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Powerful Binders for the D-Dimer by Conjugation of the GPRP Peptide to Polypeptides from a Designed Set—Illustrating a General Route to New Binders for Proteins

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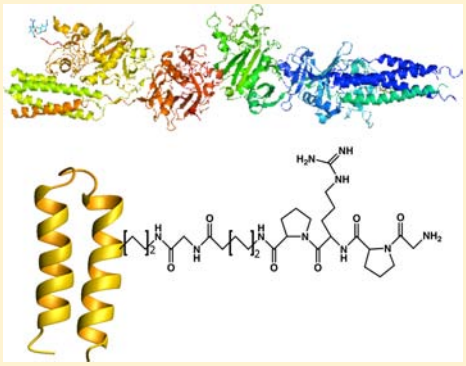
Supporting Information

ABSTRACT: The synthetic tetrapeptide GPRP based on the amino-terminal GPR sequence of the fibrin α-chain binds the D-dimer protein with a dissociation constant K_D of 25 μM. The D-dimer protein, a well-known biomarker for thrombosis, contains two cross-linked D fragments from the fibrinogen protein formed upon degradation of the fibrin gel, the core component of blood clots. In order to develop a specific high-affinity binder for the D-dimer protein, GPRP was conjugated via an aliphatic spacer to each member of a set of sixteen polypeptides designed for the development of binder molecules for proteins in general. The binders were individually characterized and ranked using surface plasmon resonance (SPR) analysis. The dissociation constant of the complex formed from the D-dimer and 4-D15L8-GPRP labeled with fluorescein was determined by fluorescense titration and found to be 3 nM, an affinity 4 orders of magnitude higher than that of free GPRP. According to SPR analysis, binding was completely inhibited by free GPRP at mM concentrations and the polypeptide conjugate was therefore shown to bind specifically to the binding site of GPRP. Affinities were further enhanced by dimerization of the polypeptide conjugates via a bifunctional linker resulting in dissociation constants that were further decreased (affinities increased) by factors of 2−4. The results suggest an efficient route to specific binders for proteins based on short peptides with affinities that need only to be modest, thus shortening the time of binder development dramatically.



INTRODUCTION

Specific, high-affinity binders for proteins are required in numerous bioanalytical applications, including the measurement of protein expression levels in the field of in vitro diagnostics. Although there are well-known problems associated with the use of antibodies, in protein recognition they are the golden standard and bind to specific epitopes on protein surfaces with high affinity and selectivity. Recently, we reported on an alternative strategy for protein recognition and binding based on the conjugation of small organic molecules to 42-residue polypeptides, showing the capacity of the technology to provide small and robust binder molecules that perform equal to or better than average antibodies.[1] To reach the affinity of average antibodies, the organic molecules were required to have only modest affinities and the polypeptides were selected from a set of only sixteen members designed to boost the affinity and selectivity for proteins in general. The conjugates formed from small molecule ligands and polypeptides were shown to bind proteins such as the C-reactive protein (CRP) and human Carbonic Anhydrase II (HCAII) with affinities that were on the order of 2 to 4 orders of magnitude higher than those of the small molecules.[2−5] The polypeptides did not have any prior relationship to CRP or HCAII and did not independently bind strongly enough for dissociation constants to be measured. The molecular weights of the polypeptide conjugates were in the range of 5−6 kD, and therefore approximately 1/30 of that of an IgY antibody. They do not have ordered structures and adapt to the surface of the biological target.[1] The concept is completely different from that of biologically generated binder molecules. While the use of small organic molecules as functional groups is an attractive strategy in the design of high-affinity binders for proteins because of their robustness and their ability to protect peptides from proteolytic degradation, the pool of available peptides is essentially endless, considering the number of sequences that can be identified, for example, from phage display techniques.[6] It is therefore of considerable interest to determine whether tight binders can also be obtained by conjugating short peptides instead of small organic molecules as this would expand the accessible pool of binder candidates dramatically. We selected the D-dimer protein for a proof-of-principle demonstration.

The process of coagulation in human blood is a cascade of molecular events, including the enzymatic cleavage of fibrinogen to form fibrin which aggregates into proteofibrils

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that upon cross-linking at the D fragment site form an insoluble gel and subsequently blood clots.[7,8] Enzymatic degradation of the clots release cross-linked D fragments, known as D-dimers, which are normally not present in blood unless coagulation has occurred, and the D-dimer therefore serves as a biomarker for thrombosis.[9,10] The measurement of D-dimer concentration in blood is a recognized and common diagnostic test, and it is commonly used to exclude the possibility of thromboembolic disease.[11]

The interaction between the D-dimer and the tetrapeptide GPRP is well-characterized with a K_D of 25 μM.[12,13] The GPRV sequence of the E domain of fibrinogen is involved in the aggregation of activated fibrinogen to form fibrin and subsequently higher aggregates, and known to bind specifically to a binding site on the D-domain.[14,15] The GPRP sequence was found after optimization to bind with higher affinity than GPRV to the D-dimer.[12] The structure of the complex between the D-dimer and GPRP has been solved and the N-terminal Gly residue has been shown to be involved in hydrogen bonding to amino acid residues in the GPRP binding cleft.[16,17] Further investigations about the structural requirements for peptide D-dimer interactions are ongoing.[18] Here, we wish to report on the conjugation of the GPRP tetrapeptide to polypeptides from the set of designed 42-residue sequences resulting in powerful binders for the D-dimer (Figure 1). The binder concept has thereby been extended to include the use of short peptides, in addition to small organic molecules, for the development of polypeptide conjugate binder molecules that recognize proteins for in vitro and in vivo applications.

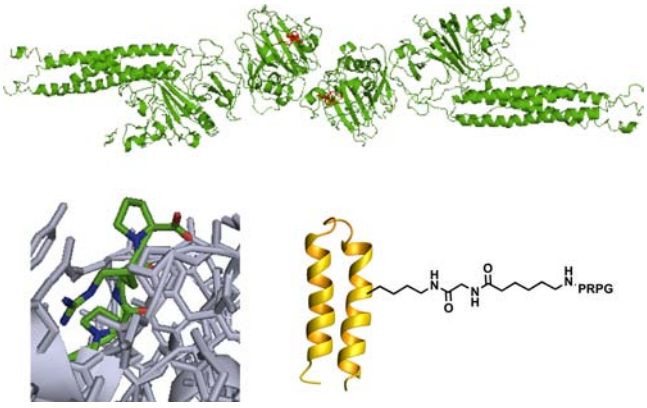


Figure 1. Illustration of principles for D-dimer binder design. The crystal structure of the D-dimer complexed to GPRP, top, reveals two binding sites for GPRP. Expansion, bottom left, shows that the C-terminal Pro residue is exposed to solvent whereas the N-terminal Gly is buried in the binding pocket of the protein. In the binder molecule, bottom right, GPRP is linked to the side chain of a lysine residue in the polypeptide chain via a Gly residue and a 6-carbon spacer attached to the C-terminal Pro. The N-terminal of the polypeptide is acetylated and no other lysine residue is present in the sequence, ensuring that the GPRP active ester reacts site specifically.

