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Sulfonated Copolymers as Heparin-Mimicking Stabiliser of Fibroblast Growth Factor – Size, Architecture and Monomer Distribution Effects.

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Abstract: Fibroblast growth factors (FGF) are involved in a wide range of biological processes such as cell proliferation and differentiation. In living organisms, the binding of FGF to its receptors are mediated through electrostatic interactions between FGF and naturally occurring heparin. Despite its prevalent use in medicine, heparin carries notable limitations, namely; its extraction from natural sources (expensive, low yield and extensive purification), viral contamination, and batch-to-batch heterogeneity. In this work a range of synthetic homopolymers and copolymers of sodium 2-acrylamido-2-methylpropane sulfonate (AMPS$^{®}$) were evaluated as potential FGF stabilisers. This was studied by measuring the proliferation of BaF3-FR1c cells, as a model assay, and the results will be compared with the natural stabilisation and activation of FGF by heparin. This study explores the structure-activity relationship of these polysulfonated polymers with a focus on the effect of molecular weight, co-monomer type, charge dispersion and polymer architecture on protein stabilisation.
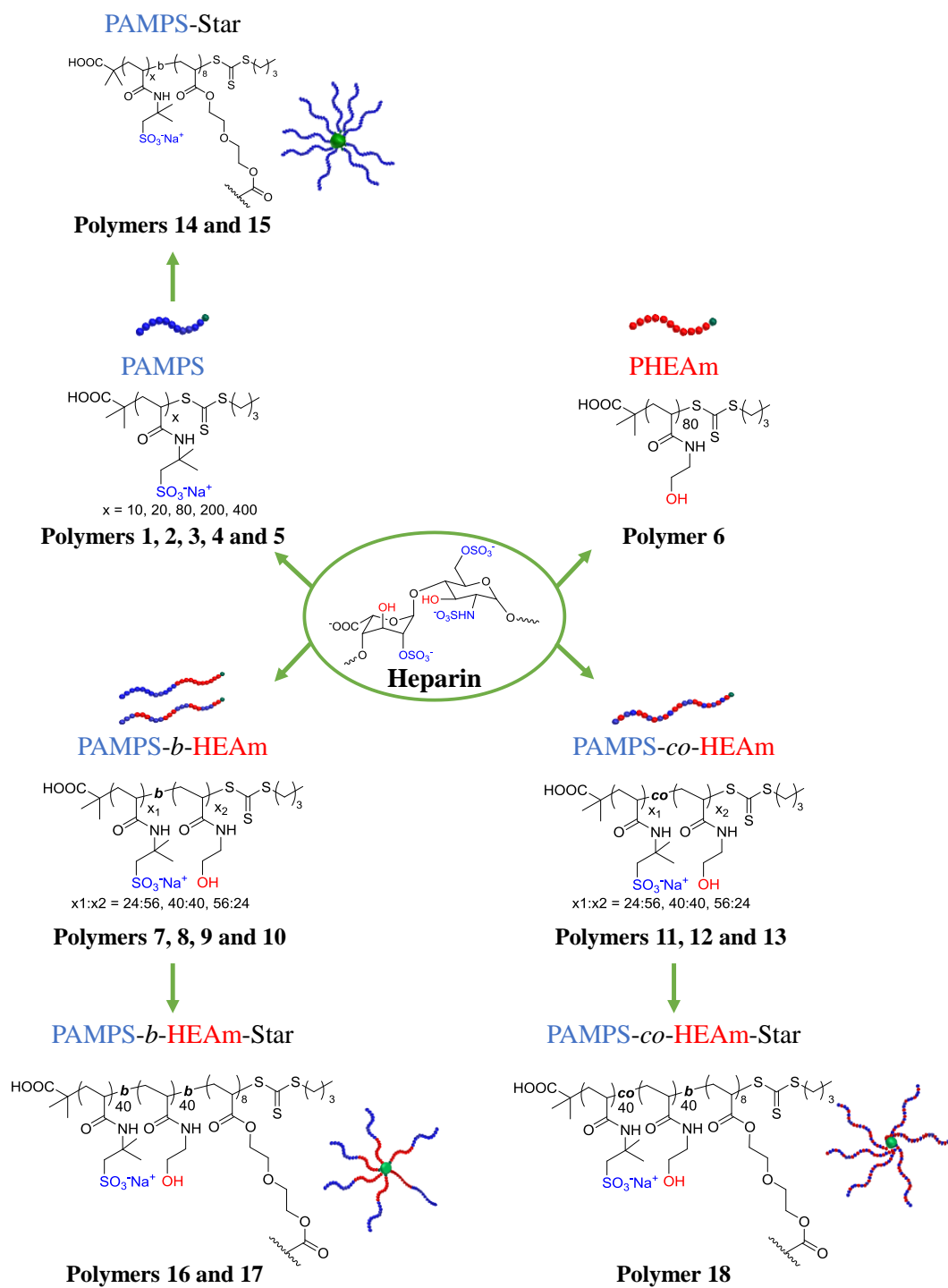
Introduction

The stabilisation of fibroblast growth factors (FGF) is a widely studied phenomenon which has implications in important cellular processes such as proliferation, differentiation and motility.[1-5] FGFs are typically activated through the dimerization of two FGF molecules via electrostatic interactions between the positively charged domains of fibroblast growth factors and the negatively charged polysaccharide, heparin. The binding of these dimers to cell surface fibroblast growth factor receptors then induces a variety of biochemical cascades, stimulating the above cellular processes.[6-7,8] The stabilisation of FGFs is therefore vital for applications such as wound healing, due to its role in fibroblast proliferation. Heparin involvement in the proliferation process is manifold; as the sulfated glycosaminoglycan not only acts as a co-factor of FGF, it was also shown to activate FGFRs and promote the dimerisation of the receptors.[2, 4, 9-14] Environmental stressors associated with storage and transport (e.g. thermal stressors) are known to lead to FGF degradation and inactivation, and heparin has been shown to protect and stabilise FGF during storage.[5, 15] Heparin uses are however limited by high cost of extraction, potential adverse side effects and most importantly, batch-to-batch dependency.[16-20] With this consideration, researchers have been extensively investigating replacing heparin with other synthetic (macro)-molecules.[16, 21]

