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Structure Activity Relationship of Heparin Mimicking Polymer p(SS-co-PEGMA): Effect of Sulfonation and Polymer Size on FGF2-Receptor Binding[†]

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Abstract

Fibroblast growth factor-2 (FGF2) is a heparin binding protein that plays a role in a range of biological functions such as wound healing and bone regeneration. Heparin, a highly sulfated glycosaminoglycan, is required for FGF2 to bind to its receptor. Therefore, polymeric mimics of heparin are widely studied for their ability to manipulate FGF2-induced biological interactions. It is known that altering the degree of sulfonated monomer incorporation and size of heparin-mimicking polymers can affect protein-receptor binding. To elucidate the relationship between degree of sulfonation and receptor binding for the heparin-mimicking polymer, poly(styrene sulfonate-co-poly(ethylene glycol) methyl ether methacrylate) (p(SS-co-PEGMA)) a library was synthesized to contain nine polymers with degrees of sulfonation ranging from 0-100%. Kinetics of the polymerization was evaluated and reactivity ratios compared to literature results. These polymers were then tested for their ability to enhance FGF2 binding with its receptor as both covalent conjugates and as excipients. In a receptor based enzyme-linked immunosorbant assay (ELISA), as well as a cell-based study, the polymer with 81% SS incorporation enhanced receptor binding compared to FGF2 alone, and to a greater extent than the other polymers. Therefore, another library of polymers was prepared maintaining the degree of sulfonation at 81% and changing the size from 41 to 390 monomer repeat units. The polymers were again tested in receptor based ELISA and cell studies, and all of the different sizes performed similarly, except for degree of polymerization 295 and 390, which had reduced response in the cellular assay. These results provide important information for the use of pSS-co-PEGMA as a potential heparin-mimicking therapeutic.

Graphical abstract

[†]Electronic supplementary information (ESI) available

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Introduction

Fibroblast growth factor 2 (FGF2) is a widely studied growth factor with important roles in cell growth, migration, and angiogenesis.¹⁻³ In its active form, fibroblast growth factor 2 (FGF2) is part of a pentameric complex that is required for mitogenic activity.^{4,5} This complex is comprised of two FGF2 proteins, two FGF receptors (FGFRs) and heparan sulfate, a naturally occurring glycosaminoglycan. The two FGF2 proteins dimerize and dock on the FGFRs with heparan sulfate threaded through the binding pocket, interacting with amino acids on both the protein and receptor.⁴ Heparin is required for receptor binding to occur and helps to facilitate protein dimerization and receptor binding, as well as stabilization of the protein.⁵⁻⁷

Because of its important biological role, heparin is an attractive therapy for treatment of diseases.⁸ However, heparin is very heterogeneous in nature.^{9,10} Polysaccharide size is one of the factors that contributes to its heterogeneity, with naturally occurring molecular weights ranging from 5 to 40 kDa.⁹ Another factor is its sulfation pattern. The placement and degree of sulfation varies widely between saccharide units, and the structure and charge density of heparin play a large role in modulating binding.^{11,12} Due to its heterogeneity, synthetic heparin mimics have been prepared as therapies to replace heparin for the purposes of either enhancing or preventing FGF2 receptor binding.^{9,13} In applications where angiogenesis and cell growth are not desired, mimics have been designed to hinder receptor binding and are intended for use in cancer therapeutics.¹⁴⁻¹⁶ Conversely, mimics that enhance receptor binding have been useful in applications where protein activity is required, such as in treatments for wound healing and ischemic conditions.^{13,17}

The ability to modulate the heparin mimicking nature of polymers is attractive for applications in therapeutics where fine-tuning of heparin mimicking properties is desired. For instance, recent work has demonstrated that changing the degree of sulfonation can affect heparin-mimicking polymer properties. Chen and coworkers found that cell growth could be tuned by manipulating styrene sulfonate (SS) incorporation in poly(styrene sulfonate-*co*-methacrylamido glucopyranose) (p(SS-*co*-MAG)).¹⁸ Maximum embryonic stem cell proliferation was obtained when the ratio of SS:MAG was approximately 1:1, and this polymer enhanced cell growth to a greater extent than heparin itself. Cell growth was also tuned to have cell proliferation lower than added heparin, in the case of higher SS incorporation, and to have cell proliferation profiles similar to that of heparin, in the case of

higher MAG incorporation. Additionally, changing the degree or location of sulfonation has been shown to influence protein binding¹⁹ and increase blood coagulation times.^{20,21} Location of sulfate moieties also plays a role in biological activity. By altering the placement and number of sulfates on sugar side chains in glycopolymers, certain biological pathways can be targeted.^{22,23}