EXPERIMENTAL PROCEDURES

General. All reagents and solvents were purchased from commercial sources and were used without further purification. Thin layer chromatography (TLC) was performed on 60 F_{254} silica and 60 F_{254} aluminum oxide plates (Merck) and spots were visualized with UV light (λ = 254 nm). ^{1}H NMR spectra were recorded on Varian Inova 500 MHz (499.9 MHz) spectrometer, and ^{13}C NMR spectra were recorded on Varian Unity 400 MHz (100.6 MHz) spectrometer. Spectra were recorded at 25 °C using deuterated chloroform as solvent. Chemical shifts (δ) in ppm are reported using TMS as an internal reference (^{1}H δ 0.0) and residual chloroform signal (^{13}C δ 77.0) and coupling constants (J) are reported in Hz. Low-resolution mass spectra were recorded on Perkin-Elmer SCIEX API 150EX spectrometer in the positive ion mode. All peptide conjugates were identified by MALDI-TOF mass spectrometry (Voyager-DE Pro, Applied Biosystem, USA); see Supporting Information. The human D-dimer protein was obtained from Abcam Inc., U.K.

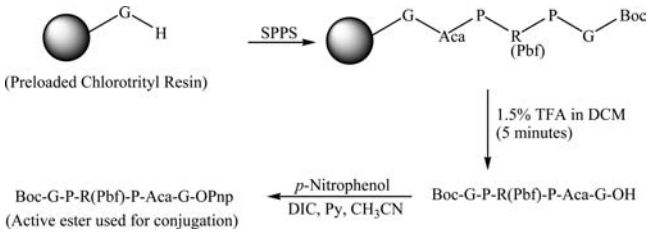
Peptide Synthesis. The peptides were synthesized on a Pioneer automated peptide synthesizer using standard fluorenylmethoxycarbonyl (Fmoc) protocols with *O*-(7-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU, Iris Biotech GmbH) and diisopropylethylamine (DIPEA, Aldrich) as activating agents. The Fmoc protection groups were removed by 20% piperidine in dimethyl formamide (DMF). The synthesis was performed on a 0.1 mmol scale with a Fmoc-glycine-poly(ethylene glycol)-polystyrene (Fmoc-Gly-PEG-PS) or a Fmoc-PAL-PEG-PS (Applied biosystems) resin, and a 4-fold excess of amino acid in each coupling. The side chains of the amino acids (Calbiochem-Novabiochem AG, Iris Biotech GmbH) were protected by the base-stable groups: *tert*-butyl ester (Asp, Glu), trityl (His, Asn, Gln), *tert*-butoxymethyl (Lys), and 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg).

Orthogonal protection of the lysine residues 15, 10, 25, and 34 by an allyloxycarbonyl (Alloc) group in the sequences C15L8, C10L17, C25L22, and C37L34, respectively, enabled the site-specific introduction of a fluorescent probe in each polypeptide. Deprotection of the allyloxycarbonyl group was performed by treatment of the resin with tetrakis-(triphenylphosphine)palladium(0) ($Pd(PPh_3)_4$) in trichloromethane, acetic acid and *N*-methylmorpholine (ratio 37:2:1 v/v; 10 mL per gram of resin) at room temperature, under N_2 during 2 h. The resin was washed sequentially with 0.5% DIPEA in DMF and 0.5% diethyldithiocarbamic acid in DMF, DMF, and DCM and desiccated.

Coupling of 7-methoxycoumarin-3-carboxylic acid to the amino group of the lysine side chain was performed in DMF during 2 h with gentle stirring at room temperature with 2-fold excess of acid activated by a coupling cocktail consisting of *N*,*N*-diisopropylethylamine (DIPEA), 1-hydroxy benzotriazole (HOBt), and diisopropyl carbodiimide (DIC) in the ratio 12:6:6. After two hours, an additional aliquot of coupling cocktail was added and the reaction was left overnight. For each peptide, deprotection and cleavage from the resin was achieved by treatment with a mixture of trifluoroacetic acid (TFA), water, and triisopropylsilane (95:2.5:2.5 v/v, 10 mL per gram of polymer) for 2 h at room temperature. After filtration and concentration, the peptide was precipitated by addition of cold diethyl ether, centrifuged, washed in diethyl ether, and dried in air. The crude peptides were purified by reversed-phase HPLC on a semipreparative Hypersil C-18 Gold column pore size 175 Å, particle size 5 μM, or a semipreparative Kromasil C8 Hichrom column pore size 100 Å, particle size 10 μM, eluted with a shallow 35−55% acetonitrile gradient in water and 0.1% TFA as additive at a flow rate of 10 mL/min. Collected fractions were identified by MALDI-TOF mass spectrometry (Bruker Daltonics Ultraflex II TOF/TOF), concentrated, and lyophilized twice.

Synthesis of the GPRP Ligand with a Spacer (Scheme 1). A 6-carbon spacer for the GPRP ligand was synthesized by

Scheme 1. Synthesis Scheme for the Assembly of the Activated GPRP Ligand



attaching a 6-aminocaproic acid residue to the proline residue at the *C*-terminus. The synthesis of the peptide was done on a 0.5 mmol scale using manual SPPS (Fmoc/^{t}Bu strategy using PyBOP for activation, 2-fold excess of amino acids and the coupling reagent was used), on a chlorotrityl resin preloaded with glycine (0.75 mmol/g). The arginine side chain and the *N*-terminus glycine were protected as Pbf and Boc groups, respectively. The hexapeptide obtained from SPPS was cleaved from the resin by treatment with 1.5% TFA in CH_2Cl_2 (10 mL) for 5 min. The resin was washed with 1% TFA in CH_2Cl_2 (2 × 5 mL) to ensure complete cleavage of the peptide. The peptide solution was washed with saturated citric acid to remove any free amines (formed by the deprotection of Pbf or Boc group) present in the solution and was dried over $MgSO_4$ and concentrated. The crude residue was directly used for the preparation of the active ester.

