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Selective toxin – lipid membrane interactions of natural, haemolytic Scyphozoan toxins analyzed by surface plasmon resonance

Heike Helmholz

GKSS Forschungszentrum Geesthacht GmbH, Institute for Coastal Research, Department for Marine Bioanalytical Chemistry, D-21502 Geesthacht, Germany

Corresponding author

Heike Helmholz

GKSS Forschungszentrum Geesthacht GmbH

Institute for Coastal Research

Department: Marine Bioanalytical Chemistry

Max-Planck-Street 1

D-21502 Geesthacht

Germany

Email: heike.helmholz@gkss.de

Tel: 0049 – 4152/871844

Fax: 0049 – 4152/871875

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Summary

A comparison of the molecular interaction of natural Scyphozoan lysins with their bioactivity in a haemolytic assay was performed by establishing an efficient, automatable and reproducible procedure for the measurement of protein-membrane interactions. The toxin-membrane interactions were analyzed utilising a chip-based technology with immobilized liposomes as artificial cell membranes. The technique was established with streptolysin O as a cholesterol–selective model toxin and its cholesterol-selectivity has been proven. The haemolytic potency of protein fractions derived from the venom of the jellyfish *Aurelia aurita* and *Cyanea capillata* was tested and EC50 values of 35.3 µg/mL and 43.1 µg/mL against sheep and 13.5 µg/mL and 8.8 µg/mL against rabbit erythrocytes were measured. Cell membrane binding as a first step in the haemolytic process was analyzed using the Biacore® technology. Major cell membrane lipids (cholesterol, sphingomyelin and phosphatidylcholine) were immobilized as pure liposomes and in binary mixtures. A preference for cholesterol and sphingomyelin of both jellyfish species was demonstrated. The specificity of the method was proven with a non-haemolytic *A. aurita* protein fraction that did not express a lipid binding. Additionally, an inactivated *C. capillata* lysine with negligible haemolytic activity showed a remaining but reduced adsorption onto lipid layers. The binding level of the lytic venom fraction of these dominant boreal jellyfish species increased as a function of protein concentration. The binding strength was expressed in RU50 values ranging from 12.4 µg/mL to 35.4 µg/mL, which were in the same order of magnitude as the EC50 values in the haemolytic assay.

1. Introduction

The toxic potency of the majority of proteinaceous toxins is based on membrane interaction, pore formation and, finally lysis and damage of cells. Such cytolysins are produced by a variety of living organisms, particularly bacteria, certain insects, poisonous reptiles and stinging marine invertebrates [1, 2]. These toxins need to be secreted as water-soluble proteins but have to be transformed into membrane proteins before penetrating the membrane. It can be assumed that the toxin is concentrated on the membrane by means of special cell surface features such as a protein receptor molecules, lipid clusters or carbohydrate side chains [3, 4].

The toxic activity of cell lysis is often measured by the lysis of red blood cells (erythrocytes) and the detection of released haemoglobin after cell disruption. Erythrocytes from diverse species have been applied for haemolytic assays, and they express different susceptibilities for the same lysine. This phenomenon indicates that the composition of erythrocyte cell membranes influences the haemolytic effect of cytolytic toxins [5 - 7].

Although haemolysis is a well-known toxic effect of jellyfish venoms, information on structure-effect relationships and even molecular interaction studies with membrane lipids are downright limited. One investigation on the haemolytic activity of *Aurelia aurita*, the Moon Jelly, shows an inhibitory effect of cholesterol and phosphatidylcholine [8]. In contrast, the inhibition of a lytic activity of crude *Cyanea capillata*, the Lion's mane jellyfish venom, could be achieved by glycoproteins [9].

While there is no detailed information available on jellyfish toxins with special emphasize on membrane-lipid interaction, membrane-active, pore-forming proteins from other Cnidaria species, mainly Anthozoa (sea anemones) have been investigated intensively [10, 11]. To date the actinoporins are the most widely studied marine pore-forming protein family [12 - 16]. The protein–monomers have an affinity to the lipids in

cell-membranes with selectivity for sphingomyelin, form di- and oligomers, and finally small cavities are engraved in the cells, thus reducing the membrane integrity [17, 18]. Contrariwise, the mode of effect of hydralysins, a group of cytolytic, pore-forming toxins having been isolated from the green hydra *Chlorohydra viridissima* (Hydrozoa, Cnidaria), is suggested to be receptor instead of lipid or carbohydrate mediated [19].

