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Kinetic Interactions between Cyclolinopeptides and Immobilized Human Serum Albumin by Surface Plasmon Resonance

Youn Young Shim*[,†,‡] and Martin J. T. Reaney*[,†,‡,§]

[†]Department of Plant Sciences, University of Saskatchewan, Saskatoon, Saskatchewan S7N 5A8, Canada

[‡]Prairie Tide Chemicals Inc., 102 Melville Street, Saskatoon, Saskatchewan S7J 0R1, Canada

[§]Guangdong Saskatchewan Oilseed Joint Laboratory, Department of Food Science and Engineering, Jinan University, 601 Huangpu Avenue West, Guangdong, Guangzhou 510632, China

Supporting Information

ABSTRACT: Cyclolinopeptides (CLs) are octa-, nona-, and decapeptides present in flaxseed (*Linum usitatissimum* L.) that may have immunosuppressive and antitumor activities, but little is known of their pharmacokinetics. Human serum albumin (HSA), the most abundant blood protein, is an important mediator of organic solute flux, and hence when compounds bind this protein, it potentially affects both their availability and efficacy. Quantitative thermodynamic analysis of the interaction of compounds with HSA is important in the development of biomedical applications. A surface plasmon resonance (SPR) biosensor was utilized to reliably determine binding constants for several CLs with HSA. The maximum binding response of [1−9-NαC]-CLA/HSA was almost 20-fold higher than that of [1−8-NαC],[1-MetO]-CLE/HSA. Through analysis of an array of peptides, it was possible to correlate the impact of structural changes on CL binding. The oxidation of sulfur in methionine (Met) residues formed methionine *S*-oxide (MetO) and reduced binding significantly. Most strikingly, the further oxidation of MetO to *S*,*S*-dioxide ($MetO_2$) produced CLs with stronger binding. The large impact on binding by relatively small modifications of methionine containing CLs suggested that small changes in methionine oxidation can disrupt hydrophobic interaction, the predominant intermolecular force stabilizing the complex between CLs and HSA. SPR binding studies may aid in understanding the fate of CLs after consumption of flaxseed or flaxseed products or the development of CLs as drugs or drug carriers.

KEYWORDS: *Linum usitatissimum L., cyclolinopeptide, orbitide, cyclic peptide, surface plasmon resonance, human serum albumin, interaction kinetics*

INTRODUCTION

Cyclolinopeptides (CLs) are plant cyclic peptides or orbitides with potential therapeutic properties.[1] CLs are extracted from flaxseed (*Linum usitatissimum* L.), which is a triglyceride source of essential polyunsaturated fatty acids.[2−4] They are composed of eight or nine mostly hydrophobic natural amino acid residues joined into a ring via an N- to C-terminal peptide bond (Table 1; Figure 1). A number of oxidized CLs have been identified in flaxseed oil.[5,6] Orbitides are distinct from cyclotides, being larger peptides with N- to C-terminal peptide bonds and cysteine bonds.[1]

There are many pharmaceutical products that are proteins or peptides or peptide derivatives.[7] Several CLs, including [1−9-NαC]-CLA (**1**),[8] [1−9-NαC]-CLB (**2**),[9] and [1−8-NαC],[1-MetO]-CLE (**8**),[10] are bioactive. CL **1** possesses immunosuppressive activities, through the inhibition of calcium-dependent T cell activation. The proposed mechanism of inhibition is similar to that of cyclosporine A (CsA).[8] Both CL **1** and CsA bind to cyclophilin to form a complex. The reaction with cyclophilin inhibits the phosphatase activity of calcineurin. In turn, the absence of an active calcineurin prevents the interleukin-1 (IL-1)- and IL-2-dependent pathway from activating T cells.[3,11,12] The binding also inhibits the peptidyl proyl *cis-trans* isomerase of cyclophilin.[13] Unlike CsA, CL **1** demonstrates a low level of toxicity at therapeutic concentrations.[14] Therefore, CL **1** might have applications in treating immunological diseases and prolonging graft survival.[15] In a related finding, the cyclotide kalata B1 inhibited IL-2 production.[11] CL **1** also inhibits hepatocytes from absorbing bile salt, phallotoxin,[16] ethanol, and cysteamine.[3] This activity is similar to that of antamanide and somatostatin, two other bioactive peptides.[17] The Pro-Phe-Phe tripeptide region, which is conserved between CL **1** and antamanide, appears to be critical for this activity.[7] CL **1** is represented by the strong interaction of the cyclopeptides with components of the cholate transport system on hepatocyte membranes.[18] This property can help to prevent cell poisoning.[19] Moreover, CL **1** was also found to have antimalarial activities.[20] In addition, CLs **2** and **8** also possess immunosuppressive properties, by which they inhibit the mitogen-induced (concanavalin A) proliferation response of peripheral blood lymphocytes.[21]

Human serum albumin (HSA) is the most abundant protein present in the human bloodstream.[22] HSA constitutes over half of the total plasma proteins, at a concentration of 35−50 g/L, in healthy individuals.[23] It is a globular protein consisting of a single peptide chain of 585 amino acid residues. As the major soluble protein constituent of the circulatory system, it has