Another factor that influences the biological properties of heparin is polymer size. To assess how size affects function, Hsieh-Wilson and coworkers polymerized heparin mimicking glycopolymers by ROMP.²⁴ They found that anticoagulant activity could be modulated by changing polymer size. Specifically, increasing glycopolymer chain length increased blood coagulation times. Smaller heparin mimics can also display useful properties. Miura and coworkers found that lower molecular weight heparin mimics were able to inhibit amyloid-beta aggregation better than the higher molecular weight heparin mimics.²⁵

We previously found that the heparin mimicking polymer p(SS-*co*-PEGMA) with a SS to PEGMA ratio of 2.2 to 1 and a number average molecular weight (M_n) of 26 kDa could stabilize FGF2, but not facilitate receptor binding.²⁶ We also demonstrated that poly(vinyl sulfonate) (pVS) could facilitate FGF2 binding to its receptor,¹⁷ but did not stabilize FGF2 well.¹³ We then showed that a block copolymer containing both segments could facilitate receptor binding in the absence of heparin, stabilize FGF2, and the FGF2-p(SS-*co*-PEGMA)-*b*-VS conjugate had superagonist activity compared to the unmodified protein.¹³ However, the block copolymer is difficult to make with controlled end groups, mostly due to the complex polymerization of activated and deactivated monomers. In order to explore potential receptor binders, we decided to further examine the stabilizing block, p(SS-*co*-PEGMA), to determine if it could be structurally manipulated to enhance FGF2-receptor binding. To accomplish this, we investigated how varying the degree of SS monomer incorporation and polymer chain length would affect the polymers ability to facilitate receptor binding. The data and results are described in detail below.

Experimental

Materials

Chemicals and reagents were purchased from Sigma Aldrich and used as received unless otherwise indicated. Prior to polymerizations 4-styrene sulfonic monomer was pretreated with Na⁺ and dried to produce the sodium salt. Before polymerization azobis(isobutyronitrile) (AIBN) was recrystallized twice from ethanol and dried. Pyridyl disulfide pPEGMA (PDS-pPEGMA) was synthesized by ATRP as previously described.²⁶ Protein was expressed and purified from the plasmid pET29c(+)-hFGF-2, which was kindly provided by Professor Thomas Scheper from the Helmholtz Centre for Infection Research (Braunschweig, Germany) according to Chen et al.²⁷ HiTrap Heparin HP columns were purchased from GE Healthcare. ELISA was performed using the ELISA Development DuoSet kit purchased from R&D Systems. Recombinant human FGFR1 α (IIIc) Fc chimera, and ELISA Development DuoSet kits were purchased from R&D Systems. Blot antibodies were purchased from CALBIOCHEM (rabbit anti-fibroblast growth factor basic) and BioRad (goat anti-rabbit IgG-HRP conjugate). BaF3-FR1C expressing FGFR1 were kindly provided by Professor David Ornitz (Washington University, Saint Louis).²⁸ Cell medium

was purchased from ATCC or Invitrogen unless otherwise indicated. Cell Titer Blue Cell Viability Assay was purchased from Promega. Polystyrene standards for gel permeation chromatography (GPC) calibration were purchased from Polymer Laboratories.

Analytical Techniques

^1H NMR and ^{13}C NMR spectroscopy were performed on Avance DRX 400 MHz. Dimethylformamide (DMF) GPC was conducted in DMF containing 0.10 M LiBr (40 °C, 0.8 mL/min) on a Shimadzu HPLC system equipped with a refractive index detector RID-10A, one Polymer Laboratories PLgel guard column, and two Polymer Laboratories PLgel 5 μm mixed D columns. Calibration was performed using near-monodisperse polystyrene standards. Chromato-grams were processed using the EZStart 7.2 chromatography software. Aqueous GPC was performed in 0.05 M Na_2SO_4 in MQ water with 10% MeOH on a Viscotek GPC_{max} VE 2001 GPC solvent/sample module equipped with Viscotek TDA 305 triple array detector. Gel electrophoresis was performed using Any kDTM Mini-PROTEAN TGXTM precast gels with SDS-Tris-glycine as running buffer (Biorad, Hercules). ELISA assays were read on an ELX800 Universal Microplate Reader (Bio-Tek Instrument Inc., Winooski) with $\lambda = 450$ nm for signal and 630 nm for background. Western blot was developed on a FluorChem FC2 System version 3.2 (Cell Biosciences, Santa Clara). CellTiter-Blue assays were read on a SpectraMax M5 microplate reader (Molecular Devices, Sunnyvale).