A membrane-affinity can serve as an indicator for a membrane-active, lytic effect and can be detected by biospecific interaction analysis as shown for the Anthozoan cytolysins equinatoxin and the sticholysins [20, 21].

The investigation of functional binding events of biomolecules is one of the key issues in modern life science research [22, 23]. Not only cytolytic effects, but also physiological cell functions including signal transduction, hormone-receptor interactions and channel formation or fusion processes are based on interactions of peptides and proteins with cellular membranes. These interactions can be resolved by the physical phenomenon of surface plasmon resonance (SPR) in real time. This methodology has been employed in a variety of applications to study artificial membrane properties and binding events [24]. Melittin, a bee toxin, was used in several studies as a model substance for analyzing toxin-membrane lipid interaction [25, 26]. Vanhoye et al (2004) investigated Gly-Leu-rich peptide orthologes in order to analyse the influence of net charge on cytotoxicity [27]. Drug/lipid membrane interactions important for the bioavailability of pharmaceuticals were performed to evaluate the degree of absorption [28, 29]. A differentiation between pore formation and lysis was demonstrated by Papo & Yechiel (2003) [30].

The SPR technology provides several advantages such as rapid and direct measurement of association and dissociation rates of the binding process. The binding of protein onto a lipid bilayer is monitored directly without needing specific labelling; and only small amounts of sample are required due to the high sensitivity of detection.

For membrane association studies, liposomes are immobilized on modified gold surfaces, and lipid bilayers in form of lamellar surface or large vesicles are built as artificial membranes on the surface of the sensor chip [31 - 33]. The protein solution is passed over this functionalized surface. Bound proteins change the refractive index near the surface. The change is expressed in Resonance Units [RU] and is linearly proportional to the amount of bound protein [34]. These molecular studies are mainly performed with isolated, recombinant or synthesized pure and well characterized compounds. Thought toxinological investigation is often accomplished with whole venoms or toxin mixtures for a primary characterization of toxic effects at in vitro bioassays. In the present paper, the molecular assay was applied for a natural toxin mixture in order to overcome this discrepancy and to show a linkage between these two test principles. The membrane-lipid interaction of natural Scyphozoan proteins analyzed by SPR in relation to a conventional haemolytic bioassay is described for the first time. Different cytotoxic effects have been observed for Scyphozoan venoms, although it is not clear whether these effects are based on lytic, pore-forming activity or other impacts on cellular processes. The affinity to as well as the influence of main lipid components of erythrocyte membranes pure and combined in binary mixtures on the binding and therefore cytolytic effect of jellyfish toxins is analyzed. Target species were the boreal jellyfish species *Aurelia aurita* and *Cyanea capillata*, which express a stinging capacity but were not capable to induce such severe envenomations like Cubozoan species and therefore these species were less well studied concerning their biochemical and toxinological properties. This study contributes to the clarification of cellular modes of toxic effects of natural, marine toxins and the advancement of biomolecular interaction assays for toxinological investigations.

2. Materials and methods

2.1. Material

The membrane lipids: cholesterol (Ch), phosphatidylcholine (PC) and sphingomyelin (SM), the ionic surfactant 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (chaps) and the model toxin streptolysin O (SLO) were purchased from Sigma (Munich, Germany). Hepes buffered saline (HBS-N) was obtained from GE Healthcare Europe (Freiburg, Germany) and the non-ionic surfactant octylglucosid (OG) from Calbiochem (Darmstadt, Germany). All other chemicals were of analytical reagent grade.

2.2. Sample preparation

Jellyfish medusae of the species *Cyanea capillata* (L.) and *Aurelia aurita* (L.) were collected at research cruises to the Scottish Orkney Islands and Western Islands. Toxin mixtures were prepared from stinging capsules (cnidocysts) by maceration of the fishing tentacle tissue in distilled water. The isolated, intact cnidocysts were suspended in ice cold 10 mM acetate buffer pH 5.5 and discharged by sonication (Branson Sonifier 450, G. Heinemann Ultraschall- und Labortechnik, Schwaebisch Gmuend, Germany) in order to obtain the crude toxin mixture (venom) [35] . A purification of this crude sample material was achieved by preparative size exclusion chromatography on a Sephadex G75 column using 10 mM ammonium acetate buffer pH 5.5 as solvent; injection volume 5.0 mL; flow 1.0 mL/min. Purified fractions were lyophilized and re-suspended in phosphate buffered saline (PBS) pH 7.2 for haemolytic assay and for SPR experiments or stored at -80°C until further use. The protein content was measured by Bradford reagent [36].

