Dynamic and equilibrium performance of sensors based on short peptide ligands for affinity adsorption of human IgG using surface plasmon resonance

Nafisa Islam a, Fei Shen a, Patrick V. Gurgel a, Orlando J. Rojas b, Ruben G. Carbonell a,c,*

a Department of Chemical and Biomolecular Engineering, North Carolina State University, 911 Partners Way Raleigh, NC 27695, USA
b Department of Forest Biomaterials, North Carolina State University, 2820 Faucette Drive, Biltmore Hall Office 3205, Raleigh, NC 27695, USA
c Biomansurfing Training and Education Center (BTEC), North Carolina State University, 850 Oval Drive, Suite 195, Raleigh, NC 27606, USA

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ABSTRACT

This paper characterizes the potential of novel hexameric peptide ligands for on-line IgG detection in bioprocesses. Surface Plasmon Resonance (SPR) was used to study the binding of human IgG to the hexameric peptide ligand HWRGWV, which was covalently grafted to alkanethiol self-assembled monolayers (SAM) on gold surfaces. Peptide coupling on SAMs was verified, followed by covalent grafting of peptides with a removable Fmoc or acetylated N-termini via their C-termini to produce active peptide SPR sensors that were tested for IgG binding. The dynamics and extent of peptide–IgG binding were compared with results from a conventional system using protein A attached on a gold surface via disulfide monolayers. IgG binding to protein A on disulfide monolayers yielded equilibrium dissociation constants of \(1.4 \times 10^{-7} \text{ M}\). The corresponding dissociation constant value for the acetylated version of the peptide (Ac-HWRGWV) supported on alkanethiol SAM was \(5.8 \times 10^{-7} \text{ M}\) and that for HWRGWV on the alkanethiol SAM (after de-protection of Fmoc-HWRGWV) was \(1.2 \times 10^{-6} \text{ M}\). Maximum IgG binding capacities, \(Q_m\), of 6.7, 3.8, and 4.1 mg m \(^{-2}\) were determined for the protein A and the two forms of HWRGWV-based biosensors, respectively. Real-time data for the kinetics of adsorption were used to determine the apparent rate constants for adsorption and desorption. The results were analyzed to understand the mechanism of IgG binding to the protein and peptide ligands. It was found that the peptide–IgG binding was reaction controlled, however the protein A–IgG binding mechanism was partially mass transfer (diffusion) controlled. The adsorption rate constants, \(k_a\), for the protein A ligand increased with decreasing concentration of analyte and the peptide ligand \(k_s\) values were constant at different IgG concentrations and flow rates.

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1. Introduction

The phenomenon of protein adsorption to surfaces is ubiquitous in systems that come in contact with biological fluids, for example prosthetics, cell culture supports, bioseparation processes, biological sensors, etc. Despite the relevance of ligand–receptor binding in clinical diagnostics, platforms that can be deployed easily and universally to monitor protein interactions at interfaces are still very limited (Dimech et al., 2008; Rivetz et al., 2009). This need is especially acute for on-line monitoring and control in the biopharmaceutical industry (Thillaivinayagalingam et al., 2010; Darcy et al., 2011; Wang et al., 2009). Real-time biosensing via quartz crystal microgravimetry (QCM), surface plasmon resonance (SPR) and capacitive techniques have afforded several advantages over traditional bioassays such as enzyme-linked immunosorbent assay (ELISA) (Loftgren et al., 2007; Swanson et al., 2004). For instance, SPR does not require reporter molecules such as enzymes/substrates, fluorochromes or radioisotopes, which may affect the binding behavior of target proteins and ligands. In addition, time-resolved information, kinetic and thermodynamic parameters can be obtained even in systems involving low sample volume or concentration, thus facilitating the determination of affinity dissociation constant \(K_d\) as well as adsorption- and desorption-kinetic rate constants \(k_a\) and \(k_d\), respectively (Karlsson et al., 2004; Mattisson et al., 2010). This is possible because SPR allows real-time analysis of interactions between analytes in solution and surface-immobilized ligands (Rich et al., 2008) by generating a continuous experimental readout of complex formation. Equilibrium affinity data help to understand the strength and specificity of interactions between biomolecules, while kinetic rate data provide insights into the binding mechanism.

* Corresponding author at: Department of Chemical and Biomolecular Engineering, North Carolina State University, 911 Partners Way, Raleigh, NC 27695, USA.
Tel.: +1 919 515 5118; fax: +1 919 515 3465.
E-mail address: ruben@ncsu.edu (R.G. Carbonell).

