, a copolymer consisting of styrene sulfonate units and methyl methacrylate units bearing poly(ethylene glycol) side chains. The bFGF conjugate of this polymer retained bioactivity after synthesis and was stable to a variety of environmentally and therapeutically relevant stressors such as heat, mild and harsh acidic conditions, storage, and proteolytic degradation, compared to native bFGF. After applied stress, the conjugate was also significantly more active than the control conjugate system where the styrene sulfonate units were omitted from the polymer structure. This research has important implications for the clinical use of bFGF and for stabilization of heparin-binding growth factors in general.

Covalent conjugation of synthetic polymers, in particular poly(ethylene glycol) (PEG), has been widely explored as a means to improve the half-lives of proteins in vivo, and to lower the immunogenicities and antigenicities of proteins. Consequently, a number of PEGylated proteins have been approved by the US Food and Drug Administration for treatment of a variety of diseases. However, therapeutic proteins often suffer from instability during storage and use, and PEG does not necessarily stabilize proteins to external stressors. Yet, there are only a few reports on conjugating polymers that promote
protein stabilization: Keefe et al. demonstrated that covalently binding poly(carboxybetaine) to α-chymotrypsin improved stability and at the same time retained the enzyme’s native binding affinity.\(^4\) We showed that polystyrene with pendent trehalose disaccharides resulted in lysozyme conjugates stable to high temperatures and repeated lyophilization.\(^5\) Herein, we demonstrate for the first time stabilization of a protein that is a member of a large class of therapeutically useful biologics, namely the heparin-binding proteins, with a polymer specifically designed to interact with the heparin-binding domain of the growth factor.

A considerable number of proteins interact with the polysaccharide heparin and make up the class of heparin-binding proteins, including proteases, growth factors, chemokines, lipid-binding proteins, pathogens, and adhesion proteins.\(^6\) Their key biological functions are wide ranging and include blood coagulation, cell differentiation, angiogenesis, inflammation, host defense and viral infection mechanisms, lipid transport and clearance, and cell adhesion and interaction. Since the introduction of heparin in the early 1900s as an anticoagulant agent, it is now known that the role of heparin in the body is far reaching. Molecular modeling and crystallography studies have defined the heparin-binding motifs on numerous proteins,\(^7\) and researchers have found that their interactions with heparin were not only critical for bioactivity, but in many cases also for stabilization. In this report, we describe that an important heparin-binding protein, basic fibroblast growth factor (bFGF), is stabilized by conjugation of a synthetic heparin-mimicking polymer.

bFGF is a therapeutic target widely investigated because of its crucial role in diverse cellular functions including: embryonic development,\(^8\) angiogenesis,\(^9\) tissue regeneration,\(^10\) bone regeneration,\(^11\) development and maintenance of the nervous system,\(^12\) stem cell self-renewal,\(^13\) and wound healing.\(^14\) bFGF is a potent stimulator of proliferation, differentiation and migration of multiple cell types.\(^15,\)\(^16\) Therefore, bFGF is promising for a wide variety of applications in regenerative medicine and others. However, due to the protein’s extreme instability in storage and delivery,\(^17,\)\(^18\) its therapeutic effectiveness is not yet widely realized.\(^19\)

Since heparin is the natural stabilizer of bFGF,\(^18,\)\(^20\) many researchers employ heparin in controlled release systems of this growth factor.\(^21\) However, heparin itself is difficult to modify, is susceptible to desulfation, suffers from batch-to-batch variation and impurities, and has significant activity in other, non-target biological pathways. In addition, it has been reported to inhibit normal growth of certain cell types including human umbilical vein endothelial cells and human dermal fibroblasts, which could possibly counteract the desirable effects of bFGF.\(^22,\)\(^23\) It is known that sulfated and sulfonated polymers can mimic heparin.\(^24,\)\(^25\) Here, we report that covalent conjugation of a heparin-mimicking polymer, poly(sodium 4-styrenesulfonate-co-poly(ethylene glycol) methyl ether methacrylate) (p(SS-co-PEGMA)),\(^26,\)\(^27\) to bFGF significantly enhances protein stability. Thus far, only PEG has been covalently conjugated to bFGF;\(^28–32\) but these conjugates either have significantly reduced protein activity, require addition of heparin to stabilize the conjugate, or require large protein concentrations. To our knowledge, this is the first example of a stabilized bFGF conjugate.
Results