Methods

Polymerization of p(SS-co-PEGMA)—RAFT polymerization was performed with varying feed ratios of [SS]:[PEGMA]:[CTA]:[AIBN] and a monomer concentration of 1.3 M. In an example polymerization, styrene sulfonate (382 mg, 1.85 mmol) was dissolved in 1.2 mL of degassed water in a Schlenk tube along with PEGMA M_n 300 (159 mg, 0.53 mmol). CTA **1** (20 mg, 0.053 mmol) and AIBN (1.74 mg, 0.011 mmol) were dissolved in 1.2 mL of degassed DMF and then transferred to the Schlenk tube. The Schlenk tube was sealed and subjected to four freeze-pump-thaw cycles before immersion in an oil bath set to 60 °C. After 4 hours the polymerization was stopped by cooling to room temperature and bubbling air through the Schlenk tube. The polymer was purified by dialysis in 3500 MWCO tubing against 1:1 v/v water:MeOH followed by 100% Milli-Q water. The resulting contents of the dialysis tubing were lyophilized to dryness. ^1H NMR 500 MHz (D_2O) δ : 8.40 (1H, s, PDS end group), 8.0–6.0 ($\text{NaSO}_3\text{C}_6\text{H}_4$ side chains), 4.5–2.7 (PEGMA side chains), 2.7–0.0 (polymer backbone). The ^1H NMR spectrum of the resulting polymer was calibrated to the peak at 8.40 ppm, and the M_n of the polymer was calculated using the formula: $M_n = [(\text{integral of } 8.0 - 6.0 \text{ ppm} / 4) * \text{MW SS monomer}] + [(\text{integral of } 4.5 - 2.7 \text{ ppm} / 20.4) * \text{MW PEGMA monomer}] + \text{MW CTA}$. See ESI for ^1H NMR spectra with integrations for each polymer.

Conjugation of p(SS-co-PEGMA) to FGF2—FGF2 (100 μg , $5.9 \times 10^{-3} \mu\text{mol}$) was diluted in 400 μL of D-PBS + 1mM EDTA and loaded onto a column containing 0.5mL-heparin Sepharose resin. The resin was placed in a 4 °C refrigerator for 30 minutes to allow the protein to bind to the resin. Next, 100 equivalents of PDS-p(SS-co-PEGMA) (various ratios or molecular weights) were dissolved in 400 μL of D-PBS + 1mM EDTA and loaded

onto the column. The column was incubated at 4 °C for 16 hours. After incubation, the column was rinsed with 8 column volumes of D-PBS + 1 mM EDTA to remove unreacted polymer. Next, the column was rinsed with 10 column volumes of D-PBS + 2M NaCl to remove conjugates and protein. Conjugate was purified using a CentriPrep® centrifugal membrane MWCO 10,000 against D-PBS 10 times at 10.2 rcf for 10 minutes/cycle. The collected conjugate was then characterized by gel electrophoresis, and the concentration was determined by ELISA prior to *in vitro* studies.

FGFR ELISA Assay—A 96 well-plate was incubated with rhFGFR1 α (IIIc) (100 μ L per well at a concentration of 0.5 μ g/mL in D-PBS) for 12 hours at 23 °C. After 16 hours the wells were washed and then blocked with 400 μ L of 1% bovine serum albumin (BSA) in D-PBS (2 hours). Next, sample solutions were prepared at 1 ng/mL and aliquoted into Eppendorf tubes. After washing the wells, 100 μ L of the sample solutions were added to each well. For protein solutions, heparin or polymer was added so that the final concentration of heparin or polymer was 1 μ g/mL. After 2 hours the wells were washed and 100 μ L of FGF2 antibody-biotin conjugate was added and incubated for an additional 2 hours before washing and incubating with streptavidin-horseradish peroxidase solution for 20 minutes. The plate was developed by incubating with 100 μ L of 1-Step Ultra 3,3',5,5'-tetramethylbenzidine (TMB) solution (Pierce Biotechnology, Rockford) for 8 minutes. The assay was terminated by the addition of 50 μ L of 1 M H₂SO₄. Absorbance was read at λ = 450 nm. Each sample was plated in triplicate.

