Poly(*N*-acryl amino acids): A New Class of Biologically Active Polyanions

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Received February 25, 2000

Poly(*N*-acryl amino acids) bearing side groups with a lipophilic character or having charged functional groups (i.e. $-NH_2$, −COOH, −SH, −OH, and phenols) were synthesized from the radical polymerization of *N*-acryl amino acid monomers. Monomers were prepared from the reaction of acryloyl chloride and amino acid esters in dry solvents. Polymers of a broad molecular weight ranging from 3 000 to 60 000 Da were obtained. The polymers were optically active, and their structures were confirmed by ^{1}H NMR and IR spectra and elemental analysis. Hydroxyl-containing polymers were sulfated in high conversion yields by SO_3/pyridine complex. The newly synthesized linear homopolyanions were tested for heparin-like activities: (i) inhibition of heparanase enzyme, (ii) release of basic fibroblast growth factor (bFGF) from the extracellular matrix (ECM), and (iii) inhibition of smooth muscle cell (SMC) proliferation. Polymers based on tyrosine and leucine were highly active in all three tests (microgram level). Polymers based on phenylalanine, *tert*-leucine, and proline were active as heparanase inhibitors and FGF release, and polymers of *trans*-hydroxyproline, glycine, and serine were active only as heparanase inhibitors. The polymer of *cis*-hydroxyproline was inactive. It was found that a net anionic charge (i.e. carboxylic acid) is essential for biological activity. Thus, methyl ester derivatives of the active polymers, zwitterionic amino acid pendent groups (lysine, histidine), and decarboxylated amino acids (tyramine, ethanolamine) were inactive. The above active polymers did not exhibit anticoagulation activity which is considered the main limitation of heparin and heparinomimetics for clinical use. These synthetic poly(*N*-acryl amino acids) may have potential use in the inhibition of heparanase-mediated degradation of basement membranes associated with tumor metastasis, inflammation, and autoimmunity.

Introduction

Regulation of physiological processes is mediated by specific interactions between macromolecules, most often by polyelectrolites. Their size, tertiary structure, and nature of charge provide them with the necessary chemical information to execute specific biological interactions. Moreover, natural polyanions such as DNA and glycosaminoglycans (GAG) are capable of binding different proteins, resulting in a wide range of biological activities. Therefore, synthetic linear polyanions have been studied since the early 1960s as potential biologically active molecules.[1] They present a wide range of biological activities such as antimicrobial,[2] antiinflammatory, and antitumor agents,[3] excitation of the reticuloendothelial system (RES),[4] and stimulation of interferon production.[5] These polymers lack a high degree of selectivity, since their activity is based primarily on their scaffold, electrical charge, or charge density along the polymeric backbone.

Two major groups of polyanionic substances have been studied during the past three decades. One is polyanionic polysaccharides, both natural and chemically modified.[6] The second group includes synthetic carboxylic acid polymers.[7]

The first polysaccharide applied in medicine was heparin. It is a natural polyanion composed of repeating disaccharide units of glucosamine and uronic acid. The amino groups of glucosamine are partially sulfated or acetylated.[8] Some of the hydroxy groups are also sulfate esters.[9] The degree of sulfation and the chain size of the polymer determine the biological activity of heparin.[10] Although heparin has a range of desired activities including inhibition of tumor metastasis and inhibition of restenosis, its clinical use is limited to treating blood-clotting disorders[11] as the anticoagulation activity of heparin may endanger the patient due to the risk of hemorrhage.

By a two-step oxidation, Claes et al.[12] have modified several polysaccharides and studied their effectiveness as interferon inducers and potential antiviral agents. Polymers produced in this way contain carboxylic groups distributed along a biodegradable polyacetal backbone. These compounds showed low toxicity and significant antitumor and antiviral activities. Sulfation of other polysaccharides such as laminarin[13] yielded potent anticoagulants.

One of the most studied synthetic polycarboxylic acids is pyran[7] a copolymer prepared from divinyl ether and maleic anhydride (1:2). Its biological activity spectrum is very wide, including the induction of interferon, antiviral, antibacterial, and antifungal activities, stimu-

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10.1021/jm000089j CCC: $19.00
Published on Web 06/13/2000

lation of immune responses, anticoagulant activity, adjuvant disease inhibition, and anticancer activity.[14]

The polymer conformation is also important in determining its biological activity. Muck et al.[15] reported that isotactic poly(acrylic acids) are more effective than atactic poly(acrylic acids) in providing protection against picornavirus infection in vivo. The optimal activity was reached at molecular weights ranging from 6 to 15 kDa.

Another interesting synthetic polyanion is aurintricarboxylic acid (ATA),[16] obtained from the acid polymerization of phenols with formaldehyde. This polymer exhibited activities such as inhibiting the cytopatic effect of HIV-1,[17] preventing the binding of interferon to its receptor,[18] and binding to nucleotides and to polynucleotide domains of glucocorticoid receptors.[19] Regan et al.[20] synthesized linear structures of polyaromatic polyanions as analogues of ATA, polymerizing properly chosen aromatic rings and avoiding the presence of strong oxidizing reagents. Polymers so obtained were shown to mimic the polyanionic framework of DNA, as revealed by their binding affinity for an anti-DNA monoclonal antibody. They were also tested as heparinoids, showing a low anticoagulant effect. Among the polymers tested, 4-hydroxyphenoxyacetic acid gave best results. It reverts autocrine cell transformation caused by basic fibroblast growth factor (bFGF),[21] abrogates bFGF receptor binding, dimerization and mitogenic activity,[22] and efficiently inhibits the proliferation of vascular smooth muscle cells (SMC).[23]

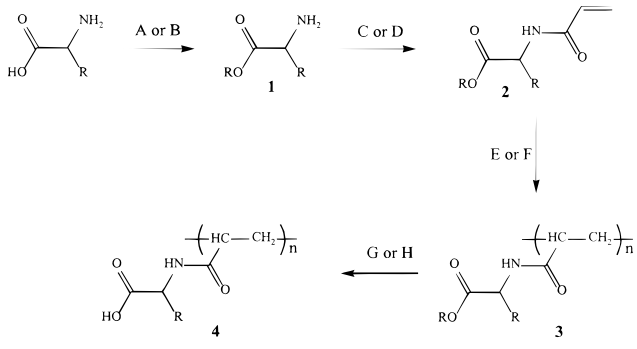
Fibroblast growth factors (FGF) are a family of structurally related polypeptides that show high affinity to heparin.[24] They are highly mitogenic for a variety of cell types, and their release from the extracellular matrix (ECM) may induce tissue repair and new blood vessel formation.[25] They are bound to heparan sulfate proteoglycans in the ECM and cell surfaces, exhibiting a high degree of stability toward proteolytic enzymes, and can be released in an active form by heparinoids and heparan sulfate-degrading enzymes.[25,26]

Heparanase is an *endo*-β-D-glucuronidase that degrades heparan sulfate side chains in basement membranes and ECMs.[27] This activity facilitates cell invasion in normal and pathological situations.[28−30] The human heparanase gene has recently been cloned and expressed,[31,32] and it has been shown to play a role in tumor progression and metastatic spread.[30,31,33] High levels of heparanase were detected in highly metastatic tumor cells and in cells of hematopoietic origin such as neutrophils and inflammatory macrophages and lymphocytes that must invade the blood vessel wall to express their physiological function.[29,30] Polyanionic molecules, sulfated polysaccharides, and some heparinoids that inhibit the enzyme also inhibit pathological processes involving cell migration such as tumor metastasis,[30,33] inflammation, and autoimmunity.[29,34]

Although these polyanions exhibited a range of desired biological activities, they lack specificity and may possess contradicting activities. Moreover, most of these polymers exhibit strong anticoagulation activity which may result in hemmohrage, thus limiting their potential clinical use.

