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# Surface Plasmon Resonance to Determine Apparent Stability Constants for the Binding of Cyclodextrins to Small Immobilized Guests

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**Abstract**

Surface plasmon resonance (SPR) has been used to determine apparent stability constants for the non-covalent interactions of cyclodextrin (CD) hosts with small organic guests. This technique allows detection of the molecular interactions by monitoring changes in refractive index at gold surfaces on which the guests are immobilized. The magnitude of an SPR response is proportional to the mass change at a surface, and thus the technique has most commonly been used in the past to study large molecules such as proteins and DNA. Now SPR has been employed to study the interactions of $\alpha$CD, $\beta$CD, $\gamma$CD, per-2,6-dimethyl-$\beta$CD and Molecusol$^{TM}$ (hydroxypropyl-$\beta$CD) with immobilized *N*-(1-adamantylmethyl)-, *N*-octyl-, *N*-benzyl-, *N*-(4-methylbenzyl)-, *N*-(4-*tert*-butylbenzyl)- and *N*-(1-pyrenylmethyl)-amides. Methods are outlined for obtaining high-quality, reproducible binding data. The magnitudes ($10^2$–$10^4$ $M^{-1}$) and trends in the apparent stability constants so observed are generally consistent with values reported for analogous solution-phase studies. The results show that SPR is suitable to study host–guest interactions of small molecules such as cyclodextrins.

## Introduction

Non-covalent interactions between molecules are important in many biological and chemical systems. In order to understand such interactions it is necessary to characterize them thermodynamically and kinetically, which is commonly done by determining parameters such as complex stability constants and rate constants for complex formation and dissociation [1]. Techniques used to determine these parameters include NMR [2] and fluorescence [3] spectroscopy, calorimetry [4], potentiometric titration [5] and chromatography [6]. The merits of these techniques vary, but limitations arise because some require large amounts of materials, some depend on at least one of the binding partners having a suitable spectroscopic probe, and some are only suitable for complexes which are highly stable.

Optical biosensors have been developed in the last decade for studying intermolecular interactions [7–10]. One of the techniques used in such biosensors is surface plasmon resonance (SPR), where a change of mass near a metal surface causes a proportional change in the refractive index signal at the surface [8]. Thus if one type of molecule is covalently immobilized on the surface, and a second type of molecule interacts with the first, the consequent refractive index change can be used to quantify the extent of interaction. There are several commercially available biosensors, including the Biacore®, IAsys® and IBIS® systems [9]. The work described in this paper has been performed on a Biacore®, which uses a flow system to bring analyte solutions containing one binding partner into contact with a metal surface on which the second binding partner is covalently immobilized. Several methods have been established to covalently attach molecules to the metal surface through a variety of functional groups, including amines, thiols and aldehydes [8]. The SPR signal, or response, is measured in response units (RU) and can be monitored in real-time, allowing the determination of both qualitative and quantitative binding data. Already it has been established that for large molecules such as DNA and proteins, the thermodynamic and kinetic parameters determined using this surface-based technique correlate well with those determined using more traditional solution methods, provided the SPR experiments are performed with care [11]. Systems involving molecules with molecular weights of less than 1000 Da are inherently more difficult to study by SPR due to the smaller magnitude of the mass change at the metal surface. Thus only a relatively few SPR studies have involved smaller molecules [7, 12–14], and the area has been noted as a significant emerging application for SPR studies [12].

Here we show that it is possible to use a Biacore® to study the complexation of small organic guests with molecular weights of 100–200 Da by small hosts such as CDs with molecular weights of 1000–1500 Da. CDs [1] are cyclic oligomers of $\alpha$-D-glucopyranose, each having a hydrophilic exterior and a hydrophobic interior cavity. The most common naturally occurring CDs are $\alpha$-, $\beta$- and $\gamma$-CD, which consist of 6, 7 and 8 $\alpha$-D-glucopyranose units, respectively,

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and have cavities which increase in size as the number of α-D-glucopyranose units increases. Under aqueous conditions organic molecules tend to form non-covalent inclusion complexes with CDs. We have studied the interaction of αCD, βCD, γCD, per-2,6-dimethyl-βCD (DMβCD) and Molecusol™ with immobilized *N*-(1-adamantylmethyl)-, *N*-octyl-, *N*-benzyl-, *N*-(4-methylbenzyl)-, *N*-(4-*tert*-butylbenzyl)- and *N*-(1-pyrenylmethyl)-amides.