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Table 1. Primary Structural Formulas of CLs

code	new name[a]	old name	amino acid sequence (NαC-)[b]	chemical formula	MW (Da)
1	[1−9-NαC]-CLA	CLA	Ile-Leu-Val-Pro-Pro-Phe-Phe-Leu-Ile	$C_{57}H_{85}N_9O_9$	1040.34
2	[1−9-NαC]-CLB	CLB	Met-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{56}H_{83}N_9O_9S$	1058.38
3	[1−9-NαC],[1-Abu]-CLB[c]	CLB-S	Abu-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{55}H_{81}N_9O_9$	1012.29
4	[1−9-NαC],[1-MetO]-CLB	CLC	MetO-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{56}H_{83}N_9O_{10}S$	1074.38
5	[1−9-NαC],[1-$MetO_2$]-CLB	CLK	$MetO_2$-Leu-Ile-Pro-Pro-Phe-Phe-Val-Ile	$C_{56}H_{83}N_9O_{11}S$	1090.38
6	[1−8-NαC]-CLE	CLP	Met-Leu-Val-Phe-Pro-Leu-Phe-Ile	$C_{51}H_{76}N_8O_8S$	961.26
7	[1−8-NαC],[1-Abu]-CLE[c]	CLP-S	Abu-Leu-Val-Phe-Pro-Leu-Phe-Ile	$C_{50}H_{74}N_8O_8$	915.57
8	[1−8-NαC],[1-MetO]-CLE	CLE	MetO-Leu-Val-Phe-Pro-Leu-Phe-Ile	$C_{51}H_{76}N_8O_9S$	977.26
9	[1−8-NαC],[1-$MetO_2$]-CLE	CLJ	$MetO_2$-Leu-Val-Phe-Pro-Leu-Phe-Ile	$C_{51}H_{76}N_8O_{10}S$	993.26

[a]All new CLs listed are proposed by Shim et al.,[46] except **3** and **7**. [b]The first positions of amino acid sequences are highlighted in Figure 1. Abbreviations are Met for methionine, Abu for 2-aminobutanoic acid, MetO for methionine *S*-oxide, and $MetO_2$ for methionine *S*,*S*-dioxide. [c]This work proposed the use of **3** and **7** for Abu-substituted forms **2** and **6**, respectively.

many physiological roles and pharmacological effects. One of its main functions is to regulate osmotic potential between the blood and tissues. It also plays a role in the maintenance of blood pH. Another very important role of albumin is its function as a transport molecule. This role is based on albumin's unique ability to bind a variety of exogenous and endogenous compounds, such as metal cations, fatty acids, amino acids, and diverse drugs.[24−26] The distribution, free concentration, and metabolism of various drugs can be significantly altered as a result of binding to HSA.[23] HSA has multiple binding sites that bind a wide range of different ligands with high affinity.[22] There are two Sudlow's sites, site I and site II, which have been shown to bind drug molecules, endogenous hormones, and ions.[27] Site I is composed of a pocket with hydrophobic walls and an opening lined with positively charged residues, allowing it to bind a variety of different ligands.[27] It binds dicarboxylic acid or bulky heterocyclic molecules with a negative charge in the middle.[27] Site II is smaller and less flexible than site I. It binds aromatic carboxylic acids with negatively charged acidic groups such as those present on nonsteroidal anti-inflammatory drugs.[27] Because the amino acid residues on CLs are primarily hydrophobic, it is probable that CL interacts with HSA when introduced to the human body.

The interaction of HSA with drugs has been investigated using fluorescence,[28,29] Fourier transform infrared spectroscopy,[30−32] and circular dichroism spectroscopy,[33] but the binding of CLs other than CL **1** to HSA has yet to be investigated. Investigation of the kinetics and equilibrium of CL/HSA binding and release is necessary as cyclic peptides are being considered as drug-delivery molecules.[34] For instance, binding information is essential to understanding how cyclic peptides can be delivered to a target environment or tissue. The information provided by surface plasmon resonance (SPR) is ideal for studying the association/dissociation kinetics of CL/HSA binding. One advantage of SPR is that the different binding kinetics of a number of different analytes to the immobilized ligand can be studied. Comparisons between the association/disassociation kinetics between different analytes can be made easily. The equilibrium constants of association (K_A) and dissociation (K_D) are determined from the association and dissociation rate constants.

MATERIALS AND METHODS

General. HSA (99% purity, fatty acid and globulin free) was purchased from Sigma-Aldrich Canada, Ltd. (Oakville, ON, Canada) and used without further purification. CLs were extracted and purified from flaxseed using previously reported procedures.[5,6,35,36] An amine coupling kit [0.4 M 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC), 0.1 M *N*-hydroxysuccinimide (NHS), and 1.0 M ethanolamine hydrochloride-NaOH (EA), pH 8.5], HBS-EP buffer [10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 150 mM NaCl, 0.005% (v/v) surfactant polysorbate 20, and 3.4 mM ethylenediaminetetraacetic acid (EDTA), pH 7.4], and a research-grade CM5 sensor chip were obtained from Biacore (GE Healthcare, Montreal, QC, Canada). The sensor chip CM5 has a 100 nm thick carboxylated dextran layer on a 50 nm thick gold layer surface. All other reagents were of analytical grade, and Milli-Q water (Millipore, Bedford, MA, USA) was used throughout the experiments.