On the basis of the fact that polyanions usually mimic heparin, we hypothesized that polyanions based on *N*-acryl L-amino acids may express heparin-like biological activities. The use of pendent amino acids along an acrylate polymer provides specific distribution of charge and of polar and hydrophobic groups arranged in a stereoselective configuration, determined by the chirality of the amino acid α-carbon. Desired hydrophobicity or charged side groups such as $-NH_2$, $-COOH$, $-OH$, and phenols can be easily introduced by choosing different amino acid residues. To test this hypothesis, we have synthesized and characterized polymers of a series of *N*-acryl L-amino acids and tested them for their heparin-like biological activity. This paper describes the synthesis and biological activity of these polymers as heparanase inhibitors, bFGF-releasing molecules, anticoagulants, and inhibitors of SMC proliferation.

Scheme 1. Synthesis of Poly(*N*-acryl amino acids)[a]



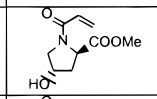
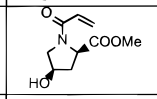
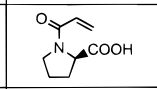
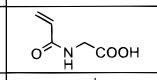
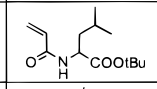
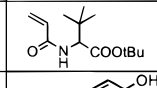
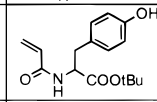
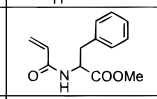
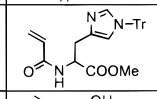
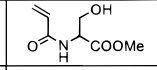
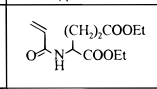
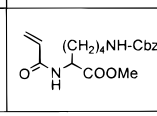
code	(R)	(R')
a	trans-4-hydroxy proline	Me
b	cis-4-hydroxy proline	Me
c	proline	H
d	glycine	H
e	Leucine	t-Bu
f	Tert-leucine	t-Bu
g	Tyrosine	t-Bu
h	Phenylalanine	Me
i	Histidine	Me
j	Serine	Me
k	Alanine	H
l	Lysine	Me
m	Glutamic acid	Et

[a] Reagents and conditions: A = dry HCl in MeOH or EtOH; B = isobutylene, dioxane, H_2SO_4; C = acryloyl chloride, $NaHCO_3$, H_2O; D = acryloyl chloride, triethylamine, CH_2Cl_2; E = ammonium persulfate/Fe^{2+}, H_2O; F = azobis(isobutyronitrile), MeCN; G = NaOH, MeOH and/or H_2O; H = trifluoroacetic acid:CH_2Cl_2 (1:1).

Chemistry

Polyacrylate Synthesis. *N*-Acryl amino acid methyl, ethyl, or *tert*-butyl esters were prepared from the reaction between acryloyl chloride and the desired amino acid ester in dry organic solvent in the presence of an acid acceptor, i.e. triethylamine. The desired acrylate derivatives were obtained quantitatively and in high purity after a short workup of the reaction mixture, which involved crystallization or precipitation (Scheme 1). The structures and the analytical data of synthesized *N*-acryl amino acids are given in Table 1. Trytil and carbobenzoxy groups were used for protection of the amine side chain functionality of histidine and lysine, respectively. No protection group was used for the hydroxyl functionality of hydroxyproline, serine, and tyrosine. Short purification by chromatography was needed for monomers containing *cis*-4-hydroxyproline, tyrosine, and histidine in order to obtain pure compounds. The ester derivatives were easily obtained by

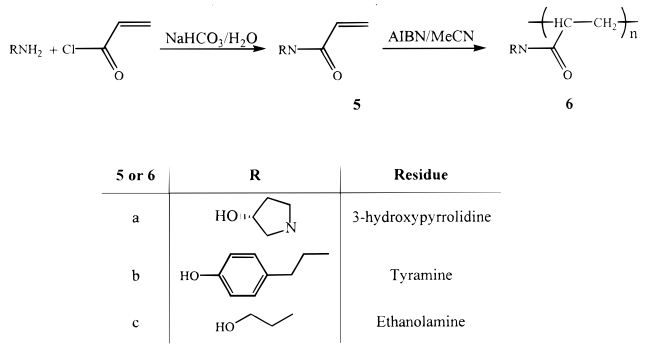
Table 1. Structures and Analytical Data of *N*-Acryl Amino Acid Monomers

Code*	Structure	MP (°C)	^{1}H-NMR
2-a		77-78	6.40 (2H, m), 5.74 (1H,m), 4.67 (2H,m), 3.87 (1H,dd), 3.75 (3H,s), 3.61 (1H,m), 2.73 (1H,broad), 2.30 (1H,m), 2.10 (1H,m)
2-b		Pasty solid	6.42 (2H,d), 5.76 (1H,m), 4.60 (1H,d), 4.45 (1H,m), 3.80 (5H,s), 2.35 (1H,m), 2.12 (1H,m), 1.63 (1H,broad)
2-c		119-120	10.68 (1H, broad); 6.43 (2H,m), 5.78 (1H,m), 4.62 (1H,d), 3.69 (1H,m), 3.58 (1H,m), 2.30 (1H,m), 2.04 (3H,m).
2-d		125-126	6.13 (2H,m), 5.65 (1H,d), 3.91 (2H,s).
2-e		63	6.28 (1H,d); 6.16-6.07 (2H,m); 5.65 (1H,d); 4.61 (1H,q); 1.64 (3H,m); 1.46 (9H,s); 0.95 (6H,d).
2-f		115	6.31 (1H,d), 6.16 (1H,m), 6,11 (1H,broad), 5.67 (1H,d), 4.45 (1H,d), 1.46 (9H,s), 1.00 (9H,s).
2-g		117	6.93 (2H,d); 6.72 (2H,d); 6.32 (1H,d); 6.11 (2H,m); 5.67 (1H,d); 4.94 (1H,q); 3.75 (3H,s); 3.10 (2H,m).
2-h		81-83	7.27-7.09 (5h,m), 6.30 (1H,d), 6.12 (1H,m), 6.05 (1H,broad), 5.65 (1H,d), 4.97 (1H,q), 3.74 (3H,s), 3.17 (2H,t).
2-i		187	7.46 (1H,d), 7.38 (1H,s), 7.32 (10H,m), 7.09 (5H,m), 6.55 (1H,s), 6.30-6.10 (2H,m), 5.63 (1H,d), 4.88 (1H,q), 3.60 (3H,s), 3.05 (2H,dq).
2-j		82	6.18 (2H,m); 5.67 (1H,d); 4.51 (1H,t); 3.80 (2H,dq); 3.64 (3H,s).
2-k		48	6.43 (1H,broad); 6.26 (1H,d); 6.15 (1H,m); 5.67 (1H,d); 4.67 (1H,q); 4.18 (2H,q); 2.39 (2H,m); 2.21 (1H,m); 1.28 (3H,t); 1.23 (3H,t).
2-l		79	7.34 (5H,s); 6.30 (2H,s); 6.15 (1H,m); 5.67 (1H,d); 5.08 (2H,s); 4.86 (1H,broad); 4.67 (1H,q); 3.75 (3H,s); 3.18 (2H,q); 1.86-1.37 (6H,m).

