

Thiocarbamate-Linked Polysulfonate–Peptide Conjugates As Selective Hepatocyte Growth Factor Receptor Binders

Soizic Besret,^{†,‡,§} Jérôme Vicogne,^{†,‡,§} Fatima Dahmani,^{†,‡,§} Véronique Fafeur,^{†,‡,§} Rémi Desmet,^{†,‡,§} Hervé Drobecq,^{†,‡,§} Anthony Romieu,^{‡,¶} Patricia Melnyk,^{*,‡,||} and Oleg Melnyk^{*,†,‡,§}

[†]UMR CNRS 8161, F-59021 Lille, France

[‡]Univ Lille Nord de France, F-59000 Lille, France

[§]Pasteur Institute of Lille, F-59000 Lille, France

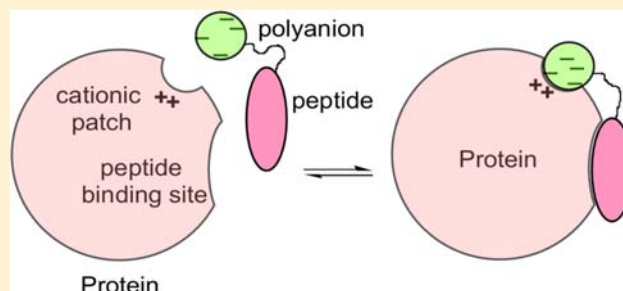
^{||}UDSL, EA 4481, UFR Pharmacie, F-59000 Lille, France

[‡]Institut de Chimie Moléculaire de l'Université de Bourgogne, UMR CNRS 6302, Université de Bourgogne, F-21078 Dijon, France

[¶]Institut Universitaire de France, F-75005 Paris, France

Supporting Information

ABSTRACT: The capacity of many proteins to interact with natural or synthetic polyanions has been exploited for modulating their biological action. However, the polydispersity of these macromolecular polyanions as well as their poor specificity is a severe limitation to their use as drugs. An emerging trend in this field is the synthesis of homogeneous and well-defined polyanion–peptide conjugates, which act as bivalent ligands, with the peptide part bringing the selectivity of the scaffold. Alternately, this strategy can be used for improving the binding of short peptides to polyanion-binding protein targets. This work describes the design and first synthesis of homogeneous polysulfonate–peptide conjugates using thiocarbamate ligation for binding to the extracellular domain of MET tyrosine kinase receptor for hepatocyte growth factor.



INTRODUCTION

Various polyanionic macromolecules such as heparin, heparan sulfate (HS) proteoglycans (HSPGs), DNA, RNA, or proteins featuring aspartic or glutamic-rich stretches are produced by living organisms. Some of these polyanions such as DNA and RNA participate in various highly specific interactions to mediate essential biological processes. However, an increasing body of evidence shows that these macromolecular polyanions participate also in a vast array of strong but nonspecific electrostatic interactions,¹ which play a critical role in almost all cellular and extracellular phenomena (for recent reviews see refs 2,3). This dual mode of binding is not exclusive in that both types of interactions, specific and nonspecific, often contribute to trigger important biological events.

A good example of a class of proteins utilizing nonspecific electrostatic interactions with polyanions is the HS-binding growth factor family. For example, the binding of vascular endothelial growth factor (VEGF) to its receptors (VEGFRs) involves the presence of HSPGs as co-receptors.^{4–6} Another example of proteins using HS as co-receptor and directly related to this study are the hepatocyte growth factor (HGF)^{7–10} and its MET tyrosine kinase receptor.¹¹ HGF/MET signaling controls cell growth, invasion, and survival. Its deregulation is associated with the acquisition of tumorigenic properties but also invasive phenotype. The involvement of

MET in numerous human tumors is now established, and interfering with its activation is therefore a potential strategy for developing therapeutics against tumorigenesis or metastatic processes.^{12,13} Both HGF and MET proteins bind heparin. The HGF binding site for heparin has been well characterized and is located within the N domain.^{14–17} In contrast, the MET binding site for heparin has not been identified yet.¹⁸

The capacity of some proteins to interact strongly with polyanions has been exploited for modulating or blocking their biological action. The HGF/MET signaling is potentiated by heparin¹⁰ or small sulfated oligosaccharides.^{19,20} Large polyanions such as carboxymethylated and/or sulfated dextran polymers were studied for their ability to mimic heparin²¹ and to inhibit^{22–25} or potentiate²⁶ the biological activity of HS-binding growth factors. However, the polydispersity of these macromolecular polyanions as well as their poor specificity is a severe limitation to their use as drugs. An emerging trend in the field is toward the synthesis of homogeneous and well-defined polyanion–peptide conjugates.^{27,28} The basic idea is to increase the selectivity and the affinity of the polyanion by its covalent association with a peptide that is specific for the target protein.

Received: March 28, 2014

Revised: April 18, 2014

Published: April 21, 2014

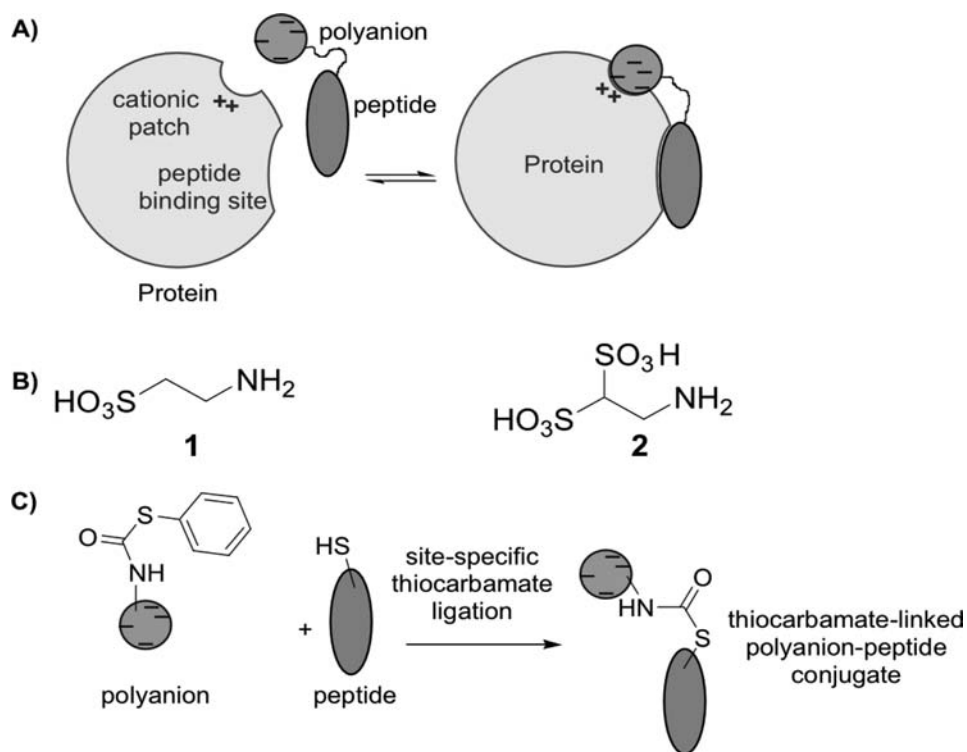


Figure 1. (A) Polyanion–peptide conjugates act as bivalent ligands. (B) Polyanions used in this study are sulfonated dendrimers synthesized starting from taurine **1** or *gem*-disulfonate **2**. (C) Polyanion and peptide modules are assembled site-specifically using thiocarbamate ligation.

These molecules can be regarded as heterobivalent ligands, the peptide part bringing the specificity of the scaffold, whose interaction with the target protein is stabilized by the ionic interaction of the polyanion with a proximal surface-exposed cationic patch (Figure 1A). Heteromultivalency, which is defined as an interaction in which two or more different types of molecular recognition events take place simultaneously between the two interacting partners, is in fact a generalization of multivalency which involves multiple molecular recognition events of the same type between two entities. Heteromultivalency is frequently encountered in biological interactions. The affinities and specificities of biological heteromultivalent interactions are greater than the monovalent interactions they are made of.²⁹ The concept of heteromultivalency is also increasingly used in the design of high affinity ligands toward proteins which do not have two similar binding sites.³⁰ For example, recent studies showed the potential of heteromultivalency for designing potent allosteric modulators of factor XIa or thrombin.^{31–34}

The aim of this proof-of-concept study was to design and select polyanion–peptide conjugates able to bind the extracellular domain of MET receptor. Instead of using polyanions related to heparin or sulfated oligosaccharides, whose synthesis may be challenging, we decided to design novel sulfonated dendrimers derived from 2-aminoethanesulfonate **1** (also known as taurine³⁵) or 2-aminoethane-1,1-disulfonate **2**³⁶ as potential HS mimetics (Figure 1B). Examination of the literature revealed that the synthesis of high molecular weight polysulfonated–peptide conjugates such as those reported here has not been addressed previously. Several reports highlight the challenge of incorporating taurine or other highly hydrophilic aminosulfonic acid derivatives into peptides.^{37,38} We therefore designed a simple, convergent, and efficient access to these amphiphilic scaffolds relying on the

chemoselective and site-specific formation of a thiocarbamate linkage between phenylthiocarbonyl functionalized HS mimetics and MET peptide binders featuring a thiol group (Figure 1C).³⁹ Importantly, this convergent approach enabled easily the variation of number and the density of the sulfonate groups within the polyanion by varying the valency of the dendrimer core or the nature of the sulfonate end-group. Moreover, this strategy also enabled variation of the position of the polyanionic moiety relative to the peptide and thus to search for a cationic patch on the protein near the peptide binding site. This possibility was of prime importance for this work because the MET binding sites for heparin and for the selected peptide binders are not known, thus precluding any rational basis for the design of the polyanion–peptide conjugates. Finally, the polyanion–peptide conjugates were screened for their capacity to bind selectively a recombinant MET-Fc chimera using a peptide microarray format.