2.3. Haemolysis

The haemolytic activity of the samples was tested according to Emura et al (2004) [37]. Sheep and rodent blood was obtained from Fiebig Nährstofftechnik (Idstein-

Niederauroff, Germany). Erythrocytes were obtained from whole blood by centrifugation and washing in sterile PBS pH 7.2. The erythrocyte concentrate was brought to a final concentration of 4% (v/v) with sterile PBS. Samples were diluted with PBS to the desired protein concentration range and 50 µL of these dilutions were filled into the wells of 96-well round-bottom microtiter plates. Erythrocyte solution measuring 50 µL was added and the plate was shaken for 1 h at 600 rpm and incubated further 20 h without shaking at room temperature. The microtiter plate was centrifuged at 700 g for 15 min, and 70 µL of the supernatant were transferred into flat-bottom microtiter plates. The released haemoglobin was measured at 550 nm in a microtiter plate reader (Victor Multilabel Counter, Perkin Elmer, Rodgau-Jügesheim, Germany). Total lysis of erythrocytes was achieved by 1% Triton X-114 solution. PBS measuring 50 µL was used as negative control inducing no haemolysis. The absorbance of total haemolysis minus the value of the negative control was set as 100%.

Inhibition assay: The assay to detect the inhibition of haemolysis was modified after Long and Burnett (1989) and Yamaji et al. (1998) [9, 38]. Liposomes of pure cholesterol, sphingomyelin and phosphatidylcholine were prepared as described below (2.4.) but the dried material was re-suspended in PBS pH 7.2 and treated several minutes in a cooled sonicator (Branson Sonifier 450, G. Heinemann Ultraschall- und Labortechnik, Schwaebisch Gmuend, Germany). Liposome measuring 25 µL were mixed with 25 µL sample and preincubated for 30 min. One sample concentration inducing 50% haemolysis was chosen (5 µg/mL). Then 50 µL of the 4% (v/v) erythrocyte suspension were added and the assay was performed as described above.

2.4. Liposome preparation

Phospholipids and cholesterol were dissolved in chloroform methanol 2:1 (v/v) in a concentration of 1 mg/mL and used pure or mixed in ratios of 1:10 and 10:1 (v/v). The

solutions were dried under nitrogen stream as a thin layer on the walls of glass vials. For liposome preparation, the mixture was re-suspended in 500 µL HBS-N (0.01 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Hepes; 0.15 M sodium chloride pH 7.4) shaken rigorously and treated with ultrasound in order to obtain a suspension. Finally, the extrusion through a 100 nm polycarbonate membrane (liposoFast, Avestin, Mannheim, Germany) was performed in order to produce unilamellar vesicles of homogenous size [39].

2.5. Protein - lipid interaction analysis

A Biacore® 3000 instrument (GE Healthcare Europe, Freiburg, Germany) was applied for the toxin-lipid interaction analysis. Liposomes were immobilized onto L1 sensor chip surfaces at low flow rates of 2 µL/min. The lipid vesicles were injected separately in the individual flow cells until an approximately equal response (7000-8000 RU) could be achieved. Prior to immobilization the flow cell surfaces were cleaned with an injection of 20 µL 20 mM chaps. The surface was allowed to stabilize after an impulse of 10 µL 50 mM NaOH for 30 min. A sample volume with changing protein concentrations of 80 µL was injected at flow rates of 20 µL/min. A desorption phase of 240 s was held before the surface was regenerated with repeating injections of 40 mM OG, 20 mM chaps and short impulses of 50 mM NaOH / i-Propanol (6/4 v/v). Each toxin concentration series was repeated at least three times. Injections of running buffer HBS-N prior to each sample injection were subtracted for base line correction. Control experiments have been conducted with several different liposome preparations, L1 chips, bovine serum albumin as control protein, different SLO charges and jellyfish-lysin preparations. Evaluation of sensograms was performed with the Biacore® evaluation software version 4.1 and RU50 values were calculated using Origin 4.1.