## Experimental

Buffers were obtained from Sigma-Aldrich, except for HBS-EP (10 mM HEPES pH 7.4, 150 mM NaCl, 3 mM EDTA, 0.005% v/v polysorbate 20) which was purchased from Biacore AB. The amines, 1-aminomethyladamantane, 1-aminooctane, benzylamine, 4-methylbenzylamine, 4-*tert*-butylbenzylamine and 1-aminomethylpyrene, were purchased from Sigma-Aldrich. αCD and βCD were generous gifts of Nihon Shokhuin Kako Company Ltd, γCD was purchased from Sigma-Aldrich, and DMβCD (per-2,6-dimethyl-βCD) and Molecusol™ (hydroxypropyl-βCD) were purchased from Cyclolab Cyclodextrin Research and Development Laboratories Ltd. Biacore® specific products such as sensor chips and chemicals required for covalent immobilization were purchased from Biacore AB. All experiments were performed at 25 °C.

The work described in this paper has been performed on a Biacore 2000®. In a typical experiment, a molecule of interest is covalently attached (immobilized) to a sensor chip, which is a glass slide covered with a thin layer of gold. This gold surface is modified with a layer of carboxymethylated dextran, which provides a hydrophilic matrix suited to the covalent attachment of proteins and other molecules. When placed in the instrument, the sensor chip surface forms one wall of a flow cell, and buffers or samples are delivered to the surface through a microfluidic system. Measurements are made under conditions of continuous liquid flow over the metal surface. The binding experiment is initiated by injecting a molecule (the analyte) into the flow system, and then monitoring the change in response due to the formation of the complex between the analyte and the molecule attached to the surface (association phase). If the analyte injection is then stopped and the continuous buffer flow restarted, the non-covalently bound analyte will gradually dissociate from the complex that has formed at the surface (dissociation phase). Once all non-covalently bound analyte has been removed (regeneration), the sensor chip containing the covalently attached molecules can be used for another binding experiment. For complexes which dissociate very slowly it may be necessary to use solutions such as salt or acid to displace all analyte from the complex within a reasonable time.

For immobilization of the amines, the continuous flow buffer was HBS-EP and the flow rate was 10 μL/min. The amines were dissolved in buffer (10 mM MES pH 6) at concentrations of around 1 mM, and the solutions were filtered before use. The surface of a carboxymethyl dextran (CM5) sensor chip was activated by injecting *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC) (0.2 M) and *N*-hydroxysuccinimide (NHS) (0.05 M) onto the sensor chip surface for seven minutes. This resulted in reactive ester groups (Figure 1, Step 1). An amine was then injected onto the activated sensor chip surface and allowed to react for four minutes (Figure 1, Step 2). The reaction of the amine with the ester groups results in an amide that is covalently attached to the carboxymethyl dextran-modified gold surface (Figure 1, Step 2). The amine injection was repeated three more times to obtain a high level of immobilization (typically 30–700 RU). Any remaining ester groups were inactivated by injection of 1 M ethanolamine pH 8.5 for seven minutes (blocking, Figure 1, Step 3). One Biacore® sensor chip contains four independent surfaces which are arranged serially and are called flow cells 1–4. Thus a typical experiment involved using flow cell 1 as a control and immobilizing three different amines using the other three flow cells. The control flow cell 1 was activated using EDC/NHS in the same manner as the other surfaces, but was blocked with ethanolamine without being exposed to any other amine.