Theoretical Aspects. The kinetic model for a 1:1 interaction is normally represented by eqs 1 and 2

$$A + B \xrightarrow{k_a} AB \quad (1)$$

$$AB \xrightarrow{k_d} A + B \quad (2)$$

where k_a and k_d represent the association and dissociation rates, respectively. The affinity constant K_A is the ratio of k_a and k_d,[37] shown in eq 3.

$$K_A = \frac{k_a}{k_d} = 1/K_D \quad (3)$$

The ligands are immobilized on the SPR sensing surface by passing HSA through a flow cell (Figure 2A). The immobilized ligands have different densities as well as different orientations on the sensor surface. The interactions are measured in real time in the flow cell (Figure 2B).

The affinity of interaction is estimated by first assuming that HSA binding sites are equally available and, second, that only high-affinity sites are occupied in the working concentration range.[38] Plots of the measured response versus different CL concentrations [CLs] are shown in Figure 3 and Figure S1A−H of the Supporting Information, respectively. K_A and the response values at the binding site saturation (R_{max}) were obtained by fitting the measured response values (R_{eq}) obtained with different CL concentrations to eq 4.

$$R_{eq} = R_{max}\frac{K_A[CLs]}{K_A[CLs] + 1} \quad (4)$$

Analyte and Buffer Preparation. Nine CLs with either 8 or 9 amino acids (915.17−1090.38 Da; Table 1 and Figure 1) were used. The CLs were purified to >98% homogeneity using reversed phase high-pressure liquid chromatography (HPLC). Analyte solutions were prepared by diluting CL stock solutions 20-fold into phosphate-buffered saline (PBS; 10 mM phosphate buffer, 2.07 mM KCl, 137 mM NaCl, pH 7.4), giving a final PBS buffer containing 5% (v/v) dimethyl sulfoxide (DMSO). The CL concentrations in the analyte solutions injected on the biosensor ranged from 40 to 150 μM. Preferably, analyte solutions were prepared just before use, but in some cases were stored at 4 °C. Running buffer for all experiments was PBS (pH 7.4) containing 5% (v/v) DMSO. Running buffers were

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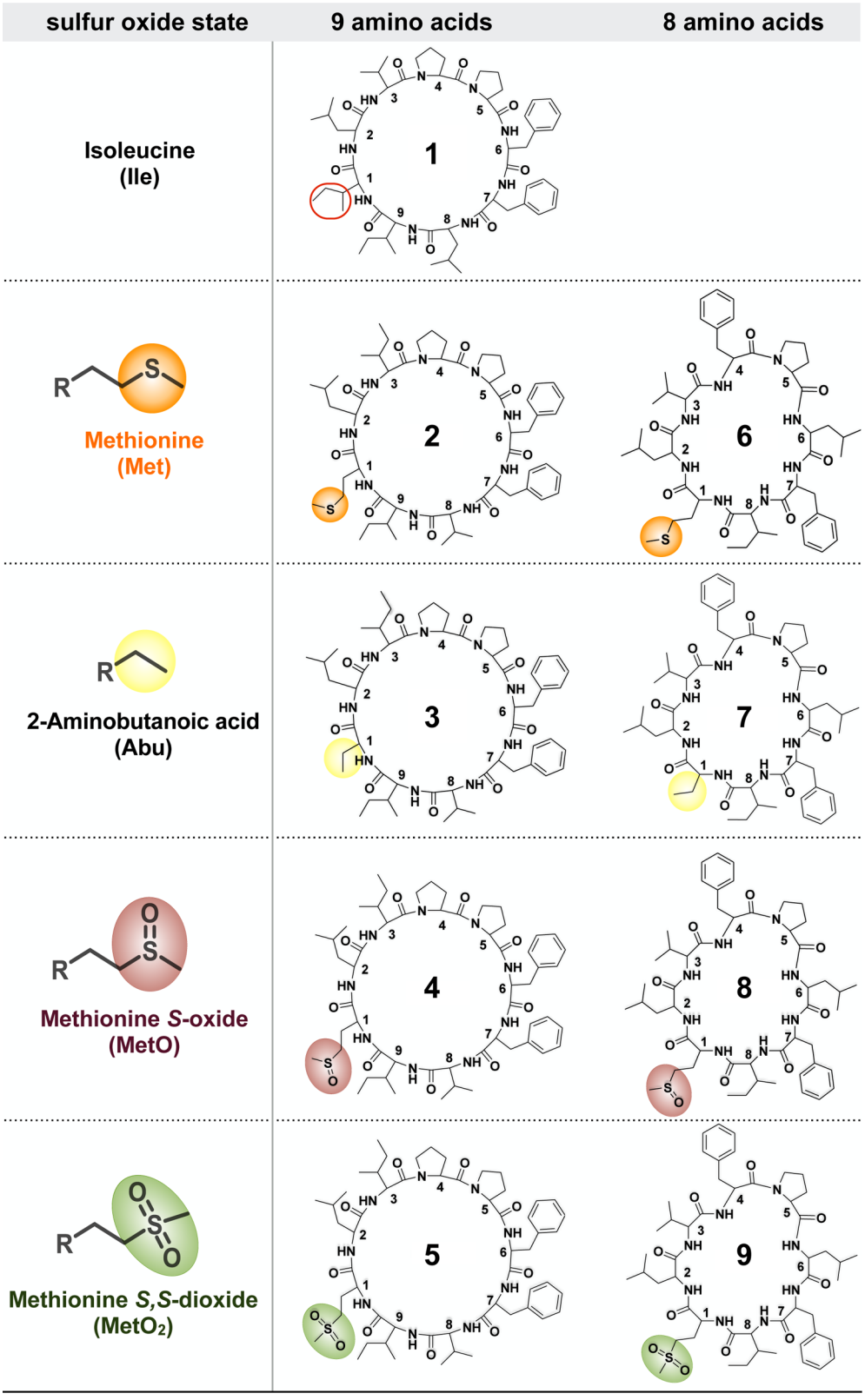