The crude hexapeptide was dissolved in anhydrous acetonitrile (10 mL) and was stirred at 0 °C. Pyridine (2 mL), DIC (0.3 mL, 4 equiv), and *p*-nitrophenol (0.28 g, 4 equiv) was added to the peptide solution, and the stirring was continued for 15 h (overnight). The reaction mixture was concentrated under vacuum, the residue was dissolved in CH_2Cl_2 (50 mL) and was washed with saturated citric acid (2 × 50 mL). The CH_2Cl_2 solution of the active ester was dried over $MgSO_4$ and the solution volume was reduced to 10 mL by evaporation. The crude product was purified by silica gel (60–90 mesh) column chromatography by eluting with 0–100% acetone in CH_2Cl_2. The active ester of the peptide was obtained as a white solid (150 mg, 30% overall yield).

Synthesis of Peptide Conjugates. The ligands were conjugated to the amino group of the lysine side chain of the 42-residue polypeptides by the addition of 3 equiv of ligand from a DMSO stock solution (50 mM) to a solution of the polypeptide in DMSO (1 mM) in the presence of 10% pyridine (v/v) and 0.5% DIPEA (v/v). The reaction was monitored by analytical HPLC and the fractions were analyzed by MALDI-TOF MS. The functionalized polypeptide was precipitated by the addition of cold diethyl ether and centrifuged. A mixture of TFA/H_2O/TIS (40:1:1) was slowly added to the precipitated polypeptide to make a 2 mM polypeptide solution which was kept at rt for 1 h to remove Boc and Pbf groups, after which the TFA was evaporated by a stream of nitrogen. The polypeptide was precipitated by the addition of cold diethyl ether and centrifuged. The pellet (1 umol) was dissolved in 1 mL TFA/anisole solution (99:1) in the presence of 100 equiv of silver trifluoromethanesulfonate (AgOTf) at 0 °C, stirred for 1 h, and allowed to reach room temperature under stirring for 2 h. The silver salt of the polypeptide conjugate was precipitated by the addition of cold diethyl ether and centrifuged. The supernatant was discarded and the precipitate dissolved in 0.5 mL MilliQ water, followed by the addition of 50 equiv of dithiothreitol (DTT) in 0.5 mL acetic acid. The mixture was stirred for 2 h at room temperature and centrifuged. The supernatant containing deprotected conjugated polypeptides was purified by HPLC. The appropriate fractions were lyophilized to afford the pure functionalized polypeptides, which were also characterized by MALDI-TOF mass spectrometry in yields of 10–65%.

Synthesis of Cross-Linked Binders. The conjugates prepared from the 3-series and 4-series of polypeptides (80–100 nM) were dissolved in HBS-EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% P20) (GE Healthcare Bio-Sciences, Uppsala, Sweden) to a final concentration of 1 mM. Reduction of potential disulfides was performed by adding immobilized TCEP disulfide reducing gel (Pierce Biotechnology, effective TCEP concentration ≥8 μmol/mL of gel) to the peptide solution, using an amount of gel corresponding to 20 times excess of TCEP over binder. The TCEP reducing gel was pre-equilibrated with HBS-EP buffer. After a brief incubation with occasional vortex, the tube was centrifuged at 3000 rpm for 1 min, and the supernatant containing the reduced binder was recovered. Gel bound sample was recovered by washing the gel with HBS-EP buffer (3 × 100 μL). A stock solution of the homobifunctional cross-linker 1,8-bis(maleimido)diethylene glycol ($BM(PEG)_2$) (Pierce Biotechnology) in DMSO was prepared, and immediately, 2 μL (1 molar equiv) was added to 200 μL of the reduced binder solution (1% DMSO v/v, final concentration). The pH of the reaction mixture was corrected to 7.4 by adding 3 × 0.5 μL 1 M NaOH, which was then incubated for 2 h with occasional vortex. The reaction was followed by analytical reversed-phase HPLC using a GraceVydac, MS C18-column (4.6 × 150 mm) with a gradient of ACN/H_2O from 10% to 60% ACN in 30 min. The identity of the product was confirmed with MALDI-TOF-MS (Voyager PRO, Applied Biosystems) using a mixture of α-cyano-4-hydroxycinnamic acid and 2-(4-hydroxy-phenylazo)-benzoic acid as matrix with detection in the positive mode. The cross-linked binders were subjected to Biacore measurement immediately after completed cross-linking reaction.

Biacore Measurements. Prior to immobilization, the storage buffer for the D-dimer protein was changed to 10 mM NaOAc at pH 4.6 with NAP5 (GE Healthcare Bio-Sciences, Uppsala, Sweden). The protein was then further diluted with 10 mM NaOAc at pH 4.6 to give a concentration of about 32 μg/mL. The D-dimer protein was covalently immobilized on the sensor chip surface by amine coupling with HBS-EP (10 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM EDTA, and 0.005% P20) (GE Healthcare Bio-Sciences, Uppsala, Sweden) as a running buffer. The CM5 sensor chip surface was activated for 7 min by injecting a solution of EDC/NHS (200 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride/50 mM *N*-hydroxysuccinimide) (GE Healthcare Bio-Sciences, Uppsala, Sweden). The D-dimer protein was injected for 1.5 min at flow rate of 5 μL/min over the activated surface and followed by 7 min pulse of 1 M ethanolamine at pH 8.5 to deactivate remaining active ester. The final immobilization levels were between 6000 and 7500 resonance units (RU).

Interaction Analysis. The interactions between the D-dimer protein and binders as well as surface competition experiments were studied using a Biacore 2000 instrument (GE