The CD binding experiments (Figure 1, Step 4) were performed using 50 mM HEPES pH 7, 150 mM NaCl as the continuous flow buffer and a flow rate of 40 μL/min. For each CD, ten concentrations in the range 5–0.01 mM were prepared by serial dilution. Once a CD sample was injected into the flow system, the association phase was monitored for 150 seconds, which was long enough for the SPR signal to reach a stable value (the steady state response) indicating that CD binding was complete. Changing the flow to buffer quickly removed all CD from the surface, so there was no need to regenerate the surface using other solutions. To ensure a stable baseline the sensor chip was left washing for fifteen minutes before the injection of another CD sample. For the ten CD concentrations in each series, the CD injections were performed randomly, and the response at each concentration was measured in duplicate. For each CD concentration, the response of the CD on control flow cell 1 was subtracted from the response observed on the surface with the immobilized amide. The average of the steady state response between approximately 120–140 s was then calculated for each CD concentration. For each immobilized amide and CD pair, this allowed construction of a curve of steady-state response due to binding as a function of CD concentration.

The data were fitted using the BiaEvaluation software version 3.0.2 (Biacore AB). The apparent stability constants ($K_a$ values) for the formation of the 1:1 CD-guest complexes were calculated by fitting the curve of steady-state response versus CD concentration to a 1:1 binding model. For each CD-guest pair, the $K_a$ value was determined at least twice using independently prepared CD samples and independent CM5 sensor chips. A dataset was included in the final $K_a$ average if the fitted data matched the experimental data, the residuals were random, the standard errors were low, the chi-squared value was less than 10% of the calculated maximum response, and duplicate runs reported similar fitted