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Proteinaceous ligands such as *Staphylococcus aureus* protein A and *Streptococcus* protein G are the most common ligands used for the affinity detection and purification of human immunoglobulin G (IgG). However, their high cost, low stability, antigenicity, and loss of antibody activity associated with harsh elution and wash conditions are reasons for realizing less expensive and more robust synthetic small molecule ligands (Roque et al., 2007). The application of short peptide ligands in the development of biosensors is an attractive alternative to protein-based biosensors due to the potentially lower synthesis costs for large amounts of highly pure ligands, their high chemical and thermal stability, and relative ease of modifications for enhanced binding (Pavan and Berti, 2010; Tothill, 2010). Synthetic peptides are also emerging as one of the most promising alternative molecules in the transducing layer of biosensors to replace larger, more complex biomolecules (Skerra, 2007). Design of peptide ligands has been reported previously by authors for detecting functional proteins, enzymes (Aili et al., 2009), toxins (Frisk et al., 2009), and small molecules (Ding et al., 2012; Lu et al., 2009; Viguier et al., 2011). The use of peptides in real applications and the optimal techniques for immobilizing peptide ligands as receptor molecules on biosensors is an ongoing area of research (Tothill, 2010). Peptide ligands for IgG binding, particularly via Fc fragment have been developed (D’Agostino et al., 2008; DeLano et al., 2000; Newcombe et al., 2005), however only a few authors have immobilized these ligands onto sensor surfaces (Bolduc et al., 2009).

A hexamer peptide ligand, HWRGWV, identified from a one-bead-one-peptide combinatorial library, was synthesized on chromatography resins and found to exhibit high affinity and specificity to the Fc fragment of human IgG (Yang et al., 2005). A chromatography resin with HWRGWV was able to purify human IgG from complete minimum essential medium (cMEM) and other complex mixtures with purities and yields as high as 95%, matching very well the results found with protein A/G as affinity ligands (Naik et al., 2011; Yang et al., 2005). In earlier work, our group has successfully demonstrated that the peptide HWRGWV may be grafted onto a random copolymer layer rich in primary amines, and has achieved success in binding IgG from buffer solutions using SPR and QCM. It involved elaborate steps for sensor fabrication but it was possible to demonstrate surface regeneration for multiple uses. The success of IgG binding on the peptide layers on polymers has demonstrated that there is a potential for construction and application of a variety of peptide based matrices for IgG separation and detection (Zhang et al., 2012). The current study aims to graft peptides on matrices where the exposure and orientation of the peptide is better defined using methods that require fewer steps to fabricate.

In this work, interactions between HWRGWV ligand and human IgG, were investigated by grafting the hexamer ligand on SPR chips with pre-adsorbed self-assembled monolayers (SAMs). The performance of the peptide ligand was compared to protein A based IgG and bovine serum albumin (BSA). The results of our studies provided insights into the differences between the interactions of large protein ligands (protein A) and small peptide ligands with antibodies and also explored the potential of hexameric peptide ligands as a viable alternative for developing sensors for on-line IgG detection. To the best of our knowledge this is the first side-by-side comparison of the binding mechanisms of IgG to a large molecule ligand (protein A) and a small molecule peptide ligand (HWRGWV).

2. Experimental

2.1. Materials

Bioresistant alkanethiol EG₆NH₂, (11-mercaptoundecyl)hexa(ethyleneglycol)ammonium-hydrochloride, HS(CH₂)₁₁(CH₂CH₂O)₆NH₂HCl was obtained from Prochimia Surfaces Inc. (Poland). Ethanol (200 proof) was obtained from Pharmaco-AAPER (Charlotte, NC). N,N-dimethyformamide (DMF, ACS reagent, 99.8%), anhydrous DMF, disopropylethylamine (DIPEA), glyceral, glacial acetic acid (ACS reagent 99.5%), bovine serum albumin (BSA, 98%), dithiobisuccinimide propionate (DSP), recombinant protein A from *S. aureus* cowan strain (expressed in *Escherichia coli*), phosphate buffered saline (PBS, pH 7.4, monobasic sodium phosphate, dibasic sodium phosphate, sodium chloride 0.138 M, potassium chloride 0.0027 M) and triethylene amine (TEA, ACS reagent 99%) were all obtained from Sigma-Aldrich (St. Louis, MO). 2-{1H-7-Azabenzotriazol-1-yl}-1,1,3,3-tetramethyl uronium hexafluorophosphate methaniminium (HATU) was purchased from ChemPept Inc. (Wellington, FL). Human immunoglobulin G (IgG, 97% purity) was obtained from Equitech-Bio, Inc. (Kerrville, TX). Deionized water (resistivity > 15 MΩ cm) and Milli-Q water (MQ water, resistivity > 18 MΩ cm) were obtained by using a Millipore water purification system (Billerica, MA). Nitrogen gas was obtained from Airgas National Welders (Raleigh, NC). Peptides Fmoc-His-Trp-Arg-Gly-Val-Ala-OH, acetylated-His-Trp-Arg-Gly-Val-Ala-OH and His-Trp-Arg-Gly-Val-Ala-Val-OH were custom synthesized by Genscript Inc (Piscataway, NJ) at > 97% purity. Dimethyl sulfoxide (DMSO) was obtained from Fisher Chemicals (Pittsburgh, PA).

