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A synthetic polypeptide conjugate from a 42-residue polypeptide and salicylhydroxamic acid binds human myeloperoxidase with high affinity

Xiaojiao Sun, Jie Yang, Thomas Norberg* and Lars Baltzer

Myeloperoxidase (MPO) is a 150 kD tetrameric heme protein consisting of two heavy chains and two light chains, which is present in neutrophils, white blood cells, at concentrations between 2% and 5% and plays an important role in the innate immune system. The MPO concentration in serum or plasma has been shown to be linked to the risk for cardiovascular diseases, and MPO is considered to be a high potential diagnostic biomarker. To develop a molecule that binds MPO, salicylhydroxamic acid (SHA), a substrate analog inhibitor of MPO with a $K_D = 2\,\mu M$, was conjugated to a designed set of 42-residue polypeptide scaffolds via 9- and 11-carbon atom aliphatic spacers to form 20 different protein binder candidates, and their interactions with MPO were evaluated by surface plasmon resonance analysis. The polypeptide conjugate 4C37L34C11SHA was found to bind to MPO with an affinity that could be estimated to have a dissociation constant of around 400 pM, nearly four orders of magnitude higher than that of SHA. Inhibition of binding to MPO by free SHA was observed in competition experiments demonstrating that the binding of the polypeptide conjugate is dominated by the interactions of SHA with the heme cavity. Although still in the future, the discovery of these new synthetic binders for MPO suggests a route to clinical diagnostic tests *in vivo* or *in vitro*, independent of antibodies. Copyright © 2012 European Peptide Society and John Wiley & Sons, Ltd.

Supporting information can be found in the online version of this article.

Keywords: polypeptide; conjugate; salicylhydroxamic acid; myeloperoxidase

Introduction

Cardiovascular diseases (CVD) remain the leading cause of death globally, and more than 17 million people died from CVD in 2008. By 2030, more than 23 million people are expected to die from CVD, mainly from heart disease and stroke [1]. Diagnostic tools are in great demand that enable early stage discovery of CVD for therapeutic intervention and monitoring of disease as a function of treatment.

Myeloperoxidase (MPO) is a tetrameric, glycosylated, basic heme protein with two heavy and two light chains and a pI of 9.2. MPO has a molecular weight of around 150 kD, depending on isoform, and is abundant [2,3] in white blood cells (leucocytes). Three isoforms are known, which only differ by the size of the two heavy chains. MPO catalyzes the formation of reactive species such as hypochlorous acid, which kills invading bacteria and other pathogens as part of the innate immune defense. During the acute inflammatory condition, MPO is released in the extracellular medium and produces large amounts of oxidants to kill the invading organisms. However, an excess of MPO-generated oxidants do not only kill invading organisms but also contribute to tissue damage during inflammation and also to potentially proatherogenic biological activities throughout the development of CVD. Correlations have been demonstrated between elevated levels of MPO and CVD, e.g. atherosclerosis, acute coronary syndrome, vascular endothelial dysfunction, and multiple sclerosis [4,5], and MPO has therefore been suggested as a diagnostic biomarker for the early detection of CVD. In a healthy individual, the MPO level is low, with a few to a hundred micrograms per liter (20–600 pM) in serum or plasma, whereas in patients with increased risks of a heart infarction, the level of MPO is higher, and a cut-off value of 350 μg/l (2.3 nM) has been suggested [6–8]. It has also been suggested that the measurement of MPO in combination with the C-reactive protein (CRP) could provide improved precision in diagnosis [5].

Diagnostic protein biomarkers for CVD are routinely used in the clinic, and antibodies remain the golden standard in bioanalytical applications including *in vitro* diagnostics. Currently, the measurement of MPO is typically carried out by immunological methods using anti-MPO antibodies as recognition elements with a detection limit of 1.6 μg/l (10 pM). Drawbacks of antibody technologies in comparison with molecules prepared by chemical synthesis include poor stability, batch-to-batch variation, non-specific interactions with native proteins, and difficulties encountered in site-specific modification. Small organic synthetic binder molecules that recognize and bind proteins with sufficient

* *Correspondence to: Thomas Norberg, Department of Chemistry-BMC, Uppsala University, PO Box 576, SE-751 23 Uppsala, Sweden. E-mail: thomas.norberg@kemi.uu.se*

Department of Chemistry-BMC, Uppsala University, PO Box 576, SE-751 23 Uppsala, Sweden

affinities and selectivities for diagnostic applications have so far proven to be cumbersome to develop, and the efforts required to develop small organic molecules that bind proteins efficiently seem only to be justified in pharmaceutical applications. New strategies for protein binder development that address these problems are of considerable interest.

Recently, we proposed a general binder concept that uses conjugates from small organic molecules and 42-residue polypeptides to form hybrid molecules that bind proteins with affinities that are enhanced by three to four orders of magnitude in comparison with those of the small molecules and selectivities that are enhanced by one to two orders of magnitude [9–11]. The concept is robust and can be used to prepare selective, high-affinity binders for, in principle, any protein in short time by organic synthesis, provided that a small molecule or peptide is available, which binds the protein with at least modest affinity and selectivity. We have previously reported on a polypeptide conjugate that binds CRP, and to develop a set of binder molecules that could in principle be used to address CVD in a fully synthetic measurement system, we have now developed a polypeptide conjugate that binds MPO. We therefore wish to report on a synthetic polypeptide conjugate binder for MPO based on salicylhydroxamic acid (SHA), a substrate analog inhibitor of MPO with a $K_D = 2\ \mu M$, which was conjugated to a set of designed polypeptides via 9 and 11 carbon aliphatic spacers. The best binder molecule was selected from the resulting set of binder candidates, and a powerful binder for MPO was identified using surface plasmon resonance (SPR) analysis. The binder concept illustrated for a binder for MPO is shown in Figure 1.

Materials and Methods

General Methods

All solvents used, including DMF, *N*-methyl-2-pyrrolidone (NMP), DCM, THF, ethyl acetate (EA), pentane (PE), chloroform, and diethyl ether, were commercially available. DCM was distilled after drying over CaH_2. THF was distilled after refluxing/drying over sodium/benzophenone. Reagents were used as obtained from commercial sources unless otherwise stated. Concentrations were performed at reduced pressure and a temperature below 40 °C. Measurement of pH was carried out using pH paper (pH range 1–14). Flash chromatography was performed using silica gel 60 (0.040–0.063 mm) as the stationary phase. TLC was performed on aluminum plates pre-coated with silica gel (Merck silica gel 60 F254), and spots were visualized by either UV light, iodine vapor, 8% ethanolic phosphomolybdic acid (dipping/heating), or 5% ethanolic sulfuric acid (dipping/heating). ^{1}H NMR and ^{13}C NMR spectra for $CDCl_3$ solutions were recorded at 25 °C on a Varian Unity Inova 400 spectrometer. Chemical shifts are reported in parts per million using either $CHCl_3$/TMS as reference ($\delta = 7.26/0.00$ for ^{1}H NMR) or $CDCl_3$ ($\delta = 77.0$ for ^{13}C NMR). Mass spectra of small molecules (MW < 1000) were recorded on a SCIEX API 150-EX mass spectrometer by direct injection of diluted samples in methanol. Conjugation reactions were monitored by analytical RP-HPLC on an analytical C18 column (Grace Vydac, pore size: 300 Å; particle size: 5 μM; length: 15 cm; diameter: 4.6 mm) using a 30-min gradient of 5–95% of 90% acetonitrile in water containing 0.1% TFA at a flow rate of 1 ml/min. The crude peptides were purified by RP-HPLC on a preparative Hypersil Gold C18 column (Thermo Scientific, pore