BaF3-FR1C Binding Assay—BaF3-FR1C cells were grown in RPMI1640 medium containing 2 mM L-glutamine, 10% newborn bovine calf serum, and 0.5 ng/mL IL-3. Before seeding cells for experiments the cells were collected and washed twice with culture medium without IL-3. Cells were plated at a concentration of 20,000 cells/well/50 μ L in the internal wells of a 96 well plate in culture medium without IL-3. Samples were prepared in culture medium without IL-3 to contain double the final concentration, and then 50 μ L of each sample was added to the corresponding well. The external wells were blocked with 100 μ L of DPBS and then incubated at 37 °C and 5% CO₂. After 48 hours of incubation CellTiter Blue assay was performed to determine the extent of cell growth. All samples were normalized to the control group, which contained only culture medium without IL-3. Each group contained six well replicates.

Results and Discussion

To understand the structure activity relationship of the p(SS-*co*-PEGMA) heparin mimicking polymer, we first investigated how varying the degree of sulfonated monomer incorporation would affect receptor binding. Reversible addition fragmentation chain transfer (RAFT) polymerization was utilized to synthesize a p(SS-*co*-PEGMA) library with varying degrees of styrene sulfonate (SS) monomer incorporation. A previously reported trithiocarbonate chain transfer agent, **CTA 1** was used to copolymerize SS and PEGMA monomers with varying initial feed ratios (Scheme 1a).²⁹ This chain transfer agent containing a pyridyl disulfide group was chosen so that the polymers could be conjugated to the protein (Scheme 1b), which presents two free cysteines.

Eight polymers were synthesized by RAFT polymerization with degrees of sulfonation ranging from 8.0% to 100% (Table 1). Polymer DP was maintained at approximately 135 monomer units in order to verify that any receptor binding observed was due to changes in degree of sulfonation and not polymer chain length. In addition, pPEGMA was homopolymerized so the library would contain a 0%SS control. Initially, RAFT polymerization was attempted to polymerize pPEGMA, however the polymerizations gelled even after manipulation of concentration and feed ratios. Therefore, atom-transfer radical polymerization (ATRP) was utilized to successfully polymerize pPEGMA as previously reported.²⁶

Polymer kinetics were monitored for p(SS-*co*-PEGMA)₈₁ by taking time points throughout the duration of the polymerization. The plot of $\ln(M_0/M_t)$ in relation to reaction time was found to be linear, which confirms that the polymerization is first order and controlled, as we previously reported for the copolymerization of SS and PEGMA with a similar CTA (Figure 1a).²⁶ Additionally, the percentage of SS incorporation was maintained between 79.1% and 82.4% throughout the duration of the polymerization (Figure 1b). Furthermore, the molecular weight dispersity remained below 1.28 for all time points taken (Figure 1c). Reactivity ratios were calculated using the curve fitting method with time points of all polymers from Table 1 taken at 10-20% conversion. The data was similar when using ~50% or ~70% conversion (data not shown). The mole fraction of SS in the copolymer (F_{SS}) was plotted against the mole fraction of SS in the monomer solution (f_{SS}) (Figure 1d). The reactivity ratios for SS (r_{SS}) and PEGMA (r_{PEGMA}) were both found to be below 1.0 ($r_{SS} = 0.57$ and $r_{PEGMA} = 0.46$), and are similar to reactivity ratios previously reported using the Fineman-Ross method ($r_{SS} = 0.70$ $r_{PEGMA} = 0.50$).³⁰ The azeotropic point was determined to be 55% SS monomer incorporation. Above the azeotropic point the compositional drift tended to prefer to add less SS than PEGMA and below the azeotropic point compositional drift tended to prefer to add more SS than PEGMA.

Next, covalent FGF2-polymer conjugates were prepared to determine if increased receptor binding could be observed. Protein-polymer conjugates can be preferable to excipients in that the polymer is used in much lower concentration as a conjugate. The polymers from Table 1 all contained a protein reactive pyridyl disulfide (PDS) end group. This end group was used to covalently attach the polymer to FGF2 through disulfide exchange, as shown in Scheme 1b. Conjugations were performed on heparin resin to prevent conjugation in the heparin binding site and conjugates were purified by centrifugation filtration. Conjugations were analyzed through sodium dodecyl sulfate poly(acrylamide) gel electrophoresis (SDS-PAGE) with protein detection by Western Blot (Figure 2). Conjugates were observed as smears at slightly higher molecular weights than free protein (Figure 2a). Because the polymer conjugation was achieved through a disulfide bond, the addition of dithiothreitol (DTT) reduced the disulfide bond producing free protein, further verifying successful conjugation (Figure 2b). While conjugates are present in the gels and show up as higher molecular weight smears, there is still unconjugated FGF2 despite repeated purification. After analyzing the conjugate concentration by ELISA, receptor binding assays were used to determine extent of binding.