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1-C15L8	*Ac*-NEADLEAKIRHLAEKLEARGPEDAEQLAEQLARAFEAFARAG-*COOH*	-7
2-C15L8	*Ac*-NEADLEAKIRHLAEKLAARGPVDAAQLAEQLARAFEAFARAG-*COOH*	-4
3-C15L8	*Ac*-NAADJEAKIRHLAEKJAARGPVDAAQJAEQLARRFEAFARAG-*$CONH_2$*	-1
4-C15L8+2	*Ac*-NAADJEAKIRHLREKJAARGPRDAAQJAEQLARRFERFARAG-*$CONH_2$*	
1-C10L17	*Ac*-NAADLEAAIKHLAEALKERGPEDCEQLAEQLARAFEAFARAG-*COOH*	-7
2-C10L17	*Ac*-NAADLEAAIKHLAEALKARGPVDAAQLAEQLARAFEAFARAG-*COOH*	-4
3-C10L17	*Ac*-NAADJEARIKHLAERJKARGPVDAAQJAEQLARAFEAFARAG-*$CONH_2$*	-1
4-C10L17+2	*Ac*-NAADJEARIKHLRERJKARGPRDAAQJAEQLARAFERFARAG-*$CONH_2$*	
1-C25L22	*Ac*-NEADLEAAIRHLAEALEARGPKDAKQLAEQLARAFEAFERAG-*COOH*	-7
2-C25L22	*Ac*-NEADLEAAIRHLAEALAARGPKDAKQLAEQLARAFEAFARAG-*COOH*	-4
3-C25L22	*Ac*-NAADJEAAIRHLAERJAARGPKDAKQJAEQLARAFEAFARAG-*$CONH_2$*	-1
4-C25L22+2	*Ac*-NAADJEARIRHLRERJAARGPKDAKQJAEQLARAFERFARAG-*$CONH_2$*	
1-C37L34	*Ac*-NEADLEAAIRHLAERLEARGPADAAQLAEQLAAKFEKFARAG-*COOH*	-7
2-C37L34	*Ac*-NAADLEAAIRHLAERLAARGPVDAAQLAEQLAAKFEKFARAG-*COOH*	-4
3-C37L34	*Ac*-NAADJEAAIRHLAERJAARGPVDAAQJAEQLARKFEKFARAG-*$CONH_2$*	-1
4-C37L34+2	*Ac*-NAADJEARIRHLRERJAARGPRDAAQJAEQLARKFEKFARAG-*$CONH_2$*	

Figure 2. Polypeptide sequences of the 16-membered set used in binder development, with amino acids represented in the one letter code and total charge shown. All N-terminals are acetylated to avoid unwanted side reactions with active esters during synthesis. The nomenclature indicates position of coumarin fluorophor and position of GPRP ligand; e.g., 1-C15L8 has a coumarin conjugated to the side chain of Lys-15, and the GPRP ligand at the side chain of Lys-8. The initial number indicates total charge; e.g., 1-C15L8 has a total charge of −7 whereas 4-C15L8 has a total charge of +2.

Healthcare Bio-Sciences, Uppsala, Sweden), equilibrated at 25 °C. The CM5 sensor chip (Research grade, Biacore) and reagents were from GE Healthcare Bio-Sciences, Uppsala, Sweden. For direct interaction studies between immobilized D-dimer protein and polypeptide conjugates, HBS-EP was used as running buffer. The polypeptide conjugates were diluted in the running buffer and injected over the immobilized protein in concentration series of 5−160 nM, or 1−100 nM, for initial screening, using 3 min contact time. For minimum sample dispersion, the samples were injected at a flow rate of 50 μL/min. After 10 min of dissociation, the surface was regenerated by 30 s pulse injection of 10 mM glycine at pH 3.0. All injections were serial and first passed over the deactivated dextran surface and then over the immobilized surfaces. Blank injections were included for each measurement series and subtracted from the data. Experiments were performed at least twice, unless stated otherwise. In the surface competition assay, a series of mixtures of the binder 4-D10L17-GPRP (100 nM) and a number of concentrations of GPRP in the range 0−1 mM were injected over the immobilized D-dimer protein. The dissociation constants, K_D, were obtained using the *Biaevaluation* program version 3.2 (Biacore) and assuming a simple 1:1 Langmuir binding mechanism.

Coupling of Fluorescein to 4-D15L8-GPRP. Peptide 4-D15L8-GPRP, containing a free cysteine in the loop region (1.27 mg, 22.0 μmol), was dissolved in 100 mM sodium phosphate buffer (N_2-flushed) containing 6 M guanidine hydrochloride at pH 7.3 (210 uL, 1 mM), and incubated with 4 mol equiv of fluorescein-5-maleimide (0.38 mg, 88 umol, Pierce Biotechnology) for 4 h at r.t, predissolved in 10 μL DMSO. The reaction was followed by analytical reversed-phase HPLC using a GraceVydac, MS C_{18} column (4.6 × 150 mm) with a shallow 30−55% acetonitrile gradient in water and 0.1% TFA as additive. The identity of the product was confirmed with MALDI-MS (Voyager PRO, Applied Biosystems) using a mixture of α-cyano-4-hydroxycinnamic acid and 2-(4-hydroxyphenylazo)-benzoic acid as matrix with detection in the positive mode. After completion of the reaction, the fluorescein−peptide conjugate was purified by reversed-phase HPLC and the pooled fractions were evaporated and lyophilized.

Affinity Determination with Fluorescence Spectroscopy. Fluorescence spectra were recorded on a Fluoromax Gemini XPS microplate reader at 25 °C. The excitation wavelength was 495 nM and the emission was monitored in the range 510−600 nM. Prior to the experimental setup, all plastic materials (tubes, tips, and the microplate) were coated with 1% aqueous solution of Pluronic F108NF Prill Polaxamer 338 (BASF) for 12 h, followed by thorough wash with water in order to minimize unspecific surface interactions. For the affinity determination, samples of 100 nM fluorescein-labeled 4-D15L8-GPRP in N_2-flushed phosphate buffered saline (50 mM NaH_2PO_4, 150 mM NaCl, pH 7.5) were set up in a 384-well black microplate (Nunc). Aliquots from solutions of D-dimer (Abcam Inc. UK, 3.0 μM, 0.6 μM, 0.1 μM, and 10 nM) were then added to the wells in the range 1.0 nM to 1.5 μM . The 0.6 μM to 10 nM solutions were diluted from the 3.0 μM stock solution with phosphate buffered saline. The total volume in all wells was 100 μL and was compensated by the buffer volume. The fluorescence intensity at 525 nm was monitored as a function of total protein concentration, and the dissociation constant K_D was determined by fitting the following equation to the experimental results under the assumption of a 1:1 binding model:

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$$F_{obs} = \frac{F_{bound}\cdot[\mathrm{Ddim}] + F_{free}\cdot K_d}{[\mathrm{Ddim}] + K_d}$$

F_{obs} is the observed fluorescence intensity, F_{bound} is the fluorescence of the peptide bound to D-dimer, F_{free} is the fluorescence of free peptide, and [Ddim] is the concentration of free D-dimer. [Ddim] can be derived from

$$[\mathrm{Ddim}] = -\frac{[P]_{tot} + K_d - [\mathrm{Ddim}]_{tot}}{2} + \sqrt{\left(\frac{[P]_{tot} + K_d - [\mathrm{Ddim}]_{tot}}{2}\right)^2 + K_d\cdot[\mathrm{Ddim}]_{tot}}$$

where $[P]_{tot}$ is the total concentration of peptide and $[\mathrm{Ddim}]_{tot}$ is the total concentration of D-dimer. Fitting was done with the *IGOR Pro 6.0* software (WaveMetrics Inc.).