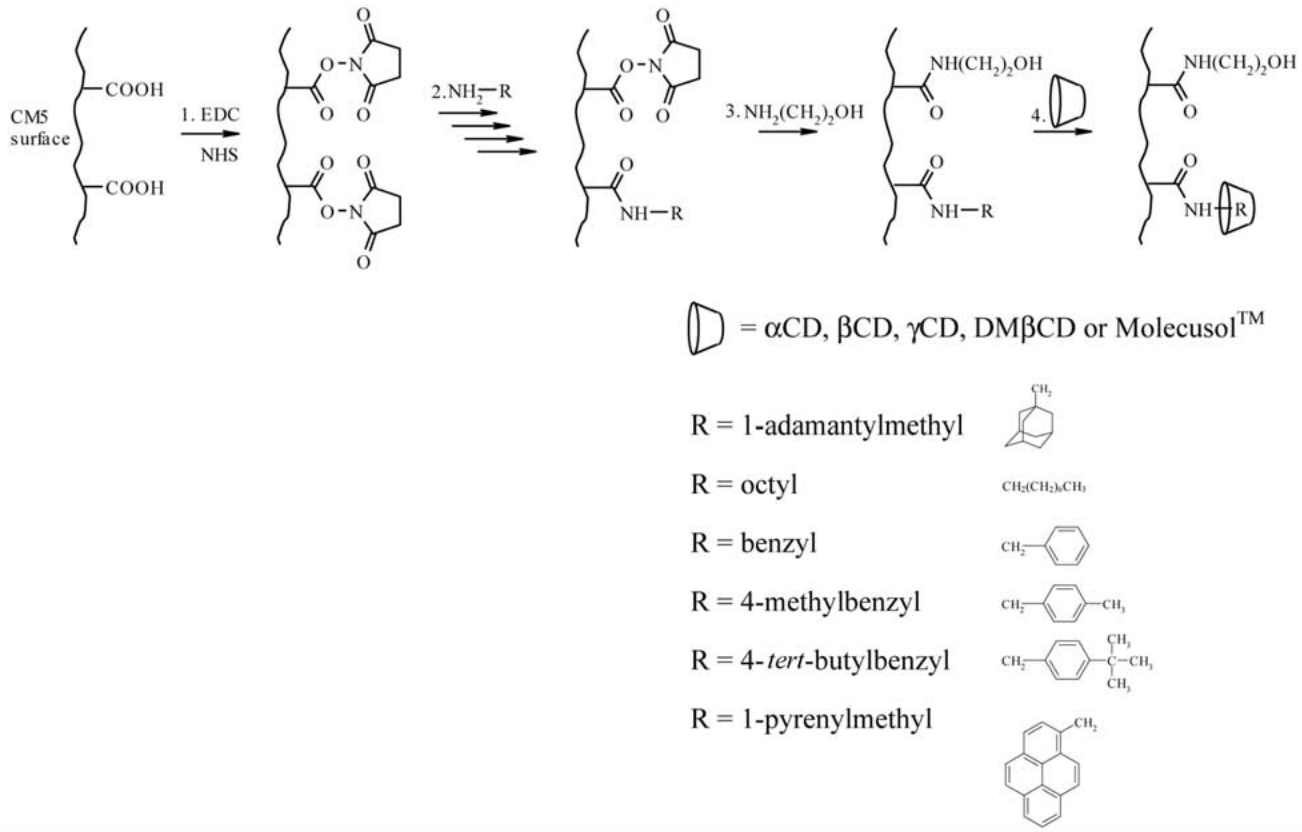


*Figure 1.* Schematic diagram showing the immobilization of an amine guest and the binding of a cyclodextrin to the immobilized amide guest.

parameters. Datasets which did not match these criteria were discarded. A reported $K_a$ value is thus the weighted mean (inversely weighted according to the square of the standard error in the individual $K_a$ values) calculated from at least two datasets from at least two independent sensor chips. The reported error in $K_a$ is the standard deviation of the mean.

## Results and discussion

### *Experimental design*

The difficulties of working with small molecules on an instrument dependent on mass changes for a signal meant that it was necessary to design experiments carefully and conduct the appropriate controls [13, 15, 16]. Since the interactions of interest typically had $K_a$ values around $10^3$–$10^4$ $M^{-1}$, it was necessary to work at high immobilization levels and high analyte concentrations of up to 10 mM to ensure that the concentrations of both binding partners were such that significant amounts of complex would form. Amine guests were chosen because it was believed that the well-established EDC/NHS chemistry (Figure 1, Steps 1–2) would be the most convenient to use. Early experiments indicated that it was possible to achieve approximately five-fold higher immobilization levels of 1-aminomethyladamantane as compared with 1-aminoadamantane. Thus other amines were chosen to have an aminomethyl group rather than an amine group. It was also found that three- to five-fold higher immobilization levels could be achieved by repeating the amine injection four times (Figure 1, Step 2).

The necessity of working at high immobilization levels and high analyte concentrations can lead to serious artifacts such as analyte depletion and bulk refractive index (BRI) changes [15–17], respectively. Depletion occurs when high immobilization levels decrease the concentration of analyte reaching flow cell 4 due to the large amounts of material used in binding to the preceding serially arranged flow cells. This artifact was excluded by showing that the response observed on flow cell 4 was the same regardless of whether the flow was directed over surfaces 1–4 or just over surface 4. BRI shifts are due to a high concentration of solute in the analyte solution causing a change in refractive index that is not due to any binding event. For example, a signal of 800 RU was observed for the injection of 10 mM $\beta$CD onto a control surface which had no guest immobilized. Note that this is a typical response for such a high concentration of carbohydrate, and is similar to the BRI shift of 600 RU that was observed for 12 mM maltose on an unmodified CM5 sensor chip [17]. Since the BRI shift could amount to more than 50% of the total observed response, it was important to correct the binding data for this effect. A possible complication was that different flow cells could have different BRI shifts, due to the different chemical and physical properties of the immobilized guests and the possibly different immobilization levels. This made it necessary to ensure that the BRI shift on the control flow cell was representative of the other flow cells before using it to correct the binding data. This

was done by measuring the response of dimethyl sulfoxide (DMSO, 0.1–2%) on a sensor chip with one control surface and three different immobilized amide guests. For each DMSO concentration, the response was the same on each surface (typically ±2 RU, data not shown), indicating that the BRI shift on all surfaces was similar. This allowed the BRI shift on the control flow cell to be subtracted from all data, resulting in corrected responses that reflected the extent of the CD binding. Finally, the response of buffer alone on all surfaces was negligible, and combined with the relatively high observed binding responses up to 500 RU, it was not necessary to perform the double reference subtraction [15] which is sometimes needed when working with small molecules.