RESULTS AND DISCUSSION

The principle of the thiocarbamate ligation^{39,40} is presented in Figure 1C. The process is based on the reaction of a thiol such as a peptide featuring a cysteine residue with a phenylthiocarbamate (PTC) component. It is formally a thiol–thioester exchange which proceeds efficiently in water at neutral pH. In this work, the PTC group was introduced on the polyanion scaffold to be ligated with the cysteinyl peptides of interest. Thus, the importance of the polyanionic moiety position relative to peptide ligand could be easily examined by just varying the position of the cysteine residue, i.e., N- or C-terminally, within the peptide ligand.

Design and Synthesis of the Sulfonated Dendrimers.

Figure 2 shows the structure of the divalent or tetravalent dendrimers which were synthesized in this study. The structure of the sulfonated dendrimers shows 2-aminoethanesulfonate or

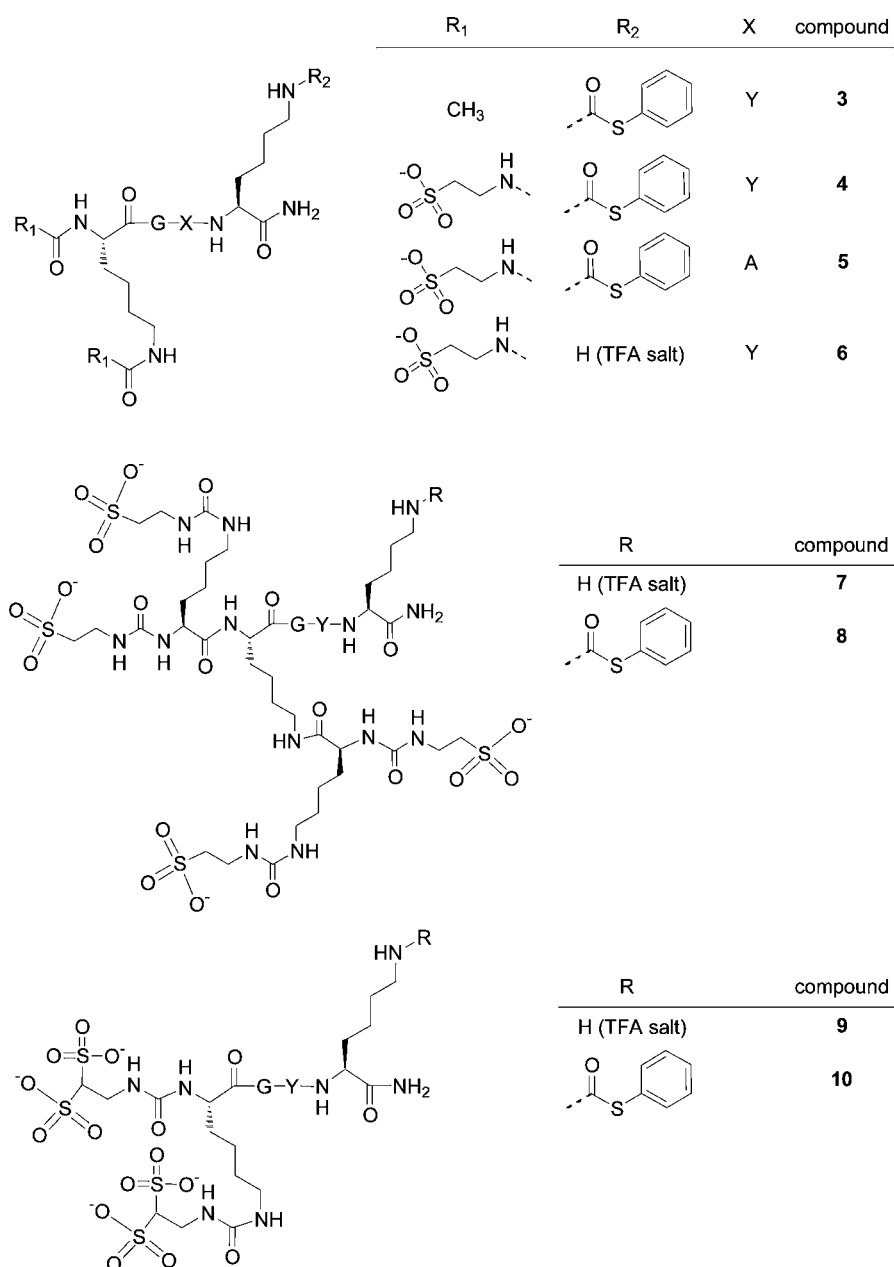


Figure 2. Structure of the sulfonated dendrimers derived from taurine **1** or *gem*-disulfonate **2**.

2-aminoethane-1,1-disulfonate residues, which are linked to peripheral α - and ϵ -amino groups of divalent or tetravalent lysinyl trees through an urea bond. The PTC group was introduced on the opposite side on the ϵ -amino group of a C-terminal lysine residue.

Note that a glycine-tyrosine dipeptidyl unit was inserted in the structure of dendrimers between the lysinyl core and the PTC group. This modification was suggested by the fact that tyrosine, together with small and highly flexible amino acids such as glycine in polyanion **4**, is particularly powerful in establishing favorable contacts within protein–protein binding interfaces.^{41,42} Obviously, nature is exploiting this property because tyrosine is substantially more frequent within the protein binding sites compared to the frequency of tyrosine generally observed in proteins.

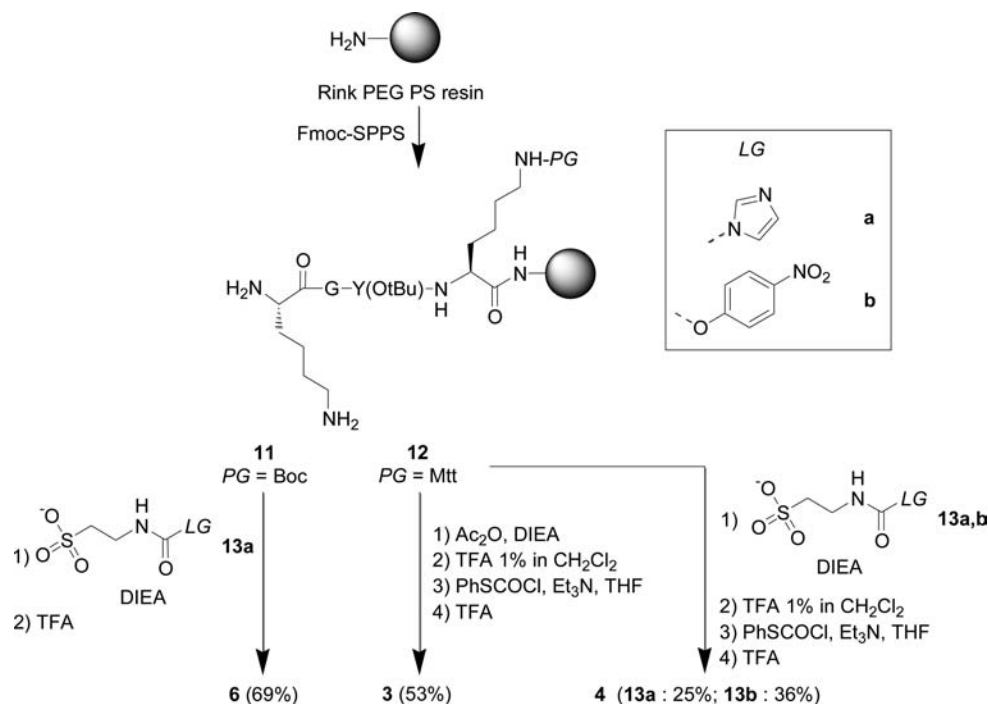
The sulfonated dendrimers **6**, **7**, and **9** lacking the PTC group or the PTC dendrimer **3** in which the sulfonate residues

were substituted by acetyl groups were used as controls in the binding studies with MET extracellular domain. Likewise, dendrimer **5** with an alanine residue in place of the tyrosine was used to evaluate the contribution of tyrosine to the binding strength.