The most popular approach to generating synthetic heparin mimics is through the full or partial sulfation of natural chitosan.[22] While these natural polymers allow for control over molecular weight, they are prone to desulfation, and consequently a loss in biological activity. Modern polymerisation techniques introduce the possibility to incorporate functional groups that enhance the versatility of these potential FGF stabilisers without the above drawbacks.[22-27] Maynard *et al.*

studied a range of synthetic sulfated polymers as heparin-mimicking polymers to stabilise FGF.[28-31] Overall, they found that PVS (poly(sodium vinyl sulfonate)) and PAHPS (poly(sodium 1-allyloxy-2 hydroxypropyl sulfonate)) stimulate BaF3-FR1c cell proliferation in a comparable way to heparin, while PAMPS acted like an inhibitor (antiproliferative) to BaF3-FR1c cell proliferation at high concentrations (100 µg/mL) while showing little to no activity at lower concentrations.[28] Garcia-Fernandez *et al.* also demonstrated this antiproliferattive effect of PAMPS but towards Balb/c 3T3 fibroblast cells in the presence of FGF. They studied two copolymers, containing 2-acrylamido-2-methylpropane sulfonate (AMPS®) and either a hydrophilic monomer (1-vinyl-2-pyrrolidinone), or a hydrophobic monomer (butyl acrylate), prepared using free radical polymerisation, as heparin mimics. The antiproliferative activity was modulated by varying either the polymer concentrations in the cell media or by varying the copolymer composition.[32-33]

Here, we report a systematic study on the effect of molecular weight, comonomer type and polymer architecture on FGF activation/stabilisation efficiency, using PAMPS homopolymers and copolymers prepared by aqueous RAFT polymerisation.[34] The efficiency of FGF stabilisation by the synthetic polymers was evaluated *in vitro* by quantifying the proliferation of IL-3 dependant murine pro B cell line (BaF3-FR1c), which has been modified to express FGFR1c but lacks extracellular heparan sulfates.[12] The thermal stabilisation of FGF by heparin-mimicking polymers was further evaluated by applying temperature stressors on FGF in the presence or absence of sulfonated polymers, and compared to heparin itself.

PAMPS-Star

Polymers 14 and 15

PAMPS

x = 10, 20, 80, 200, 400

Polymers 1, 2, 3, 4 and 5

PHEAm

Polymer 6

Heparin

PAMPS-*b*-HEAm

x1:x2 = 24:56, 40:40, 56:24

Polymers 7, 8, 9 and 10

PAMPS-*co*-HEAm

x1:x2 = 24:56, 40:40, 56:24

Polymers 11, 12 and 13

PAMPS-*b*-HEAm-Star

Polymers 16 and 17

PAMPS-*co*-HEAm-Star

Polymer 18

Scheme 1: Structures of the polymers used as heparin-mimics.

Experimental Section

Typical polymerization procedure (HEAm Homopolymer Synthesis (DP 80)).

BDMAT (16 mg, 0.06 mmol), HEAm (0.60 g, 5.1 mmol), phosphate buffer solution (2.7 mL), sodium hydroxide (3.3×10^{-2} mmol, 1.3 mg) and VA-086 (8.4×10^{-3} mmol, 2.4 mg) (from stock solution at 20.0 mg/mL) were introduced into a flask equipped with a magnetic stirrer bar and sealed with a rubber septum. The solution was deoxygenated by bubbling with nitrogen for 10 minutes, and the vial was then placed in a temperature controlled oil bath at the desired temperature (90 °C), for the duration of time required to reach nearly full conversion (~ 2 hours). At the end of the reaction, the mixture was allowed to cool down to room temperature and then opened to the atmosphere. Final materials were characterised using ^{1}H NMR spectroscopy and SEC ($M_{n,SEC}$ and $Đ$ were determined). The compound was then dialysed against water for 48 hours and freeze dried, to yield the final compound as a pale yellow powder; m.p > 300 °C; v_{max}/cm^{-1} 3272 (m, COO-H and O-H, stretch), 2931 (w, C-H, stretch), 1633 (s, C=O, stretch), 1548 (s, N-H, bend), 1057 (s, C=S, stretch).

Cell lines and cell culture

NIH-3T3 cells were cultivated in Dulbecco's modified eagle medium (DMEM) supplemented with 10 % bovine calf serum (BCS) and 1 % L-glutamine, at 37 °C and 5 % CO_2 atmosphere. Cells were passaged every 3 days when reaching approximately 80 % confluency. NIH-3T3 cells were used up passage 16. BaF3-FR1c cells were cultivated in RPMI1640 GlutaMAX media supplemented with 10 % foetal bovine serum (FBS), 2 ng/mL of recombinant mouse IL-3, 600 µg/mL of G418, at 37 °C and 5 % CO_2 atmosphere. Cell media was replaced every 2-3 days by

centrifuging at 1000 rpm for 5 minutes and kept at a density between 500,000 to 1,000,000 cells/mL and were used up to a month.