2.2. Cleaning gold slides and sensors

Glass slides coated with 100 nm of gold (25 × 6.25 × 1 mm², EMF Corporation, Ithaca, NY) or glass sensor chips (12 × 20 × 0.5 mm³) sputtered with a 50 nm gold layer on top of 2 nm chromium (KSV Instruments OY, Helsinki, Finland) were cleaned prior to use by soaking in Piranha solution (98% H₂SO₄ and 30% H₂O₂ at 7:3 v/v) for 15–20 min, followed by profuse rinsing in MQ water, further rinsing in 200 proof ethanol and finally drying under a stream of nitrogen. The surfaces were immersed in ethanol for not more than 30 min before being added to freshly prepared solutions for further modification. (Warning: Piranha solution reacts violently with organic materials and should be handled with extreme caution.)

2.3. Protein A immobilization on gold surfaces

The protein A ligand was immobilized via a disulfide linker using standard protocols, details of which are described in Supplementary information S1.

2.4. Self-assembled monolayer (SAM) on gold surfaces followed by peptide grafting

Alkanethiol EG₆NH₂, at 1 mM concentration, was dissolved in 200-proof ethanol with 3% (v/v) triethylene amine (TEA). Gold surfaces were left in the thiol solution under nitrogen for a period of 24 h. The surfaces were then removed and a rinsing protocol reported by Wang et al. (2005) was used to remove excess components from the SAM. The surfaces were rinsed with ethanol followed by soaking in a 10% (v/v) aqueous acetic acid solution. The surfaces were then sonicated in 10% acetic acid for 2 min followed by rinsing and sonication in ethanol. The surfaces were finally rinsed with ethanol and dried under a nitrogen stream in preparation for characterization, SPR experiments, or peptide grafting. The peptides were grafted via the –NH₂ group of the thiol-modified surfaces by using chemistries similar to those previously reported for chromatographic resins (Yang et al., 2005).

Acetylated peptide (Ac-HWRGWV) was coupled by immersing the slides or sensors in anhydrous DMF containing Ac-HWRGWV, HATU and DIPEA at 5 mM, 10 mM and 20 mM, respectively.
HWRGWV slides/sensors were prepared by immersing the slides or sensors in anhydrous DMF with 5 mM Fmoc-HWRGWVA instead of acetylated peptide. For comparison of ellipsometric thicknesses, slides were also prepared in the same manner with 5 mM un-capped HWRGWV. The slides/sensors and the appropriate peptide solutions were purged with nitrogen gas and the container was sealed to avoid exposure to atmospheric moisture. The container was left on a shaker at 250 rpm overnight at room temperature. After completion of the peptide grafting reaction, the slides/sensors were rinsed with ACS grade DMF followed by sonication in DMF for 5 min. For HWRGWV slides/sensors, de-protection was carried out using a solution of 20% piperidine in DMF (room temperature, 1 h) to remove the Fmoc group followed by rinsing and sonication in DMF. Finally, the slides/sensors were rinsed again in DMF and then dried under a nitrogen stream and used for further experiments. ToF-SIMS analyses of modified gold slides (1 cm × 1 cm) were performed using an ION-TOF SIMS 5 instrument (ION-TOF GmbH, Münster, Germany), details of which can be found in Supplementary information S4.