We started with the synthesis of divalent dendrimers **3–6**, which were assembled using 9-fluorenylmethyloxycarbonyl (Fmoc)-solid-phase peptide synthesis (SPPS) protocols on a Rink PEG PS resin as shown in Scheme 1. The N-terminal lysine residue was incorporated as Fmoc-Lys(Fmoc)-OH derivative, whereas the C-terminal lysine residue was incorporated as Fmoc-L-Lys(Mtt)-OH (Mtt: 4-methyltrityl) or Fmoc-L-Lys(Boc)-OH (Boc: *tert*-butoxycarbonyl) derivative depending on the structure of the target compound.

Acetylated dendrimer **3** was synthesized using peptidyl resin **12** in which the last lysine residue was protected by an Mtt group (PG = Mtt). For this, the amino groups were acetylated

Scheme 1. Synthesis of Dendrimers 3–6^a

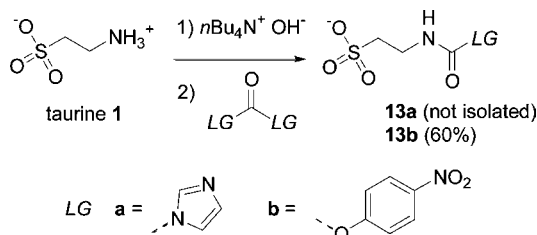


^aThe synthesis of dendrimer 5 was carried out as described for dendrimer 4 starting from peptidyl resin LysGlyAlaLys(Mtt)-Rink PEG PS 14 (39%, omitted for clarity).

with a mixture of acetic anhydride and *N,N*-diisopropylethylamine (DIEA). Then the Mtt group was removed selectively using 1% TFA in CH_2Cl_2 .⁴³ The PTC group was incorporated by treating the peptidyl resin with phenylthiochloroformate in the presence of triethylamine (TEA) as described elsewhere.³⁹ Finally, cleavage and deprotection of the peptidyl resin in trifluoroacetic acid (TFA) furnished PTC dendrimer 3, which was isolated with a yield of 53% after RP-HPLC purification.

A similar strategy was used for the synthesis of sulfonated PTC dendrimer **4** and **5**. The incorporation of the taurine residues was carried out using derivatives **13a** or **13b** (Scheme 2). First, taurine **1** was converted into the corresponding

Scheme 2. Synthesis of Taurine Derivatives 13a,b



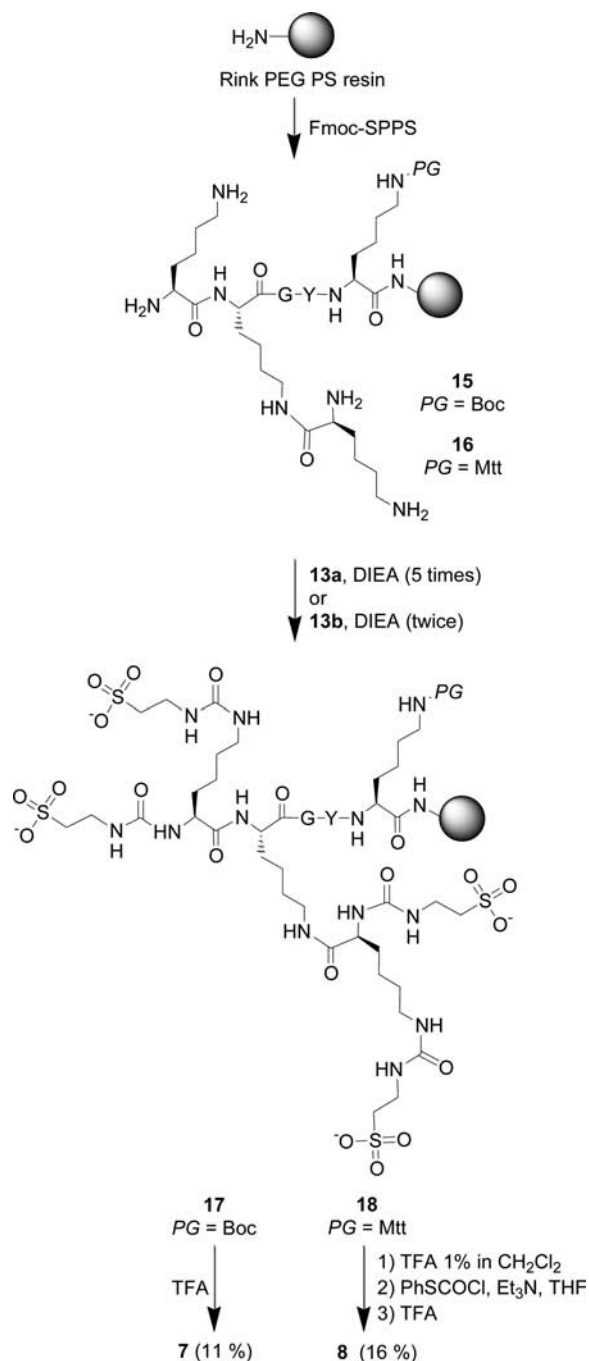
tetra(*n*-butyl)ammonium salts to allow its subsequent solubilization in organic solvents. Imidazolylcarbonyl derivative **13a** was obtained by reacting the tetra(*n*-butyl)ammonium salt of taurine **1** with *N,N'*-carbonyldiimidazole (CDI) in DMF. This solution was used immediately for the coupling step. *p*-Nitrophenylcarbonyl derivative **13b** was also prepared by reacting the tetra(*n*-butyl)ammonium salt of taurine with bis(*p*-nitrophenyl)carbonate. To the contrary of imidazolylcarbonyl derivative **13a**, *p*-nitrophenylcarbonyl derivative **13b** could be isolated (60%) and stored prior to use. A single carbamoylation

step using either of these reagents enabled the successful incorporation of taurine residues onto peptidyl resins **11** or **12**. The presence of sulfonate groups did not perturb the subsequent removal of Mtt group or the incorporation of the PTC group to a significant extent. Finally, the cleavage and deprotection step in TFA furnished dendrimers **4** and **5**, which were purified by RP-HPLC. Taurine derivatives **13a** and **13b** gave similar yields for dendrimer **4** (25 and 36% respectively). Dendrimer **5** was produced using derivative **13b** only (39%).

Dendrimer **6** was synthesized with a *tert*-butoxycarbonyl (Boc) protecting group (PG) for the C-terminal lysine residue (PG = Boc). In this case, the grafting of taurine residues was carried out using imidazolylcarbonyl derivative **13a**. Finally, the cleavage and deprotection step in TFA furnished dendrimer **6** with a 69% isolated yield.

We next examined the synthesis of tetrasulfonated dendrimers **7** and **8** using a similar solid-phase strategy (Scheme 3). Although the coupling of imidazolylcarbonyl derivative **13a** to divalent peptidyl resin **12** (Scheme 2) was straightforward, the derivatization of the tetravalent peptidyl resins **15** and **16** with the same reagent and experimental conditions proved to be problematic. Up to five coupling steps were necessary with **13a** to derivatize all the primary amino groups within the supported tetravalent lysine dendrimer. Conversely, *p*-nitrophenylcarbonyl derivative **13b** required two coupling steps. In any case, the derivatization of tetravalent peptidyl resins **15** and **16** proved to be more difficult than for divalent peptidyl resins **11** and **12**. The difficulty of obtaining sulfonated peptidyl resins **17** and **18** might be due to a significant increase of sulfonate anion concentration within the beads during the coupling step. Indeed, the initial loading of the Rink PEG PS resin is 0.25 mmol/g, meaning that the final loading in sulfonate anion for peptidyl resins **17** or **18** is expected to be as high as 1 mmol/g. Such a high concentration

Scheme 3. Synthesis of Sulfonated Dendrimers 7 and 8

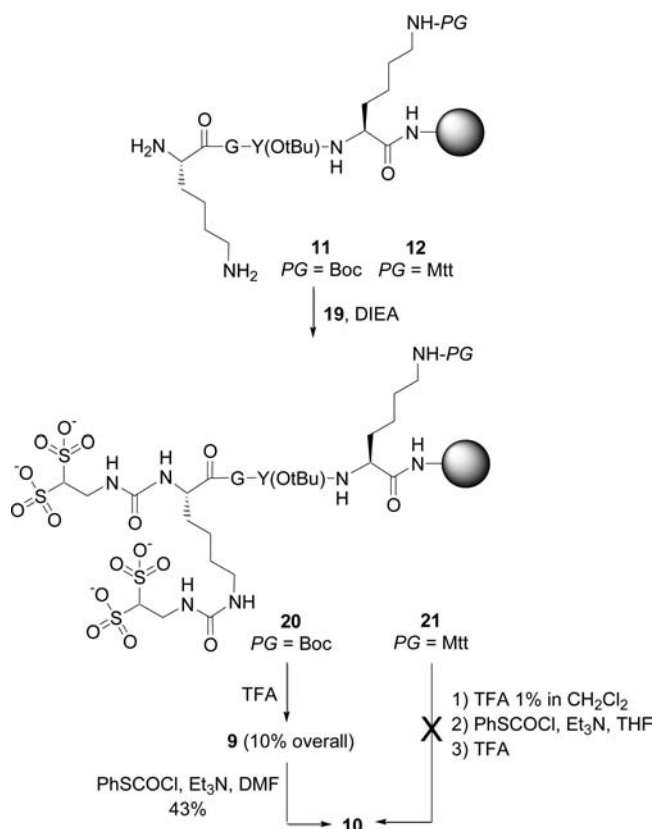


might disfavor the diffusion of reagents **13a** or **13b** within the beads due to electrostatic repulsion by the immobilized sulfonate anions.