Cytotoxicity assays on NIH-3T3 cells

NIH-3T3 cells were washed with 10 mL of PBS, trypsinized and re-suspended in media. The cells were plated at a concentration of 2,000 cells/well in a 96-well plate and allowed to attach for 12 hours. The medium was replaced with 100 µL of fresh medium containing a series of polymer dilutions ranging from 10 ng/mL to 1 mg/mL (10 ng/mL, 1, 10 and 100 µg/mL and 1 mg/mL). After an incubation of 48 hours at 37 °C and 5 % CO_2, the cells were washed and the medium replaced with fresh culture medium containing 25 µL of XTT (1 mg/mL) and PMS (25 µmol/L). Cells were further incubated for 12 hours at 37 °C, 5 % CO_2. Absorbance of each well was measured using a Synergy HTX plate reader at 475 nm and 650 nm (background) with $A = A_{475nm} - A_{475nm(blank)} - A_{650nm}$. The viability of cells was normalised to samples in which cells were incubated with medium only (positive control = 100 %). Each sample had three replicates and the experiment was repeated two times.

Haemolysis Study

Defibrinated sheep red blood cells were prepared by washing the blood (2 mL) three times with PBS (750 µL) by ultracentrifugation (4,500 g for 1 minute) and removing the plasma each time. The blood was then diluted with PBS at 1:150. Polymers were dissolved in PBS and three serial dilutions were prepared, 10, 100 and 200 µg/mL. 380 µL of blood was mixed with 20 µL of polymer samples and incubated at 37 °C for 2 hours. The samples were then centrifuged at 1,000 g for 10 minutes and then 200 µL of the supernatant was transferred into a 96-well plate and the

absorbance was read at 414 nm and normalised against a positive and negative control. A solution of 2 % of triton X-100 in PBS was used as a positive control and set at 100 % of haemolysis of red blood cells.

Cell proliferation

BaF3-FR1C cells were collected (1,000 rpm for 5 minutes) and washed twice (1,000 rpm for 5 minutes) with medium to remove traces of IL-3 and G418. Cells were plated at a concentration of 20,000 cells/well/50 µL in the internal wells of a 96-well plate in the presence of medium without IL-3 and G418. Further 50 µL of medium containing polymers or heparin and FGF at double the final desired concentration ([polymers]$_{final}$ = 100, 1 and 0.1 µg/mL and [FGF]$_{final}$ = 5 ng/mL) were added to the wells. Controls with cells only and with cells in the presence of 5 ng/mL of FGF were used as references. External wells were filled with 100 µL of PBS and a gas permeable moisture barrier seal (4titude) was used to decrease the evaporation into the plate. After incubation for 48 hours at 37 °C, 5 % CO_2, 20 µL of the CellTiter-Blue® assay were added into each wells and further incubated for 6 hours at 37 °C, 5 % CO_2. Fluorescence of each well was measured using a Synergy HTX plate reader with the excitation set to 560 nm and the emission at 590 nm. The extension of cell proliferation was calculated by using the wells containing cells in medium only as positive controls (100 %). Each sample had four replicates and the experiment was repeated four times.

Cell proliferation applying thermal stressors

Solutions of FGF alone, FGF with polymers or FGF with heparin were prepared in medium at double the final desired concentration ([polymers]$_{final}$ = 100, and 50 µg/mL and [FGF]$_{final}$ = 5

ng/mL). Solutions were then stored for 12 hours at the desired temperature either at approximately 20 °C (room temperature) or 37 °C. BaF3-FR1C cells were collected (1,000 rpm for 5 minutes) and washed twice (1,000 rpm for 5 minutes) with medium to remove traces of IL-3 and G418. Cells were plated at a concentration of 20,000 cells/well/50 µL in the internal wells of a 96-well plate in the presence of medium without IL-3 and G418. A further 50 µL of polymers or heparin solution at double the final desired concentration were added to the wells. Controls with cells only and with cells in presence of 5 ng/mL of FGF with applied thermal stressors were used as references. External wells were filled with 100 µL of PBS and a gas permeable moisture barrier seal (4titude) was used to decrease the evaporation into the plate. After incubation for 48 hours at 37 °C, 5 % CO_2, 20 µL of the CellTiter-Blue assay was added into each wells and further incubated for 6 hours at 37 °C, 5 % CO_2. Fluorescence of each well was measured using a Synergy HTX plate reader at set up at $560_{Ex}/590_{Em}$. The extension of cells proliferation was calculated by using the well with cells with medium only as positive control (100 %). Each sample had four replicates and the experiment was repeated four times.

Results and Discussion

Synthesis Polymeric Structures

To compare the influence of the sulfonated polymer structure on the stabilisation of bFGF, a library of PAMPS homopolymers and copolymers with varying size, architectures and monomer distribution was synthesised by RAFT polymerisation in aqueous solution using previously reported conditions.[34] bFGF was used in this study as it has been widely used in the literature to test the ability of polymers to mimic polymer.[16, 28, 30] A library of AMPS® homopolymers with DPs ranging from 10 (M_n = 2,500 g/mol) to 400 (M_n = 91,000 g/mol) (**Table S 1**) were synthesised. Furthermore, as literature suggests that interactions between heparin and growth factors are further enhanced through hydrogen bonding between asparagine and glutamine residues and the hydroxyl group from heparin,[8] a small library of PAMPS-*co*-PHEAm (random, diblock and octablock copolymers) was also prepared. These systems enable us to investigate the effect of monomer distribution for different ratios of AMPS® to HEAm whilst always targeting an overall DP of 80 (**Table S 2** and **Figure S 2**, **Table S 3** and **Figure S 3**).[35-36] Finally, a homopolymer bearing hydroxyl group only (PHEAm) was also prepared (**Figure S 1**) as non-sulfonated control. Reactivity ratios of AMPS® and HEAm were also determined, as monomer reactivity during polymerisation influence the monomer distribution in the final copolymer. Consequently, the apparent reactivity ratio of AMPS® and HEAm was determined using HPLC,[37] by monitoring the consumption of AMPS® and HEAm during the synthesis of the random copolymer PAMPS$_{40}$-*co*-PEAHm$_{40}$ (**Figure S 5**). The parallel decrease of each monomer peak intensity (Slope$_{AMPS}$® = 4.2x10^4 and Slope$_{HEAm}$ = 3.1x10^4) suggests that the copolymer is composed of statistically distributed AMPS® and HEAm monomers. Finally, star polymers were synthesised, using an "arm

first" approach, where a copolymer is synthesised first (the 'arms'), followed by addition of a difunctional vinyl monomer to form the 'core', as described in the literature, to investigate the influence of branching on heparin-mimicking properties.[34] All polymers were dialysed against water for 48 hours, changing the water three times. Float-A-Lyzer® with a molecular weight cut off range between 0.5-1 kDa from Spectrum were used to remove any undesirable reactants (i.e. potential monomers leftover or initiator).