After the library of conjugates was obtained, the effect of SS monomer incorporation on receptor binding was examined using a FGF-receptor enzyme linked immunosorbent assay (ELISA). In this assay, the FGF receptor FGF1 α was immobilized in the wells of a 96 well plate. The receptor was then incubated with 1 ng/mL of FGF2 (negative control) or FGF2-p(SS-*co*-PEGMA) conjugates. For the positive control, 1 ng/mL of FGF2 with 1 μ g/mL of heparin was added. After washing away unbound FGF2, remaining receptor-bound FGF2 was monitored through the use of a secondary antibody. Without heparin, the binding of FGF2 alone to FGF1 α was minimal, and as expected, the addition of heparin induced a large increase in receptor binding (Figure 3a). Out of the polymer library, the FGF2-p(SS-*co*-PEGMA)₈₁ conjugate performed the best with an increase in absorbance of 0.29 ± 0.08 AU when compared to FGF2 alone, which had an absorbance of 0.10 ± 0.05 AU (Figure 3a). The conjugate did not facilitate receptor binding as well as heparin, which gave an absorbance of 1.01 ± 0.10 AU. Furthermore, we found that PSS inhibited FGF2 binding to its receptor. This was not surprising since similar results have been previously reported and is speculated to be due to the conformation of the polymer in the heparin binding pocket, which allows tight binding to the protein, but not to the receptor.^{16,17,31}

To evaluate the ability of the polymers to assist in FGF receptor binding in a more complex system, a cell-based assay was performed using BaF3 FR1c cells. This cell line was engineered to express FGF receptors on the cell surface, but lack native heparan sulfate proteoglycans.^{28,32} For cell growth to be observed, exogenous heparin or heparin mimics are required. Therefore, this cell line is useful for assessing the ability of heparin mimics to facilitate receptor binding. Cells were incubated with 1 ng/mL of FGF2 or FGF2-p(SS-*co*-PEGMA). For the positive control, cells were incubated with 1 ng/mL of FGF2 and 1 μ g/mL of heparin. Similar results were observed in this assay, with FGF2-p(SS-*co*-PEGMA)₈₁ giving the best results with cell proliferation of $285 \pm 50\%$ when compared to FGF2 alone, which resulted in a cell proliferation of $203 \pm 12\%$ (Figure 3b). Again the cell proliferation observed for FGF2-p(SS-*co*-PEGMA)₈₁ was not as high as FGF2 with added heparin ($547 \pm 15\%$). Consistent with the ELISA results, conjugates with higher degrees of sulfonation (p(SS-*co*-PEGMA)₉₅ and PSS) exhibited statistically decreased binding when compared to FGF2 alone.

Previous computational binding models of heparin-mimics have shown that, in order for tight binding to occur to FGF2, sulfates or sulfonates must be displayed in a manner that allows for strong binding between the negative charges along the polymer backbone to the positively charged residues in the heparin-binding domain of the protein.¹⁶ Other work has verified this point by showing that the spacing of the negative charges in heparin-mimicking polymers is important for protein binding.³³ Spacing of the negative charges throughout p(SS-*co*-PEGMA)₈₁ is the most likely reason that the 81% polymer performed better than other percentages in the binding assays. Furthermore, heparin has a very high negative charge to size ratio, which contributes greatly to its binding properties. However, it has previously been reported that some heparin-mimicking polymers require an ideal sulfonate incorporation in order to achieve optimal binding, and too much sulfonate incorporation can lead to inhibition of cell proliferation or decrease in binding ability.¹⁸ Moreover, it has been reported that poly(styrene sulfonate) (pSS) inhibits cell proliferation,¹⁷ and in the case of our p(SS-*co*-PEGMA) polymer, the higher the styrene sulfonate content, the more alike the

polymer is to pSS. It is likely that the 81% incorporation of styrene sulfonate in p(SS-*co*-PEGMA)₈₁ is high enough to sufficiently mimic heparin, while not being too high as to inhibit protein activity in the same manner as pSS. Indeed the results demonstrate this in that pSS with bFGF is statistically worse than bFGF alone in the ELISA assay and both p(SS-*co*-PEGMA)₉₅ and pSS have lower cell proliferation than adding nothing to bFGF in the cell assay.