RESULTS AND DISCUSSION

Design of Conjugate Binders. The binder concept is based on the conjugation of a small organic molecule or peptide to each member of a designed set of 42-residue polypeptides to form binder candidates, where one or more are identified in a rapid screening procedure based on fluorescence as "hits". The design of the 16-membered set of polypeptides has been described in detail previously.[1] In short, the polypeptides were designed to have some propensity for forming two amphiphilic helices connected by a short loop. The concentration dependence of the CD and one-dimensional ^{1}H NMR spectra show that they indeed fold into helix−loop−helix motifs in aqueous solution at micromolar concentrations where they form helix−loop−helix dimers, whereas at nanomolar concentrations, the polypeptides exist predominantly as unordered monomers. We have shown previously that in the case of human Carbonic Anhydrase II the binder molecule binds the protein as a monomer.[3]

Hydrophobic residues were introduced to provide most of the binding energy and the design concept was based on the hypothesis that a folded amphiphilic helix presents a hydrophobic face more efficiently toward a protein than an unordered polypeptide. Consequently, the polypeptide should have the capacity to form helical segments upon binding to the target protein. In the complex formed from binder and protein, according to the design hypothesis, charged residues of the helix in neighboring positions to hydrophobic side chains are forced in close proximity to surface-exposed residues of the protein. Electrostatic repulsion between residues of like charge, for example, aspartate or glutamate residues forced in contact with aspartates or glutamates, will reduce binding affinity severely, whereas the contact between opposite charges will facilitate binding. Charge−charge interactions therefore control selectivity. The polypeptide sequences are shown in Figure 2. The polypeptides 1-D15L8, 1-D10L17, 1D25L22, and 1 D37L34, the 1-series, are the most negatively charged with total charges of −7. The 2-series of peptides have total charges of −4, the 3-series total charges of +1, and the 4-series have total charges of +2. D15 indicates that the fluorophore dansyl is attached to the side chain of Lys-15, and L8 indicates that the small organic molecule or peptide, i.e., the ligand, is attached at the side chain of Lys-8, and so on. A fluorophore, here dansyl, is typically incorporated into the sequence for quantification purposes.

There is, so far, only indirect structural evidence in support of the design principles, and it remains a hypothesis that the polypeptides form helical structures upon complexation with proteins, although indirect spectroscopic evidence is compatible with helix formation.[5]

An important aspect of the binder concept is that the small molecule or short peptide controls the interaction with the target protein, and that the polypeptide is not preorganized to bind to a predetermined epitope but adapts to the protein surface and binds to the most complementary surface area accessible within the constraints of the spacer.[1] Upon binding of the small molecule residue to its binding site, the polypeptide docks to one protein epitope or to several epitopes in rapid exchange and the size of the spacer controls to what extent the protein surface is accessible to the polypeptide. Intuitively, one would expect that selectivity decreases with increased spacer length whereas affinity might increase. While a longer spacer could be considered to reduce the entropic gain in binding affinity obtained by covalently linking the small molecule to the polypeptide, the results obtained so far with other binder molecules of this kind suggest that for spacers with lengths of 4−10 methylene groups the offset is much smaller than the affinity gained from polypeptide−protein interactions.[5] Experience suggests that aliphatic spacers generate the highest affinities perhaps because of added hydrophobic contacts with the protein, whereas poly(ethylene glycol) spacers show weaker binding.[19,20] In the design of binders for the D-dimer, we introduced a hexacarbon atom spacer for the attachment of GPRP to the polypeptides based on an analogy with binder development for the C-reactive protein. The interaction between GPRP and the D-dimer was carefully evaluated using the crystal structure of the complex, and the N-terminal Gly was found to be directly involved in binding interactions with amino acid residues in the GPRP binding site, e.g., with Asp-330.[16,17] The spacer was therefore attached to the C-terminal where a non-native Gly residue was also incorporated, mainly for ease of synthesis.

Synthesis. The synthesis of the polypeptides followed standard SPPS Fmoc protocols and has been reported in detail previously.[2] They were purified by semipreparative reversed-phase HPLC and identified by MALDI-TOF mass spectrometry. GPRP equipped with a caprylic acid spacer extended by a Gly residue transformed into a *p*-nitrophenyl ester was also synthesized on the solid phase and purified as well as identified as described in the Experimental Procedures. The conjugation reaction was carried out in DMSO in an essentially quantitative reaction, and the final binder molecules were purified and identified as the peptides described above; see Experimental Procedures.

Characterization of Affinity and Selectivity. The 16-membered set of binder candidates obtained by conjugation of each of the polypeptides to the spacered GPRP was evaluated in a surface plasmon resonance (SPR, Biacore) assay where the D-dimer was immobilized on the chip and the binder molecules were allowed to flow over the chip at concentrations of 0, 1, 10, and 100 nM in standard running buffer (see Supporting Information). The preliminary results obtained demonstrated that the binders 4D15L8-GPRP, 4D10L17-GPRP, and 4D25L22-GPRP bound strongly to the D-dimer. At 100 nM concentration, the uptake was considerable, and some uptake was also observed at 10 nM concentration, whereas no binding was observed for the 1 nM solutions. In the interest of efficiency, the binder solutions were prepared from weighing