2.5. Surface density of SAMs and grafted ligands

The thicknesses of the EG6NH2, peptide or protein A layers deposited on the gold slides were measured by ellipsometry using an Alpha-SE spectroscopic ellipsometer (J.A.Woollam, Lincoln, NE) at an angle of incidence $\Phi=70^\circ$ and a wavelength range of 380–900 nm. Five points were measured on multiple slides and the average thickness was recorded with the index of refraction of $\rho$ fixed at 1.45, unless otherwise stated. The number of molecules per unit area was estimated using the expression (Arwin, 2001)

$$\Gamma = \frac{\rho d N_A}{MW} \times 10^{-22}$$

(1)

where $\Gamma$ is the surface density in molecules nm$^{-2}$, $d$ is the ellipsometric thickness in nm, $\rho$ is the specific gravity of the species in g cm$^{-3}$, $N_A$ is the Avogadro’s constant, and $MW$ is the molecular weight of the species. The thicknesses of peptides layers were also measured 7 days after the first measurement (during which the slides were kept away from light under nitrogen at 4 °C). Authors have calculated $\Gamma$ for protein layers assuming $\rho=1.2$ (in air) and $\rho=1.35$ (in solution) (Elwing, 1998; Tengvall et al., 1998). The maximum theoretical diameter of the peptide was found using the visualization and measurement tools of a space filling model software, PYMOL (Delano Scientific Inc.). The diameter of the peptides was used to calculate the theoretical area occupied by one peptide molecule (assuming hexagonal packing of peptides assumed to behave like spheres with the calculated theoretical diameter).

2.6. Surface plasmon resonance (SPR) experiments

A KSV SPR 200 instrument (BioNavis Instruments, Helsinki, Finland) was used to detect variations in the refractive index at the interface of the sensor. The details of the experiments and measurements carried out are described in Supplementary information S2.

2.7. Equilibrium and kinetic parameters

The maximum net change of SPR signal that occurs after IgG injection at different concentrations was recorded during the experiments and the amount of analyte adsorbed per unit area calculated. Isotherms of adsorbed IgG in equilibrium with the bulk solution concentration injected were fitted to a simple Langmuir model that used as variables, the adsorbed mass per unit area Q (mg m$^{-2}$) and the solution concentration C (mg ml$^{-1}$),

$$Q = \frac{Q_m C}{K_d + C}$$

(2)

where $K_d$ is the equilibrium dissociation constant (M) and $Q_m$ is the maximum binding capacity (mg m$^{-2}$). It can be assumed that the IgG and the ligand Lg on the surface (either protein A or peptide) bind reversibly forming a complex IgG.Lg as indicated in the expression,

$$k_d$$

$$\text{IgG} + \text{Lg} \rightarrow \text{IgG.Lg}$$

(3)

The rate of adsorption can be assumed to be a second order reaction with a rate constant $k_a$ and the desorption step can be assumed to be a first order reaction proportional to the concentration of IgG.Lg complex on the surface with a desorption rate constant $k_d$.

$$\frac{d[\text{IgG.Lg}]}{dt} = k_a [\text{IgG}] [\text{Lg}] - k_d [\text{IgG.Lg}]$$

(4)

The detailed calculation steps to determine adsorption and desorption rate constant and the detailed description of the mathematical analysis to determine the mechanism of IgG binding at the SPR sensor surface can be found in Supplementary information S3. The model includes an estimation of diffusional effects of antibody transport from the bulk to the surface of the sensor, and a calculation of the change of antibody concentration in the fluid next to the surface ($C_f$) with time. If the mass transfer coefficient is $K_m$ much greater than the adsorption rate constant, the surface concentration ($C_s$) will equal the bulk concentration ($C_b$) and the binding process is rate limited and the ratio $C_b/C_s$ will approach 1. If the rate constant for adsorption is very large relative to the mass transfer coefficient, the surface concentration will approach zero and the adsorption process will be mass transfer limited.

3. Results and discussion

3.1. Characterization of ligands grafted on self-assembled monolayers (SAMs)

The hexameric peptides were grafted with uronium salt-based chemistries onto self-assembled monolayers (SAMs) of EG6NH2 on gold slides. The covalent attachment of the peptides on the slides was confirmed byToF-SIMS analyses of modified gold slides (1 cm × 1 cm) were performed using an ION-TOF SIMS 5 instrument (ION-TOF GmbH, Münster, Germany), details of which can be found in Supplementary information S4, provided direct evidence that peptide was present on the surface after the grafting step and that the de-protection was efficient.

3.1.1. Ellipsometric thicknesses of peptide on SAMs

The thickness of an adsorbed layer can be used as a parameter to quantify the amount of material on the surface if one assumes a homogeneous adsorbed layer with constant refractive index (Bae et al., 2005). The percentage of –NH$_2$ moieties that reacted can be estimated by comparing the number of grafted peptides per unit area to the number of –NH$_2$ groups in the SAM. The results are summarized in Table 1, and include calculated values for the density of ligands per unit area, $\Gamma'$; and results of 0.24–0.49 peptide molecules nm$^{-2}$ on SAMs were obtained.