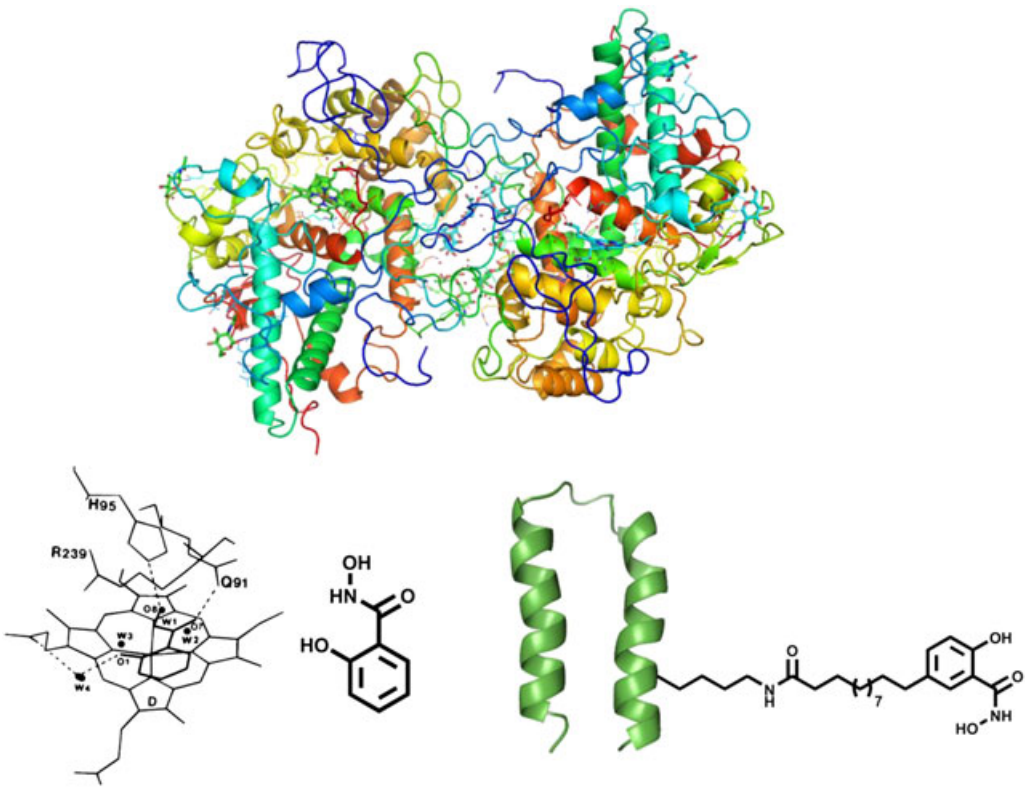

Figure 1. Illustration of the principle of myeloperoxidase (MPO) binder molecule assembly. Salicylhydroxamic acid (SHA) is known to bind to the two heme cavities with a dissociation constant, K_D, of 2 μM. The three oxygen atoms of SHA are involved in hydrogen bond formation, and the aromatic ring is important for hydrophobic interactions in the active site [16]. SHA was covalently linked to a lysine residue in each of the twenty 42-residue polypeptides by 9- or 11-carbon aliphatic spacers attached to the aromatic ring. In the sequences, no other free lysines were accessible during conjugation to ascertain that only one SHA residue was introduced. The structure of MPO was visualized by PyMOL 1.1 from X-ray structure (PDB code: 1MHL).

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size: 175 Å; particle size: 5 Å; length: 25 cm; diameter: 21.2 mm) eluted with a 40-min gradient of 25–75% of 90% acetonitrile in water containing 0.1% TFA at a flow rate of 10 ml/min. Peptides and peptide conjugates were identified by MALDI-TOF MS (Voyager-DE Pro, Applied Biosystems, Carlsbad, CA, USA), using α-cyano-4-hydroxycinnamic acid as matrix.

Small Molecule Synthesis

*Preparation of **2a** and **2b**, general*

Anhydrous aluminum chloride (7.2 g, 54.2 mmol) was added in portions to a stirred and cooled (−15 °C or below) solution of ethyl salicylate (**1**; 5 g, 30.1 mmol) in dichloroethane (9.3 ml), then sebacoyl chloride (6.4 mL, 29.9 mmol) or dodecanedioyl dichloride (8.0 g, 29.8 mmol) was added dropwise, and the mixture was kept at −15 to −20 °C for 3–4 h. The mixture was then allowed to reach room temperature and was further stirred overnight. Solvents were removed under vacuum, and the remaining orange solid was transferred into a mixture of water (200 ml) and DCM (60 ml). The aqueous solution was acidified to pH 3 with concentrated hydrochloric acid. A white precipitate was formed and filtered off. The aqueous solution was extracted with DCM (2 × 30 ml), and the organic layer was dried over anhydrous $MgSO_4$. The solvent was evaporated, and the residue was purified by flash column chromatography (PE/EA 10 : 1 to 1 : 1) to afford **2a** or **2b** as a colorless solid.

*Ethyl 5-(9′-carboxy-1′-oxononyl)-2-hydroxybenzoate (**2a**).* Yield: 4.4 g, 50%, ^{1}H NMR ($CDCl_3$, 400 MHz): δ 11.32 (s, 1H, OH), 8.49 (d, J = 2.3 Hz, 1H, ArH), 8.07 (dd, J = 8.8, 2.3 Hz, 1H, ArH), 7.02 (d, J = 8.8 Hz, 1H, ArH), 4.46 (q, J = 7.2 Hz, 2H, CH_2), 2.91 (t, J = 7.4 Hz, 2H, CH_2COPh), 2.35 (t, J = 7.6 Hz, 2H, CH_2COOH), 1.72 (m, 2H, CH_2), 1.63 (m, 2H, CH_2), 1.45 (t, J = 7.2 Hz, 3H, CH_3), 1.27–1.4 (bm, 8H, 4×CH_2); ^{13}C NMR ($CDCl_3$, 100.6 MHz): 198.3, 180.0, 169.7, 165.1, 135.1, 130.9, 128.6, 117.7, 112.2, 61.9, 38.0, 33.9, 29.1, 29.0, 28.9, 24.5, 24.2, 14.1 (one aliphatic carbon was overlapped). ESI-MS calcd for $C_{19}H_{26}O_6$ 350.1, found m/z 373.2 $[M + Na]^+$, 349.4 $[M - H]^-$.

*Ethyl 5-(11′-carboxy-1′-oxoundecyl)-2-hydroxybenzoate (**2b**).* Yield: 4.6 g, 41%, ^{1}H NMR ($CDCl_3$, 400 MHz): δ 11.32 (s, 1H, OH), 8.50 (d, J = 2.3 Hz, 1H, ArH), 8.08 (dd, J = 8.8, 2.3 Hz, 1H, ArH), 7.03 (d, J = 8.8 Hz, 1H, ArH), 4.46 (q, J = 7.2 Hz, 2H, CH_2), 2.91 (t, J = 7.6 Hz, 2H, CH_2), 2.35 (t, J = 7.5 Hz, 2H, CH_2), 1.72 (m, 2H, CH_2), 1.63 (m, 2H, CH_2), 1.45 (t, J = 7.2 Hz, 3H, CH_3), 1.23–1.39 (m, 12H, 6×CH_2). ^{13}C NMR ($CDCl_3$, 100.6 MHz) δ 198.3, 179.9, 170.0, 165.0, 135.3, 131.0, 128.8, 117.9, 111.9, 52.6, 38.1, 4.0, 29.4, 29.4, 29.3, 29.3, 24.6, 14.1. ESI-MS calcd for $C_{21}H_{30}O_6$, 378.2, found m/z 401.2 $[M + Na]^+$, 377.0 $[M - H]^-$.