Deprotection and cleavage of Boc-protected sulfonated peptidyl resin **17** furnished dendrimer **7** (11% overall). On the other hand, peptidyl resin **18** was treated with diluted TFA to remove the Mtt group and acylated with phenylthiochloroformate in the presence of TEA as usual. A final cleavage and deprotection step in concentrated TFA furnished successfully the target PTC sulfonated dendrimer **8** (16% overall).

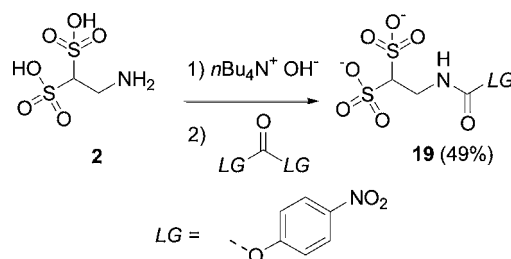
We next examined the synthesis of *gem*-disulfonated dendrimers **9** and **10** starting from peptidyl resin **12** (PG = Mtt, Scheme 4). Because *p*-nitrophenylcarbonyl derivative **13b** proved to be a good acylating reagent during the synthesis of

Scheme 4. Synthesis of PTC Dendrimer 10 by a Mixed Solid–Liquid Phase Approach



dendrimers **7** and **8**, we prepared the *p*-nitrophenoxycarbonyl derivative **19** by reacting the tetra(*n*-butyl)ammonium salt of amine **2** with *p*-nitrophenyl carbonate (isolated yield 49%, Scheme 5). *p*-Nitrophenoxycarbonyl derivative **19** was coupled

Scheme 5. Synthesis of 2-Sulfonated Taurine Derivatives 19



successfully to peptidyl resin **12** in the presence of DIEA. However, treatment of peptidyl resin **21** with 1% TFA in CH₂Cl₂ failed to remove the Mtt group as expected. This problem was not anticipated considering the successful synthesis of dendrimer **6** using a similar approach. As raising the TFA concentration for removing the Mtt group would potentially lead to a loss of orthogonality,⁴³ we devised instead a two-step procedure for accessing to dendrimer **10**. For this, Mtt group was substituted by a Boc group to give peptidyl resin **20**, which yielded dendrimer **9** after deprotection and cleavage in concentrated TFA, albeit with a modest yield (overall yield 10%). Compound **9** was subsequently reacted in solution with phenylthiocarbonyl chloride and TEA in DMF to give successfully the target dendrimer **10** (43% yield).

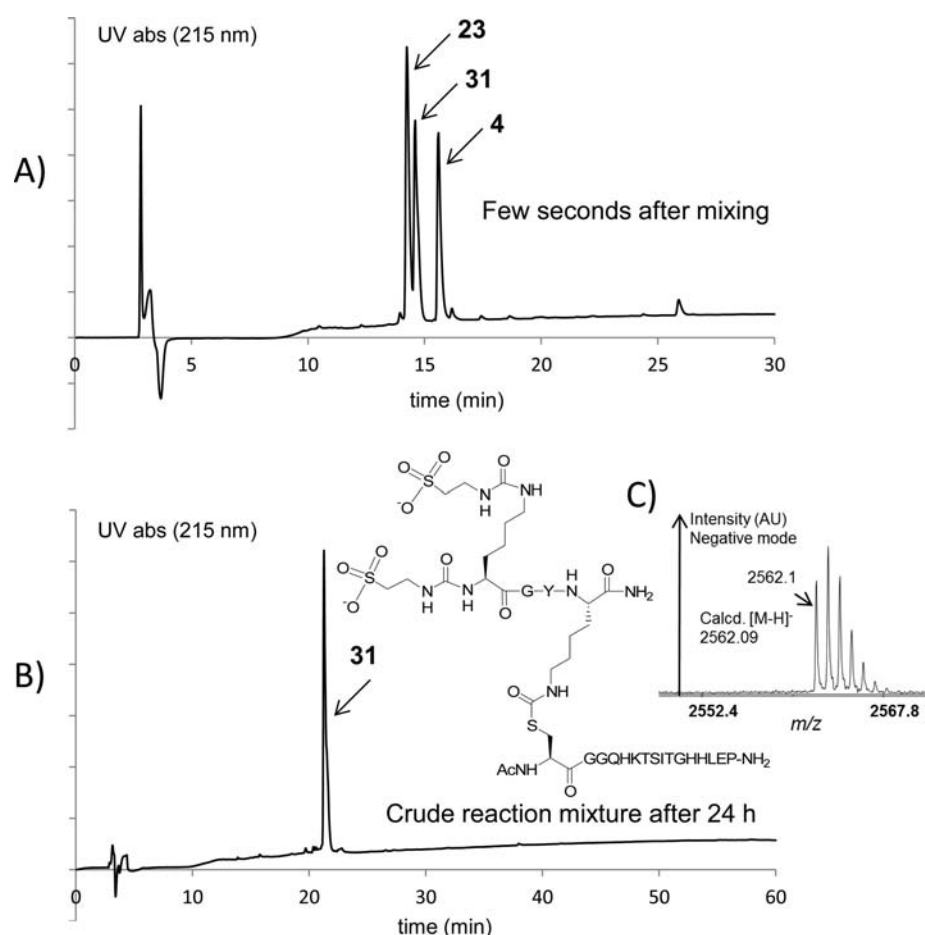


Figure 3. RP-HPLC analysis (C_{18} column, UV detection at 215 nm) of the thiocarbamate ligation reaction between sulfonated dendrimer **4** and peptide **23** to produce conjugate **31**. (A) A few seconds after mixing, linear water–acetonitrile gradient 0–80% in 30 min (0.05% TFA). (B) Crude reaction mixture after 24 h, linear water–acetonitrile gradient 0–80% in 60 min (0.05% TFA). (C) MALDI-TOF analysis of the conjugate **31** after RP-HPLC purification (negative ion mode).

Table 1. Synthesis of the Polyanion–Peptide Conjugates

$$R^1-SH + PhS-CO-NH-R^2 \xrightarrow{pH\ 7.5} R^1-S-CO-NH-R^2$$

| entry | R^1-SH | $PhS-CO-NH-R^2$ | $R^1-S-CO-NH-R^2$ | yield (%) ^a |
|-------|--|-----------------|-------------------|------------------------|
| 1 | Ac-QHKTSITGHHLEPGGC-NH ₂ 22 | 4 | 30 | 61 |
| 2 | Ac-CGGQHKTSITGHHLEP-NH ₂ 23 | 4 | 31 | 76 |
| 3 | Ac-YLFSVHWPLKAGGC-NH ₂ 24 | 4 | 32 | 44 |
| 4 | Ac-CGGYLFVHWPLKA-NH ₂ 25 | 4 | 33 | 47 |
| 5 | Ac-TLPSPLALLTVHGGC-NH ₂ 26 | 4 | 34 | 59 |
| 6 | Ac-CGGTLPSPLALLTVH-NH ₂ 27 | 4 | 35 | 44 |
| 7 | Ac-QHKMRMVLGVIVPGGC-NH ₂ 28 | 4 | 36 | 40 |
| 8 | Ac-QHKMRMVLGVIVPGRGC-NH ₂ 29 | 4 | 37 | 25 |
| 9 | Ac-QHKTSITGHHLEPGGC-NH ₂ 22 | 3 | 38 | 25 |
| 10 | Ac-QHKTSITGHHLEPGGC-NH ₂ 22 | 5 | 39 | 60 |
| 11 | Ac-QHKTSITGHHLEPGGC-NH ₂ 22 | 8 | 40 | 56 |
| 12 | Ac-QHKTSITGHHLEPGGC-NH ₂ 22 | 10 | 41 | 64 |

^aIsolated yields after RP-HPLC purification.

Optimization of the Thiocarbamate Ligation Procedure. The successful synthesis of dendrimers **3–10** set the stage for the preparation of the peptide–polyanion conjugates. We used in this study three MET binding peptides selected by Zhao and co-workers from a phage display library.⁴⁴ The peptides were synthesized with a GlyGlyCys extension at the C-terminus or a CysGlyGly extension at the N-terminus to vary

the position of sulfonated dendrimer relative to the MET binding peptide.