## Results

The experimental design outlined above allowed detection of binding responses of up to 500 RU for the interaction of various CDs with the immobilized amide guests. Typical corrected Biacore® data is shown in Figure 2 for the interaction of $\beta$CD with immobilized *N*-(1-adamantylmethyl)amide. This figure shows that the binding data appeared to be a 'square wave', with apparent association and dissociation rate constants that were too fast to be determined. However, provided the observed response was significant, the steady state responses from such a collection of binding curves (Figure 2) could still be used to determine apparent stability constants. For each CD-guest pair, graphs of steady-state response versus CD concentration were constructed (Figure 3). It was found that the binding curves could generally be classified into three categories according to the magnitude of the maximum observed response: a $K_a$ value could be determined if the maximum response was greater than 100 RU, a $K_a$ value could be estimated if the maximum response was between 50 and 100 RU, and a $K_a$ value could not be determined if the maximum response was below 50 RU. The first category, for which $K_a$ values could be determined, included the interaction of most CDs with immobilized *N*-(1-adamantylmethyl)-, *N*-octyl- and *N*-(4-*tert*-butylbenzyl)-amides (Figure 3), and the interaction of $\gamma$CD with immobilized *N*-(1-pyrenylmethyl)amide (Table 1). Representative plots of steady-state responses against free CD concentrations, including the calculated fits for formation of a 1:1 CD:guest complex, are shown in Figure 3. In the second category (datasets with maximum binding responses between 50 and 100 RU), the binding curves were close to linear and it was possible only to estimate $K_a$ values. This occurred for the interaction of $\alpha$CD with immobilized *N*-(1-adamantylmethyl)amide, $\gamma$CD with immobilized *N*-octylamide, and $\alpha$CD and $\gamma$CD with immobilized *N*-(4-*tert*-butylbenzyl)amide (Figure 3, Table 1). For datasets in the third category, which had very low maximum binding responses (<50 RU), it was not possible to determine a $K_a$ value. Such low maximum responses were observed for the interaction of all CDs with immobilized *N*-benzyl- and *N*-(4-methylbenzyl)-amide, and for all CDs except $\gamma$CD with immobilized *N*-(1-pyrenylmethyl)amide (Table 1).

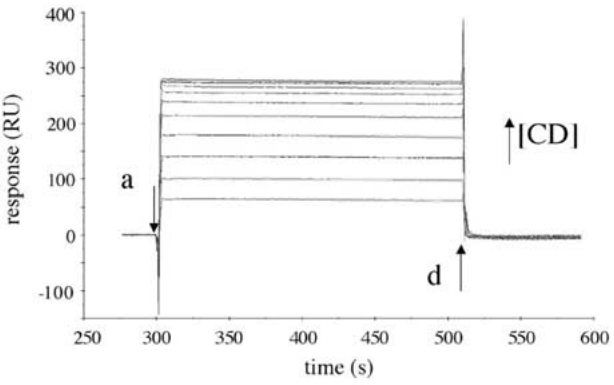


*Figure 2.* Typical raw data collected from the Biacore® for the interaction of $\beta$CD with immobilized *N*-(1-adamantylmethyl)amide. The arrows marked 'a' and 'd' indicate the association (start of cyclodextrin flow) and dissociation (start of buffer flow) phases, respectively. The arrow marked '[CD]' indicates the direction of increasing cyclodextrin concentration (0.01–5 mM). The small 'spikes' that are visible at the association and dissociation start points result from slight misalignments of the injection start point that occur in the automatic subtraction of the response on the control surface. The response data was zeroed according to the baseline prior to the injection of the cyclodextrin.