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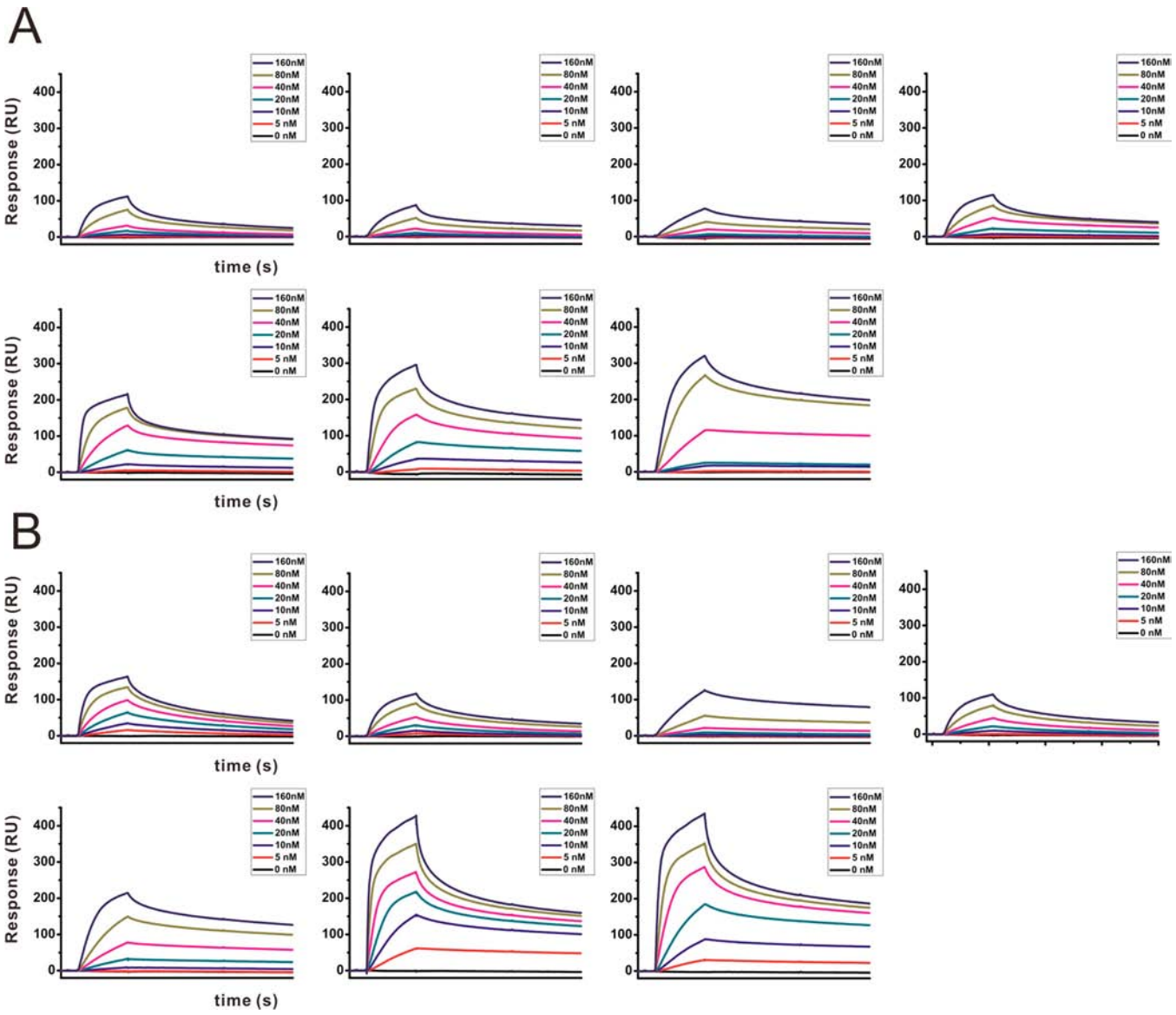

Figure 3. Panels of sensorgrams showing binding of the seven best binders to immobilized D-dimer in HBS-EP buffer at pH 7.4. Panel A shows the seven best monomeric binders. Panel B shows the corresponding binders dimerized by a PEG spacer. Panel A top row from left to right is 3-D15L8-GPRP, 3-D10L17-GPRP, 3-D25L22-GPRP, 3-D37L34-GPRP. Panel A bottom row is 4-D15L8-GPRP, 4-D10L17-GPRP, 4-D25L22-GPRP. Panel B shows the same binders dimerized by a linker. The scales of the axes are identical for direct comparison of uptake between binders. The aim was to evaluate performance of binder molecules at 0, 5, 10, 20, 40, 80, and 160 nM concentrations but minor individual variations were observed between the binder stock solutions, probably due to the difficulties encountered in weighing small amounts of polypeptides. The correct concentrations were determined by quantitative amino acid analysis of stock solutions and used for calculations of dissociation constants. The injection time was 3 min and the clearance time was 10 min.

the peptides and reported concentrations in error of 20−30% are possible. This does not affect conclusions about which binders are the tightest since the titration steps were large, but may give rise to incorrect ranking of the best binders. Seven binders were selected for further binding analysis based on the largest uptake but also on K_D values obtained from the best fit of a 1:1 binding model to the experimental data. Whereas the fit of the 1:1 model is far from excellent and the obtained dissociation constants should be taken with considerable caution, the combined ranking from estimates of uptake from Biacore and the K_D values were in reasonable agreement. For estimated dissociation constants, see Supporting Information. Binders with an uptake of more than 100 RU in the Biacore screening experiment that also had apparent K_D's of around 1 μM or less were considered medium to tight binders, whereas those with an uptake of less than 100 RU and K_D's of higher than 1 μM were considered weak binders and not considered further.

The interactions between the D-dimer protein and the seven selected binders were studied at higher resolution; pure buffer and binders at concentrations 5, 10, 20, 40, 80, and 160 nM were used for SPR interaction analysis (Figure 3A); and concentrations were determined by quantitative amino acid analysis. The sensorgrams show that the binders from the 4-series bind with higher affinity than those from the 3-series, suggesting that more positively charged polypeptides bind better to the surface area of the D-dimer in the vicinity of the binding site. In order to show that the binder molecule binds

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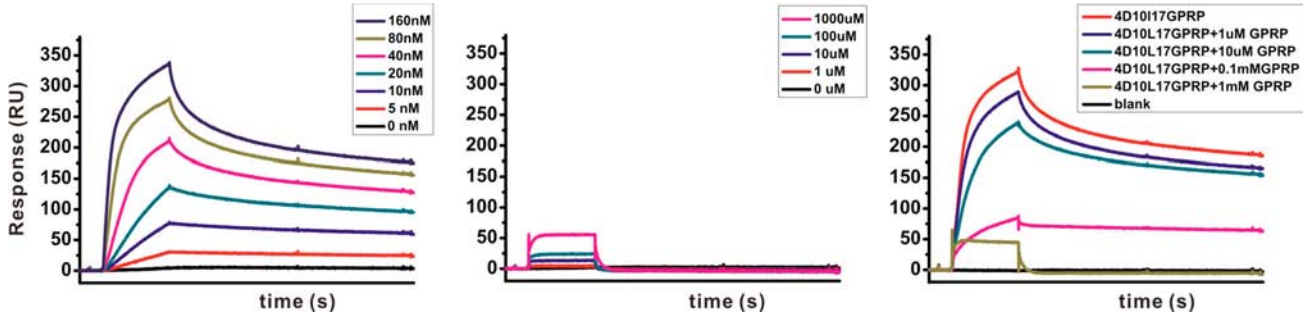