*Preparation of **3a** and **3b**, general*

To a 100 ml flask was added zinc dust (4 g, 61.5 mmol) and $HgCl_2$ (77.6 mg, 0.29 mmol) in water (4 ml). The suspension was agitated for 15 min, the liquid was then decanted, and the amalgamated zinc was washed with water. Compound **2a** (1.0 g, 2.9 mmol) or **2b** (1.1 g, 3.0 mmol) was added, followed by concentrated HCl (6 ml) and EtOH (6 ml). The mixture was refluxed with stirring for 3–5 h, then cooled to room temperature, and DCM (40 ml) was added. The aqueous layer was extracted with DCM (2 × 40 ml), washed with water and brine (3 × 20 ml), and dried with $MgSO_4$. The solvent was removed, and the residue was dissolved in ethanol (99.5%, 20 ml). Then, thionyl chloride (0.8 ml) was added dropwise to make a 2% HCl/ethanol solution. After 1 h of stirring at room temperature, the solvent was evaporated, and the residual yellow oil was purified by column chromatography (PE/EA 9 : 1 to 2 : 1) to afford pure **3a** or **3b**.

*Ethyl 5-(9′-ethoxycarbonylnonyl)-2-hydroxybenzoate (**3a**).* Yield: 0.83 g, 80%,^{1}H NMR ($CDCl_3$, 400 MHz): δ 10.68 (s, 1H, OH), 7.62 (d, J = 2.3 Hz, 1H, ArH), 7.27 (dd, J = 8.4, 2.3 Hz, 1H, ArH), 6.90 (d, J = 8.4 Hz, 1H, ArH), 4.41 (q, J = 7.1 Hz, 2H, CH_2), 4.12 (q, J = 7.2 Hz, 2H, CH_2), 2.53 (t, J = 8.0 Hz, 2H, CH_2Ph), 2.28 (t, J = 7.7 Hz, 2H, CH_2COOEt), 1.58 (m, 4H, 2×CH_2), 1.42 (t, J = 7.1 Hz, 3H, CH_3), 1.25 (t, J = 7.2 Hz, 3H, CH_3), 1.27–1.33 (bm, 10H, 5×CH_2); ^{13}C NMR ($CDCl_3$, 100.6 MHz): 173.9, 170.2, 159.7, 135.9, 133.4, 128.9, 117.3, 112.2, 61.3, 60.1, 34.4, 31.6, 29.4, 29.3, 29.2, 29.1, 29.0, 24.9 14.2. ESI-MS m/z calcd for $C_{21}H_{32}O_5$ 364.2, found m/z 387.2 $[M + Na]^+$, 751.6 $[2M + Na]^+$.

*Ethyl 5-(11′-ethoxycarbonylundecyl)-2-hydroxybenzoate (**3b**).* Yield: 0.90 g, 76%, ^{1}H NMR ($CDCl_3$, 400 MHz): δ 10.66 (s, 1H, OH), 7.62 (d, J = 2.3 Hz, 1H, ArH), 7.26 (dd, J = 8.5, 2.3 Hz, 1H, ArH), 6.88 (d, J = 8.5 Hz, 1H, ArH), 4.40 (q, J = 7.1 Hz, 2H, CH_2), 4.12 (q, J = 7.1 Hz, 2H, CH_2), 2.53 (t, J = 8.0 Hz, 2H, CH_2), 2.28 (t, J = 7.9 Hz, 2H, CH_2), 1.58 (m, 4H, 2×CH_2), 1.42 (t, J = 7.1 Hz, 3H, CH_3), 1.17–1.34 (bm, 17H, 7×CH_2 and CH_3); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 173.9, 170.3, 159.7, 135.9, 133.4, 129.0, 117.3, 112.3, 61.3, 60.1, 34.9,34.3, 31.6,29.5, 29.4, 29.4, 29.2, 29.2, 29.1, 24.9, 14.2. ESI-MS m/z calcd for $C_{23}H_{36}O_5$ 392.2, found m/z 415.2 $[M + Na]^+$.

*Preparation of **4a** and **4b**, general*

To a dried 100 ml round bottom flask was added **3a** (0.58 g, 1.6 mmol) or **3b** (0.63 g, 1.60 mmol), sodium hydride (60% dispersion in mineral oil, 0.16 g, 4.0 mmol), followed by dry THF (24 ml) in a nitrogen atomosphere. The suspension was stirred and cooled in ice for 30 min. Methoxyethoxymethyl chloride (0.45 ml, 4.0 mmol) was added dropwise. After stirring for 2 h at 0 °C, the reaction mixture was warmed to room temperature and stirred overnight. The mixture was concentrated, and then a saturated aqueous solution of sodium bicarbonate was added. The resulting mixture was extracted with DCM (3 × 10 ml). The combined organic layers were washed with H_2O and brine, then dried with $MgSO_4$, and concentrated. The residue was purified by flash chromatography (PE/EA 2 : 1) to afford **4a** or **4b** as a faint yellow oil.

*Ethyl 5-(9′-ethoxycarbonylnonyl)-2-[(2″-methoxy)-ethoxymethyloxy] benzoate (**4a**).* Yield: 0.54 g, 76%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.54 (d, J = 2.2 Hz, 1H, ArH), 7.21 (dd, J = 8.4, 2.2 Hz, 1H, ArH), 7.12 (d, J = 8.4 Hz, 1H, ArH), 5.29 (s, 2H, OCH_2O), 4.33 (q, 2H, CH_2CH_3), 4.10 (q, 2H, CH_2CH_3), 3.86 (m, 2H, OCH_2CH_2O), 3.54 (m, 2H, OCH_2CH_2O), 3.35 (s, 3H, CH_3), 2.54 (t, J = 7.1 Hz, 2H, CH_2Ph), 2.26 (t, J = 7.2 Hz, 2H, CH_2COOEt), 1.57 (m, 4H, 2×CH_2), 1.36 (t, J = 7.1 Hz, 3H, CH_3), 1.23 (t, J = 7.2 Hz, 3H, CH_3), 1.23–1.32 (bm, 10H, 5×CH_2); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 173.7, 166.2, 154.5, 136.0, 133.0, 129.7, 121.4, 116.6, 94.2, 71.4, 67.7, 60.7, 60.0, 58.8, 34.8, 34.2, 31.3, 29.3, 29.2, 29.1, 29.0, 28.9, 24.8, 14.2, 14.1. ESI-MS m/z calcd for $C_{25}H_{40}O_7$ 452.2, found m/z 475.0 $[M + Na]^+$.

*Ethyl 5-(11′-ethoxycarbonylundecyl)-2-[(2″-methoxy)-ethoxymethyloxy] benzoate (**4b**).* Yield: 0.54 g, 71%. Crude ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.55 (d, J = 2.4 Hz, 1H), 7.23 (dd, J = 8.5, 2.4 Hz, 1H), 7.14 (d, J = 8.5 Hz, 1H), 5.30 (s, 2H), 4.34 (q, J = 7.1 Hz, 2H), 4.12 (q, J = 7.1 Hz, 2H), 3.90–3.85 (m, 2H), 3.58–3.53 (m, 2H), 3.37 (s, 3H), 2.58 (t, 2H), 2.24 (t, 2H), 1.66–1.50 (m, 4H), 1.38 (t, J = 7.1 Hz, 3H), 1.30–1.21 (m, 17H). ESI-MS m/z calcd for $C_{27}H_{44}O_7$ 480.3, found m/z 503.2 $[M + Na]^+$.

*Preparation of **5a** and **5b**, general*

To a stirred solution of **4a** (289 mg, 0.6 mmol) or **4b** (306 mg, 0.6 mmol) in MeOH (2.6 ml) was added aqueous 1 M NaOH (2.6 ml, 2.6 mmol). The resulting mixture was kept at 60 °C for 4 h. After cooling, the organic solvent was removed under reduced pressure, and the aqueous layer was washed with EA (3 × 20 ml). The resulting solution was then acidified with aqueous 10% citric acid to pH 4.5 and then extracted with EA (3 × 5 ml). The combined organic extracts were washed with H_2O (3 × 5 ml), dried over anhydrous $MgSO_4$, and concentrated to afford **5a** or **5b** as a colorless liquid.