* Codes are related to Scheme 1.

bubbling dry HCl through the methanolic or ethanolic amino acid suspension or by acid-catalyzed reaction of the amino acid with isobutene. Attempts to synthesize *N*-acryl amino acid by direct amidation of acryloyl chloride with amino acids in aqueous solutions afforded satisfactory results only when glycine and proline were used. The structures obtained were corroborated unambiguously by ^{1}H NMR and elemental analysis. The ^{1}H NMR of all monomers synthesized showed the same pattern for the vinyl moiety. Proton Hc is downfield shifted because of its proximity to the carbonyl group. Protons Ha and Hb are nonequivalent due to the planar geometry of the amide bond. Hence, their coupling to Hc produced split doublet signals. The integration of acrylic protons is in good correlation with the ester ones. This is an indication that no additional acrylic group was attached to the unprotected side chain functionality of compounds **2a**, **2b**, **2g**, and **2j** (Table 1). Acrylate monomers lacking the carboxylic groups of tyramine, ethanolamine, and 3-hydroxypyrrolidone which correspond to tyrosine, serine, and hydroxyproline but without the carboxylic acid were prepared by direct amidation in water by the same procedure used to synthesize the hydroxyproline methyl ester derivatives and purified by chromatography (see Experimental Section). The IR spectra of the monomers and polymers exhibited absorption peaks confirming the structure of functional groups. For example, poly(*N*-acrylhydroxyproline methyl ester) showed peaks at: 3412 (hydroxyl, narrow), 1742 (ester, sharp), and 1627 cm^{-1} (amide, sharp), whereas the free carboxylic acid derivative showed peaks at: 3504 (hydroxyl, broad), 1731 (carboxylic acid, sharp), and 1634 cm^{-1} (amide, sharp). Almost all monomers were stable at room temperature. Their ^{1}H NMR remained unchanged after 1 year of storage at room temperature. Monomers **2b** (Scheme 1) and **5c** (Scheme 2) were unstable, polymerizing within hours at 4 °C.

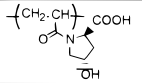
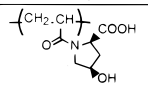
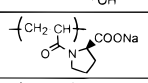
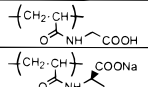
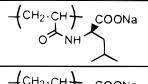
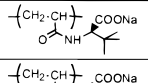
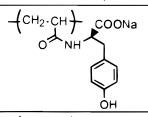
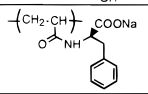
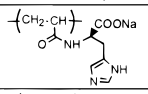
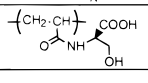
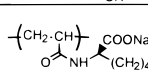
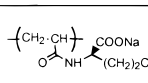
Scheme 2. Synthesis of N-Substituted Acryl Amides



The polymerization was achieved in high yields (>90% conversion) by radical initiation either by azobis(isobutyronitrile) in acetonitrile or by ammonium persulfate/Fe(II) in water or water−methanol mixtures, depending on monomer solubility (Scheme 1). Tables 2 and 3 summarize the structures and analytical data for the poly(*N*-acryl amino acids) and the corresponding decarboxylated polymers, respectively. The polymers melted (T_{max}, by DSC) at temperatures between 80 and 160 °C with a narrow heat absorption peak and heat of fusion ranging between 60 and 140 J/g, indicating relatively crystalline polymers. Most polymers had negative optical rotation, except for the tyrosine (**4g**, Table 2) and phenylalanine (**4h**) based polymers. Molecular weights were determined by GPC analysis. Purification by precipitation afforded polymers with narrower polydispersity than the bulk. Methyl and ethyl esters were cleaved by basic hydrolysis and *tert*-butyl esters by hydrolysis with trifuoroacetic acid (TFA). Sodium or potassium salts of all polymers described in Tables 2−4 were water-soluble, while the free carboxylic acid polymers were slightly soluble. The ester derivatives were soluble in aliphatic alcohols, chlorinated hydrocarbons, and acetonitrile. The methyl esters and free acids of hydroxyproline (**2a**, **2b**) and serine (**2j**) polymers were water-soluble. Although the ^{1}H NMR spectra of the polymers showed poor resolution, it was possible to monitor the polymerization process by following the disappearance of the signals representing the vinyl protons.

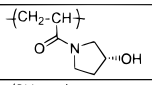
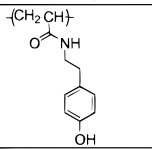
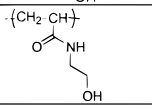
To increase the anionic nature of the polymers in biological media, sulfation of hydroxyl side groups was performed. Polymers based on *trans*-4-hydroxyproline (**3a**), tyrosine (**3g**), serine (**3j**), and 3-hydroxypyrrolidine (**6a**) were sulfated on the hydroxyl side group using SO_3/pyridine complex followed by known procedures described for polysaccharides.[12,35] Sulfate content was

Table 2. Structures and Analytical Data of Poly(*N*-acryl amino acids)

Code	Structure	^{1}H NMR	Molec Weights Mw	Molec Weights Mn	$[\alpha]_D$	Tmax (°C)	Anticoagulation (APTT)*
4-a		4.6-4.4 (2H,broad); 3.4-3.8 (3H,broad); 2.5-2.1 (2H,broad); 1.60 (1H,broad).	38,000	16,000	-104	149	37
4-b		4.4-4.1 (2H,broad); 3.9-3.4 (3H,broad); 2.7-2.3 (2H,broad); 1.90 (1H,s).	3,300	1,000	-31	131	35
4-c		4.30 (2H,broad); 3.70 (2H,broad); 2.1-1.9 (5H,broad); 1.50(2H,broad).	52,000	12,000	-78	141	55 (109)
4-d		3.8-3.32 (2H,broad); 2.0-1.8 (1H,broad); 1.5-1.1 (1H,broad).	34,000	11,500	0	143	40
4-e		3.90 (1H, broad); 2.1 (1H, broad); 1.35 (4H, broad); 1.1 (1H, s); 0.75 (6H, broad).	31,500	23,400	-75	86	36
4-f		3.90 (1H,s); 1.00 (3H,s); 0.75 (9H,s).	24,000	12,000	-9	69	36
4-g		6.70 (2H,broad); 6.25 (2H,broad); 4.00 (1H,broad); 2.60 (2H,broad); 2.00 (1H,broad); 1.4-1.0 (2H,broad).	18,000	8,700	+84	86	32
4-h		7.35 (5H,broad); 4.40 (1H,broad); 2.90 (2H,broad); 2.15 (1H,broad); 1.30 (2H,broad).	61,000	28,000	+38	169	56 (109)
4-i		8.00 (1H, broad); 6.95 (1H, broad); 4.25 (1H, broad); 2.90 (2H, broad); 2.00 (1H, broad); 1.25 (2H, broad).	5,800	2,700	+17	86	30
4-j		4.25 (1H, broad); 3.75 (2H, broad); 2.20 (1H, broad); 1.40 (2H, broad).	55,300	15,400	-6	79	39
4-k		4.15 (1H,broad); 2.90 (2H,broad); 2.10 (1H,broad); 1.60 (4H,broad); 1.30 (3H,broad).	36,500	5,000	-8	133	33
4-l		4.00 (1H,broad); 2.20 (2H,broad); 1.90(3H,broad); 1.50 (1H,broad); 1.2 (1H,broad).	47,000	17,000	-5	164	56 (75)