It is worth noting that the reproducibility and quality of the data was good. For those CD-guest pairs where $K_a$ values could be determined, datasets were assessed according to the criteria outlined in the Experimental Section, and were only rejected occasionally for having unacceptable statistical parameters (such as high chi-squared values or large errors on the calculated value of the maximum response), and even in these cases the $K_a$ values generally agreed with the values determined in other independent runs. The observation that almost all fits obtained using this 1:1 CD:guest model easily satisfied the stringent criteria outlined in the Experimental Section confirmed that the 1:1 stoichiometry was the most appropriate model. Individual determinations of $K_a$ for a particular guest and CD typically varied by 10–20% with an error in the calculated maximum binding response of around 10–40%, except in the case of immobilized *N*-(1-adamantylmethyl)amide, where these values were 20–40% and 40–70%, respectively.

## Discussion

The results in Table 1 indicated that the BIACORE® can be used to determine $K_a$ values in the range $10^2$–$10^4$ $M^{-1}$. It was not possible to reliably determine $K_a$ values that were lower than around 400 $M^{-1}$, due to the lack of a significant observed binding response. The highest $K_a$ value determined was $3 \times 10^5$ $M^{-1}$, although it is expected that the technique could be used for studying CD–guest interactions with even higher $K_a$ values. This expectation is based on the many studies that have been performed with larger molecules which have reported $K_a$ values up to $10^{10}$ $M^{-1}$ [9, 18].

In order to evaluate further the results obtained using the BIACORE®, there are two useful comparisons that can be made with the solution studies of CD binding that have

*Table 1.* Apparent stability constants[a] for interactions of the cyclodextrins $\alpha$CD, $\beta$CD, $\gamma$CD, DM$\beta$CD and Molecusol™ with immobilized *N*-(1-adamantylmethyl)-, *N*-octyl-, *N*-benzyl-, *N*-(4-methylbenzyl)-, *N*-(4-*tert*-butylbenzyl)- and *N*-(1-pyrenylmethyl)-amides in 50 mM HEPES buffer pH 7, 150 mM NaCl at 298 K

| Cyclodextrin | Immobilized Amide | | | | | |
|---|---|---|---|---|---|---|
| | *N*-(1-adamantyl methyl)- | *N*-octyl- | *N*-benzyl- | *N*-(4-methyl benzyl)- | *N*-(4-*tert*-butyl benzyl)- | *N*-(1-pyrenyl methyl)- |
| | ($K_a$, $M^{-1}$)[a] | | | | | |
| $\alpha$CD | ~100[b] | 1700 ± 80 | –[c] | –[c] | ~100[b] | –[c] |
| $\beta$CD | 28000 ± 3000 | 780 ± 30 | –[c] | –[c] | 9400 ± 600 | –[c] |
| $\gamma$CD | 640 ± 50 | ~200[b] | –[c] | –[c] | ~200[b] | ~200[d] |
| DM$\beta$CD | 11000 ± 1000 | 640 ± 50 | –[c] | –[c] | 5200 ± 300 | –[c] |
| Molecusol™ | 9000 ± 1000 | 440 ± 30 | –[c] | –[c] | 5800 ± 500 | –[c] |

[a]Assumes a 1:1 CD:guest stoichiometry.
[b]SPR response was low (50–100 RU) so $K_a$ values were not reliably determined and are thus reported as approximate values only.
[c]SPR response was too low (<50 RU) to allow a $K_a$ value to be measured.
[d]SPR response was around 300 RU, but the $K_a$ value is reported as an approximate value only as there was some instability in the baseline.

been reported in the literature – the general trends in $K_a$ values with varying CD cavity size, and the actual magnitude of the $K_a$ values. In relation to the former, the results in Table 1 and corresponding solution studies each show that the $\alpha$CD cavity is preferred for guests with an *N*-octyl substituent [19–22], the $\beta$CD cavity is preferred for guests with a *tert*-butylbenzyl [23] or adamantyl [4, 19, 20] substituent, and the $\gamma$CD cavity is preferred for guests with a pyrenyl substituent [6, 19, 20, 24].