Table 1. Molecular weights and haemolytic activity of heparin and heparin mimicking polymers used in this study.

Polymer	Structure	$M_{n,SEC}$ ($Đ$) [a] (g/mol)	10 µg/mL [b]	100 µg/mL [b]	200 µg/mL [b]
Heparin	Heparin	16,400	-0.5 ± 0.1	0.0 ± 0.4	0.1 ± 0.1
1	PAMPS$_{10}$	-	0.1 ± 0.6	0.7 ± 0.7	1.7 ± 0.8
2	PAMPS$_{20}$	4,100 (1.10)	0.9 ± 0.7	-0.3 ± 0.3	0.3 ± 0.3
3	PAMPS$_{80}$	14,400 (1.19)	0.3 ± 0.6	0.0 ± 0.6	0.7 ± 1.1
4	PAMPS$_{200}$	30,500 (1.25)	-0.2 ± 0.5	-0.3 ± 0.3	-0.3 ± 0.3
5	PAMPS$_{400}$	64,800 (1.51)	0.0 ± 0.3	-0.2 ± 0.9	0.0 ± 0.8
6	PHEAm$_{80}$	-	1.1 ± 0.4	-0.3 ± 0.6	1.8 ± 0.5
7	(PAMPS$_{10}$-b-PHEAm$_{10}$)$_4$	24,700 (1.25)	1.1 ± 0.2	0.6 ± 0.4	0.4 ± 1.0
8	PAMPS$_{24}$-b-PHEAm$_{56}$	13,100 (1.18)	0.6 ± 0.5	0.5 ± 0.4	1.2 ± 0.3
9	PAMPS$_{40}$-b-PHEAm$_{40}$	13,300 (1.21)	0.5 ± 0.2	0.8 ± 0.1	1.3 ± 0.4
10	PAMPS$_{56}$-b-PHEAm$_{24}$	14,400 (1.23)	0.5 ± 0.5	0.8 ± 0.7	1.4 ± 0.3
11	PAMPS$_{24}$-co-PHEAm$_{56}$	9,500 (1.15)	1.1 ± 0.7	1.6 ± 0.1	2.1 ± 0.2
12	PAMPS$_{40}$-co-PHEAm$_{40}$	12,000 (1.15)	0.8 ± 0.5	0.8 ± 0.2	1.1 ± 0.9
13	PAMPS$_{56}$-co-PHEAm$_{24}$	14,000 (1.16)	0.5 ± 0.4	0.8 ± 0.2	1.9 ± 0.7
14	PAMPS$_{50}$-Star	316,200 (1.01)	-0.1 ± 0.4	0.5 ± 0.5	0.6 ± 0.6
15	PAMPS$_{100}$-Star	526,000 (1.01)	-0.1 ± 0.2	0.0 ± 0.3	-0.2 ± 0.7
16	(PAMPS$_{10}$-b-PHEAm$_{10}$)$_4$-Star	1,482,000 (1.22)	1.4 ± 0.6	1.7 ± 0.5	2.0 ± 0.6
17	PAMPS$_{40}$-b-PHEAm$_{40}$-Star	714,000 (1.01)	1.4 ± 0.2	2.1 ± 0.3	1.7 ± 0.9
18	PAMPS$_{40}$-co-PHEAm$_{40}$-Star	436,000 (1.01)	1.6 ± 0.3	1.6 ± 1.2	0.2 ± 0.1

[a] Experimental $M_{n,SEC}$ values were determined by size-exclusion chromatography in 20 % MeOH / 80 % 0.1M NaNO$_3$ in Milli-Q water eluent using the triple detection options in Agilent GPC/SEC Software which uses a combination of a Refractive Index (RI), a Multi-Angle Light Scattering (MALS) and a Viscometer (VS) detectors.; [b] Haemolysis of heparin mimicking polymers against defibrinated sheep blood red cells at 37 °C for 2 hours, Triton X was used as positive control (100 %).