Synthesis of Polymers and Analysis of Cytotoxicity

p(SS-co-PEGMA) was selected because we previously demonstrated that the polymer bound to bFGF in cell culture media, likely through interaction with the heparin-binding domain.\textsuperscript{26, 27} bFGF has two free cysteines; thus, the polymer was prepared with a pyridyl disulfide (PDS) end group that reacts with thiols. Reversible addition-fragmentation chain transfer polymerization has been widely employed for preparation of protein-polymer conjugates.\textsuperscript{33–35} RAFT polymerization of SS and PEGMA monomers in the presence of a PDS-functionalized trithiocarbonate chain transfer agent (CTA) produced the desired polymer (Fig. 1a). Since the trithiocarbonate moiety can exhibit cytotoxicity at high polymer concentrations,\textsuperscript{36} this group was removed by radical exchange with 2, 2’-azobisisobutyronitrile (AIBN). The resulting copolymer PDS-p(SS-co-PEGMA) had a number-average molecular weight ($M_n$) of 26.1 kDa by NMR and a polydispersity index (PDI) of 1.16 (Supplementary Fig. S1). The analogous control polymer, PDS-pPEGMA, was prepared via atom transfer radical polymerization (ATRP) in the presence of an PDS-functionalized initiator, CuBr and 2, 2’-bipyridine (Fig. 1b). It had a $M_n$ of 23.0 kDa by NMR and a PDI of 1.13 (Supplementary Fig. S3).

Next, the cytotoxicity of the p(SS-co-PEGMA) without the PDS end group was evaluated and compared to nontoxic PEG, control polymer pPEGMA and heparin. PEG 4-kDa was chosen as the control because it has a comparable number of repeating units to p(SS-co-PEGMA) (DP = 91 and 98, respectively). The cytotoxicity study was performed with normal human dermal fibroblast (HDF) cells. HDF cells play an important role in the wound healing process of skin, and the proliferation of these cells is largely stimulated by bFGF.\textsuperscript{15} HDF cells were exposed to either p(SS-co-PEGMA), pPEGMA, PEG, or heparin at increasing concentrations from 1 ng/ml to 1 mg/ml for 24 hours in the absence of bFGF before assessment with the LIVE/DEAD® viability assay. The percent cell viability was the same in the presence of all of the polymers at all concentrations tested, and the same as no polymer added (Fig. 1c). This demonstrated that the heparin-mimicking polymer p(SS-co-PEGMA) is non-cytotoxic to HDF cells to at least 1 mg/ml.

Preparation of Conjugates

An on-column conjugation technique was utilized to synthesize bFGF-p(SS-co-PEGMA) and bFGF-pPEGMA.\textsuperscript{37} Briefly, the conjugations were conducted by adding the corresponding polymer solution to heparin sepharose-bound bFGF, prior to eluting with increasing salt in Dulbecco’s phosphate buffered saline (D-PBS). In this way, the excess polymer was completely removed (Supplementary Fig. S5); the typical yield of the isolated conjugate was 50% (Supplementary Table S1). Western blotting of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed diffuse bands at higher MW typical of protein-polymer conjugates (Fig. 2b left, lane 2 and Supplementary Fig. S4). Native PAGE showed a smear corresponding to the heparin-mimicking polymer conjugate due to the change in its overall surface charge (Fig. 2b right, lane 2). Furthermore, the bands corresponding to the conjugates disappeared while the signals corresponding to the
unconjugated bFGF were more intense under reducing conditions, as expected for conjugates prepared via disulfide bonds.