* Codes are related to Scheme 1. T_{max} was determined by DSC; optical activity was determined in water (40 mg/mL) at 25 °C at the D-line of sodium. APTT (in s) was determined for 25 μg/mL polymer in pooled normal plasma; the data in parentheses are for polymer concentration 250 μg/mL. Control: 32 s. No coagulation was observed with heparin (positive control).

Table 3. Analogues of Poly(*N*-acryl amino acids) Lacking Carboxylic Acid Group*

Code	Structure	^{1}H NMR	Molecular Weight Mw	Molecular Weight Mn	Tmax (°C)
6-a		4.35 (1H, broad); 3.30 (4H, broad); 2.40(1H, broad); 1.90 (2H, broad); 1.50 (2H, broad).	18,000	2,700	106
6-b		6.60 (2H, broad); 6.20(2H, broad); 4.35 (1H, broad); 2.95 (2H, broad); 2.30 (2H, broad); 1.40 (2H, broad)	29,000	3,100	130
6-c		3.55 (2H, broad); 3.15 (2H, broad); 2.9-2.1 (1H, broad); 1.8-1.4 (2H, broad)	29,000	5,000	93

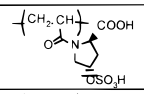
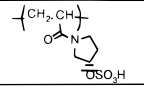
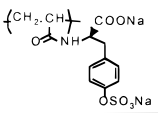
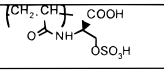
* Codes are related to Scheme 2. T_{max} was determined by DSC; molecular weight was determined by GPC.

determined by sulfur analysis. The data analysis for these polymers is given in Table 4. Sulfation of between 40% and 74% of the hydroxyls was obtained. These polymers were water-soluble.

Biology

Heparanase Inhibition. The effect of the polymers on heparanase activity was tested using $[^{35}S]O_4^{2-}$ labeled ECM produced by cultured endothelial cells and prepared as previously described.[29−31,36] Incubation of partially purified human placental heparanase[37] with this ECM releases into the medium high- and low-molecular-weight sulfate-labeled degradation products. The high-molecular-weight material is composed primarily of nearly intact heparan sulfate proteoglycans, serving as a readily accessible heparanase substrate.[29,30,38] In the absence of heparanase inhibitors, low-molecular-weight sulfate-labeled degradation frag-

Table 4. Physical and Biological Data Obtained for Sulfated Poly(*N*-acryl amino acids)

Code	Structure	Molec. Weight		%SO_4	Tmax (°C)	Anticoagulation (APTT)*	
		Mw	Mn			Sulfated	Non sulfated
7-a		69,000	7,000	74	107	100	37
7-b		27,000	15,000	53	141	32	39
7-c		44,000	10,000	40	112	45	35
7-d		13,000	5,000	45	90	35	32

* Codes are related to Scheme 3. APTT is expressed in seconds (s). Pooled normal plasma was used as control with 32 s. Polymers were tested at 25 μg/mL.

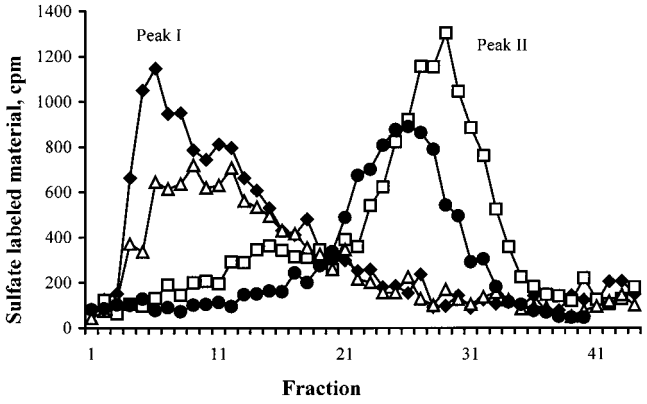


Figure 1. Effect of heparin and polyanions on heparanase activity. Sulfate-labeled ECM-coated 35-mm dishes were incubated (37 °C, 48 h, pH 6.2) with human placental heparanase in the absence (control) (□) and presence of 5 μg/mL heparin (◆), poly(*N*-acryl-*trans*-4-hydroxy-L-proline) (△), or poly(*N*-acryl-*cis*-4-hydroxy-L-proline) (●). Sulfate-labeled material released into the incubation medium was analyzed by gel filtration over Sepharose 6B columns. Heparanase activity is represented by the amount of radioactivity eluted in peak II (fractions 23−35).

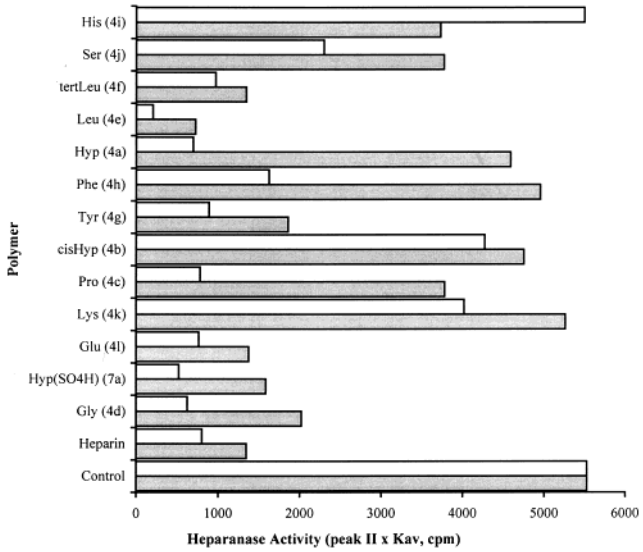


Figure 2. Inhibition of heparanase by poly(*N*-acryl amino acids). Sulfate-labeled ECM-coated 35-mm dishes were incubated (37 °C, 48 h, pH 6.2) with human placental heparanase in the absence (control) and presence of 1 μg/mL (gray bars) or 5 μg/mL (white bars) polyanionic compounds. Sulfate-labeled material released into the incubation medium was analyzed by gel filtration over Sepharose 6B columns. Heparanase activity is expressed by the amount of radioactivity eluted in peak II (sum of cpm in the highest nine fractions of peak II) multiplied by the K_{av} of this peak.[30]

ments of heparan sulfate are released into the incubation medium[29−31,38] (Figure 1, peak II). Heparin and N-substituted nonanticoagulant species of low-molecular-weight heparin are inhibitors of heparanase, preventing the degradation of heparan sulfate.[28,30,31,38] All polymers were assayed for heparanase inhibition activity (Figure 2). Poly(*N*-acrylates) of glycine (**4d**), tyrosine (**4g**), glutamic acid (**4l**), leucine (**4e**), *tert*-leucine (**4f**), and sulfated hydroxyproline (**7a**) yielded 70−80% inhibition of heparanase activity already at a concentration of 1 μg/mL, similar to heparin. The most effective compound was poly(*N*-acrylleucine) (**4e**) (Figure 2). Polymers containing *trans*-hydroxyproline (**4a**) (Figure 1), proline (**4c**), and phenylalanine (**4h**) inhibited the enzyme only at 5 μg/mL. Polyanions bearing *cis*-hydroxyproline (**4b**) (Figure 1), histidine (**4i**), and lysine (**4k**) were inactive. The corresponding decarboxylated poly(acryl amino acids) (series 6) were inactive even at 25 μg/mL (not shown).