The procedure initially described for thiocarbamate ligation used thiophenol as an additive to minimize the oxidation of cysteine residues.^{39,40} However, thiophenol is toxic and malodorous and requires an extraction procedure prior to the RP-HPLC purification step. Thus, we examined the possibility

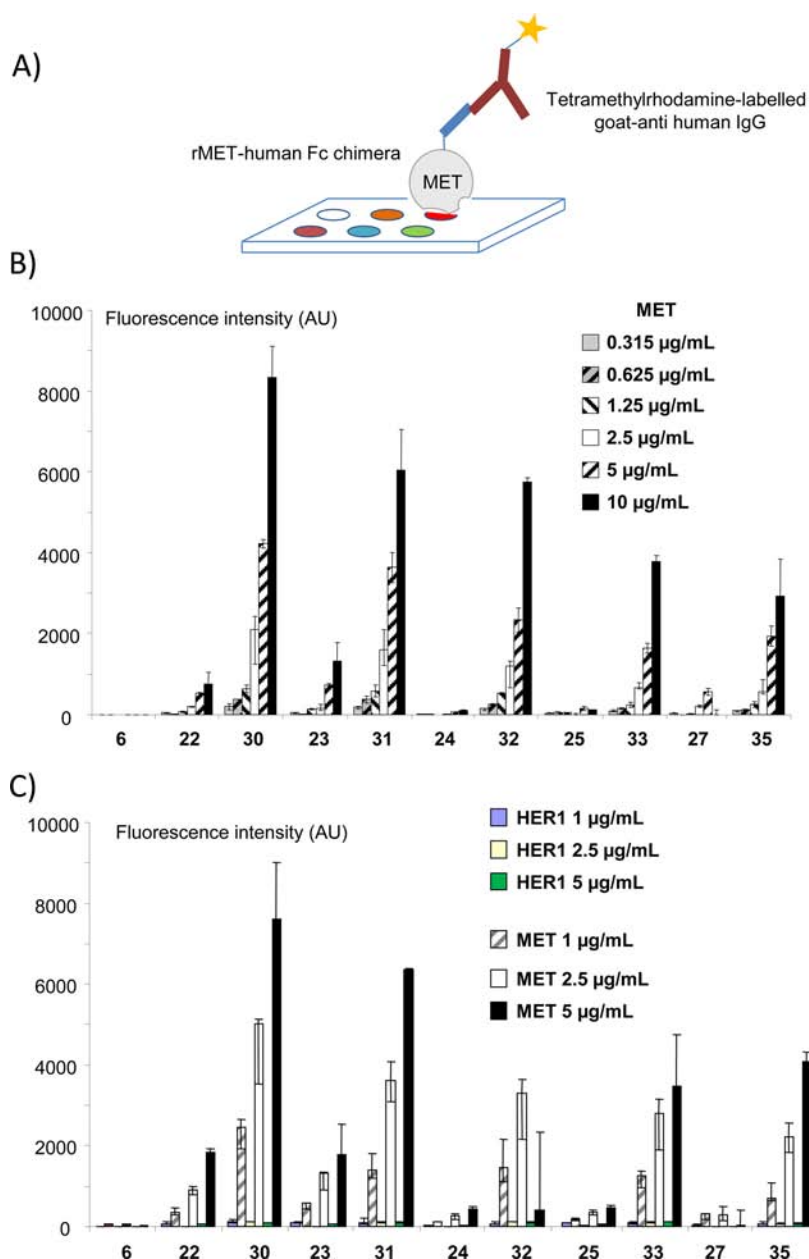


Figure 4. (A) Principle of the microarray binding experiment. (B) Fluorescence data for binding experiments with MET (rMET-hFc) or HER1-hFc chimeras, 16 bits intensities in arbitrary units after background subtraction, $n = 3$, median and interquartile range).

of performing the ligation in the absence of thiophenol to facilitate the preparation of the conjugates. Interestingly, the thiocarbamate ligation reaction proceeded highly efficiently at room temperature in the absence of thiophenol when the reaction was performed under argon atmosphere at 10 mM concentration for each ligation partner. A typical example is shown in Figure 3, which corresponds to the RP-HPLC trace of the crude ligation mixture for the reaction between peptide 23 and sulfonated dendrimer 4. The reaction yielded exclusively conjugate 31 after 24 h at room temperature, which was isolated with a 76% yield after RP-HPLC purification (entry 2 of Table 1). The efficiency of the ligation reaction combined with the use of the reactants in stoichiometric amounts yielded crude polyanion–peptide conjugates in excellent purity. All the synthesized conjugates were purified by RP-HPLC with satisfactory yields (Table 1). Conjugates 30–35 were prepared by reacting MET binding peptides 22–27 with dendrimer 4

(entries 1–6 of Table 1). The other entries were synthesized for further structure–function relationships and will be discussed later.

Microarray Screening of the Polyanion–Peptide Conjugate Chemical Library. The successful synthesis of conjugates 30–35 enabled setting up of the first binding experiments with recombinant MET extracellular domain. For this, sulfonate dendrimer 6, MET binding peptides 22–27 and conjugates 30–35 were microarrayed on microscope glass slides (0.1 mM, $n = 3$). This screening method enabled to assay all the compounds in a single experiment while minimizing the consumption of the different peptide probes. Because the oriented covalent immobilization of the polyanion–peptide conjugates on the glass slides would require installing an additional functional group on the synthesized compounds and to set up an orthogonal ligation scheme, we chose instead to immobilize the capture probes by physisorption. For this, we

took advantage of our previous studies on sulfated polysaccharide microarrays for performing binding experiments with HS-binding growth factors,²⁶ which showed the interest of semicarbazide coated glass slides^{45–48} for immobilizing anionic probes by physisorption. Indeed, semicarbazide groups are neutral at pH 7 and unable to mask the anionic groups by electrostatic interactions. Moreover, we have verified that the modification of the peptides by the sulfonated dendrimers had no significant effect on their physisorption properties by using a series of small biotinylated peptides and conjugates derived from peptide 22 and sulfonated dendrimers 4 and 9 (see Supporting Information Figure S91). The peptide microarrays were incubated with recombinant MET-human Fc chimera (MET, 0.3–10 $\mu\text{g/mL}$, $n = 3$) or with recombinant HER1 human epidermal growth factor receptor-human Fc chimera used as control (HER1, 1–5 $\mu\text{g/mL}$) and then with goat antihuman IgG antibodies labeled with tetramethylrhodamine (Figure 4A). The fluorescence intensity for each peptide spot was quantified at 532 nm using a 16 bits confocal microarray scanner. The data are presented in Figure 4B,C (data for peptide 26 and its conjugate 34 are missing due to solubility problems).

Figure 4B shows that the signal displayed by the polyanion–peptide conjugates was significantly greater than the signal displayed by their individual constituents (i.e., the MET binding peptides 22–25, 27, or the sulfonate dendrimer 6). The highest signals were produced by polyanion–peptide conjugates 30 and 31 which present the same MET binding sequence QHKTSITGHHLEP. In comparison, the intensities obtained after incubating the microarrays with HER1 corresponded to the background and suggested a selective interaction between the conjugates and MET extracellular domain.

Next, several modifications were performed starting from the peptide conjugate 30, which was among the best MET binders, to further assess the specificity of the interaction and identify the structural features that are important for binding to MET receptor.

We first examined the sequence of the MET binding peptide 22. Intriguingly, the MET binding sequence with the GGC extension at the C-terminus showed 40% of identity with HGF 681–698, i.e. QHKMRMVLGVIPGRGC, which contain several residues implicated in the HGF β chain–MET SEMA domain binding interface (underlined).⁴⁹ Besides V692, G694, G696, and C697, the other residues are located in the interior of the HGF β domain. The fact that most of the residues contained within HGF 681–698 are buried in the interior of HGF β chain and lack the main binding determinants such as Y673 makes the binding of this peptide to MET extracellular domain unlikely to occur. Nevertheless, peptides 28 and 29 corresponding to HGF 681–693 with an extra GGC sequence and HGF 681–698 respectively were synthesized and conjugated to dendrimer 4 to produce conjugates 36 and 37 (entries 7–8, Table 1).

Second, taurine residues of the polyanion tail 4 were substituted by acetyl groups (4 \rightarrow 3, Figure 2). Ligation of acetylated dendrimer 3 with peptide 22 yielded conjugate 38 (entry 9, Table 1), which enabled evaluating the importance of the sulfonate groups in the binding properties of conjugate 30. Third, substitution of tyrosine residue within the polyanion by alanine (4 \rightarrow 5, Figure 2) enabled the synthesis of conjugate 39 (entry 10, Table 1). Finally, the importance of the number and density of the sulfonate groups was examined by using phenylthiocarbonyl polyanions 8 and 10 (Figure 2), which

yielded successfully conjugates 40 and 41, respectively, upon reaction with peptide 22 (entries 11 and 12, Table 1).