Analyses of the conjugates via matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization mass spectrometry (ESI-MS) were unsuccessful due to difficulties with ionization and the sample heterogeneity. Therefore, electrospray ionization-gas-phase electrophoretic mobility molecular analysis (ESI-GEMMA) of the conjugates was employed. ESI-GEMMA separates macromolecules based on their electrophoretic mobility in air that is directly related to their electrophoretic mobility diameters (EMD); we have shown previously that the technique can be applied to polymer conjugates.\textsuperscript{38, 39} The ESI-GEMMA spectrum of bFGF (MW=16 kDa) showed a major peak at 4.45 nm corresponding to 16,116 Da (Fig. 2c, top). Utilizing the signals for the bFGF-p(SS-co-PEGMA) and bFGF-pPEGMA (Fig. 2c bottom, Supplementary Fig. S4), the calculated MWs were 39 kDa and 30 kDa, respectively, when assuming one polymer was attached to the protein (see ref. 38 for details of how the molecular weights were calculated). This agreed reasonably well with the theoretical MWs of 42 kDa and 39 kDa, respectively. The small discrepancy in the MWs was attributed to the polydispersity of the conjugated polymers. The results were significantly different if instead the calculation assumed two polymers were attached to one protein (for example for bFGF-p(SS-co-PEGMA), the calculated MW of this conjugate was 41 kDa compared to the theoretical MW of 68 kDa). The results strongly support that one polymer was conjugated to bFGF for both conjugates.

**Stability Studies**

HDF cells were used as the in vitro model to evaluate the bioactivity of the bFGF-heparin-mimicking polymer conjugate before and after exposure to various stresses. With careful storage at −80 °C before use for all samples, an addition of 1 ng/ml of bFGF-p(SS-co-PEGMA) conjugate stimulated cell growth to 213 ± 13%, which was not statistically different than when 1 ng/ml of bFGF (192 ± 9%), bFGF (+)1 µg/ml of heparin (205 ± 10%), bFGF (+)1.5 ng/ml of heparin (197 ± 4%), bFGF (+)1.5 ng/ml of pPEGMA (179 ± 14%), 1 ng/ml of bFGF-pPEGMA (190 ± 6%), or bFGF (+)1.5 ng/ml of p(SS-co-PEGMA) (207 ± 5%) were applied (Fig. 3). Thus, all samples had similar activity prior to application of stress.

Stressors were chosen to investigate the stability of the bFGF-p(SS-co-PEGMA) conjugate in comparison to other groups. The chosen treatments represent environmental stresses that the growth factor (or any heparin-binding protein) could be exposed to such as storage in the unfrozen form and exposure to heat during delivery, transport, or use (represented by “Storage at 4 °C for 16 hrs” and “Heat at 55 °C for 30 mins” in Fig. 3). Incubation of the protein in pH 4.7 buffer for 16 hours is relevant because mildly acidic conditions are present during wound healing.\textsuperscript{40} Furthermore, the stability of the bFGF-heparin-mimicking polymer conjugate was challenged against proteolytic degradation and extreme acidic conditions by incubating with 0.1% w/v trypsin for 16 hours and 1% trifluoroacetic acid (TFA) for 2 hours, respectively. The starting concentrations of bFGF and bFGF in the conjugates were equivalent and the concentrations of the unconjugated polymers and heparin were the same.
at approximately stoichiometric ratios to bFGF. The positive control group contained 700 fold molar excess of heparin to bFGF. The negative control was native bFGF alone.

In all cases, the bioactivity of bFGF-p(SS-co-PEGMA) was significantly higher than all the other groups including bFGF-pPEGMA, and the same as the positive control (Fig. 3). Upon exposure to storage and mildly acidic conditions, the bioactivity of bFGF-p(SS-co-PEGMA) (210 ± 10% and 183 ± 13%, respectively) was statistically the same as before treatment (213 ± 13%). The bFGF-heparin-mimicking polymer conjugate also showed no loss of bioactivity for at least 6 weeks when stored at 4 °C (Supplementary Fig. S7). The percent cell growth of bFGF-p(SS-co-PEGMA) under heat, trypsin and 1% TFA treatments were 167 ± 12%, 176 ± 11%, and 162 ± 17% respectively. In each case, the bFGF-heparin-mimicking polymer conjugate was statistically the same as the positive control, bFGF with a 700 molar excess of heparin. The bFGF-pPEGMA conjugate did exhibit some stability under storage, mildly acidic and trypsin treatments (p < 0.01). However, the bFGF-p(SS-co-PEGMA) conjugate had a significantly better stability profile than the bFGF-pPEGMA conjugate for all treatments (p < 0.001 for storage, p < 0.05 for others). This result demonstrates the importance of a heparin-mimicking polymer for the observed protective effect.