It is more difficult to compare the magnitudes of the $K_a$ values, due to the difficulties in finding an appropriate guest to correspond to an immobilized amide, and also due to the large variations in the $K_a$ values reported in the literature for solution studies. For example, the reported $K_a$ values for the formation of a 1:1 complex between $\gamma$CD and pyrene range from 20 to 1104 $M^{-1}$ [6, 19, 20, 24], depending on factors such as the particular solution conditions and the physical method used. Although these factors make direct literature comparisons difficult, there are still some useful comparisons that can be made. No significant binding was observed for the interaction of any of the CDs with immobilized *N*-benzyl- and *N*-(4-methylbenzyl)-amide, suggesting that the $K_a$ values are less than 200 $M^{-1}$. This matches the literature values of 200 $M^{-1}$ or less typically reported for the binding of CDs to benzyl alcohol, benzene or toluene [19, 20, 22, 25]. For adamantyl guests, the $K_a$ values measured here are within the expected range of around $10^2$ and $10^4$–$10^5$ $M^{-1}$ for $\alpha$CD and $\beta$CD, respectively [4, 19, 20]. The $K_a$ values for the binding of octyl guests to various CDs are in the range typically reported for solution studies in the literature, namely $10^3$–$10^4$ $M^{-1}$ for $\alpha$CD and $10^2$–$10^3$ $M^{-1}$ with $\beta$CD and $\gamma$CD [19, 20]. The $K_a$ value for the interaction of the pyrenyl guest with $\gamma$CD is in the expected range of $10^2$–$10^3$ $M^{-1}$ [6, 19, 20]. Thus the $K_a$ values measured using the BIACORE® (Table 1) are of the same order of magnitude as those obtained in free solution.

The literature includes a few other SPR studies involving small molecules which also report good agreement between the binding or kinetic parameters determined by Biacore® and those determined by other techniques. These include some thorough studies of the binding of antibacterial agents to immobilized DNA gyrase [26], antitumour antibiotics to immobilized DNA [27], and oligosaccharides to immobilized antibodies [17]. The measured $K_a$ values are in the range $10^3$–$10^9$ $M^{-1}$ [17, 26, 27], and are similar to those measured by solution methods, including fluorescence quenching [27], titration calorimetry and rapid gel filtration [26], and weak affinity chromatography [17]. These studies also indicate that the SPR method can be used to determine $K_a$ values that agree with values determined by other physical methods, provided the SPR experiments are designed carefully [11].

There are only a few SPR reports involving CDs or other oligosaccharides, and none of these used direct binding to determine a series of apparent stability constants for a variety of CDs and guests. In particular, there are several reports by one group of the interaction of oligosaccharide-branched CDs with immobilized small and large molecules [28–31]. This group used competitive binding with cyclohexanol to determine the $K_a$ values for the interaction of modified CDs with immobilized doxorubicin (molecular weight 580, $K_a$ = 0.3–6.2 × $10^4$ $M^{-1}$) and cholic acid (molecular weight 409, $K_a$ = $10^3$–$10^7$ $M^{-1}$) [28, 31]. Other studies examined the binding of oligosaccharide-branched CDs to immobilized peanut lectin or concanavalin A [28–31], and reported $K_a$ values in the range $10^2$–$10^8$ $M^{-1}$ for different modified CDs. These studies were performed on aminosilane surfaces using an IAsys instrument, and in some cases kinetic parameters were also noted, but few details of these experiments were reported. A Biacore® was used to study the binding of oligosaccharides such as maltose, maltotriose and tetraglucose to an immobilized IgG antibody, and determined $K_a$ values in the range $10^3$–$10^4$ $M^{-1}$ [17]. The rapid kinetics of these weak interactions resulted in binding curves that were effectively 'square-waves', from which the dissociation rate constant was estimated as being greater than 0.1 $s^{-1}$. Finally, some studies have used SPR to study self-assembled monolayers on gold surfaces that involve CDs and guests [32,