During reactions of the Fmoc-HWRGWVA (with subsequent de-protection) and the uncapped HWRGWV, only 10–12% of –NH$_2$ of the SAMs were converted to peptides as shown in Table 1. Meanwhile the
acetylated peptides showed a higher percentage of NH$_2$ conversion at 18%. After 7 days, the HWRGWV slides showed an increase in length of approximately 1 Å, while the Ac-HWRGWV slides did not show significant changes. The overall peptide densities were similar to those obtained when grafting molecules of similar molecular mass onto SAMs by other authors (Ding et al., 2012; Xiao et al., 2004).

3.1.2. Comparison to theoretical values
The theoretical surface density of peptide molecules with efficient packing can be calculated using the theoretical diameter of influence as provided by the space filling model software PYMOL. After the structure of the peptide molecule was introduced, the theoretical diameter was calculated by PYMOL to be 15 Å, yielding a theoretical surface peptide density for hexagonal close packing on the surface of 0.51 molecules nm$^{-2}$. The experimental surface density calculated was 0.49 molecules nm$^{-2}$ for the Ac-HWRGWV peptide, which suggests that the acetylated peptide molecules are well packed on the surface in a brush-like structure consistent with the SAM layer below the peptides. The surface densities of the other peptide molecules are lower than the values of 0.51 molecules nm$^{-2}$ suggesting that the peptide molecules are situated further from each other due to the lower efficiency of the NH$_2$ conversion.

3.1.3. Surface density of protein A immobilized on disulfide layer supports
The total ellipsometric thicknesses of the protein A and the underlying DSP layer were measured, giving a thickness of 31.7 Å, and a surface density of 0.0466 molecules nm$^{-2}$ on the dried surface. Experiments to measure surface density of protein A on the disulfide monolayer were also done by injecting protein A (at a concentration of 0.25 mg ml$^{-1}$) into the SPR flow cell onto the surface of a sensor modified by DSP. The calculated protein A surface density by the SPR experiment was 0.047 molecules nm$^{-2}$, and matched well with the ellipsometric density obtained with our experiments. Schmid et al. (2006) immobilized protein A at different concentrations (0.1 mg ml$^{-1}$ and 0.5 mg ml$^{-1}$) on DSP-modified gold surfaces and measured the resulting surface densities. Their results were interpolated to find the surface density that would result for a coupling reaction at 0.25 mg ml$^{-1}$ of protein A, giving a value of 0.0448 molecules nm$^{-2}$, which corresponded well with our results.

3.2. SPR results of human immunoglobulin G (IgG) and bovine serum albumin (BSA) binding on protein A and peptide ligands

3.2.1. Maximum binding and equilibrium dissociation constant of protein A sensors
SPR experiments with various concentrations of analytes were carried out on protein A sensors, which are conventionally used for IgG detection. The amount of analyte bound to the surface was calculated from the net angle change shown by the sensorgrams before and after analyte injections. The data points for IgG binding were fitted to a Langmuir Eq. (2) as shown in Fig. 1(a) to yield values of $K_d$ of $(1.43 \pm 0.26) \times 10^{-7}$ M and $Q_m$ of $6.72 \pm 0.26$ mg m$^{-2}$. Comparing IgG and BSA binding results, it can be observed that the maximum amount of IgG bound to the protein A biosensor is almost 40 times that of the maximum BSA binding, thus demonstrating the high specificity of protein A biosensor towards IgG.

Wang and Jin (2003) immobilized IgG on silanized silica surfaces with a cross-linker and protein A, and found a maximum binding capacity similar to ours at 5.7 mg m$^{-2}$ with a $K_d$ of $1 \times 10^{10}$ M. Saha et al. (2003) immobilized protein A directly on gold and found IgG equilibrium dissociation constant, $K_d$, equal to $3.4 \times 10^{-8}$ M which is lower than what was found in this study. The protein A–IgG equilibrium binding parameters of our study are in a similar range as those cited by other authors.