**Inhibition study**

To confirm that the bFGF-heparin-mimicking polymer conjugate promoted cell growth by the same signal transduction pathway as native bFGF, a potent inhibitor for FGF receptor 1 (FGFR1) was included in the medium. PD173074 competes with adenosine-5'-triphosphate (ATP) for binding to FGFR1, a key step for FGFR phosphorylation, and shuts down the signal transduction pathways associated with cell proliferation. HDF cells express primarily FGFR1 and PD173074 has been well demonstrated to deactivate FGFR1 phosphorylation at low concentrations. The following groups were tested: 1 ng/ml of bFGF, 1 ng/ml of bFGF with 1 µg/ml of heparin, and 1 ng/ml bFGF-p(SS-co-PEGMA). The data was normalized to the blank group containing no PD173074. Figure 4 shows that for 1 ng/ml of bFGF with no inhibitor present, the percent cell growth was 178 ± 6%; while in the presence of 125 nM PD173074, the percent cell growth was decreased to 88 ± 5%. Similarly for bFGF-p(SS-co-PEGMA), the percent cell growth without and with the addition of PD173074 were 177 ± 6% and 85 ± 11%, respectively. In the presence of 1 µg/ml of heparin, cell proliferation at 197 ± 14 % was reduced to 108 ± 5% when 125 nM of PD173074 was added. This data indicated that the bFGF-heparin-mimicking polymer conjugate triggered HDF cell proliferation through transmembrane tyrosine kinase receptor (FGFR) activation similar to native bFGF, since activity was abrogated by an inhibitor for this receptor.

**Proliferation study of BaF3 cells**

While bFGF interacts directly with cells by binding to FGFRs, signal transduction requires simultaneous interaction with heparan sulfate (HS) proteoglycan cell surface receptors. To investigate whether the heparin-mimicking polymer p(SS-co-PEGMA) stabilized the growth factor as anticipated or whether the observed activity resulted from participation of the polymer in the binding site, BaF3 cells (FR1C-11) were used. This BaF3 cell line is
engineered to express FGFR1 and lacks cell-surface HS; addition of soluble heparin is required to activate the receptors with bFGF. A 1 µg/ml concentration of heparin effectively stimulated cell proliferation as expected; percent cell growth was almost four times the blank control (384 ± 35%) (Fig. 5). An addition of 1 µg/ml of the p(SS-co-PEGMA) did not show an increase in proliferation; the percent cell growth (128 ± 13%) was similar to the sample group where bFGF was added without heparin (127 ± 12%). Likewise, 1 ng/ml of the bFGF-p(SS-co-PEGMA) did not stimulate significant cell growth (147 ± 14%), suggesting that the heparin-mimicking polymer did not participate in receptor binding of the protein to FGFRs. Thus the role of the polymer was to stabilize the growth factor, not to activate the receptor.

Discussion

For the past three decades, PEG conjugates have been widely utilized as biologic drugs; yet these conjugates still suffer from environmental instability issues, and often require addition of large concentrations of excipients. We demonstrate herein that by careful design of a polymer to mimic a natural polysaccharide, a protein that is normally unstable can be rendered stable to numerous stresses that would typically inactivate the protein. bFGF has far reaching biological activity from wound healing to cardiac repair and as a result is an important drug target; thus, this stable construct may be useful therapeutically. Indeed, prior results have demonstrated that clinical trials of bFGF in wound healing have failed, potentially due to issues with stability. The results also suggest that the bFGF-heparin-mimicking polymer conjugate does not require restrictive storage temperatures (freezing) or require loading of excipients as does the native protein. This is important when considering at-home patient use of any clinically relevant biologic, where storage in a freezer until just before use is not desirable and may not be feasible.