Since there was unconjugated FGF2 visible in the gels, it was possible that this left over unconjugated FGF2 was attributing to the results observed. For example, FGF2-p(SS-*co*-PEGMA)₈₁ was among those that had the least amount of residual FGF2 in the gel, and it had the best result in the above assays. We anticipated that adding a large excess of polymer would account for any residual unmodified FGF2 since if the polymer is heparin-like it should facilitate binding of the growth factor to the receptor whether in the native protein or conjugate form. First, we tested if this approach was feasible, by adding 1 $\mu\text{g/mL}$ of heparin to p(SS-*co*-PEGMA)₈₁, and cell proliferation was monitored in BaF3-FR1c cells. As shown in Figure 4, the conjugate plus 1 $\mu\text{g/mL}$ heparin exhibited similar binding as FGF2 alone plus 1 $\mu\text{g/mL}$ heparin. Thus, adding an excess of each polymer should indeed provide data on whether or not the polymers can facilitate FGF2 binding to its receptor.

The FGF-receptor ELISA assay was performed after adding 1 $\mu\text{g/mL}$ of the pyridyl disulfide polymers to 1 ng/mL of FGF2 and used directly in the assays without purification. This should result in some conjugate with a large excess of nonconjugated polymer as excipient. The data showed that there was an increase in receptor binding when p(SS-*co*-PEGMA)₈₁ was added, but not for the other polymer ratios (Figure 5a). Additionally, the samples were evaluated for receptor binding in the BaF3 assay. As shown in Figure 5b, when 1 $\mu\text{g/mL}$ of heparin is added there was a large increase in cell proliferation up to $401.7 \pm 10\%$ compared to $190 \pm 13\%$ for FGF2 alone. When p(SS-*co*-PEGMA)₈₁ was incubated with the cells an increase in proliferation of $255 \pm 9\%$ was observed. All other polymers were the same as FGF2 alone.

Taken together these results demonstrate that 81% styrene sulfonate provided the best results out of the polymers. This was true whether conjugate was tested alone or with excess added polymer. Further when heparin was added to p(SS-*co*-PEGMA)₈₁, full cellular response was obtained as when heparin was added to FGF2. This demonstrates that the protein in the 81% conjugate is still bioactive and able to attain full efficacy with exogenous heparin. Thus, the conjugation procedure did not decrease FGF2 activity, but rather the polymer is not able to facilitate receptor binding to the same extent as heparin itself.

We next determined if there was an optimal size for receptor binding of FGF2-p(SS-*co*-PEGMA). A small library of p(SS-*co*-PEGMA) was polymerized by RAFT polymerization with **CTA 1** (Scheme 1a). The degree of sulfonation was maintained close to 81%, the best performing styrene sulfonate incorporation from the studies described above. DP was varied from 41 to 390 monomer units. This was done by maintaining the ratio of [SS]:[PEGMA] at approximately 5.5:1 in the initial feed ratio, but changing the ratio of total monomer concentration to CTA. Molecular weights of the polymers were calculated by ¹H NMR analysis relative to PDS end group (Table 2). Additionally, right angle light scattering

(RALS) GPC was utilized to determine accurate polymer molecular weight. All GPC traces were monomodal, except for p(SS-*co*-PEGMA)DP78, which contained a higher molecular weight shoulder (Figure 6). In general, the molecular weights by RALS-GPC and those calculated by ^1H NMR were similar. However, as molecular weight increased, the difference between ^1H NMR M_n and GPC M_n increased. This is likely due to inaccuracies from calculating the molecular weights from the ^1H NMR spectra when the concentrations of the end groups are relatively small. The molecular weights calculated by RALS-GPC are likely more accurate, and therefore the DPs provided in the polymer names in Table 2 are based on the RALS-GPC data.

The polymers from Table 2 were tested for receptor binding with excess polymer at a concentration of 1 $\mu\text{g/mL}$ and 1 ng/mL of FGF2 as described above. No further increase in receptor binding was observed in relation to size. In the FGFR ELISA (Figure 7a) the polymers contributed to an increase in receptor binding up to 0.42 ± 0.03 AU compared to FGF2 alone, which exhibited a response of 0.14 ± 0.02 AU. FGF2 with 1 $\mu\text{g/mL}$ of added heparin gave a response of 1.0 ± 0.05 AU. The BaF3 receptor assay (Figure 7b) showed similar results with the polymers contributing to an increase in cell proliferation up to $215 \pm 12\%$ when compared to FGF2, which had cell proliferation of $155 \pm 17\%$. These results were compared to heparin, which gave a cell response of $334 \pm 9\%$. Larger molecular weight polymers (DP295 and DP390) did not promote any receptor binding and were actually not statistically different from FGF2 alone. This data suggests that the size of the polymer does not matter, except for degrees of polymerization greater than or equal to 295.