Toxicity of Polymers

None of the materials displayed cytotoxicity at concentrations up to 1 mg/mL after 48 hours incubation with murine embryonic fibroblasts (NIH-3T3; in the absence of bFGF), as determined using an XTT assay.[38] Of importance for wound healing formulations, the haemolytic activity of the synthesised polymers was then evaluated using PBS and Triton-X solutions as negative (0 %) and positive (100 %) controls, respectively. Following incubation with red blood cells for 2 hours at 37 °C at three different concentrations (10, 100 and 200 μg/mL), none of the polymers displayed any significant haemolytic activity (< 2.5 %) (**Table 1**). These results, being comparable to previously reported data, confirm that the polymers are non-toxic and non-haemolytic.[39-40]
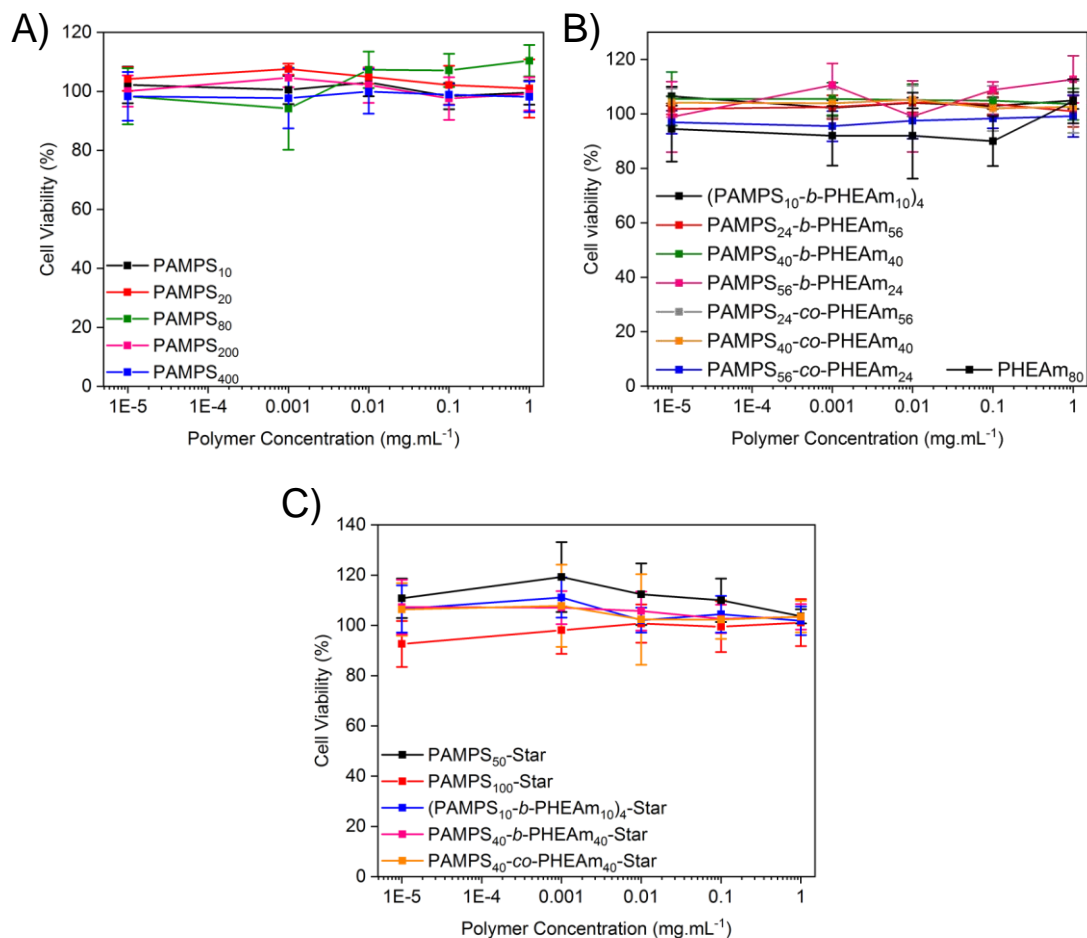
Figure 1: Cell viability of NIH-3T3 fibroblasts incubated for 48 hours in the presence of varying concentrations (10 ng/mL, 1, 10 and 100 µg/mL and 1 mg/mL) of A) PAMPS with various DPs; B) PAMPS and PHEAm copolymers with varying percentages of charges, and C) star (co-) polymers. Cell viability was determined using typical protocol for XTT assay. Each data point represents the means of triplicates from two independent experiments (N = 6). The error bars represent the standard deviation of the mean.