Release of ECM-Bound bFGF. It has been demonstrated that heparin and heparin-like molecules induce release of bFGF from the ECM.[25,26,39] The released bFGF promotes, among other effects, proliferation of vascular endothelial and smooth muscle cells. As shown in Figure 3, polymers containing proline (**4c**), leucine (**4e**), tyrosine (**4g**), and sulfated hydroxyproline (**7a**) were highly active at 5 μg/mL, releasing 40−50% of the ECM-bound bFGF, compared to about 85% release of bFGF induced by heparin under the same conditions. A significant bFGF release was also exerted by polyanions bearing *tert*-leucine (**4f**), phenylalanine (**4h**), and glutamic acid (**4l**), releasing 30−45% and 55−65% of the ECM-bound bFGF at 5 and 25 μg/mL, respectively. Other poly(*N*-acryl amino acids) tested (**4b**, **4d**, **4j**, **4k**) were inactive and yielded percent release similar to the spontaneous release of bFGF obtained in

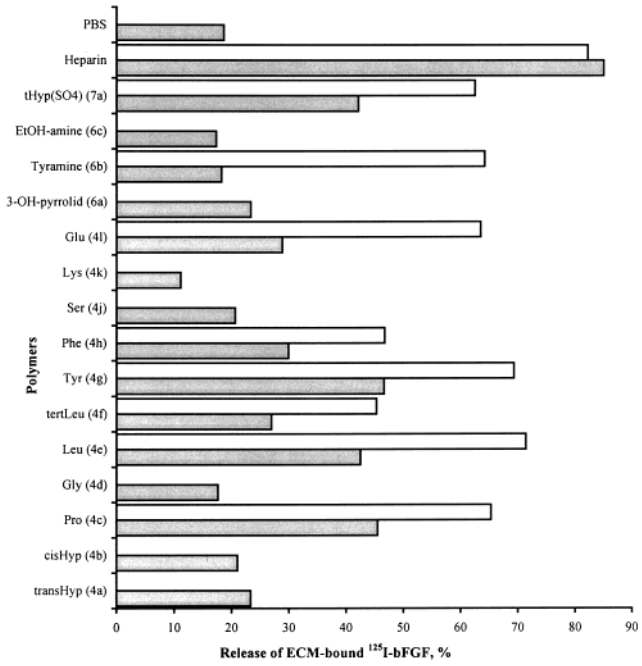


Figure 3. Release of ECM-bound bFGF. ECM-coated wells (4-well plates) were incubated (16 h, 25 °C) with [^{125}I]bFGF (4×10^4 cpm/well). The ECM was washed (×3) to remove the unbound bFGF and incubated (3 h, 37 °C) with 5 μg/mL (gray bars) and 25 μg/mL (white bars) heparin and poly(*N*-acryl amino acids). Released [^{125}I]bFGF was counted in a γ-counter. Released radioactivity is expressed as percent of total ECM-bound [^{125}I]bFGF ($\sim 1 \times 10^4$ cpm/well).[26,39] Each data point represents the mean of four wells, and the variation between wells did not exceed ±10%.

precense of PBS alone. Among the polymers lacking carboxylic groups (series 6) only poly(acryltyramide) (**6b**) showed activity at 25 μg/mL. The other polymers of this series and those bearing zwitterionic residues (lysine and histidine) were also inactive (Figure 3).

SMC Proliferation. To test the antiproliferative activity toward vascular SMC, the cells were first arrested by incubation in medium containing 0.2% fetal calf serum (FCS) followed by stimulation with 1 ng/mL bFGF in the absence or presence of heparin or the indicated poly(*N*-acryl amino acids). Among the synthesized polymers, only substances that were found to be active in the heparanase inhibition or bFGF release tests [i.e. polymers based on *trans*-4-hydroxyproline (**4a**), glycine (**4d**), leucine (**4e**), tyrosine (**4g**), serine (**4j**), and glutamic acid (**4l**)] were selected for this assay. As shown in Figure 4, polymers **4e** and **4g** effectively inhibited the incorporation of [^{3}H]thymidine into DNA at 5 μg/mL, a concentration in which heparin was inactive or slightly stimulatory.

Anticoagulant Activity. The high anticoagulant activity of heparin is attributed to its sulfate groups since partially or fully desulfated heparin shows low or no anticoagulant activity. The polymers were screened for anticoagulant activity by the standard activated partial thromboplastin time (APTT) method.[40] All polymers had a very low anticoagulant activity at a relatively high concentration (25 μg/mL) (Table 2). In the sulfated polymer series (Table 4), only sulfated hydroxyproline (**7a**) exhibited significant anticoagulation activity.

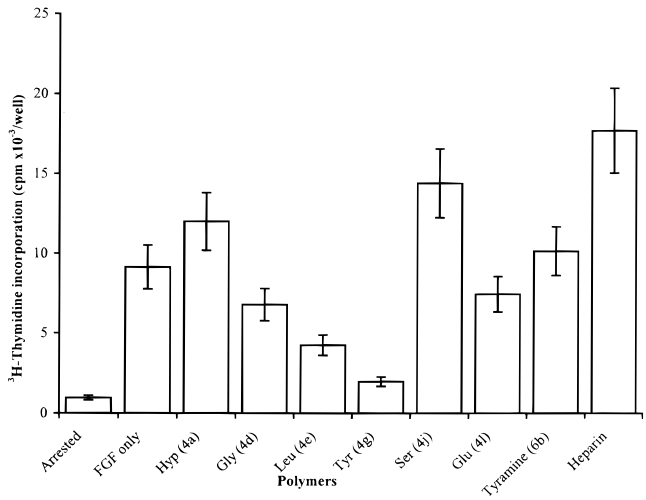


Figure 4. Effect of heparin and polyanionic compounds on proliferation of SMC. Twenty-four hours after seeding, the SMC were arrested by 48 h incubation in medium containing 0.2% FCS, followed by addition of heparin or polymers (5 μg/mL), 1 ng/mL bFGF, and [^{3}H]thymidine, as described in the Experimental Section. Cells were incubated for an additional 48 h and tested for thymidine incorporation into trichloroacetic acid-insoluble materials. The background level of thymidine incorporation by growth arrested SMC was 1000−1500 cpm in different plates. Each data point represents the mean of four wells, and the variation between different determinations did not exceed ±15%.