These novel compounds enabled to perform additional microarray preparations and binding experiments (Figures 5

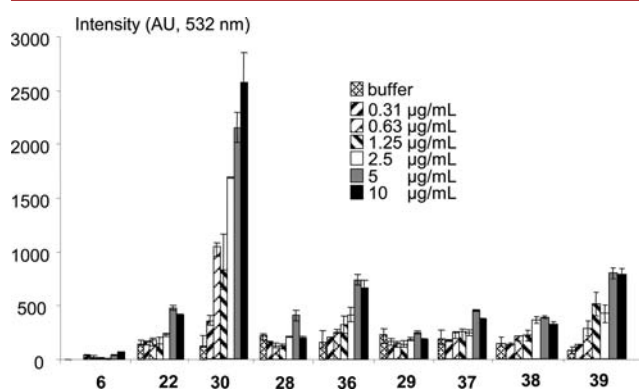


Figure 5. Role of the peptide sequence, of the sulfonate groups, and of the tyrosine residue within the polyanion for the binding of the conjugates to MET extracellular domain (rMET-hFc). Fluorescence data at 532 nm, 16 bits intensities in arbitrary units after background subtraction, $n = 3$, median and interquartile range).

and 6). As expected, the modification of the peptide sequence (conjugate 30 \rightarrow conjugates 36 and 37, Figure 5) resulted in a dramatic decrease of the signal intensity upon binding with MET extracellular domain. Moreover, the replacement of sulfonate groups by acetyl groups (conjugate 30 \rightarrow conjugate 38) or changing tyrosine residue within the polyanion by alanine (conjugate 30 \rightarrow conjugate 39) resulted in a significant decrease of the signal intensity upon incubation with rMET-Fc protein (Figure 5). The contribution of tyrosine to the binding strength is noteworthy and suggests as discussed before that electrostatic and hydrophobic interactions, and perhaps hydrogen bonds with the phenol group, synergize in the interaction with MET extracellular domain.

Figure 6 details the data obtained by varying the number or the density of the sulfonate groups within the polyanion (conjugate 30 \rightarrow conjugates 40 and 41). For this last experiment, the microarrays were incubated with MET and HER1 proteins but also with the KDR recombinant VEGFR-hFc chimera. EGF-induced receptor activation is independent of heparin.⁵⁰ In contrast, HS molecules are important cofactors for the activation of KDR by VEGF 165 isoform. Moreover, an heparin-binding site has been identified on the KDR molecule, which is essential for receptor activation.⁵

Conjugate 40 presenting four 2-aminoethanesulfonate groups on a tetravalent lysinyl core displayed lower signal intensities compared to conjugate 30. The result obtained with tetrasulfonated conjugate 40 might be due to the bulkiness of the dendrimeric part of the molecule, which might impair the access to the MET peptide and/or to the Gly-Tyr dipeptide unit. In contrast, *gem*-disulfonate conjugate 41, which is very similar in structure to conjugate 30, gave strong signal intensities upon binding with MET extracellular domain. As in the previous experiments (Figure 4), incubation with HER1 extracellular domain resulted in poor signal intensities even for *gem*-disulfonate conjugate 41. Conjugates 30, 40, and 41 bound to KDR molecule too, albeit with a signal strength about 3-fold less than for MET extracellular domain.

Finally, the capacity of conjugate 30 to inhibit the binding of HGF to the extracellular domain of MET receptor was assayed

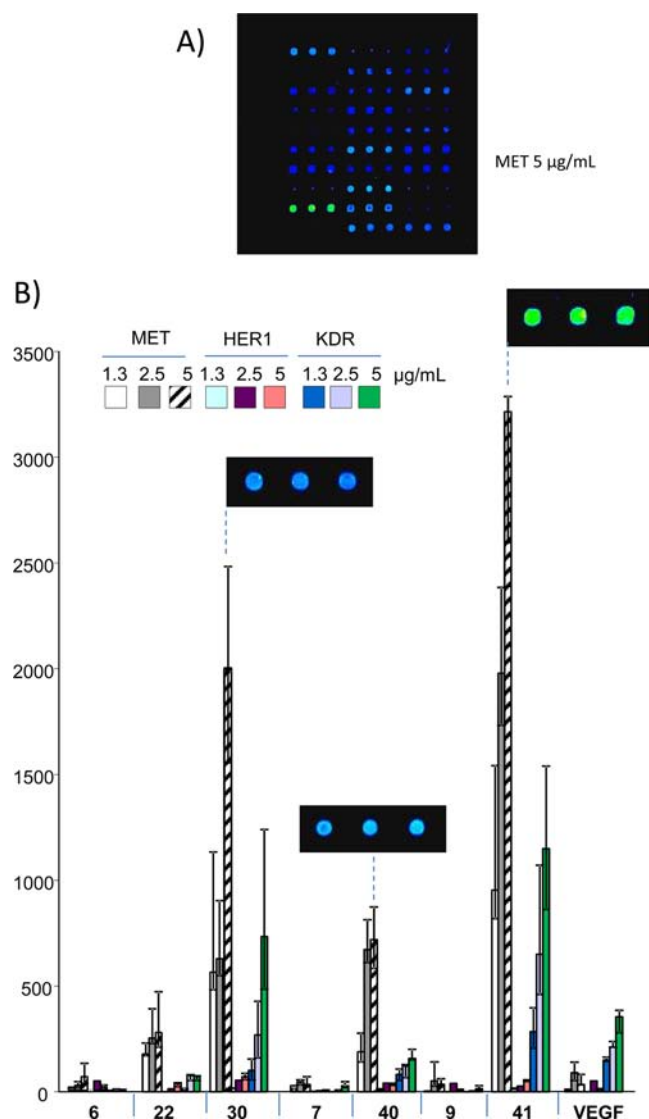


Figure 6. Role of the number and density of sulfonate groups for the binding of the conjugates to MET extracellular domain (rMET-hFc). (A) Typical microarray image (MET-Fc 5 µg/mL, fluorescence image at 532 nm, 16 bits, false color scale). (B) Incubations with MET-Fc, HER-Fc, or KDR-Fc proteins, intensities in arbitrary units after background subtraction, 3 slides, 3 spots per slide, median and interquartile range).

using the Alphascreen technology, which relies on a bead-based homogeneous approach (Figure 7).⁵¹ In this assay, light-sensitive (donor) beads coated with streptavidin and light-emitting (acceptor) beads coated with protein A are brought in close proximity due several specific biomolecular interactions: the biotin–streptavidin interaction, the capture of HGF by a biotinylated antibody raised against HGF, the interaction of HGF with the MET extracellular domain, and finally the interaction of protein A with the human Fc domain of MET-Fc recombinant chimera (Figure 7A). Irradiation of the light-sensitive beads generates singlet oxygen close to the light-emitting beads only when the series of interactions take place. In this case, the short distance between the beads enables singlet oxygen to diffuse and react with the light-emitting beads despite its short half-life. The separation of the beads due to the inhibition of the HGF/MET interaction makes unlikely the emission of light due to the decomposition of singlet oxygen

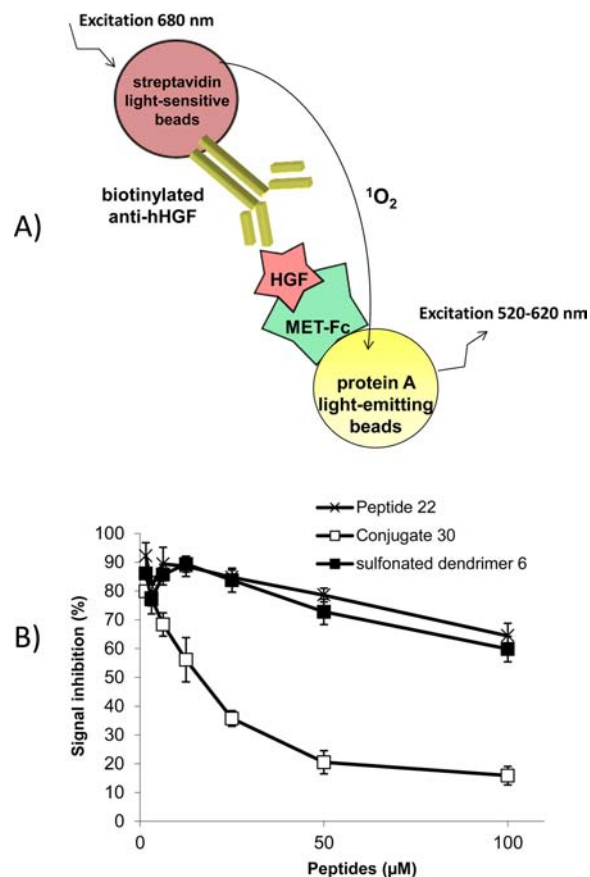


Figure 7. Inhibition of HGF/MET interaction was probed using Alphascreen HGF/MET binding assay.

before it can react with the light-emitting beads. The data presented in Figure 7B show that unlike sulfonated dendrimer 6 and peptide 22, conjugate 30 was able to inhibit the interaction of HGF with its receptor, thereby validating previous microarray experiments and the potential of the approach for improving the binding capacity of small peptides for their target proteins. The inhibitory activity of conjugate 30 on HGF/MET binding was moderate ($IC_{50} \sim 15 \mu M$) but not unexpected given the complexity of the HGF/MET interaction and the involvement of an extensive binding interface.¹³ Indeed, HGF is a 90 kDa disulfide-linked α/β heterodimer. The 60 kDa α chain is composed of a 10 kDa N-terminal domain (N domain) followed by four kringle domains (K1 to K4). The N domain contains the binding site for heparin or HS. Contiguous N and K1 domains constitute the high affinity HGF binding site for MET. The 30 kDa β chain consists of an enzymatically inactive serine protease homology domain (SPH) which corresponds to the secondary HGF binding site for MET. Therefore, the inhibition of HGF/MET binding with small molecules or peptides is highly challenging. Small heparin mimics were shown to inhibit the HGF-induced MET activation in in vitro cellular assays, but no direct binding of the compounds to HGF or MET has been demonstrated.⁵²