Proliferation Study

The propensity of PAMPS synthetic polymers to stabilise bFGF and promote cellular proliferation in the presence of bFGF was investigated. In these experiments BaF3-FR1c cells that have been modified to express the FGFR1c receptor but lack heparan sulfate proteoglycans on their surface were used. Absence of heparan sulfate proteoglycans is important as these may interfere with the activity of our heparin mimicking polymers.[12] An overall increase in cell proliferation indicates an increase of bFGF stabilisation and dimerisation. Control experiments revealed no increase of cellular proliferation when BaF3-FR1c cells were incubated with bFGF alone, whilst the presence of heparin resulted in a concentration dependent increase in cellular proliferation of about 300 to 500 %, (**Figure 2 – A** and **Table S 5,** , 115 ± 17 %, $p < 0.001$), in agreement with literature.[28]
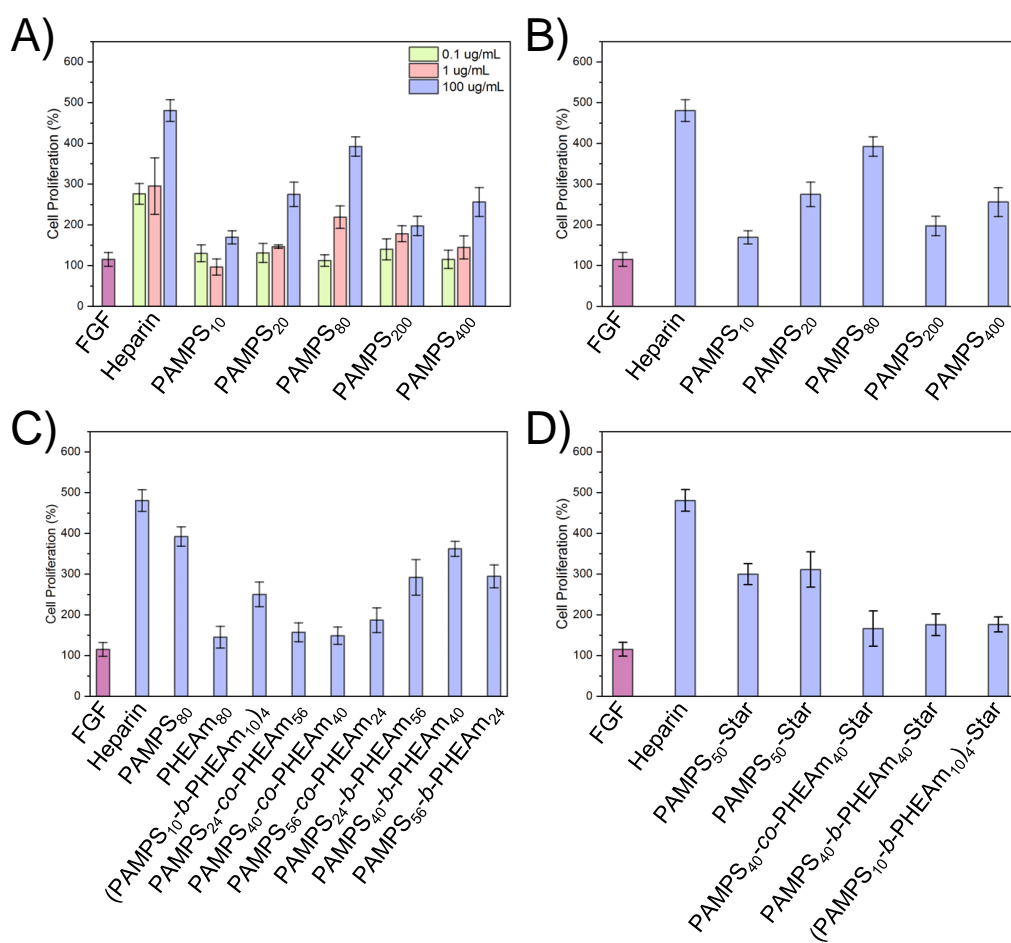
Figure 2: Proliferation of BaF3-FR1c cells incubated for 48 hours at 37 °C in the presence of 5 ng/mL of bFGF A) PAMPS homopolymers with various DPs at various concentrations (0.1, 1 and 100 µg/mL); B) PAMPS homopolymers with various DPs at 100 µg/mL; C) PAMPS and PHEAm copolymers with varying percentages of charges and monomers segmentations at 100 µg/mL; D) star-shaped (co-)polymers at 100 µg/mL. Cell growth was determined using typical protocol for CellTiter-Blue® assay. Data were normalised (100 %) to values obtained for cells incubated with culture medium only. Each data point represents four replicates of four independent experiments (N = 16). The error bars represent the standard deviation from the mean. Statistical analyses were performed with an ANOVA, then Tukey HSD post-hoc test using SPSS software, results can be found in the supporting information.

16

Effect of Molecular Weight

The effect of molecular weight on bFGF stabilisation was studied using AMPS® homopolymers with increasing degrees of polymerisation from 10 to 400. The variation of proliferation for various AMPS homopolymers appeared to be both concentration and molecular weight dependant. Considerably higher proliferation was observed when incubating bFGF with 100 µg/mL of polymers rather than with 0.1 or 1 µg/mL (**Table S 5**), which is similar to what is observed in the case of heparin. The maximum proliferation value of synthetic polymers was reached for PAMPS$_{80}$ (392 ± 24 %). The proliferation obtained with PAMPS$_{80}$ was comparable to that obtained for heparin at 100 µg/mL (481 ± 27 %, $p < 0.001$ (heparin)) (**Table S 6**). To understand this phenomenon, the experimental average molecular weight of the various synthetic AMPS® homopolymers was compared to that of heparin (**Figure 3 − A** and **B**). The results confirm that PAMPS$_{80}$ experimental molecular weight is closest to that of heparin (~ 15,000 g/mol), suggesting that there is an optimal size range for bFGF activation.[1, 16] The importance of size could be linked to differences in the propensity of polymers with different lengths to induce dimerisation of the growth factor, a phenomenon which has been observed in the past for heparin.[12] Chains smaller than the optimal length are less likely to bind to two bFGF units and provoke their dimerization. Additionally, they are less likely to bind to both the dimeric form of bFGF and FGFR1c. In contrast, while longer polymeric chains can bind to multiple copies of bFGF, their flexible nature does not constrain the proteins into near proximity, which in turn does not promote the dimerisation of bFGF and its binding to FGFR1c. Interestingly, these results are in contrast with those obtained by Maynard *et al.,* who demonstrated that the molecular weight of P(SS-*co*-PEGMA) copolymers did not affect cellular proliferation.[30] This difference could be due to the PEGMA monomer the

authored used, as its high molecular weight may induce significantly higher steric hindrance. However, PSS and PAMPS are different polymers and cannot be compared directly but only a trend can be deduced. Polymers made by the same technic and with similar molecular weights (or DP) would be easier to compare with each other's.
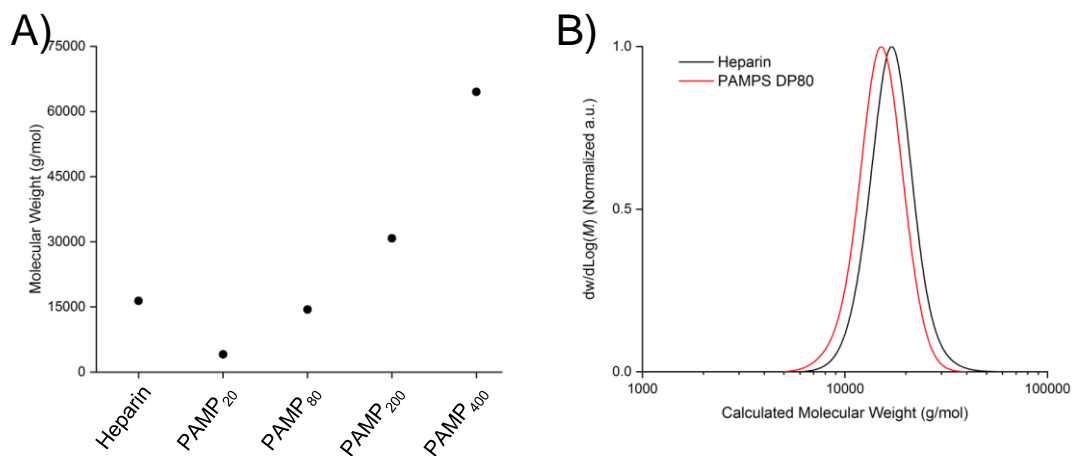
Figure 3: A) Molecular weight of selected linear PAMPS and heparin, as determined using size-exclusion chromatography with triple detection; B) Molecular weight distributions of heparin sample used in this study and PAMPS$_{80}$, as determined using size-exclusion chromatography with triple detection.