Figure 1. Structure of CLs used in the HSA binding studies.

filtered and degassed daily before use. A 10 mM sodium acetate buffer (pH 5.3) is generally adequate for immobilizing amine ligands onto a CM5 sensor chip and was used in all experiments. All buffer solutions were freshly prepared, degassed, and filtered with a 0.22 μm filter.

Surface Plasmon Resonance Analysis. SPR measurements were performed using a Biacore X instrument (Biacore AB, Uppsala, Sweden) equipped with an internal injection system (500 μL Hamilton syringe). This instrument has a flow injection system to enable real-time measurement of molecular binding. The SPR angle shift is used as a response unit (RU) to quantify the binding of biomolecules to the sensing surface (Figure 2B). The fluidic unit of the instrument was cleaned prior to experiments by injecting 0.5% sodium

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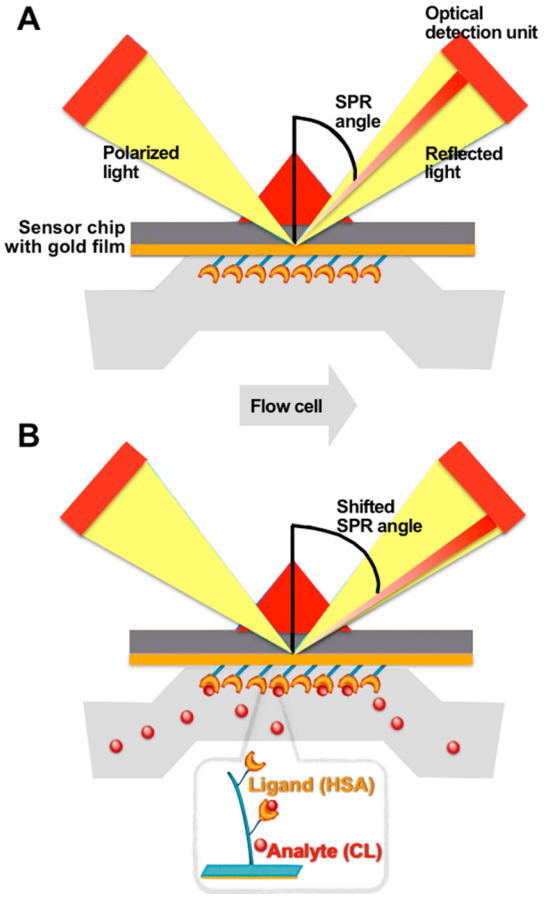

Figure 2. Principle of SPR phenomenon. (A) Polarized light is applied to the surface of the sensor chip and is reflected. The intensity of the reflected light is reduced at a certain incident angle, the SPR angle. (B) Interacting substances near the surface of the sensor chip increase the refractive index, which alters the SPR angle. The optical detection unit detects position changes in the wedge of the reflected light, corresponding to the SPR angle. The signals produced are measured in RU.

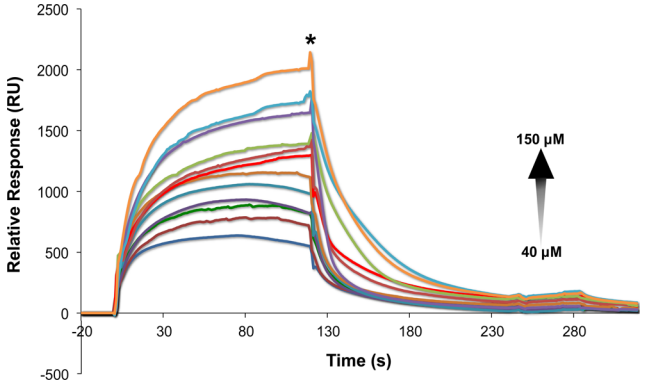

Figure 3. Concentration-dependent analysis of CL **1** complexes binding to HSA. All color plots indicate a spot exposed to 30 μg/mL HSA ligand and with various concentrations of CL **1** on the surface, respectively, with duplicates (omitting the regeneration stage). The response baseline is set to equal the level of HSA whereas time zero is set at the time of injection of CL **1**. One representative experiment is shown. All experiments are performed on the same sensor chip. The asterisk marks the end of the CL **1** injection and the beginning of CL **1** desorption.