CONCLUSION

This proof-of-concept study shows that short peptides can be modified chemoselectively by synthetic HS mimetics for improving their ability to interact with HS-binding proteins. In this work, MET binding peptides were site-specifically

modified with lysine-based dendrimers decorated with sulfonate groups using chemoselective thiocarbamate ligation. The ligation reaction proved highly efficient and enabled the rapid synthesis of a small conjugate library aimed at studying the importance of several parameters such as the dendrimer structure or the number and density of the negatively charged sulfonate groups on the binding strength. The interaction experiments of the conjugates with recombinant MET, HER1 EGFR, or KDR VEGFR extracellular domains showed an interesting selectivity for MET protein. One of the most promising sulfonated-peptide conjugate inhibited the interaction of HGF with the extracellular MET receptor domain in an Alphascreen assay. Further work for improving the binding selectivity for MET and characterize the biochemical properties of the conjugates is in progress.

■ ASSOCIATED CONTENT

Supporting Information

Procedures and characterization for all new compounds, procedure for microarray and Alphascreen assays. Materials and methods section including synthesis and characterizations of peptides and related conjugates. This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Authors

*E-mail: oleg.melnyk@ibl.cnrs.fr.

*E-mail: patricia.melnyk@univ-lille2.fr.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

Dr. Oleg Melnyk and Pr. Patricia Melnyk thank the Lille 2 University, CNRS, the Pasteur Institute of Lille, and Région Nord Pas de Calais for financial support. Prof. Anthony Romieu thanks the Institut Universitaire de France (IUF) for financial support. We thank Gauthier Goormachtigh for useful discussions.

■ REFERENCES

- (1) Salamat-Miller, N.; Fang, J.; Seidel, C. W.; Assenov, Y.; Albrecht, M.; Middaugh, C. R. (2007) A network-based analysis of polyanion-binding proteins utilizing human protein arrays. *J. Biol. Chem.* 282, 10153–10163.
- (2) Jones, L. S.; Yazzie, B.; and Middaugh, C. R. (2004) Polyanions and the proteome. *Mol. Cell. Proteomics* 3, 746–769.
- (3) Urbinati, C.; Chiodelli, P.; and Rusnati, M. (2008) Polyanionic drugs and viral oncogenesis: a novel approach to control infection, tumor-associated inflammation and angiogenesis. *Molecules* 13, 2758–2785.
- (4) Fairbrother, W. J.; Champe, M. A.; Christinger, H. W.; Key, B. A.; and Starovasnik, M. A. (1998) Solution structure of the heparin-binding domain of vascular endothelial growth factor. *Structure* 6, 637–648.
- (5) Dougher, A. M.; Wasserstrom, H.; Torley, L.; Shridaran, L.; Westdock, P.; Hileman, R. E.; Fromm, J. R.; Anderberg, R.; Lyman, S.; Linhardt, R. J.; Kaplan, J.; and Terman, B. I. (1997) Identification of a heparin binding peptide on the extracellular domain of the KDR VEGF receptor. *Growth Factors* 14, 257–268.
- (6) Gitay-Goren, H.; Soker, S.; Vlodavsky, I.; and Neufeld, G. (1992) The binding of vascular endothelial growth factor to its receptors is dependent on cell surface-associated heparin-like molecules. *J. Biol. Chem.* 267, 6093–6098.
- (7) Gohda, E.; Tsubouchi, H.; Nakayama, H.; Hirono, S.; Takahashi, K.; Koura, M.; Hashimoto, S.; and Daikuhara, Y. (1986) Human hepatocyte growth factor in plasma from patients with fulminant hepatic failure. *Exp. Cell. Res.* 166, 139–150.
- (8) Stoker, M.; Gherardi, E.; Perryman, M.; and Gray, J. (1987) Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. *Nature* 327, 239–242.
- (9) Weidner, K. M.; Arakaki, N.; Hartmann, G.; Vandekerckhove, J.; Weingart, S.; Rieder, H.; Fonatsch, C.; Tsubouchi, H.; Hishida, T.; Daikuhara, Y.; and Birchmeier, W. (1991) Evidence for the identity of human scatter factor and human hepatocyte growth factor. *Proc. Natl. Acad. Sci. U. S. A.* 88, 7001–7005.
- (10) Kemp, L. E.; Mulloy, B.; and Gherardi, E. (2006) Signalling by HGF/SF and Met: the role of heparan sulphate co-receptors. *Biochem. Soc. Trans.* 34, 414–417.
- (11) Naldini, L.; Weidner, K. M.; Vigna, E.; Gaudino, G.; Bardelli, A.; Ponzetto, C.; Narsimhan, R. P.; Hartmann, G.; Zarnegar, R.; Michalopoulos, G. K.; Birchmeier, W.; and Comoglio, P. (1991) Scatter factor and hepatocyte growth factor are indistinguishable ligands for the MET receptor. *EMBO J.* 10, 2867–2878.
- (12) Birchmeier, C.; Birchmeier, W.; Gherardi, E.; and Vande Woude, G. F. (2003) Met, metastasis, motility and more. *Nature Rev. Mol. Cell Biol.* 4, 915–925.
- (13) Gherardi, E.; Birchmeier, W.; Birchmeier, C.; and Vande Woude, G. (2012) Targeting MET in cancer: rationale and progress. *Nature Rev. Cancer* 12, 89–103.
- (14) Zhou, H.; Casas-Finet, J. R.; Heath Coats, R.; Kaufman, J. D.; Stahl, S. J.; Wingfield, P. T.; Rubin, J. S.; Bottaro, D. P.; and Byrd, R. A. (1999) Identification and dynamics of a heparin-binding site in hepatocyte growth factor. *Biochemistry* 38, 14793–14802.
- (15) Zhou, H.; Mazzulla, M. J.; Kaufman, J. D.; Stahl, S. J.; Wingfield, P. T.; Rubin, J. S.; Bottaro, D. P.; and Byrd, R. A. (1998) The solution structure of the N-terminal domain of hepatocyte growth factor reveals a potential heparin-binding site. *Structure* 6, 109–116.
- (16) Lietha, D.; Chirgadze, D. Y.; Mulloy, B.; Blundell, T. L.; and Gherardi, E. (2001) Crystal structures of NK1-heparin complexes reveal the basis for NK1 activity and enable engineering of potent agonists of the MET receptor. *EMBO J.* 20, 5543–5555.
- (17) Raibaut, L.; Vicogne, J.; Leclercq, B.; Drobecq, H.; Desmet, R.; and Melnyk, O. (2013) Total synthesis of biotinylated N domain of human hepatocyte growth factor. *Bioorg. Med. Chem.* 21, 3486–3494.
- (18) Gherardi, E.; Youles, M. E.; Miguel, R. N.; Blundell, T. L.; Iamele, L.; Gough, J.; Bandyopadhyay, A.; Hartmann, G.; and Butler, P. J. (2003) Functional map and domain structure of MET, the product of the c-met protooncogene and receptor for hepatocyte growth factor/scatter factor. *Proc. Natl. Acad. Sci. U. S. A.* 100, 12039–12044.
- (19) Zioncheck, T. F.; Richardson, L.; Liu, J.; Chang, L.; King, K. L.; Bennett, G. L.; Fugedi, P.; Chamow, S. M.; Schwall, R. H.; and Stack, R. J. (1995) Sulfated oligosaccharides promote hepatocyte growth factor association and govern its mitogenic activity. *J. Biol. Chem.* 270, 16871–16878.
- (20) Delehedde, M.; Lyon, M.; Vidyasagar, R.; McDonnell, T. J.; and Fernig, D. G. (2002) Hepatocyte growth factor/scatter factor binds to small heparin-derived oligosaccharides and stimulates the proliferation of human HaCaT keratinocytes. *J. Biol. Chem.* 277, 12456–12462.
- (21) Huynh, R.; Chaubet, F.; and Jozefonvicz, J. (2001) Anticoagulant properties of dextranmethylcarboxylate benzylamide sulfate (DMCBSu); a new generation of bioactive functionalized dextran. *Carbohydr. Res.* 332, 75–83.
- (22) Bittoun, P.; Bagheri-Yarmand, R.; Chaubet, F.; Crepin, M.; Jozefonvicz, J.; and Fermandjian, S. (1999) Effects of the binding of a dextran derivative on fibroblast growth factor 2: secondary structure and receptor-binding studies. *Biochem. Pharmacol.* 57, 1399–1406.
- (23) Bagheri-Yarmand, R.; Kourbali, Y.; Rath, A. M.; Vassy, R.; Martin, A.; Jozefonvicz, J.; Soria, C.; Lu, H.; and Crepin, M. (1999) Carboxymethyl benzylamide dextran blocks angiogenesis of MDA-MB435 breast carcinoma xenografted in fat pad and its lung metastases in nude mice. *Cancer Res.* 59, 507–510.