3.3. Maximum binding and equilibrium dissociation constants of peptide sensors
Peptide HWRGWV and its acetylated version Ac-HWRGWV were investigated for IgG and BSA binding using SPR biosensors. The binding isotherms of IgG and BSA to the SAM-modified gold surface and the surface containing the two versions of ligands are shown in Fig. 1(b, c). It is evident that the IgG binding on the sensors is effective in the presence of peptides, displaying maximum adsorbed IgG values of 4.02 and 3.75 mg m$^{-2}$ on HWRGWV and Ac-HWRGWV, respectively. On the sensors with only SAM layers, the IgG and BSA bound at comparable levels of approximately 1 mg m$^{-2}$. This can be attributed to non-specific binding by electrostatic interactions with the unreacted –NH$_2$ groups of the SAMs. The presence of the peptides enhanced the IgG binding on the surfaces, indicating the specificity of the peptides towards IgG. BSA binding at 0.5 and 1.0 mg m$^{-2}$ on HWRGWV and Ac-HWRGWV can be also attributed to the non-specific binding onto the free –NH$_2$ groups. It should be noted however that non-specific binding arising from electrostatic interactions was minimized by carrying out all the binding experiments using buffers with high NaCl concentrations. A reduction of charge on the surfaces themselves should further contribute to reduction of non-specific binding. This can be achieved by diluting the SAMs to form mixed monolayers, with the dilutor thiol of SAMs having end groups which contribute to reduction of non-specific binding, for instance, –OH terminated thiols.

Fitting the isotherm data to the Langmuir Equation, Eq. (2) for IgG binding, the HWRGWV peptide exhibited a $K_d$ = $(1.2 \pm 0.3) \times 10^{-6}$ M and a value of $Q_m$ = $4.09 \pm 0.24$ mg m$^{-2}$ whereas the Ac-HWRGWV peptides resulted in a $K_d$ of $(5.8 \pm 1) \times 10^{-7}$ M and $Q_m$ of $3.75 \pm 0.19$ mg m$^{-2}$. Thus the Ac-HWRGWV and HWRGWV ligands do not show much difference in IgG binding capacities or in affinities. A similar observation was reported in other work using polymethacrylate resins with both forms of peptides immobilized via C-termini (Yang et al., 2009).

The specificity of the sensors can be indicated by the ratio of the maximum IgG binding to the maximum BSA binding. This ratio for protein A was about 40:1, higher than that for peptide sensors with a calculated ratio of up to 10:1. The binding affinities of the peptide-based biosensors are four times lower than those of protein A sensors. It should be noted that the lower $K_d$ value for
the protein A sensor enables it to measure IgG concentrations well below 1 mg ml$^{-1}$. The higher $K_a$ value for peptide-based sensors still allows them to detect IgG concentrations of 0.01 mg ml$^{-1}$ or above. Given that titers of IgG in CHO cell cultures are commonly below 1 mg ml$^{-1}$, the protein A sensor enables it to measure IgG concentrations well above 0.01 mg ml$^{-1}$, as demonstrated on work done with chromatographic resins, due to the high $K_a$ value of the ligand-IgG binding experiments were utilized in the calculations of the adsorption and desorption rate constants and a comparison of these rate constants for large and small ligands.

The $K_d$ values from the isotherm and the SPR dynamic responses of the ligand-IgG binding experiments were utilized in the calculation of $k_o$. The value of the adsorption rate constant $k_a$ was calculated by fitting the sensogram data (SPR response in arbitrary units, $R$ vs time, $t$) to the numerical solution of the differential equations, described in the Supplementary information S3. The fitting of theoretical models (solid lines) to the experimental data (dotted lines) for IgG binding to the peptide and protein ligands pertaining to three concentrations ($C_o > K_d$, $C_o \sim K_d$ and $C_o < K_d$) are shown in Fig. 2 for (a) protein A, (b) Ac-HWRGWV and (c) HWRGWV sensors. The resultant $k_a$ values are summarized in Table 2.

The best fits were observed for protein A sensors for all concentrations, and for the Ac-HWRGWV sensors for concentrations of IgG equal to or less than $K_d$. The experimental runs on HWRGWV sensors (dotted lines, Fig. 2(c)) show more variability among runs, as well as with the theoretical fittings. HWRGWV sensors have a lower peptide density, 0.24 molecules nm$^{-2}$ compared to those of the Ac-HWRGWV. The presence of higher number of unreacted –NH$_2$ as well as the free –NH$_2$ at the peptide terminus, may contribute to increased variability due to non-specific binding.