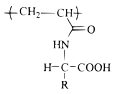
Discussion

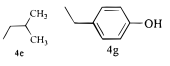
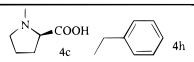
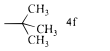
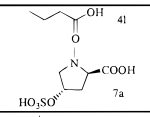
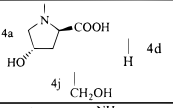
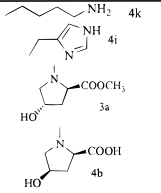
In the present study, polyanionic molecules based on amino acids have been synthesized and tested for their biological activity as heparin mimetics. Amino acids were conjugated to polyacrylate via the amino group. The polyanions obtained from the polymerization of *N*-acryl amino acids or the corresponding decarboxylated derivatives were tested for their ability to (i) inhibit the heparanase enzyme, (ii) release ECM-bound bFGF, and (iii) inhibit SMC proliferation. The polymers of *N*-acrylates of tyramine, 3-hydroxypyrrolidine, and ethanolamine were used as the decarboxylated analogues of *N*-acryltyrosine, hydroxyproline, and serine, respectively. Polymers obtained from these monomers have the same pendent residues as the poly(acryl amino acids) but without the carboxylic acid moiety. Sulfation of polymers having hydroxyl side groups such as tyrosine, serine, and hydroxyproline yielded polymers with enhanced anionic character that may confer additional similarity to native heparin.

None of the polymers tested in this study expressed a significant anticoagulant activity, indicating that these polymers will not suffer from the main limitation of heparin, i.e. powerful anticoagulation effect.

The amino acid-containing polyacrylates showed some correlation between the polymer structural features and biological activity. These are summarized in Table 5. In general, active polymers should display a net negative charge, hydrophobicity, and stereoregularity. Polymers having at least one carboxylate group, providing one negative charge per amino acid residue, were active as heparanase inhibitors and bFGF-releasing agents. Accordingly, methyl ester derivatives and zwitterionic amino acid side groups such as poly(*N*-acryl-*trans*-hydroxyproline methyl ester), poly(*N*-acryllysine), and

Table 5. Structures of Polyelectrolytes versus Biological Activity



Biological activity	Residue (R)	Features
Heparanase Inhibitor b-FGF release SMC Proliferation inhibitor		High lipophilicity
Heparanase Inhibitor b-FGF release		High lipophilicity
		(and high steric hindrance)
		Enhanced charge density
Heparanase Inhibitor		High hydrophilicity
Inactive		Zwitterionic or masked carboxylic acid or steric requisites unfulfilled

poly(*N*-acrylhistidine) were inactive. Also, the corresponding decarboxylated poly(acryl amino acid) derivatives, poly(acryltyramide), poly(acryl-3-hydroxypyrrolidineamide), and poly(acrylethanolamide), were found to be inactive, while their carboxylated analogues, poly(*N*-acryltyrosine), poly(*N*-acrylhydroxyproline), and poly(*N*-acrylserine), strongly inhibited heparanase activity. Poly(*N*-acryltyrosine) also efficiently released bFGF from ECM and inhibited SMC proliferation. Moreover, polyacrylates with glutamic acid or O-sulfated hydroxyproline side groups exhibiting enhanced density of negative charges per amino acid residue were potent heparanase inhibitors and highly active in releasing bFGF from ECM. In the case of the hydroxyproline bearing polymer, the latter activity was attributed to sulfation since the corresponding nonsulfated polymer showed only heparanase inhibiting activity (Table 5).

A second observation is related to the nature of the amino acid side groups. Polymers bearing hydrophilic residues showed only heparanase inhibition activity, while polymers having more lipophilic residues also induced bFGF release. These findings may indicate that the inhibition of heparanase is caused primarily by electrostatic interactions between the enzyme and the polyanion. On the other hand, in the absence of the strongly anionic sulfonamide groups (existing in heparin), release of ECM-bound bFGF required both electrostatic and some kind of lipophilic interactions.

Only poly(*N*-acryltyrosine) and poly(*N*-acrylleucine) efficiently inhibited SMC proliferation. These polymers were 5−10-fold more active than heparin which, under the experimental conditions applied in this study, failed to inhibit and even stimulated thymidine incorporation by SMCs at concentrations of 1 and 5 μg/mL (Figure 4). This activity was more pronounced in the presence of bFGF, suggesting that these polymers interfere with the mitogenic pathway of bFGF. Hence, poly(*N*-acryltyrosine or -leucine) may be potentially applied to inhibit SMC proliferation and intimal thickening following balloon deendothelialization and bypass coronary surgery.

The polymer stereoregularity is also important for determining its biological activity. We have found that poly(*N*-acryl-*cis*-4-hydroxy-L-proline) (**4b**) was biologically inactive at the assays performed in this study, as opposed to the trans homologue (**4a**) which efficiently inhibited heparanase activity (Figure 1). This finding indicates that not only the anionic charge but also some specific structural features have to be fulfilled to elicit significant biological activity.

Altogether, some of the poly(*N*-acryl amino acids) synthesized and characterized in this study may be of clinical significance. For example, heparanase inhibitors may have significant clinical applications in preventing tumor metastasis and inflammatory/autoimmune processes due to the involvement of this enzyme in the extravasation of blood-borne tumor cells and activated cells of the immune system.[29−32] The fact that only one species of heparanase was identified makes the heparanase enzyme a promising target for potential antimetastatic drugs.[41] Molecules that efficiently compete with heparan sulfate and release active bFGF and other heparin-binding growth factors from their storage in ECMs and basement membranes may accelerate neovascularization and tissue repair. Compounds which efficiently inhibit SMC proliferation may be applied, systemically or when immobilized on intravascular metal stents, to inhibit restenosis and accelerated atherosclerosis.

Experimental Section

Materials. Amino acids were purchased from Aldrich (Milwaukee, WI), Fluka (Buch, Switzerland) and Sigma (St. Louis, MO). Analytical grade solvents were purchased from Frutarom (Haifa, Israel) and were used without further purification. Triethylamine (Riedel-de Haen, Seelze, Germany) was refluxed over KOH, distilled and stored over previously desiccated molecular sieves (type 4A). Sepharose 6B was from Pharmacia (Uppsala, Sweden). Sodium heparin from porcine intestinal mucosa (PM-heparin, M_r 14 000, anti-FXa, 165 IU/mg) was obtained from Hepar Industries (Franklin, OH). Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), newborn calf serum (NBCS), penicillin, streptomycin, L-glutamine and saline containing 0.05% trypsin, 0.01 M sodium phosphate (pH 7.4) and 0.02% EDTA (STV) were obtained from Biological Industries (Beit-Haemek, Israel). Tissue culture dishes were obtained from Falcon Labware Division, Becton Dickinson (Oxnard, CA). Four-well tissue culture plates were obtained from Nunc (Roskilde, Denmark). $Na_2[^{35}S]O_4$ was obtained from Amersham (Buckinghamshire, England). Recombinant human bFGF was kindly provided by Takeda Chemical Industries (Osaka, Japan). Anticoagulation tests were performed using IL Test APTT Liquid Silica kit from Instrumentation Laboratory Co. (Lexington).