dodecyl sulfate, followed by 50 mM glycine (pH 9.5). To reuse a chip for SPR analysis, bound analyte must be regenerated by stripping prior test compounds from the surface after each injection, usually with mild detergents. However, standard regeneration treatments did not effectively remove CLs from the HSA-immobilized chip. The only reagent that reproducibly did so was 6 M guanidine hydrochloride (GdnHCl) applied as a brief pulse (10 μL at 10 μL/min flow rate), followed by continued HBS-EP buffer flow (>5 min at 25 μL/min) to stabilize HSA prior to the next injection.

Immobilization of HSA. HSA was covalently immobilized onto a research-grade CM5 sensor surface at 25 °C using standard amine-coupling chemistry.[37] Briefly, CM5 sensor chips were preconditioned prior to HSA immobilization by priming the instrument three times with HBS-EP buffer, followed by three successive 1 min injections of 50 mM NaOH at 50 μL/min. Flow cell 1 (Fc1) and flow cell 2 (Fc2) were activated by a 7 min injection of a 1:1 mixture of 0.1 M NHS and 0.4 M EDC using a flow rate of 5 μL/min. HSA has an isoelectric point (p*I*) of 5.67 and is positively charged below this pH. Immediately after activation, HSA (30 μg/mL) was diluted in 10 mM acetate buffer (pH 5.3) to impart a positive charge on the HSA (Figure 4). Thereafter, the HSA is attracted to the negatively charged

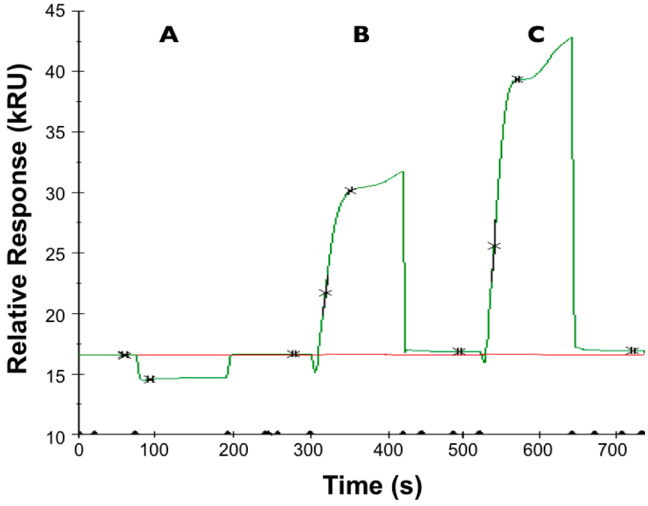

Figure 4. PH scouting of three successive electrostatic preconcentration assays for HSA (66 kDa MW and p*I* 5.67) as ligand. All HSA was diluted in 10 mM sodium acetate to a final concentration of 30 μg/mL, at three different pH values, (A) 5.5, (B) 5.3, and (C) 5.0, respectively.

matrix of the sensor chip and immobilized onto Fc2 (Figure 5). A solution of 10 mM acetate buffer without HSA was passed over Fc1, to act as the reference cell. A 7 min injection of 1.0 M EA (pH 8.5) using a flow rate of 5 μL/min was passed over Fc1 and Fc2 to deactivate unbound areas of the sensor surfaces. The thickness of the bilayer was calculated by assuming that 1 kRU corresponded to a 1 nm thick layer.[39] This bilayer was subsequently used as a model membrane surface to study the CL/HSA interactions. When kinetic analyses are performed, the ligand density should be as low as possible, provided signal-to-noise ratios are adequate. Direct detection of CL (ca. 1.0 kDa) binding to the immobilized HSA ligand (ca. 66 kDa) on a Biacore X generally requires immobilization responses.

CL Binding to HSA. For all CLs, 40 μL of analyte solution (40–150 μM) was injected, in duplicate, and flowed over reference and HSA surfaces for 2 min at a flow rate of 20 μL/min, followed by a dissociation phase of 2 min for all experimental conditions. At the end of the CL injection, the flow solution was switched to the running buffer, and a decrease in the response toward the baseline was observed within minutes. This decrease in response indicated that CLs were dissociating from the HSA immobilized to the sensor surface. All experiments were performed at 25 °C. When the baseline RU was not readily achieved, a short pulse of 50% (v/v) DMSO solution was injected at a high flow rate to remove any remaining CLs. Repeated