For the acetylated peptides average values $k_a$ of $2.1 \pm 0.7$ m$^3$ mol$^{-1}$ s$^{-1}$ and $K_d$ of $(1.2 \pm 0.4) \times 10^{-3}$ s$^{-1}$ were calculated. The average $k_d$ of HWRGWV sensors was $2.18 \pm 0.4$ m$^3$ mol$^{-1}$ s$^{-1}$ and $k_o$ was $(3.1 \pm 0.5) \times 10^{-5}$ s$^{-1}$. The $k_a$ and $K_d$ values obtained were independent of concentrations as well as flow rates used, for these peptide sensors. For the protein A sensors the $k_o$ values ranged from 2.4 to 10 m$^3$ mol$^{-1}$ s$^{-1}$, with $k_a$ values ranging from 0.3 to $1.5 \times 10^{-3}$ s$^{-1}$ and the values showed a decreasing trend with increasing concentration at flow rates of 10 μl min$^{-1}$. This trend was also prevalent at higher flow rates of 100 μl min$^{-1}$ with $k_o$ values in the range from 3.1 and 9.3 m$^3$ mol$^{-1}$ s$^{-1}$ for $C_o > K_d$ and $C_o < K_d$, respectively, as shown in Table 2.

Protein A experiments were carried out in our study using IgG concentrations from 0.001 to 5 mg ml$^{-1}$. The results showed a higher value of adsorption rate constants for concentrations ranging from 0.001 to 0.05 mg ml$^{-1}$ than those in the range of 0.05–5 mg ml$^{-1}$. In quartz crystal microbalance (QCM) studies by Ogi et al. (2007) to evaluate the effect of concentration on the affinity between protein A and IgG, they observed different affinities for different concentration ranges. Similar to our results,  

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the *K_d* value they observed for the 0.005–0.02 mg ml⁻¹ concentration range is 1.6 × 10⁻⁷ M; for lower concentration ranges, a smaller *K_d* value was estimated.

Saha et al. (2003) calculated *k_∞* values of 8.02 m³ mol⁻¹ s⁻¹ for protein A–IgG experiments with IgG concentration in the range of 0.001–0.05 mg ml⁻¹, similar to our values 8.83 ± 2.6 m³ mol⁻¹ s⁻¹ taken for the same concentration range (*C_b ≤ K_d*); and reported a smaller *k_∞* value for a broader concentration range 0.001–0.1 mg ml⁻¹.

The cited studies indicate a decrease of *k_∞* for higher concentrations or at a higher concentration range, which corresponded with our observations. The variation in affinity at various analyte concentrations can be attributed to different structures of the IgG–protein A complex, which occur at different IgG:protein A concentration ratios, and the fact that these different complex structures may possess different affinities. The difference in affinity has been attributed to allosteric/cooperative binding of IgG to protein A; at lower concentrations of analyte, a neighboring second protein A molecule is able to bind to an already bound IgG molecule with greater affinity than the first (Hanson and Schumaker, 1984).

To analyze effects of mass transport, according to guidelines described in Supplementary information S3, the calculated ratio of surface to bulk IgG concentrations, *C_s/C_b*, were plotted as a function of

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**Table 2**

Average *k_∞* values of protein A and peptide ligands (Ac-HWRGWV and HWRGWV) at different IgG concentrations and flow rates.

<table>
<thead>
<tr>
<th>Concentration range</th>
<th>Average <em>k_∞</em> at flow rate 10 μL min⁻¹ (m³ mol⁻¹ s⁻¹)</th>
<th>Average <em>k_∞</em> at 100 μL min⁻¹ (m³ mol⁻¹ s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Protein A</strong></td>
<td><strong>Ac-HWRGWV</strong></td>
<td><strong>HWRGWV</strong></td>
</tr>
<tr>
<td><em>C_b ≤ K_d</em></td>
<td>10.3</td>
<td>2.54</td>
</tr>
<tr>
<td>*C_b ~ <em>K_d</em></td>
<td>5.89</td>
<td>2.11</td>
</tr>
<tr>
<td>*C_b &gt; <em>K_d</em></td>
<td>2.15</td>
<td>3.1</td>
</tr>
</tbody>
</table>

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**Fig. 2.** Experimental (dotted) and theoretical fittings (solid lines) yielding *k_∞* values for (a) protein A (b) Ac-HWRGWV and (c) HWRGWV.