Interestingly, the bFGF-heparin-mimicking polymer conjugate, bFGF-p(SS-co-PEGMA), had superior stability compared to the control conjugate, bFGF-pPEGMA. bFGF-pPEGMA was significantly degraded after exposure to all stress conditions. With exposure to trypsin and mild acid, the experimental set-up required keeping samples refrigerated rather than frozen. Thus, it cannot be ruled out that the typical testing conditions for these stressors, i.e., keeping the samples at 4 °C for 16 hours, caused the degradation, rather than the enzyme or acidic conditions. Yet, the data clearly showed that the heparin-mimicking polymer conjugate was superior to the PEGylated one, in that it could be heated to high temperature or stored in the refrigerator for weeks without loss of activity. The conjugate was also stable to acidic conditions (that may be found in wounds for example), and to at least one proteolytic enzyme, which is a typical advantage of a PEGylated conjugate.

In the current study, the resulting conjugate outperformed addition of the same equivalent of non-conjugated polymer or heparin to bFGF. This suggests that the close proximity of the heparin-mimicking polymer protected bFGF from denaturation. This result is also useful because it shows that a very small amount of polymer can be used for stabilization. A large concentration of heparin stabilized the protein as expected. However, heparin itself has significant cross-bioactivity, and thus adding large amounts of the polysaccharide in vivo is not desirable.
Other heparin-binding proteins bind to heparin for stabilization. For example, non-conjugated PEG-polyanions (pentosan polysulfate and dextran sulfate) have been utilized to increase stability of a related protein keratinocyte growth factor-2 (KGF-2);\(^4^9\) histidine-tagged FGF-8a has been non-covalently associated with nitrilotriacetic acid-nickel-modified poly(acrylamide).\(^5^0\) These complexes are non-covalent. Non-covalent conjugates may not be useful in vivo because of the likelihood of detachment of the polymer upon dilution before reaching target sites. Thus, the strategy described herein, whereby a heparin-mimicking polymer is covalently, yet reversibly, attached to the protein, may be useful to stabilize other heparin-binding growth factors such as KGF-2. These studies are underway.

Although the inhibition assay indicated that the bFGF-heparin-mimicking polymer conjugate triggered HDF cells proliferation via the same signal transduction pathway as native bFGF, the heparin-mimicking polymer did not participate in receptor binding as does heparin. Heparin added at high concentrations to normal cells can inhibit the activity of these cells,\(^2^2,\,2^3\) and this has been proposed to occur by competition of heparin with the HS. That p(SS-co-PEGMA) mimicked heparin in that it bound to bFGF to stabilize it, but did not participate in receptor binding, could be advantageous: high concentrations of the polymer may not inhibit cellular activity as does heparin. It may be that the sulfonate groups do not bind to the receptor or that the PEG side chains prevented the polymer from fitting into the HS groove of the bFGF/FGFR tetramer complex.\(^4^5\) Thus, it would also be interesting to identify a polymer that would stabilize the bFGF and bind in the HS receptor site, and this work is underway.

**Conclusions**

In this report, we describe a protein-polymer conjugate that has superior stability while retaining native activity after a variety of stressors. A cysteine-reactive heparin-mimicking polymer, PDS-p(SS-co-PEGMA), was prepared via RAFT polymerization. Conjugation of the polymer to bFGF via disulfide exchange was accomplished using an on-column technique to produce bFGF-p(SS-co-PEGMA). The conjugate exhibited significantly enhanced stability against heat, mild and harsh acidic conditions, storage, and proteolytic degradation compared to native bFGF and the analogous bFGF-pPEGMA conjugate. The conjugate targeted the same receptor as unmodified bFGF. While one equivalent of added polymer or heparin did not stabilize the growth factor, the same amount of conjugated polymer did. The results together demonstrate that the strategy of conjugating heparin-mimicking polymers to bFGF is valuable as a means to stabilize this clinically important protein.