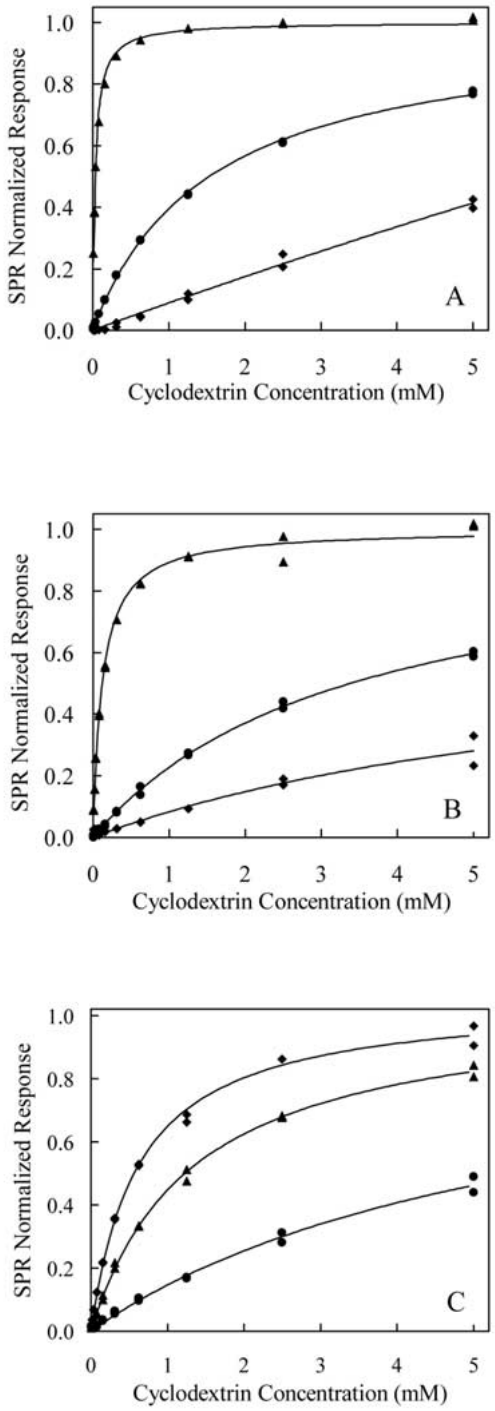


*Figure 3.* Graph showing the steady-state response of varying concentrations of cyclodextrin interacting with immobilized A. *N*-(1-adamantylmethyl)amide B. *N*-(4-*tert*-butylbenzyl)amide, and C. *N*-octylamide. The experimental data are shown as shapes ($\alpha$CD ♦, $\beta$CD ▲ and $\gamma$CD ●), with the response for each cyclodextrin concentration measured in duplicate. The line of best fit to the experimental data is shown and was calculated according to a 1:1 interaction model. The data were normalized according to the calculated maximum response value. The data for DM $\beta$CD and Molecusol$^{TM}$ (not shown) are very similar to that for $\beta$CD.

33]. For example, SPR was used to determine $K_a$ values for the interaction of 1-anilinonaphthalene-8-sulfonic acid with monolayers of CDs modified with alkylthiol chains [32].

The present study shows that it is possible to use SPR to determine apparent stability constants for the interaction of hosts such as CDs with guest molecules, even when the molecular weights of the interacting molecules are relatively low. It should also be possible to use SPR to study other hosts, such as calixarenes and crown ethers, and extend the range of guests using some of the other surface immobilization chemistries that are available. The technique should also be useful for rapid screening experiments, where a sensor chip is created with a control surface and three guests of a different size or nature, and then used to screen a range of hosts for the desired binding properties.

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