3. Results

The standardized chip surface functionalization and the routinely performed affinity process were shown exemplary in figure 1 with PC and SM liposomes and Ccap prep 2 as injected sample at a concentration of 100 µg/mL. The optimal procedure for the present purpose was an immobilization of a liposome preparation at a final concentration of 2 mg/mL at low flow rates of 2 µL/min until the desired (>7000 RU) surface coverage is reached, followed by a short impulse of 50 mM NaOH to remove loosely bound liposomes and a stabilization phase of 30 min. It was observed that the immobilization and stability of liposomes consisting of pure lipids is not as good as of lipid mixtures. Larger volumes of such pure liposomes were necessary to obtain the same level of surface coverage. Unfortunately, a complete removal of the toxins was impossible without destroying or even affecting the lipid layer. Therefore, an extensive regeneration of the L1 chip surface with surfactants was necessary to ensure a reproducible functionalization.

3.1 Interaction of streptolysin O

The pore-forming bacterial toxin streptolysin O was used as model toxin to establish the analytical procedure and to show the selectivity of lipid-toxin interactions. The interaction of SLO with liposomes containing a cholesterol majority was remarkably stronger than with liposome mixtures of Ch:SM 1:10 and Ch:PC 1:10 (Fig. 2), indicating the cholesterol-selectivity of SLO. Due to the negligible low specific adsorption of SLO onto the Ch:SM 1:10 and Ch:PC 1:10 these liposome surfaces were used as reference flow cells and subtracted from the corresponding Ch:SM 10:1 and Ch:PC 10:1, respectively. The binding of SLO onto the immobilized liposomes was concentration-dependent and nearly irreversible as shown in figure 3 A and B. Within this concentration range, a steady state binding was not achieved and a reasonable fit (1:1 binding, and two-step model,

respectively) could not be performed. Comparing the response values at equilibrium for the corrected Ch:SM 10:1 and Ch:PC 10:1 surfaces it could be pointed out that the binding to Ch:PC was stronger than to Ch:SM. At the highest concentration of 150 nM SLO an average response value at equilibrium Req of 377 RU was measured for Ch:PC whereas only 167 RU were reached at the same concentration for Ch:SM.

3.2 Interaction of Scyphozoan lysine

Purified protein fractions of *Aurelia aurita* and *Cyanea capillata* were obtained by extracting the fishing tentacle cnidocysts and size exclusion chromatography. The first, major fraction (indicated in figure 4) of the venom of both Scyphozoa species expressed a concentration dependent haemolytic activity against sheep and rabbit erythrocytes measured in a microtiter haemolytic assay. EC50 values are compared in table 1. Rodent red blood cells showed an increased sensitivity compared to the ruminant erythrocytes. The freshly prepared *C. capillata* fraction (prep1) induced a slightly increased lysis of rabbit erythrocytes than *A. aurita* lysine, but a higher EC50 value for the lysis of sheep erythrocytes was measured. In order to prove a loss of activity with storage, a second preparation of *C. capillata* stored at -80°C for 24 month was tested and showed no lytic activity against sheep and only a marginal activity of 7.6% lysis of rabbit erythrocytes at a protein concentration of 50 µg/mL. The haemolytic activity of *C. capillata* lysine could not be inhibited by the three membrane lipids Ch, SM and PC whereas the activity of *A. aurita* lysine was reduced in relation to the buffer control (100%) by PC (reduced to 13.2 % sheep and 20.1% rabbit), SM (reduced to 45.3 % sheep and 30.6% rabbit) and to a lesser extent by Ch (reduced to 83.8 % sheep and 57.2% rabbit).

The results of the in vitro bioassay have been associated to molecular membrane-protein interaction studies by applying the established SPR method. The concentration at a 50% binding level (RU50) was calculated from the Req versus sample protein

concentration plots and used for comparison of the affinity strength (Tab. 2). The RU50 values represent the sample concentration at 50% of the maximum response analogue to the EC50 values. SPR sensograms of a concentration series of haemolytic toxin mixtures obtained from the two Scyphozoa species illustrating the weakest and strongest adsorption onto lipid-mixture liposomes and the resulting Req vs. concentration plots were presented in figure 5 and 6. A concentration dependency was detected in the SPR experiments for both species samples. The curvature of the sensograms showed a reduced dissociation at lower concentrations up to 10 µg/mL, supposing a stronger binding with a membrane insertion. The ongoing adsorption and faster dissociation at higher concentrations implied an oligomerization with a weaker multilayer formation.