*5-(9′-Carboxynonyl)-2-[(2″-methoxy)-ethoxymethyloxy]benzoic acid (**5a**).* Yield: 0.23 g, 89%. ^{1}H NMR ($CDCl_3$, 400 MHz): δ 7.94 (d, *J* = 2.1 Hz, 1H, ArH), 7.33 (dd, *J* = 8.4, 2.1 Hz, 1H, ArH), 7.19 (d, *J* = 8.4 Hz, 1H, ArH), 5.46 (s, 2H, OCH_2O), 3.89 (m, 2H, OCH_2CH_2O), 3.57 (m, 2H, OCH_2CH_2O), 3.36 (s, 3H, CH_3), 2.58 (t, *J* = 7.9 Hz, 2H, CH_2), 2.34 (t, *J* = 7.5 Hz, 2H, CH_2),1.60 (m, 4H, 2×CH_2), 1.21–1.37 (bm, 10H, 5×CH_2); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 179.5, 166.5, 154.2, 137.5, 134.9, 132.9, 118.0, 115.0, 94.7, 71.4, 68.9, 59.0, 34.8, 34.0, 31.3, 29.3, 29.2, 29.1, 29.0, 28.9, 24.6. ESI-MS *m/z* calcd for $C_{21}H_{32}O_7$ 396.2, found *m/z* 419.2 $[M + Na]^+$.

*5-(11′-Carboxyundecyl)-2-[(2″-methoxy)-ethoxymethyloxy]benzoic acid (**5b**).* This product was not purified but was directly used in the next step. Crude ^{1}H NMR ($CDCl_3$, 400 MHz): δ 10.42 (bs, 1H, COOH), 7.91 (d, *J* = 2.3 Hz, 1H, ArH), 7.31 (dd, *J* = 8.5, 2.3 Hz, 1H, ArH), 7.18 (d, *J* = 8.5 Hz, 1H, ArH), 5.44 (s, 2H, OCH_2O), 3.88 (ddd, *J* = 2.7 Hz, 1.8 Hz, 4.5 Hz, 2H, OCH_2CH_2O), 3.56 (ddd, *J* = 2.7 Hz, 1.8 Hz, 4.5 Hz, 2H, OCH_2CH_2O), 3.35 (s, 3H, CH_3), 2.57 (t, *J* = 7.8 Hz, 2H, CH_2), 2.34 (t, *J* = 7.6 Hz, 2H, CH_2),1.59 (m, 4H, 2×CH_2), 1.15–1.36 (bm, 14H, 7×CH_2). ESI-MS *m/z* calcd for $C_{23}H_{36}O_7$ 424.2, found *m/z* 447.2 $[M + Na]^+$.

*Preparation of **7a** and **7b**, general*

Dry DCM (33 mL) was added to **5a** (189 mg, 0.47 mmol) or **5b** (203 mg, 0.47 mmol), 4-nitrophenol (67 mg, 0.47 mmol), *N*,*N*-dicyclohexylcarbodiimide (99 mg, 0.47 mmol), and 4-*N*,*N*-dimethylaminopyridine (5.8 mg, 0.096 mmol) under a nitrogen atmosphere. The reaction mixture was stirred overnight, washed with brine and water, dried with $MgSO_4$, and concentrated. The residue was purified by column chromatography (DCM/methanol 30 : 1) to afford a mixture of 4-nitrophenyl monoesters (aliphatic and aromatic), which was used in the next step without further purification. To an ice-cooled solution of this mixture in DCM (15 ml) under an atmosphere of nitrogen were added, consecutively, hydroxylbenzotriazole (68 mg, 0.503 mmol), diisopropylcarbodiimide (DIPCI; 77.9 µl, 0.503 mmol), *O*-tritylhydroxylamine (115 mg, 0.419 mmol), and TEA (23.4 µl, 0.168 mmol). The resulting mixture was then stirred for 15 h at room temperature, diluted with DCM (15 ml), washed with aqueous 1% citric acid solution (3 × 15 ml), saturated aqueous $NaHCO_3$ (3 × 15 ml), and water (3 × 15 mL), dried over anhydrous $MgSO_4$, and concentrated. The residue was purified by flash chromatography (DCM/MeOH 35 : 1) to afford **7a** or **7b** as a colorless liquid.

5-[9′-(4‴-Nitrophenyloxycarbonyl)-nonyl]-2-[(2″-methoxy)-ethoxymethyloxy]-N-trityloxybenzamide (**7a**). Yield: 102 mg, 28% (total yield of final two steps). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.71 (s, 1H, NH), 8.26 (dm, *J* = 9.2 Hz, 2H, *p*-NO_2ArH), 7.87 (d, *J* = 2.0 Hz, 1H, ArH), 7.56 (m, 6H, Trityl), 7.39–7.19 (bm, 11H, Trityl and *p*-NO_2ArH), 7.14 (dd, *J* = 8.6, 2.0 Hz, 1H, ArH), 7.03 (d, *J* = 8.6 Hz, 1H, ArH), 4.89 (s, 2H, OCH_2O), 3.43 (m, 2H, OCH_2CH_2O), 3.42 (m, 2H, OCH_2CH_2O), 3.32 (s, 3H, CH_3), 2.59 (t, *J* = 7.6 Hz, 2H, CH_2), 2.53 (t, *J* = 7.7 Hz, 2H, CH_2), 1.75 (m, 2H, CH_2), 1.55 (m, 2H, CH_2), 1.44–1.18 (bm, 10H, 5×CH_2); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 171.3, 163.0, 152.6, 142.2, 137.0, 132.8, 131.5, 128.9, 128.0, 127.7, 125.2, 122.4, 120.1, 114.4, 93.4, 71.3, 68.0, 59.0, 34.8, 34.3, 31.3, 29.3 (2 carbons), 29.1 (2 carbons), 29.0, 24.7. (3 quaternary carbons were not found). ESI-MS calcd for $C_{46}H_{50}N_2O_9$ 774.3, found *m/z* 797.4 $[M + Na]^+$.

5-[11′-(4‴-Nitrophenyloxycarbonyl)-undecyl]-2-[(2″-methoxy)-ethoxymethyloxy]-N-trityloxybenzamide (**7b**). Yield: 56 mg, 15% (total yield of final two steps). ^{1}H NMR ($CDCl_3$, 400 MHz): δ 9.70 (s, 1H, NH), 8.26 (m, 2H, *p*-NO_2ArH), 7.87 (d, *J* = 2.4 Hz, 1H, ArH), 7.56 (bm, 5H, Trityl and *p*-NO_2ArH), 7.38–7.22 (m, 12H, Trityl), 7.14 (dd, *J* = 8.6, 2.4 Hz, 1H, ArH), 7.03 (d, *J* = 8.6 Hz, 1H, ArH), 4.89 (s, 2H, OCH_2O), 3.44 (multiplet, 2H, OCH_2CH_2O), 3.42 (multiplet, 2H, OCH_2CH_2O), 3.33 (s, 3H, CH_3), 2.60 (t, *J* = 7.8 Hz, 2H, CH_2), 2.53 (t, *J* = 7.6 Hz, 2H, CH_2), 1.76 (m, 2H, CH_2), 1.55 (m, 2H, CH_2), 1.46–1.18 (bm, 14H, 7×CH_2); ^{13}C NMR ($CDCl_3$, 100.6 MHz): δ 171.3, 163.0, 155.5, 152.6, 142.2, 137.0, 132.6, 131.5, 128.9, 128.0, 127.6, 125.2, 122.4, 120.1, 114.3, 93.4, 92.6, (95.4), 71.3, 68.0, 59.0, 34.8, 34.3, 31.3, 29.5, 29.4, 29.3, 29.1, 29.0, 24.7. ESI-MS calcd for $C_{48}H_{54}N_2O_9$ 802.3, found *m/z* 825.4 $[M + Na]^+$.