**Fig. 3.** Surface concentration to bulk concentration ratio (**C_s/C_b**) plots for (a) protein A, (b) Ac-HWRGWV and (c) HWRGWV at flow rate 10 μL min⁻¹. Plots for IgG concentrations higher than dissociation constant are denoted by large dashed lines (— — —), those similar to dissociation constant by solid lines (—) and those lower than dissociation constant by small dashed lines (— —).
time, as shown in Fig. 3 for (a) protein A ligands, (b) Ac-HWRGWV and (c) HWRGWV ligands at a fixed flow rate of 10 μL min⁻¹. Each plot shows the behavior at three concentrations: long dashed lines for concentrations greater than \( K_a \), short dashed lines for concentrations less than \( K_a \) and solid lines for concentration similar to \( K_a \). It can be concluded that the IgG-peptide binding is rate-controlled as is evident from the IgG–peptide binding profiles, where \( C_i/C_b \) is greater than 1 from the onset of the adsorption process. However for IgG–protein A binding, the \( C_i/C_b \) plots do not approach unity immediately, leading to the conclusion that the binding reaction in this case (10 μL min⁻¹ flow rate) is partly mass transport controlled.

This experiment was repeated by increasing the flow rate to 100 μL min⁻¹ in order to increase the \( K_m \) value, and the corresponding analysis is shown in Fig. 4. The experiments were carried out at two concentrations of IgG with 1 mg ml⁻¹ (\( C_b > K_d \)) and 0.01 mg ml⁻¹ (\( C_b < K_d \)) on protein A and 1 mg ml⁻¹ IgG on peptide sensors; but at a higher flow rate of 100 μL min⁻¹ in order to increase \( K_m \). Increasing the flow rate did not affect the \( C_i/C_b \) plots for 1 mg ml⁻¹ (long dashed lines) for either protein A sensors (Fig. 4(a)) or the peptide (Fig. 4(b)). In these cases the ratio of \( C_i/C_b \) is close to 1 at all times, unlike the ratio of \( C_i/C_b \) obtained with protein A sensors at an IgG concentration of 0.01 mg ml⁻¹. In this latter case the ratio of surface to bulk concentration \( C_i/C_b \) is less than 1 from the onset of the adsorption step.

Thus, the peptide binding mechanism to IgG tends to be reaction rate controlled regardless of flow rate. The protein A–IgG binding shows two major characteristics: binding shows distinctly different affinities at different concentration ranges of IgG; and the reaction is partially diffusion controlled. The mass transport limitations are more evident at lower concentrations of the analyte, or in other words, at lower stoichiometric analyte:ligand ratios.

The partially diffusion controlled situations are typical in biological systems like the SPR microfluidic channel (Gervais and Jensenk, 2006). Since the peptide ligands are much smaller than protein A and have no tertiary structure, it is not unreasonable that the binding kinetic rate is relatively fast since there are minimal molecular rearrangements required during binding. Highly specific binding of IgG to protein A might require additional time to adjust the orientation of sites in the binding regions of the proteins and this slower process can introduce some mass transfer limitations during the SPR experiments.

4. Conclusions

Applicability of novel hexameric peptide ligands for IgG detection was studied and compared to the results of protein A based sensors. Both protein A and small peptide sensors were compared side-by-side in IgG binding experiments using SPR. Kinetic parameters for binding of IgG were obtained by fitting the experimental data to a theoretical model for IgG to ligand binding on the surface which included mass transfer and kinetic effects.

HWRGWV peptides were grafted onto the alkanethiol monolayers on gold sensors and showed specific binding with high affinity towards IgG, as did the protein sensors. Peptide sensors can be suitable for IgG detection as low as 0.01 mg ml⁻¹ IgG, and can be potentially constructed at lower costs as compared to protein A sensors.

The affinity properties of protein A are concentration-dependent, and at high IgG concentrations, the adsorption rate constants closely approach that of hexameric peptide ligands. The change in affinity properties of protein A are possibly due to the allosteric effects which are attributed to multiple binding sites on either ligand or analyte. The peptide, which lacks a tertiary structure, on the other hand, displays constant \( K_a \) values at all concentrations of IgG, as well as at high flow rates.

Among the two versions of the peptide based sensors, the HWRGWV sensors showed variability of peptide density, lower value of peptide density, as well as higher variability among experimental runs in IgG binding. The maximum binding capacity and equilibrium constants of the HWRGWV and Ac-HWRGWV sensors are statistically similar. The desorption rate constant for the Ac-HWRGWV sensors however are lower than for their non-acetylated counterparts, thus indicating that in buffer, the IgG desorption would be slower and the binding would be stronger. The evidence thus favors the use of small molecule ligands such as Ac-HWRGWV rather than their non-acetylated counterparts, without further modifications, for developing peptide based biosensors. However, issues such as selectivity and performance in complex mixtures must be looked at if the peptide based biosensor is to be made viable in a variety of industrial settings.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2014.02.069.