Figure 4. Panel of sensorgrams showing GPRP inhibition of D-dimer binding by 4-D10L17-GPRP. Left sensorgram, binding of immobilized D-dimer by 4-D10L17-GPRP in running buffer at pH 7.4 and 0, 5, 10, 20, 40, 80, and 160 nM of binder. Middle sensorgram, binding of immobilized D-dimer by GPRP in running buffer at pH 7.4 and 0, 1, 10, 100 and 1000 μM and 1 mM of GPRP. Right sensorgram, binding of immobilized D-dimer by 100 nM 4-D10L17-GPRP in running buffer containing 0, 1, 10, 100, and 1000 μM GPRP. Scales of axes are identical for direct comparison of uptake between binders. The injection time was 3 min and the clearance time was 10 min.

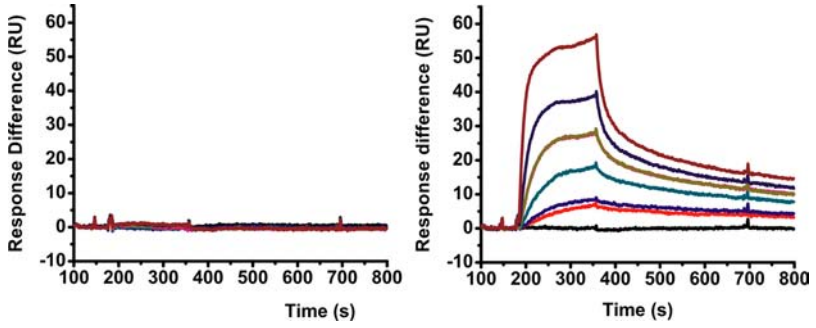

Figure 5. Panel of sensorgrams showing no binding of immobilized D-dimer by polypeptide 4-D15L8 (left) and binding of immobilized D-dimer by polypeptide conjugate 4-D15L8-GPRP (right) in running buffer at pH 7.4 and 0, 5, 10, 20, 40, 80, and 160 nM of polypeptide and binder, respectively. The scales of the axes are identical for direct comparison.

according to design, competition experiments were carried out using Biacore where GPRP over a range of concentrations was included in the running buffer to inhibit binding to the D-dimer (Figure 4). As a control, GPRP binding was also monitored. Binding of 100 nM 4-D10L17-GPRP was completely inhibited by 1 mM GPRP in standard running buffer. The results show that the GPRP residue is critical for binding, that the binder molecule binds specifically to the GPRP binding site, and that conjugation of the polypeptide to GPRP gives rise to a binder with dramatically increased affinity for the D-dimer. In contrast, the polypeptides without conjugated GPRP do not bind to the D-dimer at concentrations used for affinity analysis, showing that the synergy between GPRP and the polypeptides in recognizing the D-dimer is responsible for the high affinity, in good agreement with the binder concept (Figure 5 and Supporting Information). In order to determine a dissociation constant in solution unaffected by surface effects, a fluorescein fluorophore was conjugated to the Cys side chain of the binder 4-C15L8-GPRP. The binder molecule was carefully titrated with the D-dimer protein followed by data analysis where the best fit of an equation describing the dissociation of a 1:1 complex to the experimental results was determined (Figure 6). The dissociation constant was found to be 3 nM, or 4 orders of magnitude larger than that of the solution affinity of the GPRP tetrapeptide.[12]

In order to further improve the binding performance, the binders of the 4-series were dimerized by reacting deprotected Cys residues in the loop regin of the helix−loop−helix motifs with a bifunctional linker to form poly(etheylene glycol) linked helix−loop−helix dimers. The seven binders from the 3- and 4-

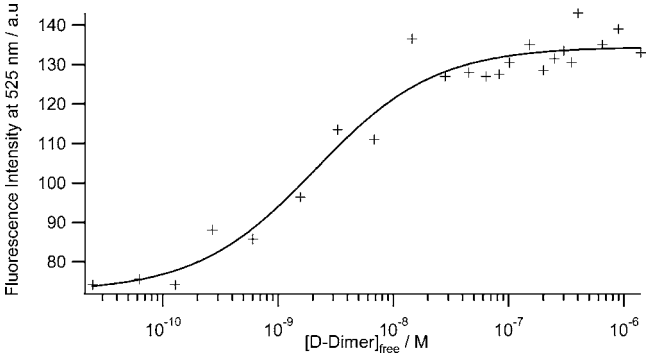

Figure 6. Titration of 4-C15L8-GPRP with the D-dimer protein in phosphate buffered saline at pH 7.5. The best fit of an equation describing the dissociation of a 1:1 complex to the experimental data give a dissociation constant K_D of 3 nM.

series shown to be the strongest binders were dimerized and their binding to the D-dimer analyzed by Biacore (Figure 3B). In comparison with the monomeric binder molecules, increased binder performance was in general observed for all binders based on the observation of increased uptake. It is difficult from the Biacore data to determine quantitatively actual increases in affinity from decreased dissociation constants because of the poor fits of the 1:1 Langmuir model to the experimental data. However, the uptake was clearly increased and the dissociation constants obtained by fitting the 1:1 Langmuir model to the experimental data suggested relative affinity increases by factors of 2−4 as a result of dimerization (see Supporting Information). For the binder 4-C25L22-GPRP, a disulfide

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bridge gave better performance than the PEG spacer and a further affinity boost by a factor of 2, and thus a total of nearly an order of magnitude (data not shown). Dimerization of the binder molecules was thus shown to further increase affinities by an overall factor of 5−10, enabling the development of peptide-based binder molecules with high picomolar affinities from low to medium micromolar affinity peptides, that are readily available, e.g., from early phase phage display generation of peptides. Attaching more well-developed phage display peptides to the designed polypeptides is expected to give rise to proportionally higher affinity binder molecules.

The sensorgrams obtained from binding of the D-dimer are similar to those obtained from sensor analysis of other proteins in that they do not show saturation and application of standard kinetic models to the experimental results do not give good fits.[1] In contrast, fluorescence titration experiments of binding as well as RIFS, reflectance interference spectroscopy,[21] show the expected behavior, and the reasons for the poor fits obtained from Biacore measurements are not clear. The kinetic curves do not show single exponential behavior, but very complex models are needed to elucidate the binding kinetics.