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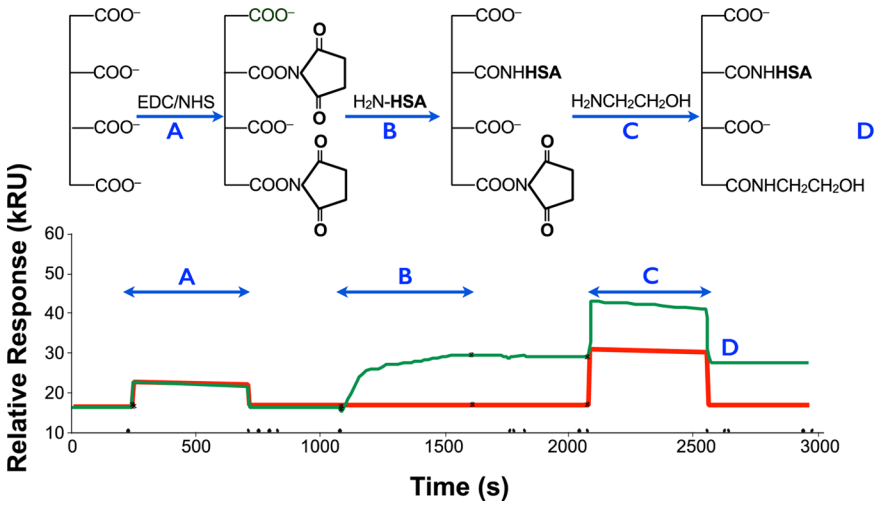

Figure 5. Steps in the immobilization of HSA ligand on a CM5 research-grade sensor chip via the amine coupling reaction (red line, reference surface; green line, HSA surface). The labeled portions of the graph indicate (A) injection of the EDC/NHS mixture (1:1, v/v) onto the surface, activating the carboxymethyl group by forming a highly reactive succinimide ester, followed by termination of the injection; (B) after surface activation, injection of ligand sample diluted in buffer of the appropriate, predetermined pH, with continuation of injection resulting in covalent binding of ligand to the reactive surface to yield; (C) blockage of remaining nonreacted-activated carboxymethyl groups by injection of ethanolamine solution, followed by cessation of injection; and (D) point of attained immobilization of ligand. Small triangles on time line below designate events such as injection, end of injection, and refilling.

Table 2. CL/HSA Equilibrium Binding Constants of Dissociation Determined from SPR Sensorgrams Using a 1:1 Interaction Model with a Drifting Baseline[a]

interaction CL[b]	k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_A (mM^{-1})	K_D (mM)	R_{max} (RU)	χ^2
1	6.55×10^{-1}	2.38×10^{-4}	2.75×10^{0}	3.64×10^{-1}	6.27×10^{3}	7.04×10^{2}
2	5.88×10^{0}	2.62×10^{-2}	2.25×10^{-1}	4.45×10^{0}	1.92×10^{4}	1.94×10^{3}
3	8.47×10^{0}	1.21×10^{-1}	7.01×10^{-2}	1.43×10^{1}	7.86×10^{4}	9.64×10^{2}
4	1.91×10^{2}	5.88×10^{-2}	3.25×10^{0}	3.08×10^{-1}	1.17×10^{3}	5.75×10^{2}
5	1.94×10^{2}	1.60×10^{-2}	1.21×10^{1}	8.27×10^{-2}	2.78×10^{2}	2.00×10^{1}
6	2.78×10^{0}	2.05×10^{-2}	1.35×10^{-1}	7.38×10^{0}	9.05×10^{4}	8.90×10^{3}
7	3.51×10^{0}	2.70×10^{-2}	1.30×10^{-1}	7.68×10^{0}	1.70×10^{4}	5.14×10^{2}
8	1.96×10^{2}	3.47×10^{-2}	5.64×10^{0}	1.77×10^{-1}	4.38×10^{3}	2.99×10^{1}
9	7.06×10^{-1}	1.20×10^{-3}	5.89×10^{-1}	1.70×10^{0}	1.01×10^{4}	1.02×10^{3}

[a]All experiments were performed on the same HSA-immobilized CM5 chip. Values are means of SPR assays performed in duplicate. [b]The slopes were determined from curve-fitting dose-response curves in Figure 3 and Figure S1 of the Supporting Information.

experiments indicated that the regeneration procedure did not change the binding characteristics of the immobilized HSA (data not shown).

The sensorgrams were analyzed by the global fitting procedure using a 1:1 (Langmuir type) drifting baseline model-derived equation[26] available in the BIA evaluation software (version 4.2). RU curves taken from the sensorgrams 10−30 s after association and the dissociation were used in the model. The association rate constant (k_a), the dissociation rate constant (k_d), and the dissociation equilibrium constants (K_D) were determined from the model at the minimal chi-squared (χ^2) value.

RESULTS AND DISCUSSION

Preconcentration Assays. Preconcentration assays were performed to determine the best immobilization buffer. Different preconcentration pH values were used to determine optimal binding of HSA to the sensor chip surface (Figure 4). Using ca. 12 kRU as a reasonable immobilization level for the direct kinetic assay of CL binding to a HSA surface, pH 5.5 produces inefficient preconcentration, resulting in low levels of the ligand binding to the carboxymethyl-dextran matrix of the sensor chip. At pH 5.3, efficient HSA preconcentration occurred, producing a satisfactory final response level (Figure 4B). In contrast, HSA uptake by the surface is too rapid at pH 5.0, resulting in an excessive final response (Figure 4C). Thus, for immobilization of HSA via the amine coupling reaction, a preconcentration pH of 5.3 was chosen.