Polypeptide Synthesis

The polypeptides were synthesized on a 433A peptide synthesizer (Applied Biosystems, Carlsbad, CA, USA) using standard Fmoc chemistry with HBTU (Iris Biotech GmbH, Marktredwitz, Germany) and DIPEA (Iris Biotech GmbH) as activating agents. Fmoc deprotection of the amino terminal was performed using 20% piperidine in NMP. The synthesis was performed on a 0.1 mmol scale using Fmoc-PAL-polyethyleneglycol-polystyrene resin (Fmoc-PAL-PEG-PS, Applied Biosystems) with a substitution level of 0.19 mmol/g. A 10-fold excess of amino acid was used in each coupling, and activation was with a mixture of HBTU (0.5 M in DMF) and DIPEA (2 M in NMP). The side chains of the amino acids (Iris Biotech GmbH) were protected by base-stable groups: *tert*-butyl ester (Asp and Glu), Trt (His, Asn, and Gln), 2,2,4,6,7-pentamethyldihydrobenzofuran-5-sulfonyl (Arg), and acetamidomethyl (Cys).

For the site-selective incorporation of a fluorescent probe and ligands, the side chains of lysine residues were N-protected by an Alloc group, which can be selectively deprotected by treatment with tetrakis(triphenylphosphine) palladium(0) ($Pd(PPh_3)_4$), a Boc group, which can be deprotected by TFA, or an Mtt group, which can be deprotected by a mixture of TFA, triisopropylsilane (TIS), and DCM (v/v/v 1 : 1 : 500). The N-terminal of the polypeptide was capped manually with a mixture of acetic anhydride, DIPEA, and NMP (v/v/v 10 : 5 : 85) for 20 min. After complete machine synthesis, the resin was rinsed with DMF and DCM and dried in a desiccator under vacuum.

Before the cleavage of the peptide from the resin, the Alloc-protected lysine was deprotected by three equivalents of tetrakis(triphenylphosphine)palladium(0) in a mixture of acetic acid, N-methylmorpholine, and chloroform (v/v/v 2 : 1 : 17; 10 ml/g of resin) at room temperature under N_2 for 2.5 h. The resin was then sequentially washed with 30 mM DIPEA in DMF, 20 mM diethyldithiocarbamic acid in DMF, DMF, and DCM and then desiccated. For the chromophore coupling, the resin was mixed with 4 equiv of 7-methoxycoumarin-3-carboxylic acid, 4 equiv of HBTU, and 6 equiv of DIPEA in DMF (4 ml/g of resin) and was gently shaken for 4 h at room temperature in a mini-rocker. Alternatively, the resin was mixed with 4 equiv of dansyl chloride and

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8 equiv of DIPEA in DMF (4 ml/g of resin) and gently shaken for 2 h. The resin was then washed with DMF and DCM and desiccated. The peptide was cleaved from the resin using a mixture of TFA, TIS, and water (v/v/v 95 : 2.5 : 2.5; 10 ml/g of resin) for 2 h at room temperature. The mixture was filtered, and TFA was then evaporated from the filtrate using a stream of nitrogen. The polypeptide was precipitated by the addition of cold ether, centrifuged, redissolved in water, and lyophilized. The crude peptides were purified by RP-HPLC and characterized by MALDI-TOF MS.

Conjugation Procedure

The ligands were conjugated to the free lysyl amino group of the 42-residue polypeptides by the addition of 2.5 equiv of **7a** or **7b** in DMSO stock solution (50 mM) to a solution of the polypeptide in DMSO (2 mM) in the presence of 30 equiv of DIPEA or TEA. 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 (95 : 2.5 : 2.5) was slowly added to the precipitated polypeptide to make a 2 mM polypeptide solution, which was kept at room temperature for 2–3 h, after which the TFA was evaporated by a stream of nitrogen. The deprotected polypeptide was precipitated by the addition of cold ether and centrifuged. The pellet was purified by semi-preparative HPLC, and the appropriate fractions were lyophilized to afford the pure functionalized polypeptides, which were characterized by MALDI-TOF MS (see supporting information) in yields of 10–65%.

Screening of Polypeptide Conjugates with Immobilized MPO

Human myeloperoxidase (MW = 145 kDa) or human myeloperoxidase isoform C (MW = 146 kDa) was obtained commercially (Biodesign Inc., Täby, Sweden, and Lee Biosolution, Inc. St Louis, MO, USA, respectively). The 42-residue polypeptide-SHA conjugates were synthesized as described. Human myeloperoxidase (1 mg/ml) in 50 mM sodium acetate, 0.1 M NaCl (pH 6.0) and myeloperoxidase isoform C (1.9 mg/ml) in sodium acetate buffer (pH 6.0) were diluted in de-ionized water or 10 mM sodium acetate (pH 6.0) to give a concentration of about 50 μg/ml. The MPO was covalently immobilized to the sensor chip surface by amine coupling with PBS (10 mM phosphate, 2.7 mM KCl, 137 mM NaCl, and 0.005% P_{20}, pH 7.4) (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/*N*-hydroxysuccinimide (200 mM EDC/50 mM *N*-hydroxysuccinimide) (GE Healthcare Bio-Sciences). The MPO was injected for 5–7 min at a flow rate of 3–5 μl/min over the activated surface and followed by 7-min pulse of 1 M ethanolamine at pH 8.5 to deactivate the remaining active ester groups. The final immobilization levels were between 11 000 and 28 000 RU. The interaction experiments between MPO and binders and the competitive experiments were carried out using a Biacore 2000 instrument (GE Healthcare Bio-Sciences), equilibrated at 25 °C. The CM5 sensor chip (Research grade, Biacore) and reagents were from GE Healthcare Bio-Sciences. For direct interaction studies between immobilized MPO and polypeptide conjugates, PBS containing 1–3% DMSO and 0.005% P20 was used as a running buffer. The polypeptide conjugates were diluted from a 50 uM stock solution in the running buffer and injected over the immobilized enzyme in concentration series of 5–640 nM for 4-series 9-carbon linked conjugates, 5–320 nM for 4-series 11-carbon linked conjugates, and 0.5–32 μM for 1-, 2-, 3-series 9-carbon linked conjugates using 3 min contact time. For minimum sample dispersion, the samples were injected at a flow rate of 50 μl/min. After 10 min dissociation, the surface was regenerated by 30-s pulse injection of 10 mM glycine, 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.

Competition Experiments

Human myeloperoxidase isoform C was immobilized to the sensor chip in a final immobilization level of 15 491 RU. The detailed procedure was as described previously. A total of 0.5, 5, and 50 μM of 4C37L34C11SHA in running buffer were prepared from 0.731 mM 4C37L34C11SHA DMSO solution. We used 6.52 mM SHA in running buffer for the preparation of the different concentrations. In these binding assays, to reach the saturation of binding, the injection time was 8 min in a flow rate of 20 μl/min, and after 10 min dissociation, the surface was regenerated by dissociation in running buffer for 20 min. A series of concentration of SHA (0, 0.125, 0.25, 0.5, 1, 2, and 4 mM) and 4C37L34C11SHA (0, 5, 10, 20, 40, 80, 160, and 320 nM) were flowed over the immobilized MPO C surface as positive controls. The mixtures of 100 nM 4C37L34C11SHA with a series of concentration of SHA were injected. The sensograms were obtained by subtraction of individual SHA concentration responses and blank injections. The experiments were carried out in duplicate.