**Methods**

**Synthesis of bFGF-p(SS-co-PEGMA) conjugate**

bFGF (25 µg, 1.6 × 10\(^{-3}\) µmol) was diluted into 900 µl of D-PBS + 1mM ethylenediaminetetraacetic acid (EDTA), and loaded onto a hand-packed 1ml-heparin Sepharose column.\(^9\) PDS-p(SS-co-PEGMA) (3.6 mg, 0.14 µmol) was dissolved in 900 µl of D-PBS + 1mM EDTA, and loaded onto the column; the flow-through volume was collected for analysis. The column was allowed to incubate at 4 °C for 16 hours. The unconjugated
polymer, and weakly bound bFGF were washed off the column with 2 × 6 ml of D-PBS and 1 × 3 ml of 0.5 M NaCl D-PBS, respectively. The conjugate was eluted off the column using 2 × 6 ml of 2 M NaCl D-PBS. All of the fractions, except for the 2 M NaCl fraction, were then desalted, concentrated using a CentriPrep® centrifugal membrane MWCO 3000 with D-PBS, and stored at -80 °C. To purify the conjugate, the 2M NaCl fractions were subjected to dialysis against D-PBS using MWCO 26,000 tubing for 12 hours at 4 °C, then washed for 10 cycles using a CentriPrep® centrifugal membrane MWCO 30,000 with D-PBS at 12.0 rcf for 8 minutes/cycle. The collected conjugate was then characterized by Western blot (see Supplementary Methods online). ELISA (see Supplementary Methods online) was carried out three times and averaged to determine the concentration of bFGF in the conjugate prior to cell studies.

Stability study of bFGF-p(SS-co-PEGMA) and controls under stressors

An amount of 15 µl of each experimental group in D-PBS was made up to contain 0.5 ng/µl of bFGF or either of the conjugates (bFGF-p(SS-co-PEGMA) and bFGF-pPEGMA). The control experimental groups were prepared to contain 0.5 ng/µl of bFGF with either 0.75 ng/µl of heparin, 0.5 µg/µl of heparin, 0.75 ng/µl of p(SS-co-PEGMA), or 0.75 ng/µl of pPEGMA. The blank group contained 15 µl of D-PBS. The 15 µl/experimental group was divided into 4 vials of 3 µl/vial for four separate treatments. In one set of the 3 µl-vials, 27 µl of D-PBS was added to each vial and the samples were stored at 4 °C for 16 hours. In another set of 3 µl-vials, 27 µl of pH 4.7 PBS was added to each vial and the samples were stored at 4 °C for 16 hours. In the third set of 3 µl-vials, 27 µl of 0.1% Trypsin was added to each vial and the samples were stored at 4 °C for 16 hours. The fourth set of 3 µl-vials were stored at 4 °C for 16 hours before 27 µl of 1% TFA was added and treated for 2 hours at 4 °C. The samples under heat treatment were prepared fresh in the same manner towards the end of the 16-hour treatment of the other samples. The samples were placed in a dry bath at 55 °C for 30 minutes. The concentrations of native bFGF and bFGF in the conjugates under treatment were 0.05 ng/µL. The concentrations of heparin under treatment were 0.075 ng/µl or 0.05 µg/µl, while the concentrations of the unconjugated polymers were 0.075 ng/µl. To stop all treatments, the samples were diluted to 1.5 ml of the UltraCULTURE™ serum-free medium to bring final concentrations to 1 ng/ml of bFGF/bFGF-p(SS-co-PEGMA)/bFGF-pPEGMA, 1.5 ng/ml or 1 µg/ml of heparin, 1.5 ng/ml of p(SS-co-PEGMA)/pPEGMA. The untreated samples were prepared fresh to the indicated final concentrations in the working medium without serial dilutions. Then, the medium samples were used in the cell proliferation assay as described in the Supplementary Methods. After incubation for 72 hours at 37 °C, 5% CO₂, the CellTiter-Blue® assay was carried out to evaluate the extent of cell growth. All experimental groups were normalized to the blank control of that treatment set, which had only treated buffer in medium. Each sample had six replicates, and the whole experiment was repeated eight times including one blinded study, except (+)bFGF (+)1.5 ng/ml pPEGMA and (+)bFGF-pPEGMA which were repeated four times, and the “pH 4.7 for 16 hrs” treatment set of other groups which was repeated seven times. A two-way ANOVA and Student’s t-test were performed for statistical analysis.
**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.
Syntheses and cytotoxicity study of the polymers. a, Synthesis scheme of heparin-mimicking polymer PDS-p(SS-co-PEGMA). b, Synthesis scheme of PDS-pPEGMA. c, Cytotoxicity study of the heparin-mimicking polymer compared to controls: 5,000 human dermal fibroblast (HDF) cells/well in a 48-well plate were incubated with various concentrations of each polymer for 24 hours at 37 °C, 5% CO₂. Each well was washed twice with Dulbecco’s phosphate buffered saline (D-PBS) and subsequently incubated with 1 µM calcein AM and 4 µM ethidium homodimer-1 for 20 minutes at 37 °C, 5% CO₂. The percentage of live cells was calculated by dividing the number of live cells by the total number of live and dead cells. The experiment was repeated four times. Error bars represent standard errors of the mean (SEM).