- (24) Dheur, J., Dendane, N., Desmet, R., Dahmani, F., Goormachtigh, G., Vicogne, J., Fafeur, V., and Melnyk, O. (2012) Polysaccharide microarrays: application to the identification of heparan sulphate mimetics. *Methods Mol. Biol.* 808, 231–240.
- (25) Hamma-Kourbali, Y., Vassy, R., Starzec, A., Le Meuth-Metzinger, V., Oudar, O., Bagheri-Yarmand, R., Perret, G., and Crepin, M. (2001) Vascular endothelial growth factor 165 (VEGF(165)) activities are inhibited by carboxymethyl benzylamide dextran that competes for heparin binding to VEGF(165) and VEGF(165) KDR Complexes. *J. Biol. Chem.* 276, 39748–39754.
- (26) Carion, O., Lefebvre, J., Dubreucq, G., Dahri-Correia, L., Correia, J., and Melnyk, O. (2006) Polysaccharide microarrays for polysaccharide-platelet-derived-growth-factor interaction studies. *ChemBioChem* 7, 817–826.
- (27) Connell, B. J., Baleux, F., Coic, Y. M., Clayette, P., Bonnaffe, D., and Lortat-Jacob, H. (2012) A synthetic heparan sulfate-mimetic peptide conjugated to a mini CD4 displays very high anti- HIV-1 activity independently of coreceptor usage. *Chem. Biol.* 19, 131–139.
- (28) Baleux, F., Loureiro-Morais, L., Hersant, Y., Clayette, P., Arenzana-Seisdedos, F., Bonnaffe, D., and Lortat-Jacob, H. (2009) A synthetic CD4-heparan sulfate glycoconjugate inhibits CCR5 and CXCR4 HIV-1 attachment and entry. *Nature Chem. Biol.* 5, 743–748.
- (29) Mammen, M., Choi, S.-K., and Whitesides, G. M. (1998) Polyvalent interactions in biological systems: Implications for design and use of multivalent ligands and inhibitors. *Angew. Chem., Int. Ed.* 37, 2754–2794.
- (30) Krishnamurthy, V. M., Estroff, L. A., and Whitesides, G. M. (2006) Multivalency in ligand design. In *Fragment-based Approaches in Drug Discovery* (Mannhold, R., Kubinyi, H., and Folkers, G., Eds.) pp 11–39, Wiley-VCH Verlag GmbH & Co., Weinheim, Germany.
- (31) Argade, M. D., Mehta, A. Y., Sarkar, A., and Desai, U. R. (2014) Allosteric inhibition of human factor XIa: discovery of monosulfated benzofurans as a class of promising inhibitors. *J. Med. Chem.* 57 (8), 3559–3569.
- (32) Sidhu, P. S., Abdel Aziz, M. H., Sarkar, A., Mehta, A. Y., Zhou, Q., and Desai, U. R. (2013) Designing allosteric regulators of thrombin. Exosite 2 features multiple subsites that can be targeted by sulfated small molecules for inducing inhibition. *J. Med. Chem.* 56, 5059–5070.
- (33) Karuturi, R., Al-Horani, R. A., Mehta, S. C., Gailani, D., and Desai, U. R. (2013) Discovery of allosteric modulators of factor XIa by targeting hydrophobic domains adjacent to its heparin-binding site. *J. Med. Chem.* 56, 2415–2428.
- (34) Al-Horani, R. A., Ponnusamy, P., Mehta, A. Y., Gailani, D., and Desai, U. R. (2013) Sulfated pentagalloylglucoside is a potent, allosteric, and selective inhibitor of factor XIa. *J. Med. Chem.* 56, 867–878.
- (35) Chandra Gupta, R., Win, T., and Bittner, S. (2009) Taurine analogues: a new class of therapeutics. In *Frontiers in Medicinal Chemistry* (Atta-Ur-Rahman, Reitz, A. B., Iqbal Choudhary, M., Eds.) pp 183–213, Bentham Science Publishers, Amsterdam.
- (36) Romieu, A., Tavernier-Lohr, D., Pellet-Rostaing, S., Lemaire, M., and Renard, P.-Y. (2010) Water solubilization of xanthene dyes by post-synthetic sulfonation in organic media. *Tetrahedron Lett.* 51, 3304–3308.
- (37) Altamura, M., and Agnes, G. (1988) Easy approach to N-(aminoacyl)taurine derivatives. *J. Org. Chem.* 53, 1307–1309.
- (38) Vertesaljai, P., Biswas, S., Lebedyeva, I., Broggi, E., Asiri, A. M., and Katritzky, A. R. (2014) Synthesis of taurine-containing peptides, sulfonopeptides, and N- and O-Conjugates. *J. Org. Chem.* 79, 2688–2693.
- (39) Besret, S., Ollivier, N., Blanpain, A., and Melnyk, O. (2008) Thiocarbamate-linked peptides by chemoselective peptide ligation. *J. Pept. Sci.* 14, 1244–1250.
- (40) Melnyk, O., Ollivier, N., Besret, S., and Melnyk, P. (2014) Phenylthiocarbamate or N-carbothiophenyl group chemistry in peptide synthesis and bioconjugation. *Bioconjugate Chem.* 25, 629–639.
- (41) Koide, S., and Sidhu, S. S. (2009) The importance of being tyrosine: lessons in molecular recognition from minimalist synthetic binding proteins. *ACS Chem. Biol.* 4, 325–334.
- (42) Fellouse, F. A., Wiesmann, C., and Sidhu, S. S. (2004) Synthetic antibodies from a four-amino-acid code: a dominant role for tyrosine in antigen recognition. *Proc. Natl. Acad. Sci. U. S. A.* 101, 12467–12472.
- (43) Bourel, L., Carion, O., Gras-Masse, H., and Melnyk, O. (2000) The deprotection of Lys(Mtt) revisited. *J. Pept. Sci.* 6, 264–270.
- (44) Zhao, P., Grabinski, T., Gao, C., Skinner, R. S., Giambernardi, T., Su, Y., Hudson, E., Resau, J., Gross, M., Vande Woude, G. F., Hay, R., and Cao, B. (2007) Identification of a met-binding peptide from a phage display library. *Clin. Cancer Res.* 13, 6049–6055.
- (45) Melnyk, O., Olivier, C., Ollivier, N., Lemoine, Y., Hot, D., Huot, L., and Gouyette, C. (2005) Preparation of α -oxo semicarbazone oligonucleotide microarrays. *Curr. Protoc. Nucleic Acid Chem.* 19, 12.6.1–12.6.19.
- (46) Duburcq, X., Olivier, C., Desmet, R., Halasa, M., Carion, O., Grandidier, B., Heim, T., Stievenard, D., Auriault, C., and Melnyk, O. (2004) Polypeptide semicarbazide glass slide microarrays: characterization and comparison with amine slides in serodetection studies. *Bioconjugate Chem.* 15, 317–325.
- (47) Olivier, C., Hot, D., Huot, L., Ollivier, N., El-Mahdi, O., Gouyette, C., Huynh-Dinh, T., Gras-Masse, H., Lemoine, Y., and Melnyk, O. (2003) Alpha-oxo semicarbazone peptide or oligodeoxynucleotide microarrays. *Bioconjugate Chem.* 14, 430–439.
- (48) Melnyk, O., Duburcq, X., Olivier, C., Urbes, F., Auriault, C., and Gras-Masse, H. (2002) Peptide arrays for highly sensitive and specific antibody-binding fluorescence assays. *Bioconjugate Chem.* 13, 713–720.
- (49) Stamos, J., Lazarus, R. A., Yao, X., Kirchhofer, D., and Wiesmann, C. (2004) Crystal structure of the HGF beta-chain in complex with the Sema domain of the Met receptor. *EMBO J.* 23, 2325–2335.
- (50) Aviezer, D., and Yayon, A. (1994) Heparin-dependent binding and autophosphorylation of epidermal growth factor (EGF) receptor by heparin-binding EGF-like growth factor but not by EGF. *Proc. Natl. Acad. Sci. U. S. A.* 91, 12173–12177.
- (51) Taouji, S., Dahan, S., Bosse, R., and Chevet, E. (2009) Current Screens Based on the AlphaScreen Technology for Deciphering Cell Signalling Pathways. *Curr. Genomics* 10, 93–101.
- (52) Raiber, E. A., Wilkinson, J. A., Manetti, F., Botta, M., Deakin, J., Gallagher, J., Lyon, M., and Ducki, S. W. (2007) Novel heparin/heparan sulfate mimics as inhibitors of HGF/SF-induced MET activation. *Bioorg. Med. Chem. Lett.* 17, 6321–6325.