Instrumentation. Melting point of monomers was determined on an Electrothermal apparatus (Electrothermal, England). T_{max} of polymers was determined on a Metler TA 4000-DSC differential scanning calorimeter, calibrated with Zn and In standards, at a heating rate of 10 °C/min. Molecular weights of the polymers were estimated on a gel permeation chromatography (GPC) system consisting of a Spectra Physics (Darmstadt, Germany) P1000 pump with RI detection (ERMA Inc.; ERC-7510), a Rheodyne (Coatati, CA) injection valve with a

20-μL loop, and a Spectra Physics data jet integrator. Samples were eluted with 0.05 M $NaNO_3$ through a linear Shodex OHpak KB-804 column at a flow rate of 1 mL/min. The molecular weights were determined relative to pullulan standards (Showa Denko KK, Japan) with a molecular weight range of 5 800−350 000 Da using a WINner/286 computer program.

^{1}H NMR spectra were recorded on a Varian 300-MHz spectrometer. $CDCl_3$ (99.5% D) and D_2O (99.75% D) were purchased from Merck (Darmstadt, Germany). Scale position was determined by chloroform or water signals, respectively, and results are reported in ppm. Optical activity was measured on an automatic polarimeter apparatus from Optical Activity (England). InfraRed spectra were recorded on a Perkin-Elmer FTIR 2000 (Columbia), and results are reported in cm^{-1}. Samples were prepared as KBr pellets at a concentration of 1−2%.

***N*-Acryl Amino Acids (2, R′ = H).** 10.0 mmol of amino acid was dissolved in 10 mL of 2 M KOH (2.0 equiv); 1.0 mL of acryloyl chloride (1.2 equiv) was added dropwise with vigorous stirring to the chilled solution. The solution was stirred at room temperature for 1 h and then washed with ether (20 mL × 2). The water layer was then acidified with 5 M HCl to pH ∼3. The solution was extracted with chloroform (20 mL × 3) and dried over $MgSO_4$ and the solvent was evaporated to an oily residue. Products usually crystallized from chilled mixtures of ethyl acetate−ether. See Table 1 for structures and physical data of monomers.

***N*-Acryl-4-*trans*-hydroxyproline Methyl Ester (2a).** Acryloyl chloride (1.8 mL, 20 mmol) was added dropwise to a vigorously stirred chilled solution of 4-*trans*-hydroxyproline methyl ester hydrochloride (3.63 g, 20 mol) and $NaHCO_3$ (3.36 g, 40 mol) in water. The stirring was continued at room temperature for 30 min. Then, the reaction mixture was acidified with 1 M $NaHSO_4$ solution to pH∼3. The solution was extracted three times with ethyl acetate. The washings were dried over $MgSO_4$ and filtrated and the solvent removed by flash evaporation. The oily residue was recrystalized from ethyl acetate and ether, yielding 1.83 g (48%).

***N*-Acryl-4-*cis*-hydroxyproline Methyl Ester (2b).** 3.63 g of 4-*cis*-hydroxyproline methyl ester hydrochloride (20 mmol) was treated as described above for the trans derivative. The product did not crystallize and the residue was purified by chromatography (silica gel 60, 70−230 mesh, eluent: gradient of 0 to 5% MeOH in dichloromethane): 2.5 g of pasty solid was obtained (65%); low stability and polymerizes readily.

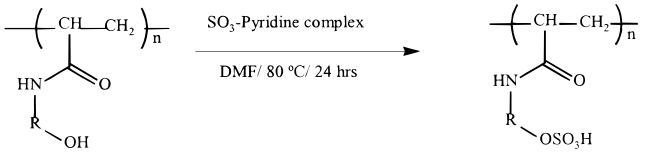
***N*-Acryl-3-hydroxypyrrolidine (5a):** ^{1}H NMR ($CDCl_3$) 6.32 (2H,m), 5.66 (1H,d), 4.45 (1H,d), 4.10 (1H, broad), 3.60 (4H,m), 2.01 (2H,m); mp = 48 °C. Anal. ($C_7H_{11}NO_2$) Calcd: C, 59.56; H, 7.85; N, 9.92. Found: C, 59.51; H, 7.93; N, 9.73.

***N*-Acryltyramide (5b):** ^{1}H NMR ($CDCl_3$) 7.05 (2h,d), 6.78 (2H,d), 6.25 (1H,d), 6.05 (1H,m), 5.60 (1H,d), 5.58 (1H,broad), 3.59 (2H,q), 2.80 (2H,t); mp = 101 °C. Anal. ($C_{11}H_{13}NO_2$) Calcd: C, 69.09; H, 6.85; N, 7.33. Found: C, 68.98; H, 6.95; N, 7.23.

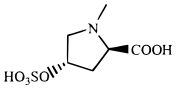
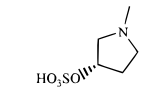
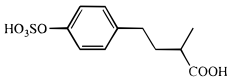
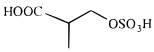
***N*-Acrylethanolamide (5c):** ^{1}H NMR (D_2O: 6.75 (1H, broad), 6.20 (2H,m), 5.65 (1H,d), 3.74 (2H,t), 3.25 (2H,q); syrup; low stability and polymerizes readily. Anal. ($C_5H_9NO_2$) Calcd: C, 52.16; H, 7.88; N, 12.17. Found: C, 53.27; H, 7.54; N, 11.79.

***N*-Acryl Amino Acid *tert*-Butyl (2, R′ = Bu^t) or Methyl (2, R′ = Me) Esters.** 20 mmol of amino acid ester was dissolved in 100 mL of dichloromethane; 3.3 mL of triethylamine (24 mmol, 1.2 equiv) was added. The solution was chilled and acryloyl chloride (1.80 mL, 1.1 equiv) in 10 mL of dichloromethane was added dropwise over 1 h. The cooling bath was removed and the mixture was stirred overnight. The solvents were removed in vacuum and the residues dissolved in ethyl acetate (about 200 mL). The crystals (triethylamine hydrochloride) were filtered out and the solution was washed three times with 1 M $NaHSO_4$ and 5% $NaHCO_3$ and once with brine (about 20 mL each wash). The organic layer was dried over $MgSO_4$, filtered and concentrated by flash evaporation. Crudes were purified by crystallization, triturating with hexane or liquid chromatography (silica gel 60, 70−230 mesh). See Scheme 1.

Scheme 3. Sulfation of Hydroxyl-Containing Poly(*N*-acryl amino acids) and Poly(acryl amides)



7

7	R	Residue
a		trans-4-hydroxyproline
b		3-hydroxypyrrolidine
c		tyrosine
d		serine

Polymerization in Aqueous Solution. 2.0 mmol of *N*-acryl amino acid ester was dissolved in 5 mL of methanol and 1 mL of water was added. A stream of N_2 was bubbled trough the solution for 3 min; 1% ammonium persulfate solution (450 mL, 1% mol/mol monomer), 2% sodium methabisulfite (450 mL) and 2% ferrous ammonium sulfate (250 mL) were added. Polymerization was carried out at room temperature for 10 h. Solvents were removed by flash evaporation and the residue was precipitated in ether from methanolic solution. Precipitates were collected by vacuum filtration, washed with ether and air dried.