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Figure 2.
Conjugation of the polymers to bFGF and characterization. a, Synthesis scheme of bFGF-p(SS-co-PEGMA) (similar for bFGF-pPEGMA). b, Western blots of bFGF-p(SS-co-PEGMA) from SDS-PAGE (left) and Native PAGE (right), lane 1: bFGF-p(SS-co-PEGMA) with 54 mg/ml dithiothreitol (DTT), lane 2: bFGF-p(SS-co-PEGMA) without DTT (diffuse bands = conjugates), lane 3: bFGF without DTT. c, ESI-GEMMA spectra of bFGF (top, EMD = 5.43 nm, d = 0.58 g/cm$^3$, MW$_{\text{calculated}}$ = MW$_{\text{theoretical}}$ = 16 kDa), bFGF-p (SS-co-PEGMA) (bottom, EMD = 5.43 nm, d = 0.78 g/cm$^3$, MW$_{\text{calculated}}$ = 39 kDa, MW$_{\text{theoretical}}$ = 42 kDa) in 20 mM ammonium acetate.
Figure 3.
Stability study of bFGF-heparin-mimicking polymer conjugate, bFGF-p(SS-co-PEGMA), compared to control groups. Preparation of the treated and untreated samples is detailed in the Methods section. The concentrations of bFGF and bFGF conjugates under treatment and later in the medium were 0.05 ng/µL and 1 ng/ml, respectively. Incubation of HDF cells with the samples was carried out for 72 hours. CellTiter®-Blue assay was performed to quantify the extent of cell growth. Data was normalized to the blank group (no bFGF added) in that same treatment set. Each sample had six repeats, and the whole experiment was repeated eight times including one blinded study, except (+)bFGF (+)1.5 ng/ml pPEGMA and (+)bFGF-pPEGMA which were repeated four times, and the “pH 4.7 for 16 hrs” treatment set of other groups which were repeated seven times. Error bars are SEM. Statistical analysis was done using two-way ANOVA and Student’s t-test. ** Denotes p < 0.01 and *** Denotes p < 0.001 for (+)bFGF-p(SS-co-PEGMA) group or positive control group (+)bFGF (+)heparin 1 µg/mL compared to the negative control group (+)bFGF (-)heparin for each stressor. See Supplementary Fig. S6 for statistical analysis of other groups. Denotes no induced proliferation by bFGF.
Figure 4.
Inhibition of FGFR1 activation induced by bFGF and bFGF-heparin-mimicking polymer conjugate, bFGF-p(SS-co-PEGMA). The samples were prepared with and without 125 nM of PD173074. 2,000 HDF cells/well was incubated with 100 µl of each of the samples for 72 hours. CellTiter®-Blue assay was performed to quantify percent cell growth. Data was normalized to the blank sample group containing no PD173074. Each sample group was measured with six replicates, and the entire experiment was repeated three times. Error bars represent SEMs. Denotes no induced proliferation by bFGF.
Figure 5.
Proliferation study with BaF3 cells in response to the addition of the heparin-mimicking polymer and its bFGF conjugate. BaF3 cells (FR1C-11), which express FGFR1 but lack HS proteoglycans, were seeded at a density of 20,000 cells/well in culture medium lacking IL-3 in a 96-well plate. The samples were prepared in the working medium with the final concentrations: 1 ng/ml of bFGF, 1 ng/ml of bFGF with either 1 µg/ml of heparin or 1 µg/ml of p(SS-co-PEGMA), and 1 ng/ml of bFGF-p(SS-co-PEGMA). The cells were incubated for 42 hours. CellTiter®-Blue assay was performed to quantify percent cell growth. Data was normalized to blank the sample group. Each group was done with six replicates. Error bars are standard deviations. Denotes no induced proliferation by bFGF.