Effect of Comonomer and Monomer Distribution

Incubation in the presence of a non-sulfonated polymer control, PHEAm homopolymer (DP = 80), showed a relatively small increase in cellular proliferation (145 ± 27 % with 100 µg/mL of PHEAm$_{80}$, $p < 0.001$ (heparin) and $p = 0.71$ (FGF)) as compared to heparin (**Figure 2 – C** and **Table S 7)**. This result is in accordance with the accepted mechanism of interaction between heparin and bFGF, which mostly relies on electrostatic interactions between heparin's sulfated

18

groups and the positively charged arginine and lysine rich domain of bFGF.[41] It is noteworthy that the theoretical molecular weight of PHEAm (9,500 g/mol) is lower than PAMPS$_{80}$ (18,600 g/mol), meaning that at equivalent mass concentrations, twice as many molecules of PHEAm are present in the system. Next, the activity of copolymers of AMPS$^{®}$ and HEAm was investigated using a library of copolymers with varying segmentation (random, octablock and diblock) and percentage of charges (30 %, 50 %, and 70 %) (**Figure S 4**). At 100 μg/mL and in the case of copolymers with 50 % of AMPS$^{®}$, differences in cell proliferation were observed with changing block segmentation, from random (**12**, 149 ± 21 %) to octablock (**7**, 250 ± 30 %) and finally diblock (**9**, 362 ± 18 %) copolymers (**Figure 2 – C** and **Table S 7,** $p < 0.001$). While the overall proliferation remains still lower than heparin or PAMPS$_{80}$, the highest results are obtained with the diblock copolymer, yet with only half the number of sulfate groups. This can be attributed to the compartmentalisation of anionic charge in a particular section of the polymer chain (i.e for the diblock compared to the octablock or random copolymers). This could increase the affinity for the cation-rich heparin-binding section of bFGF and FGFR1c. A similar hypothesis was suggested by Garcia-Fernandez *et al.*[32] who attributed the enhanced heparin-mimicking activity of partially segmented poly(BA-co-AMPS), synthesised by free radical polymerisation, to a higher propensity of the AMPS-rich blocks to form the helical conformation required to interact with the heparin binding site of bFGF.[42] Interestingly, increasing the percentage of AMPS compared to HEAm from 30 to 70 % had a negligible influence on the proliferation activity induced by both the random and diblock copolymeric system (**Figure 2 – C** and **Table S 7**, $p < 0.001$). On one hand, this further indicates that hydrogen bonding does not play an important role in the interaction process.[43-44] On the other hand, the degree of charge present on the chain may not matter as much as charge segregation.

19

Effect of Polymer Architecture

To further study the importance of size on bFGF stabilisation, a range of larger branched star-shaped copolymers were investigated. The experiments, shown in **Figure 2 – D**, showed lower proliferative activity (300 ± 26 % for PAMPS$_{50}$-Star (**14**) and 311 ± 43 % for PAMPS$_{100}$-Star (**15**)) compared to the linear polymers (392 ± 24 % for PAMPS$_{80}$ (**3**)). Unlike their linear counterparts, none of the star copolymers (random *versus* diblock *versus* octablock star copolymers) showed higher cellular proliferation compared to when heparin is used (**Table S 8**, $p = 1.000$). Direct comparison between star and linear polymers is difficult due to the dramatic difference in molecular weight. However, the lower proliferation observed is likely due to the lower mobility of the arms in the star structure, in comparison with their unbound linear homopolymer counterparts. The steric hindrance of the polymeric arms in the star architecture potentially results in bFGF being only able to bind onto the surface of the polymer structure, thus decreasing opportunities for bFGF dimerisation. In addition, for the star copolymers the surface is covered by a mixture of AMPS and HEAm, this is expected to further reduce opportunity for bFGF dimerization

Effect of Temperature

bFGF is known to degrade during storage and delivery, thus reducing its efficacy and applicability caused by denaturation at around 40 °C.[29, 45] The electrostatic stabilisation between the protein and heparin has been shown to significantly increase the denaturation temperature,