Results and Discussion

Binder Design

The binder concept has been described in detail previously [9–11]. In short, it is based on the idea that if a small molecule that is known to bind to a protein, albeit with poor affinity and selectivity, is conjugated to a polypeptide with hydrophobic as well as charged residues, a dramatic increase in affinity and selectivity is achieved although the polypeptide itself binds to the protein with poor affinity. Because the entropic penalty has been 'paid' by the small molecule, the polypeptide has to contribute very little binding energy to increase the overall affinity by, say, four orders of magnitude. The interactions between two leucine side chains and hydrophobic patches on the protein surface would be enough to provide the required amount of binding energy. Alternatively, charge–charge interactions and hydrogen bonding could contribute similar amounts. The polypeptides have been shown to fold into helix-loop-helix motifs that dimerize to form four-helix bundles at micromolar concentrations but exist as unordered monomers at low nanomolar concentrations. The polypeptides are therefore capable of adopting a large number of conformations capable of interacting with the protein and according to the design hypothesis bind by adapting to the protein surface to form the binding interaction. Because only a few contacts between polypeptide and protein are needed, the number of possible binding modes is very large; thus, the small set of polypeptides can interact with a very large number of proteins, possibly all. The concept is unlike that of binder molecules prepared by molecular biology technologies that form pre-organized structures that are shape complimentary to the target protein. The set of 16 polypeptide sequences was described in detail previously (Figure 2) [9–11]. The sequences were designed to have some propensity for forming helix-loop-helix motifs and were varied with regard to the number

Seq. name	N-Terminus	C-terminus	Charge
1C15L8	AcNEADLEAKIRHLAEKLEARGPEDAEQLAEQLARAFEAFARAG	-OH	-7
1C10L17	AcNAADLEAAIKHLAEALKERGPEDCEQLAEQLARAFEAFARAG	-OH	-7
1C25L22	AcNEADLEAAIRHLAEALEARGPKDAKQLAEQLARAFEAFERAG	-OH	-6
1C37L34	AcNEADLEAAIRHLAERLEARGPADAAQLAEQLAAKFEKFARAG	-OH	-5
2C15L8	AcNEADLEAKIRHLAEKLAARGPVDCAQLAEQLARAFEAFARAG	-OH	-4
2C10L17	AcNAADLEAAIKHLAEALKARGPVDCAQLAEQLARAFEAFARAG	-OH	-4
2C25L22	AcNEADLEAAIRHLAEALAARGPKDCKQLAEQLARAFEAFARAG	-OH	-4
2C37L34	AcNAADLEAAIRHLAERLAARGPVDCAQLAEQLAAKFEKFARAG	-OH	-3
3C15L8	AcNAADJEAKIRHLAEKJAARGPVDCAQJAEQLARRFEAFARAG	-NH2	-1
3C10L17	AcNAADJEARIKHLAERJKARGPVDCAQJAEQLARAFEAFARAG	-NH2	-1
3C25L22	AcNAADJEAAIRHLAERJAARGPKDCKQJAEQLARAFEAFARAG	-NH2	-1
3C37L34	AcNAADJEAAIRHLAERJAARGPVDCAQJAEQLARKFEKFARAG	-NH2	-1
4C15L8	AcNAADJEAKIRHLREKJAARGPRDCAQJAEQLARRFERFARAG	-NH2	+2
4C10L17	AcNAADJEARIKHLRERJKARGPRDCAQJAEQLARAFERFARAG	-NH2	+2
4C25L22	AcNAADJEARIRHLRERJAARGPKDCKQJAEQLARAFERFARAG	-NH2	+2
4C37L34	AcNAADJEARIRHLRERJAARGPRDCAQJAEQLARKFEKFARAG	-NH2	+2

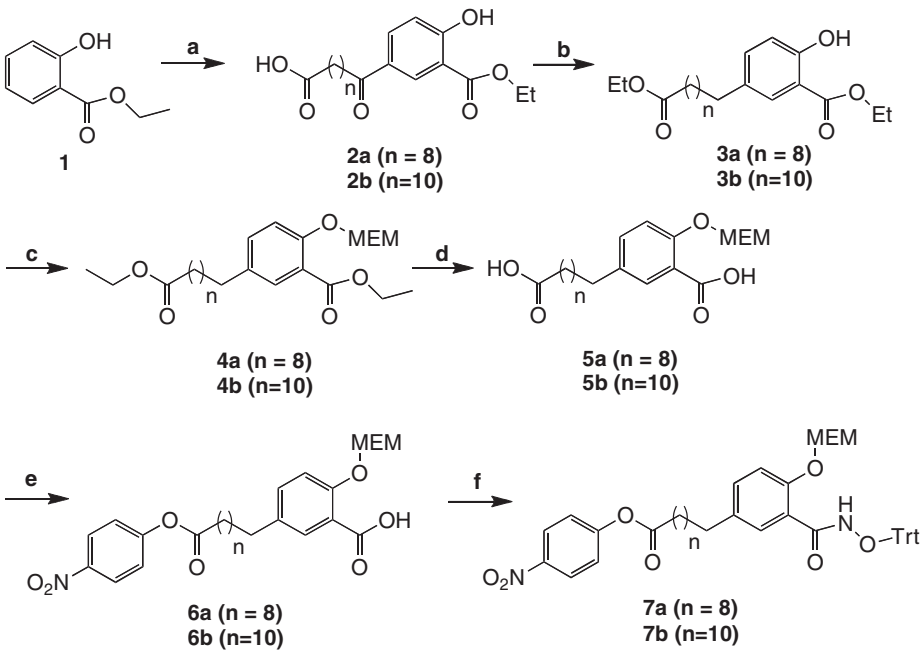
Figure 2. The 42-residue polypeptides used for binder development in the one-letter code (J is norleucine, Nle). The total charge, the site of incorporation of SHA, and the chromophore site are the main variables. The lysine residue K shown in green is the attachment site of the chromophore and that shown in red is the intended site of SHA conjugation. All N-terminals were acetylated, and all C residues were Acm-protected. The C-terminals were either carboxylates (1- and 2-charge types) or amides (3- and 4-charge types). In the sequence names, the first number indicates the charge type of the peptide, e.g. all peptides beginning with '4' carry a total charge of +2 (at pH 7 and when amidated at both lysines). The second and third numbers indicate, respectively, the lysine carrying the chromophore and that carrying a free side chain amino group, e.g. 4C37L34 has a 7-methoxycoumarinyl chromophore group at the side chain of lysine-37 and a free amino group at the side chain of lysine-34 (available for conjugation). Sometimes, for future purposes, the alanine-41 residue was replaced with an ε-*N*-trifluoroacetylated lysine residue (see Supporting Information for details and characterizations of peptides and conjugates).

and position of charged residues and also with regard to the site of incorporation of the small molecules. The hydrophobic residues Leu, Ile, and Phe remain the same throughout the sequences.

In the crystal structure of MPO, the heme group is located in a crevice, about 15 Å in depth, with access to the solvent via an open channel, approximately 10 Å in diameter, and the hydroxyl oxygen and NH nitrogen of SHA are 3.1 and 3.5 Å away from the iron, respectively [16]. SHA is a known substrate mimic inhibitor of MPO with a K_D of 2 μM in sodium phosphate buffer at pH 6.0 [12]. It was therefore selected for conjugation to the polypeptide. On the basis of the SHA location and the depth of the heme cavity, 9 and 11 carbon aliphatic spacers were selected for MPO (Figure 1).