CONCLUSION

The example of the D-dimer shows that in solution binder affinities of polypeptide−peptide conjugates based on the designed set of polypeptides can be more than 4 orders of magnitude higher than that of the short peptide itself and that the technology offers a rapid route to powerful binders from mediocre ones. The experimental results show strong uptake and tight binding of binders based on the GPRP peptide, thus widening the scope of binder development considerably as peptides obtained from various techniques used in molecular biology promises to be rich sources of short peptide ligands for protein binder development. The time saved in preparing efficient binder molecules should enable a more rapid development of tools for the advancement of proteomics and diagnostic applications. The binder concept also provides an opportunity to monitor the fate of small organic molecules or peptides in vivo as well as in vitro for medical imaging, target validation, and drug development at an early stage of development. Endogenous peptides with biological functions can be used to develop much improved binders to explore their biological function and protein epitopes with unclear functions can be analyzed by improving their binder performance toward biological targets. The binder molecules can be used in direct pharmaceutical applications.

ASSOCIATED CONTENT

Supporting Information

SPR panels of sensorgrams from initial screening and tables of dissociation constants obtained by fitting an equation describing a 1:1 interaction model to the experimental results. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare the following competing financial interest(s): A patent application has been submitted covering the binder reported here and the IP is the property of a biotech company in which Lars Baltzer owns more than 5% of the shares.

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REFERENCES

(1) Baltzer, L. (2011) Crossing borders to bind proteins − a new concept in protein recognition based on the conjugation of small organic molecules or short peptides to polypeptides from a designed set. *Anal. Bioanal. Chem. 400*, 1653−1664.

(2) Tegler, L. T., Nonglaton, G., Büttner, F., Caldwell, K., Christopeit, T., Danielson, U. H., Fromell, K., Gossas, T., Larsson, A., Longati, P., Norberg, T., Ramapanicker, R., Rydberg, J., and Baltzer, L. (2011) Powerful protein binders from designed polypeptides and small organic molecules-a general concept for protein recognition. *Angew. Chem., Int. Ed. 50*, 1823−1827.

(3) Enander, K., Dolphin, G. T., and Baltzer, L. (2004) Designed, functionalized helix-loop-helix motifs that bind human carbonic anhydrase II: a new class of synthetic receptor molecules. *J. Am. Chem. Soc. 126*, 4464−4465.

(4) Tegler, L. T., Fromell, K., Jonsson, B.-H., Viljanen, J., Winander, C., Carlsson, J., and Baltzer, L. (2011) Polypeptide conjugate binders that discriminate between two isoforms of Human Carbonic Anhydrase in human blood. *ChemBioChem. 12*, 559−566.

(5) Andersson, T., Lundquist, M., Dolphin, G. T., Enander, K., Jonsson, B.-H., Nilsson, J. W., and Baltzer, L. (2005) The binding of Human Carbonic Anhydrase II by functionalized folded polypeptide receptors. *Chem. Biol. 12*, 1245−1252.

(6) Furie, B., and Furie, B. C. (2005) Thrombus formation in vivo. *J. Clin. Invest. 115*, 3355−3362.

(7) Davie, E. W. (1964) Waterfall sequence for intrinsic blood clotting. *Science 145*, 1310−1312.

(8) Lippi, G., Cervellin, G., Franchini, M., and Favaloro, E. J. (2010) Biochemical markers for the diagnosis of venous thromboembolism: the past, present and future. *J. Throm. Thrombolysis 30*, 459−471.

(9) Wada, H., and Sakuragawa, N. (2008) Are fibrin-related markers useful for diagnosis of thrombosis? *Semin. Thromb. Hemost. 34*, 33−38.

(10) Linkins, L.-A., Bates, S. M., Ginsberg, J. S., and Kearon, C. (2004) Use of different D-dimer levels to exclude venous thromboembolism depending on clinic al pretest probablility. *J. Throm. Haemost. 2*, 1256−1260.

(11) Smith, G. P., and Petrenko, V. A. (1997) Phage display. *Chem. Rev. 97*, 391−410.

(12) Laudano, A. P., and Doolittle, R. F. (1980) Studies on synthetic peptides that bind to fibrinogen and prevent fibrin polymerization. Structural requirements, number of binding sites, and species differences. *Biochemistry 19*, 1013−1019.

(13) Laudano, A. P., and Doolittle, R. F. (1981) Influence of calcium ion on the binding of fibrin amino terminal peptides to fibrinogen. *Science 212*, 457−459.

(14) Olexa, S. A., and Budzynski, A. Z. (1980) Evidence for four different polymerization sites involved in human fibrin formation. *Proc. Natl. Acad. Sci. U.S.A. 77*, 1374−1378.

(15) Pratt, K. P., Cote, H. C. F., Chung, D. W., Stenkamp, R. E., and Davie, E. W. (1997) The primary fibrin polymerization pocket: three-dimensional structure of a 30-kDa C-terminal γ chain fragment complexed with the peptide GLy-Pro-Arg-Pro. *Proc. Natl. Acad. Sci. U.S.A. 94*, 7176−7181.

(16) Spraggon, G., Everse, S. J., and Doolittle, R. F. (1997) Crystal structures of fragment D from human fibrinogen and its crosslinked counterpart from fibrin. *Nature 389*, 455−462.

(17) Everse, S. J., Spraggon, G., Veerapandian, L., Riley, M., and Doolittle, R. F. (1998) Crystal structure of fragment double-D from human Fibrin with two different bound ligands. *Biochemistry 37*, 8637−8642.

(18) Doolittle, R. F., and Pandi, L. (2007) Probing the β-chain hole of fibrinogen with synthetic peptides that differ at their amino termini. *Biochemistry 46*, 10033−10038.
(19) Gao, J., Qiao, S., and Whitesides, G. M. (1995) Increacing binding constants of ligands to carbonic anhydrase by using "greasy" tails. *J. Med. Chem. 38*, 2292−2301.
(20) Jain, A., Huang, S. G., and Whitesides, G. M. (1994) Lack of effect of the length of oligoglycine- and oligo(ethylene glycol)-derived para-substituents on the affinity of benzenesulfonamides for carbonic anhydrase II in solution. *J. Am. Chem. Soc. 116*, 5057−5062.
(21) Albrecht, C., Fechner, P., Honcharenko, D., Baltzer, L., and Gauglitz, G. (2010) A new assay design for clinical diagnostics based on alternative recognition elements. *Biosens. Bioelectron. 25*, 2302−2308.