While the heparin mimicking polymers did show a significant increase in receptor binding when compared to FGF2 alone, they were not able to facilitate receptor binding to the same extent as heparin. We speculate that complete binding of the polymer to FGF2 is not observed due to the conformation of the sulfonates along the polymer backbone and this observation may be due to the lowest energy conformation of the polymer. It could be that the p(SS-*co*-PEGMA) polymer is not able to display the sulfonates in the same locations that are necessary for receptor binding. Binding pockets are often specific in nature, so if the sulfonates are not displayed in the correct orientation, then binding will be reduced or not occur. In order to improve protein binding of heparin-mimicking polymers, researchers have incorporated sugar moieties to better mimic the polysaccharide backbone of heparin, and others have introduced rigid spacers along the polymer backbone to better mimic the sulfate spacing of heparin.^{18,34}

While previous reports have shown that heparin-mimicking polymers can be tuned for receptor binding, this is the first report showing the tunable nature of p(SS-*co*-PEGMA) specifically. We have found that the degree of styrene sulfonate monomer incorporation is important for receptor binding, and thus, can likely influence other biological outcomes. Specifically, a degree of sulfonate monomer incorporation of approximately 81% is necessary to increase protein activity in cells lacking heparin sulfate proteoglycans. Polymer size did not contribute to an increase in receptor binding, as all but the highest molecular weights facilitated FGF2 binding to its receptor to the same extent. These data together are important for understanding how to facilitate receptor binding of the known heparin mimicking polymer p(SS-*co*-PEGMA).

Conclusions

A small library of p(SS-*co*-PEGMA) polymers was prepared by RAFT polymerization to contain polymers of similar sizes but varying degrees of styrene sulfonate incorporation. Polymers were conjugated to FGF2 and verified by SDS-PAGE stained with Western Blot. Both conjugates and FGF2 with polymers added in excess were assayed in ELISA based receptor binding assays and BaF3 cell based receptor binding assays. p(SS-*co*-PEGMA)₈₁ was identified to facilitate receptor binding of FGF2 to its receptors better than the other polymers, but not to the same extent as heparin. In order to determine the effect of degree of polymerization, another library of polymers was synthesized keeping the degree of sulfonation the same at approximately 81% and altering the size of the polymer. Ultimately, changing the size of the polymers did not increase receptor binding. This information study shows that the heparin-mimicking nature of p(SS-*co*-PEGMA) can be increased, specifically by altering degree of sulfonated monomer incorporation rather than changing polymer size.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Polymer kinetics performed on p(SS-*co*-PEGMA) polymerizations. (a) Kinetic studies of the RAFT polymerization of p(SS-*co*-PEGMA)₈₁ at 70 °C. Conversion, M_0 , and M_t were calculated using ^1H NMR spectroscopy. (b) Percent styrene sulfonate incorporation over time. (c) Theoretical versus experimental M_n from right angle light scattering in aqueous running buffer. (d) F_{SS} versus f_{SS} curves.

Figure 2. SDS-PAGE with Western Blot of FGF-p(SS-*co*-PEGMA) conjugates in both (a) non-reducing conditions and (b) reducing conditions. Lane 1: FGF2, Lane 2: FGF2-p(SS-*co*-PEGMA)_{8,0}, Lane 3: FGF2-p(SS-*co*-PEGMA)₂₈, Lane 5: FGF2-p(SS-*co*-PEGMA)₅₅, Lane 6: FGF2-p(SS-*co*-PEGMA)₈₁, Lane 7: FGF2-pPEGMA, Lane 8: FGF2-p(SS-*co*-PEGMA)₇₆, Lane 9: FGF2-p(SS-*co*-PEGMA)₉₅, Lane 10: FGF2-pSS.

Figure 3.

FGF2-p(SS-co-PEGMA) conjugates in FGF2 receptor binding assays. (a) FGF Receptor based ELISA. The experiment was repeated three times with $n = 3$ for each experiment. Error bars represent the standard deviation. (b) Cell growth of heparin-mimicking polymer conjugates in BaF3-FR1C cells. Data was normalized to the blank medium group, which was set at 100%. Each sample contained six replicates and the experiment was repeated three times. Error bars represent standard deviation. Statistical analysis was done using Student's t test. * $p < 0.01$ compared to FGF2, # $p < 0.03$ compared to FGF2.

Figure 4. Determination of FGF2-p(SS-co-PEGMA)₈₁ receptor binding in BaF3-FR1c with the addition of 1 $\mu\text{g/mL}$ heparin. Data was normalized to the blank medium group, which was set at 100%. Each sample contained six replicates. Error bars represent standard deviation. Statistical analysis was done using Student's t test. * $p < 0.01$ compared to FGF2.

Figure 5.