HSA Covalent Immobilization. Typical SPR response sensorgrams of HSA immobilization are shown in Figure 5. In a first stage (A), the EDC/NHS activating mixture is injected with the consequent increase in the SPR signal due to a change in the bulk refractive index. The HSA solution is then injected, and the binding event (B) can be followed in real time. Once an adequate binding level is reached, the remaining active carboxyl-NHS esters are blocked with ethanolamine hydrochloride (C), causing a significant change in the bulk refractive index. The biospecific HSA surface is then ready to be used (D). The final immobilized HSA response is 12−13 kRU at the sensorgram.

Binding of the Bioactive Compound CLs to HSA. Interactions between **1** and immobilized HSA were measured (Figure 3). As the concentration of **1** increased from 40 to 150 μM, the binding signal steadily increased in RU. This result was similar to that reported by Rempel et al., although the response is significantly stronger in this study.[40] Interestingly, when the chip surface was saturated with analyte, the response

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normalized by the concentration-dependent analysis should have yielded equal maximum responses. A CL:HSA (1:1) binding stoichiometry was observed, using a drifting baseline model (Table 2 and Figure S1 of the Supporting Information). Residual values from Figure 6 also support the closeness of this

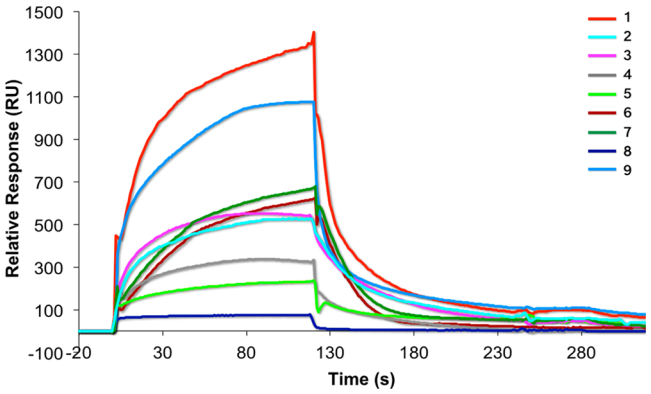

Figure 6. SPR sensorgram responses for 100 μM of different CLs binding to HSA. Colors of sensorgram traces are assigned in the legend.

1:1 ratio between the experimental and theoretical curves. All plots illustrated the experimental curve-fitting methodology for a simple binding model (1:1 Langmuir).

Data Processing and Evaluation. The concentration dependencies of different CLs binding to immobilized HSA for concentrations ranging between 40 and 150 μM were determined (Figure S1 of the Supporting Information). Sensorgrams corresponding to a nonspecific CL **8** injected on a HSA surface are shown in Figure 6 and Figure S1G. The sensorgrams are square-wave shaped due to a refractive index jump, which is confirmed by the fact that no CL **8** is bound to the HSA at the beginning of the dissociation phase: (1) CL **8** did not appreciably bind HSA; (2) the increase in RU caused by an increase in refractive index is largely the result of buffer injection; and (3) all other CLs examined formed HSA complexes as the association curve is relatively higher than observed in CL **8**.

In contrast, other sensorgrams correspond to a specific interaction between an injected CL and the immobilized HSA. Compared to the other CLs tested, CL **1** has slow association kinetics with HSA (Figure 6). At higher concentrations, the steady-state plateau was not reached after 120 s. The gradual increase of response to a steady state during CL **1** injection indicates slow recognition kinetics of CL **1** with the HSA binding site compared to other CLs tested (Figure 6). Quantitative kinetic data were initially extracted using the kinetics profiling within the Biacore Evaluation software. The slow recognition may be due to CL becoming available for interaction with HSA. It is likely that CL **1** has a large hydration sphere, which must dissociate before interaction with HSA is possible.

HSA has seven known fatty acid binding sites, each of which is known to bind other small, hydrophobic molecules. Two of the binding sites for small molecule drugs are known as Sudlow site I (warfarin binding site) and Sudlow site II (indole-benzodiazepine binding site).[41] Site I binds drug molecules that are structurally bulky, heterocyclic compounds with centrally localized negative polar/charges, and hydrophobic substitutions. It has been suggested that binding is through hydrophobic interactions between the analyte and a hydrophobic crevice within the binding cavity of site I.[42] The nature of CL binding was not proven in the present study.