Polymerization in Organic Solvents. 2.0 mmol of *N*-acryl amino acid ester and 4 mg of azobis(isobutyronitrile) were dissolved in about 3 mL of acetonitrile in a borosilicate ampule. The solution was degassed by bubbling N_2 for 2 min. The ampule was sealed and heated overnight to 70 °C. Ampule was smashed; the polymer was ground in a mortar, washed on a sinter funnel with ether and dried by air stream.

Methyl Ester Hydrolysis. Poly(*N*-acryl amino acid methyl esters) were dissolved in methanol or acetonitrile and 1 M sodium hydroxide (2 equiv per ester group) was added. Solutions were stirred overnight at room temperature and neutralized by 1 M sodium bisulfate. Organic solvent was removed and the aqueous solutions were freeze-dried. The solids were dissolved in methanol and salts were removed by filtration. Pure polymers were obtained by evaporation of the clear methanolic solutions or by precipitation with ether or petroleum ether. Poly(*N*-acryl diethyl glutamate) was hydrolyzed by the same procedure.

***tert*-Butyl Ester Hydrolysis.** Poly(*N*-acryl amino acid *tert*-butyl esters) were dissolved in dichloromethane:trifluoroacetic acid (1:1). Solutions were left at room temperature for 3−4 h. Most of the solvent was removed by flash evaporation. Ether or petroleum ether was added to precipitate the residue. The bulk polymers were grounded and triturated with petroleum ether, ether or hexane.

O-Sulfated Poly(*N*-acryl amino acids). 1.0 mmol of poly(*N*-acryl amino acid) was suspended in dry DMF and 0.5 mL of dry pyridine (6 equiv). The suspension was heated to 80 °C and 0.480 mg of SO_3/pyridine complex (3 equiv) was added. The mixture was refluxed for 2 h. Solvent was removed by flash evaporation and the residue dissolved in 1 M KOH (about 10 mL). The solution was dialyzed against DDW for 3 days at 4 °C and freeze-dried. Extent of sulfation was measured by elemental analysis (Scheme 3). The analytical and biological data are compiled in Table 4.

Preparation of Dishes Coated with ECM. Cultures of bovine corneal endothelial cells were established from steer eyes as previously described.[36] Stock cultures were maintained in DMEM (1 g of glucose/L) supplemented with 10% newborn calf serum, 5% FCS, 50 U/mL penicillin, and 50 μg/mL streptomycin at 37 °C in 10% CO_2 humidified incubators. Partially purified brain-derived bFGF (100 ng/mL) was added every other day during the phase of active cell growth.[36] For the preparation of sulfate-labeled ECM coated dishes, bovine corneal endothelial cells were dissociated from stock cultures (second to fifth passage) with STV and plated into 35-well plates at an initial density of 2×10^5 cells/mL. Cells were maintained as described above except that 5% dextran T-40 was included in the growth medium and the cells were maintained without addition of bFGF for 12 days. $Na_2[^{32}S]O_4$ (540−590 mCi/mmol) was added (40 μCi/mL) 2 and 5 days after seeding and the cultures were incubated with the label without medium change.[36] The subendothelial ECM was exposed by dissolving (5 min, room temperature) the cell layer with PBS containing 0.5% Triton X-100 and 20 mM NH_4OH, followed by four washes in PBS. The ECM remained intact, free of cellular debris and firmly attached to the entire area of the tissue culture dish.[36]

Heparanase Activity. Sulfate-labeled ECM was incubated (24 h, 37 °C, 10% CO_2 incubator) with 1 μg/mL partially purified human placental heparanase[37] at pH 6.2. To evaluate the occurrence of proteoglycan degradation, the incubation medium was collected and applied for gel filtration on Sepharose 6B columns (0.9 × 30 cm). Fractions (0.2 mL) were eluted with PBS at a flow rate of 5 mL/h and counted for radioactivity using Bio-fluor scintillation fluid. The excluded volume (V_o) was marked by blue dextran and the total included volume (V_t) by phenol red. The latter was shown to comigrate with free sulfate.[29−31,38] Degradation fragments of HS side chains were eluted from Sepharose 6B at $0.5 < K_{av} < 0.8$ (Figure 1, peak II).[29−31,38] A nearly intact HSPG released from ECM by trypsin or plasmin was eluted next to V_o ($K_{av} < 0.2$, Figure 1, peak I). Recoveries of labeled material applied on the columns ranged from 85 to 95% in different experiments. Each experiment was performed at least three times and the variation in elution positions (K_{av} values) did not exceed ±15%.

Release of ECM-Bound [^{125}I]bFGF. Recombinant bFGF was iodinated using chloramine T, as previously described.[21] The specific activity was $0.8-1.2 \times 10^5$ cpm/ng bFGF and the labeled preparation was kept for up to 3 weeks at −70 °C.

For bFGF binding to ECM, 4-well plates coated with ECM were incubated (3 h, 24 °C) with [^{125}I]bFGF (2.5 ng/mL in PBS containing 0.02% gelatin). Unbound bFGF was washed away and the ECM incubated at room temperature with 5 or 25 μg/mL heparin or each of the polyanionic molecules mentioned. The incubation media were collected and counted in a γ-counter to determine the amount of released iodinated material. The remaining ECM was incubated (3 h, 37 °C) with 1 N NaOH and the solubilized radioactivity counted in a γ-counter. The percentage of released [^{125}I]bFGF was calculated from the total ECM-associated radioactivity.[26,39] "Spontaneous" release of [^{125}I]bFGF obtained in the presence of incubation medium alone did not exceed 17% of the total binding.

SMC Proliferation. SMCs were isolated from the bovine aortic media as previously described.[23] Briefly, the abdominal segment of the aorta was removed and the fascia cleaned away under a dissecting microscope. The aorta was cut longitudinally and small pieces of the media were carefully stripped from the vessel wall. Two or three such strips with average dimensions of 2 × 2 mm were placed in 60-mm tissue culture dishes that contained DMEM (4.5 g of glucose/L) supplemented with 10% FCS, 100 U/mL penicillin and 100 μg/mL streptomycin. SMC proliferation was evaluated by [^{3}H]thymidine incorporation. For this purpose, SMCs were plated (4×10^4 cells/16-mm well/mL) in DMEM supplemented with 10% FCS. Twenty-four h after seeding, the medium was replaced with medium containing 0.2% FCS, and 48 h later, the cells were exposed to bFGF (1 ng/mL) and [^{3}H]thymidine (1 μCi/well) was added. DNA synthesis was assayed 48 h afterward by measuring the radioactivity incorporated into trichloroacetic acid (TCA) insoluble material.[23]

Acknowledgment. This work was supported by grants from the German−Israeli Cooperation in Cancer Research sponsored by the Deutsches Krebforschungszentrum (DKFZ) and the Israeli Ministry of Science, the Israeli Academy of Science.

Supporting Information Available: Typical ^{1}H NMR spectrum of an *N*-acryl amino acid ester. This material is available free of charge via the Internet at http://pubs.acs.org.

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JM000089J