Examination of the ability of p(SS-*co*-PEGMA) to enhance FGF2 receptor binding. (a) FGF Receptor based ELISA. The experiment was repeated 3 times with $n = 3$ for each experiment. Error bars represent the standard deviation. (b) Cell growth of heparin-mimicking polymers in BaF3-FR1C cells. Data was normalized to the blank medium group, which was set at 100%. The experiment was repeated 3 times with $n = 6$ for each experiment. Error bars represent the standard deviation. Statistical analysis was done using Student's *t* test. * $p < 0.01$ compared to FGF2.

Figure 6.
Light scattering GPC traces showing varying molecular weight polymers with 81% SS

Figure 7.

Determining the ability of p(SS-co-PEGMA) with varying polymer sizes to enhance FGF receptor binding. (a) FGF receptor based ELISA. The experiment was repeated 3 times with $n = 3$ for each experiment. Error bars represent standard deviation. (b) Cell growth of heparin-mimicking polymers in BaF3-FR1C cells. Data was normalized to the blank medium group, which was set at 100%. Each sample contained 6 replicates and the experiment was repeated 2 times. Error bars represent standard error of the mean (SEM). Statistical analysis was done using Student's t test. * $p < 0.01$ compared to FGF2.

Scheme 1.

Synthesis of heparin mimicking polymer-protein conjugate, FGF2-p(SS-*co*-PEGMA). (a) Polymerization of p(SS-*co*-PEGMA) via RAFT polymerization. (b) Conjugation of p(SS-*co*-PEGMA) to FGF2 via disulfide exchange. Protein structure modified from PDB 1CVS using PyMOL software.

Table 1

p(SS-co-PEGMA) library with varying styrene sulfonate monomer incorporation.

Polymer	Feed Ratio [SS]:[PEGMA]: [CTA 1]:[AIBN]	[SS]:[PEGMA] in Polymer	SS%	DP (NMR)	M_n (NMR)	M_n (GPC) ^b	$\bar{D}$
pPEGMA	66:1:4:3:8:3 ^a	0:1	0%	129	43.6	53.2	1.16
P(SS-co-PEGMA) ₈₀	20:142:1:0:5	1:11.5	8.0%	141	41.6	51.0	1.20
P(SS-co-PEGMA) ₂₈	38:118:1:0:5	1:2.5	28.4%	124	34.1	43.4	1.24
P(SS-co-PEGMA) ₅₅	117:63:1:0:5	1:2:1	54.5%	136	34.2	34.8	1.26
P(SS-co-PEGMA) ₆₃	148:42:1:0:5	1.7:1	62.9%	135	33.4	24.8	1.26
P(SS-co-PEGMA) ₇₆	155:41:1:0:5	3.2:1	76.3%	135	31.3	23.6	1.24
P(SS-co-PEGMA) ₈₁	166:30:1:0:5	4.4:1	81.4%	139	31.4	23.2	1.26
P(SS-co-PEGMA) ₉₅	195:10:1:0:5	18:1	94.7%	139	29.7	22.5	1.29
pSS	205:0:1:0:5	1:0	100%	114	23.0	14.4	1.31

^a [PEGMA]:[Initiator]:[CuBr]:[bipy]

^b GPC values calculated in DMF using an RI detector with polystyrene standards

Table 2

p(SS-co-PEGMA) library with varying sizes and 81% SS incorporation.

Polymer	Feed Ratio [SS]:[PEGMA] ^a	DS (¹ H NMR)	M _n (¹ H NMR)	DP (¹ H NMR)	DP (GPC) ^b	M _n (GPC) ^b	D
p(SS-co-PEGMA) DP41	48:8	82.7%	8.8 kDa	38	41	9.3 kDa	1.15
p(SS-co-PEGMA) DP78	112:19	82.6%	16.6 kDa	73	78	17.5 kDa	1.37
p(SS-co-PEGMA) DP161	183:33	82.1%	31.7 kDa	141	161	35.4 kDa	1.13
p(SS-co-PEGMA) DP223	332:60	82.1%	53.1 kDa	236	223	49.7 kDa	1.14
p(SS-co-PEGMA) DP263	448:81	81.6 %	60.3 kDa	268	263	57.9 kDa	1.14
p(SS-co-PEGMA) DP295	608:109	80.9%	84.0 kDa	373	295	64.9 kDa	1.16
p(SS-co-PEGMA) DP390	767:138	82.0%	95.8 kDa	428	390	85.6 kDa	1.19

^a Feed ratios of [CTA]:[AIBN] = 1:0.5 for all polymers

^b GPC values calculated in aqueous buffer using a right angle light scattering detector