Synthesis

The strategy used for the synthesis of the polypeptide conjugates was to first synthesize the protected ligands **7a** and **7b**, then to conjugate them in protected form to the side chain amino groups of polypeptides (obtained by Fmoc-type solid-phase synthesis), and finally to subject the conjugates to acidic deprotection conditions. Acid-labile methoxyethoxymethyl (MEM)- and Trt-protecting groups were used for protection of the ligands. The synthesis of the protected ligands **7a** and **7b** from ethyl salicylate **1** (14% and 3.5% overall yields, respectively) is shown in Scheme 1. Friedel–Crafts acylation with sebacoyl or didodecanedioyl dichloride afforded compounds **2a** and **2b** in 50% and 41% yields, respectively [13–15].



Scheme 1. Synthesis of compounds **7a** and **7b**. Conditions: (a) $AlCl_3$, $ClCO(CH_2)_{8\ or\ 10}COCl$, dichloroethane, −20 to −15 °C to rt; (b) i: Zn/Hg, 6*N* HCl, ethanol, reflux, ii: HCl/abs. ethanol; (c) NaH, THF, MEMCl, 0 °C; (d) 1*N* NaOH, H_2O/MeOH (1 : 1), 60 °C; (e) 4-nitrophenol, DCC, DMAP, dry DCM, rt, o.v. (f) HOBt, DIPCI, NH_2OTrt, NEt_3, dry DCM, r.t., o.v.

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Clemmensen reduction [13] and subsequent esterification of **2a** and **2b** gave compounds **3a** and **3b** (80% and 76%, respectively). The phenolic hydroxyl group was protected by a MEM group to give derivatives **4a** and **4b** (76% and 71%), followed by saponification to give diacids **5a** and **5b**. DCC-promoted partial esterification of **5a** and **5b** gave predominantly the aliphatic monoesters **6a** and **6b**. The site of esterification in **6a** was determined by comparing the H-NMR spectrum of **6a** to that of the starting material **5a** (see the Supporting Information). The CH2COO methylene proton triplet was significantly shifted downfield when going from the dicarboxylic acid **5a** (2.33 ppm) to **6a** (2.59 ppm), indicating that the ester group was on the aliphatic chain. Analogous shift changes were observed for **6b**. Finally, treatment of the monoesters with *N*-tritylhydroxylamine, DIPCI, and HOBt together with TEA, afforded compounds **7a** and **7b** (28% and 15%). That Trt-hydroxylamidation had indeed taken place at the aromatic carboxyl group was again evident from the H-NMR spectra of **7a** and **7b** (see the Supporting Information), which showed significant chemical shift difference for the MEM methylene protons between **6a** and **7a**, indicating that trityloxyamidation had occurred at the aromatic carboxylic acid. Finally, **7a** and **7b** were successfully conjugated to the specified (Figure 2) free lysine side chain amino group of the various peptides in DMSO solution to afford peptide conjugates. The MEM- and Trt-protecting groups of the conjugates were deprotected simultaneously by a cocktail mixture of TFA, TIS, and water in a ratio of 95 : 2.5 : 2.5 to afford the final peptide conjugates, which were purified by HPLC and characterized by MALDI-TOF MS. A total of 20 peptide conjugates were prepared in a yield of 10–65% (for structures and characterization, see Figure 2 and the Supporting Information).

Evaluation of Binder Performance

The interactions between the 20 polypeptide-SHA conjugates and MPO were investigated by SPR biosensor analysis (Figures 3 and 4). MPO was immobilized on a CM-5 chip (Biacore) using standard protocols, and each one of the binder candidates were allowed to flow over the immobilized protein for evaluation of binding. Within the series of binder candidates equipped with the shorter 9-carbon atom spacer, the highest uptake was observed for the four polypeptide sequences that were highly positively charged (four series), and the same phenomenon was found for the series of binder candidates with the longer 11-carbon

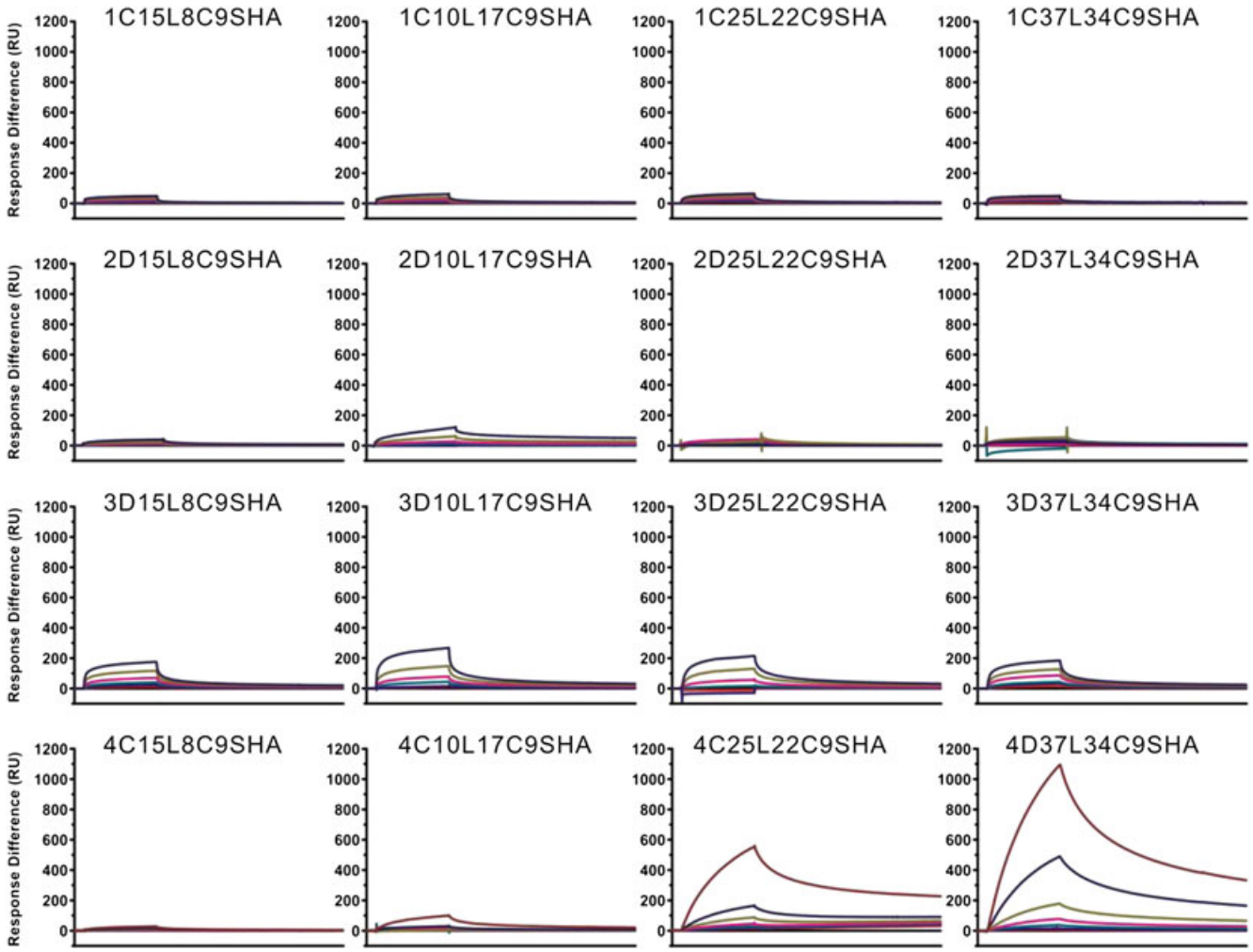

Figure 3. Panel of sensograms showing screening of MPO conjugates carrying 9-carbon atom aliphatic spacer for binding to immobilized MPO in PBS buffer containing 0–1% DMSO and 0.005% P20 at pH 7.4. The concentrations of polypeptide conjugates in the top 12 panels were 0.5, 1, 2, 4, 8, 16, and 32 μM, and the concentrations used for the bottom four panels were 5, 10, 20, 40, 80, 160, 320, and 640 nM. Axes are drawn to scale for direct comparison of uptake between binders. The injection time was 3 min, and the clearance time 10 min. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci

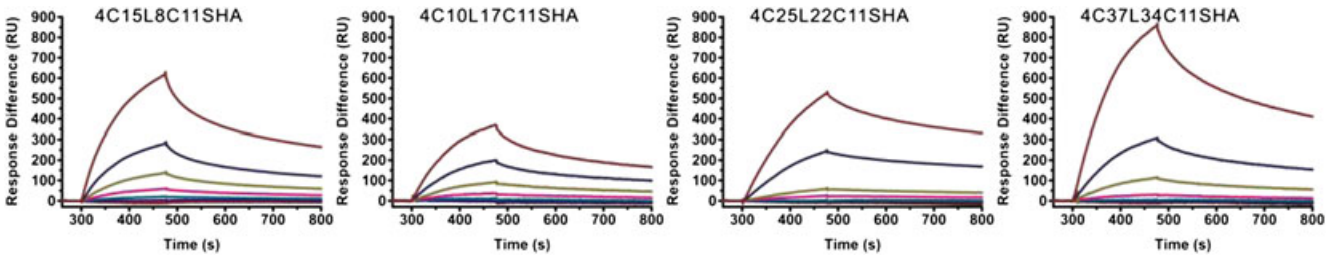

Figure 4. Panel of sensogram for the conjugates with 11-carbon atom aliphatic spacer that bind the strongest to immobilized MPO isoform C in PBS buffer containing 3% DMSO and 0.005% P20 at pH 7.4. Axes are drawn to scale for direct comparison of uptake between binders. The injection time was 3 min, and the clearance time 10 min. This figure is available in colour online at wileyonlinelibrary.com/journal/jpepsci

atom spacer. The weaker binders were not found to bind significantly at low micromolar concentrations, whereas the strongest binders showed strong uptake even at low nanomolar concentrations. The polypeptide conjugate 4D37L34C9SHA was found qualitatively to bind the most strongly to immobilized MPO among the 9-carbon spacer conjugates (Figure 3), and the analogous 4C37L34C11SHA conjugate bound strongest among the 11-carbon spacer ones (Figure 4). The dissociation constant K_D of SHA was reported previously to be 2 μM in sodium phosphate buffer at pH 6.0 [12]. The sensogram panel of four series binders with 11-carbon spacers is shown in Figure 4. Strong uptake at low nanomolar concentrations suggest higher affinity than that of SHA, but as has been reported previously, binding does not follow single exponential kinetics, and determination of dissociation constants by SPR is associated with large errors. We therefore do not report dissociation constants from simple SPR analysis.

To demonstrate that the polypeptide conjugate binds MPO by anchoring the small molecule SHA to its binding site, a competition experiment was carried out using SPR analysis where a mixture of 100 nM of the strong binder 4C37L34C11SHA and SHA over a range of concentrations from 0 to 4 mM was used to probe uptake by freshly immobilized MPO on a sensor chip (Figure 5; see also Figure S2 in the Supporting Information).

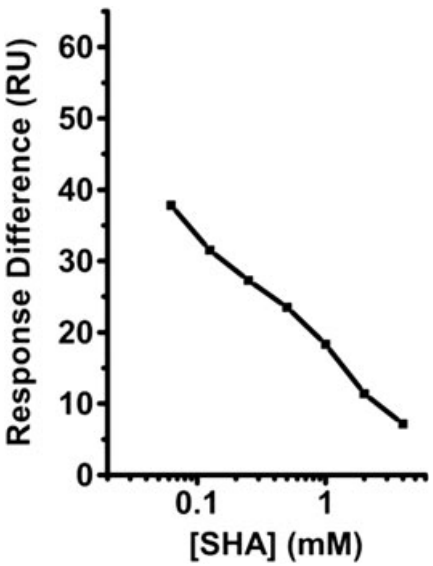

Figure 5. Inhibition of 4C37L34C11SHA binding to immobilized MPO isoform C by 0.0625, 0.125, 0.25, 0.5, 1, 2, and 4 mM concentrations of salicylhydroxamic acid (SHA). The maximum solubility of SHA in the buffer was around 6.5 mM, therefore higher concentrations than 4 mM were not used. The data were obtained from the right sensogram in Figure S2 (supplementary information).

Although lower and higher SHA concentrations would have been needed to produce a complete S-shaped inhibition curve (the solubility of SHA was the limiting factor at the high end), the diagram clearly shows that increasing concentrations of SHA reduced the binding of 4C37L34C11SHA to the chip, with an approximate 50% inhibition of binding at around 0.25–0.5 mM concentration. The results show that the interaction between the polypeptide conjugate and MPO is driven by the conjugated SHA and that the polypeptide provides further interactions to boost the affinity of the binder molecule. A rough estimate of the dissociation constant K_D for the MPO-binder complex can be obtained from the competition experiments. At 50% inhibition of binding, the concentrations of the binder–MPO complex and the SHA–MPO complex are approximately equal, and the ratio of concentrations is directly related to the ratio of dissociation constants. We observe 50% inhibition at around 0.25–0.5 mM of SHA and 100 nM of 4C37L34C11SHA, indicating a 2500- to 5000-fold higher concentration of SHA in comparison with 4C37L34C11SHA and therefore a 2500- to 5000-fold higher affinity of the polypeptide conjugate compared with that of SHA. The K_D of SHA has been reported [12] to be 2 μM, and an estimated dissociation constant of 4C37L34C11SHA is therefore 400–800 pM. Although this estimate is a rough one, it nevertheless shows that conjugation to the polypeptide provides a binder molecule that binds to the site of the small molecule but with dramatically enhanced affinity.

Mode of Binding

According to the crystal structure of SHA complexed with MPO, the aromatic ring of SHA binds to a hydrophobic region at the entrance to the distal heme pocket, and the hydroxamic acid moiety is hydrogen bonded to both the distal histidine and the adjacent Glu or Arg but is not coordinated to heme iron [16]. The structural data suggested that the spacer should be introduced on the aromatic ring in the para position relative to the phenolic hydroxy group. Although the crystal structure suggested where to introduce the spacer, the required spacer length could not be estimated. Two spacer lengths were therefore evaluated, and the longer one was found to provide slightly higher affinity (data not shown).

Myeloperoxidase is a cationic basic protein, with an isoelectric point of over 9.2. The total charge of MPO is positive. The results from the SPR analysis showed that positively charged polypeptide conjugates bound strongly to MPO, whereas negatively charged ones did not. These facts seem to be contradictory but instead illustrate that the overall charge of the protein is not critical for strong interactions between polypeptide and protein. Instead, the interactions are local and most likely depend on

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hydrophobic patches on the surface or hydrophobic patches that are exposed as a result of conformational changes.

Conclusion

The active esters of protected SHA ligands were successfully synthesized, and the ligands successfully conjugated to polypeptides to afford 20 conjugates for MPO recognition and binding. The interactions between polypeptide-SHA conjugates and MPO were evaluated by SPR, and competition experiments indicated that the SHA group of the strong binder 4C37L34C11SHA bound to the active site of MPO. The polypeptide provided further contacts with the protein to form a binder molecule with an affinity for MPO that was more than four orders of magnitude higher than that of SHA. The reported binder molecule offers a starting point for the development of a robust point of care test for a cardiovascular risk that does not rely on antibodies. The combination with a previously developed synthetic binder for CRP provides an opportunity for an improved clinical diagnostic test designed to address simultaneously two biomarkers that have been suggested to provide an improved level of precision in predicting risk for heart infarction.

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