The specificity of the lipid-interaction was demonstrated by utilizing a protein fraction (indicated in fig. 4) of the *A. aurita* venom that did not show any haemolytic activity neither to sheep nor to rabbit erythrocytes even at high protein concentrations up to 75 µg/mL. At protein concentrations of 5 and 50 µg/mL no specific interaction with liposomes consisting of pure cholesterol, sphingomyelin or phosphatidylcholine and to a 10:1 mixture of Ch:PC was detected (Fig. 7: SM; Ch:PC 10:1; PC and Ch data not shown). Storage in solubilised form can alter the activity of jellyfish venoms. Therefore, a 24 month old preparation of *C. capillata* stored solubilised at -80°C was tested. Although there was only a negligible lysis of rabbit erythrocytes and no effect on sheep red blood cells, there is still an affinity to the different membrane lipids detectable. However, the RU50 values of *C. capillata* prep 2 were higher compared to *C. capillata* prep 1 (Tab. 2), thus indicating a reduced membrane binding activity.

Comparing the RU50 values of two Scyphozoa species a remarkable higher affinity for *A. aurita* haemolysin to the mixed surfaces compared to the *C. capillata* lysine was observed. However, this could not be detected for the interaction studies with the pure

lipids. This reflected the comparable haemolytic activity of the investigated jellyfish species.

In order to find out the specificity of lipid-lysine interaction different pure membrane lipids and binary lipid mixtures were immobilized onto L1 chip surfaces and used as artificial cell membranes. The RU50 values were compared in table 2. The affinity of the lysine from both jellyfish species towards phosphatidylcholine was the lowest. Whereas the lysine of *C. capillata* expressed an equal affinity towards cholesterol and sphingomyelin, there was a preference of the *A. aurita* lysine to cholesterol. The same tendencies were detected utilizing different sample preparations. Due to the SPR experiments with pure and mixed liposomes an affinity strength of *A. aurita* lysine for Ch>SM>>PC and of *C. capillata* lysine Ch; SM >> PC was shown.

4. Discussion

4.1. Toxin – lipid interactions with immobilized liposomes

Protein-membrane interactions play a crucial role in physiological cell function processes. But also non-physiological agents like bacterial, insecticidal or cnidarian toxins act via the lipid components of cell membranes. Therefore, liposomes mimicking artificial cell membranes are a useful tool to characterize the effects of pore-forming toxins [40]. Since the chip-based technology with immobilized lipid layers was introduced, biomolecular studies in this field have been improved [24, 34, 41]. Although the applicability has been shown for a variety of protein-lipid systems applying isolated, pure compounds investigation on the lipid – affinity of natural toxins especially in mixtures are still limited and up to now were not performed for jellyfish venoms.

Bacterial toxins are widely studied in terms of structure-function relationships and membrane binding due to their medicinal and infectious relevance [42]. Streptolysin O, a

pore-forming, glycosylated protein with an approximate molecular weight of 61.5 kDa derived from the gram positive microorganism *Streptococcus pyrogenes* is one example for such well investigated bacterial toxins. It has been chosen as model toxin for the present membrane interaction analysis because it was classified as a prototype of cholesterol-binding toxins [43, 44]. SLO is able to form very large pores in a two step oligomerization process. Wilkop et al. applied this molecule in SPR studies utilizing cholesterol-phosphatidylcholine liposomes to introduce a novel supported lipid bilayer membrane to study toxin membrane interactions in principle, but direct binding of SLO onto immobilized membrane-lipid liposomes have not been performed before [45, 46].