improving performance.[46-47] The stabilisation of bFGF with the highest performing polymers (i.e. $PAMPS_{80}$, $PAMPS_{40}$-b-$PHEAm_{40}$, $PAMPS_{50}$-Star and heparin itself) was studied at different temperatures (20 °C or 37 °C for 12 hours) applied to mimic potential storage conditions, and compared to bFGF alone (**Figure 4**). After storage at room temperature and at 37 °C for 12 hours, all of the linear polymers (heparin, $PAMPS_{80}$, $PAMPS_{40}$-b-$PHEAm_{40}$) showed a 20-40 % decrease in relative cellular proliferation compared to when no stressors were applied. These were similar to what was observed for bFGF alone (**Table S 9**, $p \leq 0.05$).
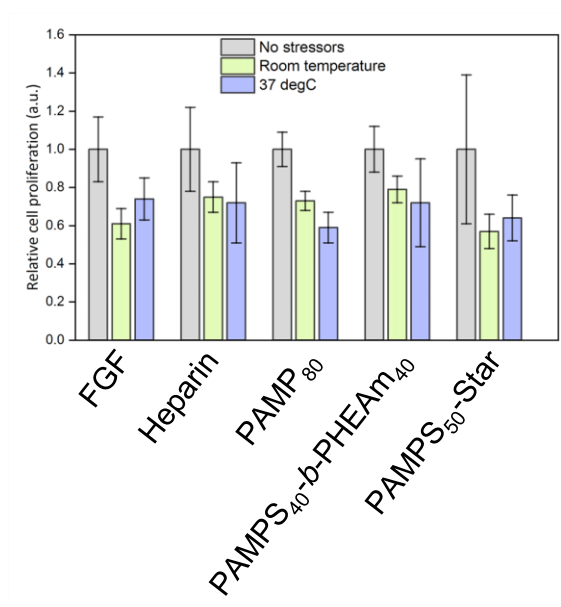
Figure 4: Relative cell proliferation of BaF3-FR1c compared to when no stressors were applied on bFGF with or without polymer or heparin. bFGF (5 ng/mL) was pre-incubated at the indicated temperatures for 12 hours with or without polymers or heparin at 100 µg/mL. Cell growth was determined using typical protocol for CellTiter-Blue® assay. Data were normalised to cellular proliferation without stressors. Each data point represents four replicates of two independent experiments (N = 8). The error bar represents the standard deviation from the mean. Statistical

analyses were performed with an ANOVA, then Tukey HSD post-hoc test using SPSS software, results can be found in the supporting information.

After storage of bFGF at 37 °C during 12 hours with heparin, a decrease in bioactivity was observed from 1 ± 0.18 to 0.78 ± 0.02 and 0.71 ± 0.15 at 100 µg/mL. This is in accordance with a previous observation that heparin acts as a natural stabiliser for bFGF.[3] Overall, the relative cell proliferation was shown to decrease when the storage temperature was increased respectively to room temperature and finally 37 °C. Finally, no improvement of bFGF stability was observed in the presence of the star polymers during storage at any temperatures. This can be attributed to a weakened affinity between the star polymers compared to when linear polymers are used with bFGF at increased temperature due to the potential breakdown of electrostatic interactions.[48-49] Additionally, it was previously hypothesised that the binding of the star polymers happen only onto their surface which is likely due to the difference of conformation between linear and star polymers. This probably weakened the interaction between bFGF and star polymers compared to linear polymers.

Conclusions

Here, the efficiency of PAMPS-based synthetic heparin-mimicking polymers for the stabilisation of bFGF, determined using the proliferation of BaF3-FR1c cells as a model assay, has been demonstrated. The results indicate that the stabilising properties of AMPS® homopolymers are length-dependant, with the optimal length matching that of naturally occurring heparin. Investigating a range of copolymers of AMPS® and HEAm showed that charge distribution has a

significant influence, with the highest charge density showing the highest proliferation activity (i.e. diblock > octablock > random copolymer). Branched architectures in the form of star polymers showed an overall decrease in bFGF stabilisation activity, which we attribute to the binding of bFGF onto the surface of the star polymers only, affecting both the binding of two copies of bFGF and consequently the dimerisation. However no significant improvement in stabilisation was evident after applying thermal stressors to bFGF in the presence of any of the synthesised polymers.

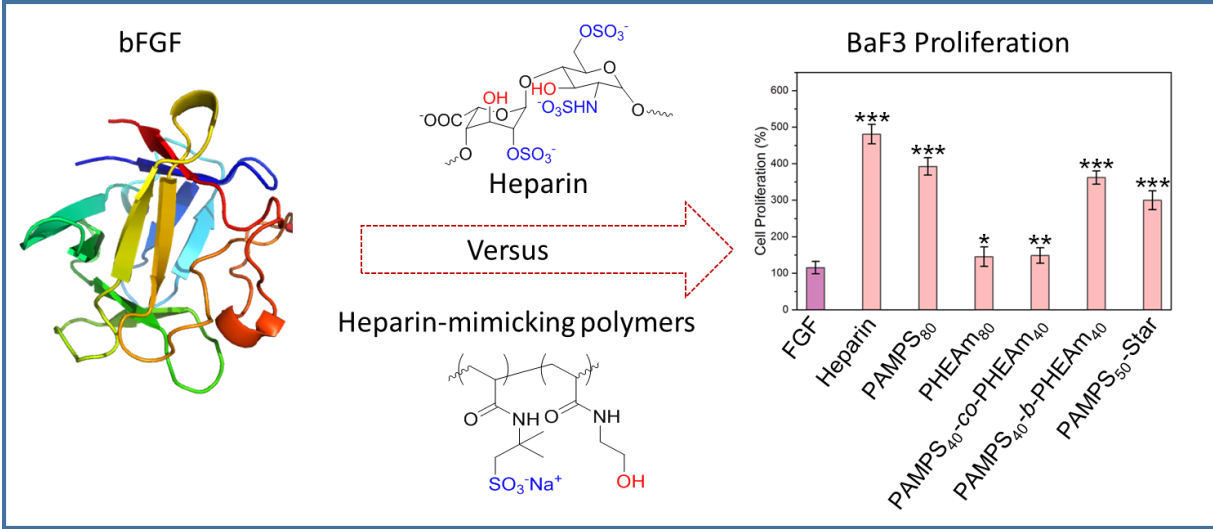## Supporting information

SI contain Materials, Instrumentations, Equations, Synthesis Details, NMR results, GPC spectrum and statistical test.

Acknowledgements

bFGF

OSO₃⁻

Heparin

Versus

Heparin-mimicking polymers

BaF3 Proliferation

24

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