The equilibrium parameters for several CL binding reactions with immobilized HSA were determined (Table 2). The K_A value indicated that the affinity tends to increase as the CL becomes more hydrophobic, although there are significant exceptions. The K_D showed more rapid dissociation of CLs **4**, **5**, and **8** than of CL **1** (Table 2 and Figure 6). This suggests that there is more binding of **1** at the highly hydrophobic crevice. All CLs tested were able to bind, showing more than an order of magnitude increase in the **8**/HSA binding (RU 71.0) and **1**/HSA, which was almost 20-fold higher (RU 1370.6) than **8**/HSA (Figure 6). Also, the oxidation state of the sulfur-containing amino acid residue in position 1 of CLs (**2**−**9**) was a component critical to binding. To examine the importance of this sulfur-containing residue (*S*), we synthesized CLs with 2-aminobutanoic acid (Abu) instead of methionine residues at this position (Table 1 and Figure 1). These modified compounds are named [1−9-NαC],[1-Abu]-CLB (**3**)[35] and [1−8-NαC],[1-Abu]-CLE (**7**)[36] according to established conventions. Each of the S-free [1-Abu]-CLs had similar binding strengths to their 2-Met-containing counterparts (Figure 6). Most strikingly, the conversion of methionine sulfoxide to the sulfone peptide [1−8-NαC],[1-MetO_2]-CLE (**9**) had the greatest effect on binding (Figure 6 and Figure S1 of the Supporting Information). It can be speculated that this was an effect of structural differences between CLs **6** and **9** and CLs **8** and **9** induced by the oxygen−sulfur bonds or the dipole moment of the *S*-oxide group. A similar but less pronounced effect was observed with CLs **2**−**5**. The conservative introduction of an oxygen group to **2** leads to a partial disruption of the HSA interaction and weak binding of compound **4**, whereas the addition of two oxygen groups results in **5** with 4-fold stronger binding than that of **4** (the most polar CL tested).

CLs are produced in plants via expression of genes that code for multiple CLs.[1] The peptides produced in the same reading frame are usually produced in constant ratios in the plant determined by transcription and post-translational modification of the peptide gene. In an attempt to observe the sensorgram of peptides encoded by the same gene after different post-translational modification, we compared three mixtures of three peptides each with a similar modification of methionine. The slow release of the stronger binding groups (CL mixtures **1_2_6**; **1_3_7**; and **1_5_9**) suggested that parameters would also be important in understanding their HSA binding (Figure 7). The fast dissociation of CL mixture **1_4_8** showed weak binding with HSA. This is due to the contribution from breaking of the solution complex and hydrophobic binding. It has been observed that CL mixture **1_2_6** is observed in flaxseed meal products with prolonged storage. This mixture is the major product of a single gene located on scaffold 1198 coded on the 3′ to 5′ strand bases 162067−161526 available via Phytozome portal (version 9.1). Flaxseed oil CLs **2** and **6** become oxidized to **4** and **8**, respectively. Finally, excessive oxidation will produce **5** and **9**.[43]

In conclusion, the K_D of CL/HSA solution is correlated with both the methionine oxidation state and polarity. The binding of methionine-containing CLs with HSA was similar to that of peptides that had Abu in place of methionine but greater than that of peptides with methionine *S*-oxide. The dipole moment of the *S*-oxide likely contributes to decreased affinity.

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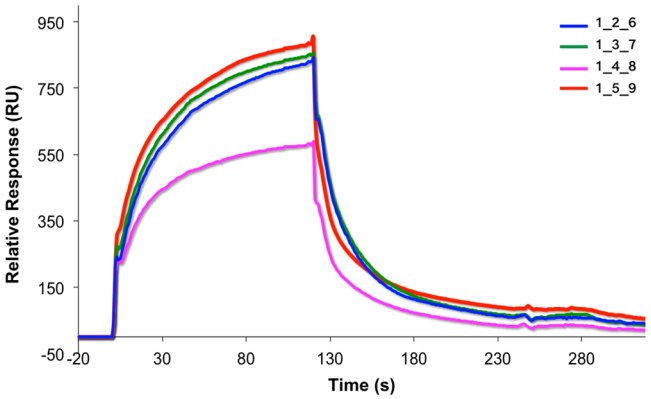

Figure 7. SPR sensorgram responses for 100 μM of each CL mixture binding to HSA.

Conversely, increased oxidation of the methionine *S*-oxide to form the *S*,*S*-dioxide produces a molecule with restored HSA binding. These findings show that cyclic peptides and particularly CLs can be used to study the discrete effects of changing a single amino acid on receptor binding. Other studies on more complex molecules may not allow the examination of a single substitution. For example, oxidation of methionine residues in human IgG2 Fc decreased SPR binding affinities to protein A.[44] Comparison of the equilibrium dissociation constants to other literature compounds indicates that CL binding to HSA is not comparatively strong when compared with that of other drugs, except that the values of K_D for **5** and **8** were similar to those reported for pyrimethamine and rifampicin bindings, respectively.[45] In this study the impact of sulfur oxidation and elimination was observed for orbitides containing methionine and its modified analogues.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Concentration-dependent analysis of different CLs (**2**–**9**) binding to immobilized HSA (Figure S1A–H). This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Authors

*(Y.Y.S.) Phone: (306) 966-5050. Fax: (306) 966-5015. E-mail: younyoung.shim@usask.ca.

*(M.J.T.R.) Phone: (306) 966-5027. Fax: (306) 966-5015. E-mail: martin.reaney@usask.ca.

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Notes

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