On the other hand Cocklin et al. (2006) investigated another cholesterol-dependent cytolysine derived from *Bacillus anthracis* and were able to demonstrate the specificity of cholesterol binding by SPR experiment [47]. A comparable pivotal role of cholesterol in the membrane binding of SLO was detected in the present SPR study. The applied combination of cholesterol with other natural cell membrane lipids showed an increased affinity of SLO onto phosphatidylcholine compared to sphingomyelin indicating that different lipid binding sites with different affinities may exist depending on the lipid micro-environment. It could be shown by using SLO as model toxin, that a lipid-binding specificity can be detected under the established analytical and experimental conditions.

4.2. Bioactivity of Scyphozoan lysine

The toxic potential of different jellyfish venoms has been analyzed using among others haemolytic assays. This includes species causing fatal systemic responses like various species of Cubozoa [48 - 52], Mediterranean [53] and Pacific [54, 55] Scyphozoa, inducing serious envenomations but also less dangerous species from the temperate zones like *Aurelia aurita* and *Cyanea* spec. [8, 9, 35, 56]. Mechanisms for the haemolytic effect could be an enzymatic activity by a phospholipase A_2 cleaving fatty acid chains from

membrane glycerophospholipids and destabilizing cell membranes [57]. But also a pore-forming activity via affinity to cell membrane lipids or glycoproteins may be possible. The later hypothesis is supported by the different susceptibilities of erythrocytes against jellyfish venoms [6, 49, 55, 58]. Although there are indications for a selective affinity of jellyfish lysins, inhibition assays to investigate the cell membrane component specificity are limited [9, 49, 59].

Gusmani et al. investigated polypeptides in the venom of *Rhopilema nomadica* and found a lack of specificity comparing the lytic activity against human, rabbit, sheep and guinea-pig erythrocytes [59]. An inhibition of the haemolysis could be induced by the membrane lipids phopshatidylserine and phosphatidylethanolamin, but neither sphingomyelin nor cholesterol showed that inhibitory effect. The opposite case was described by Rottini et al. for a cytolytic toxin from *Carybdea marsupialis*. The haemolytic activity against sheep erythrocytes could be blocked by sphingomyelin and phosphatidylinositol [49]. Human and rabbit red blood cells were not susceptible for this cytolysine. Contrarily Marino et al. found an increased susceptibility of rabbit and human erythocytes against the venom of *Pelagia noctiluca* [60].

In the present investigation erythrocytes from whole rabbit and sheep blood were tested and, alike to the investigation on *P. noctiluca* an increased activity against the rodent erythrocytes has been found. This was in contrast to the investigation of Long-Rowe and Burnett (1994) on the venom of *Chrysaora quinquecirrha* detecting also a slightly increased susceptibility of sheep erythrocytes [58]. The composition of the erythrocyte membrane differs mainly in the sphingomyelin and phosphatidylcholine content, whereas the cholesterol fraction contributes about 25-29% to the total lipid in all mammalian species [61]. Human and rabbit erythrocytes contain approximately 30% phosphatidylcholine of the total phospholipid content and no sphingomyelin. In contrast, sheep and other ruminants have no phosphatidylcholine but 50% sphingomyelin. Because

of the detected increased susceptibility of rodent erythrocytes for *A. aurita* as well as for *C. capillata* lysins a stronger affinity to phosphatidylcholine was expected in the SPR experiments. Different components of the sensitive red blood cell membrane of rabbits may play a role for pore-formation. Long and Burnett (1989) achieved a strong inhibitory effect of the lysis process by utilizing glycophorin, and they suggest a more specific, glycoconjugate-mediated affinity [9]. This is supported by the inability of Ch, SM and PC to block the lytic activity of *C. capillata* toxin, found in the present investigation. In general, the haemolytic and especially the inhibition assays applied and consequently the results in the literature suffer from a lack of standardization and make an evaluation and comparison more difficult.

Due to the lack of data concerning jellyfish toxin - cell membrane interactions only a comparison to the well studied sea anemone toxins was possible. The isolated equinatoxin II and also the sticholysins expressed a selective binding onto sphingomyelin that was also detected for the Scyphozoan lysins in the present biomolecular interaction analysis. However, investigation of Alvarez-Valcarcel et al. (2001) showed that also minor lipid components of cell membranes may have an effect on membrane-permealization and that the formation of lipid – microdomains is important for a stable toxin aggregation and pore formation [62]. This effect could explain the remarkably improved binding of *A. aurita* lysine onto the mixed liposomes in comparison to the pure compounds as documented in table 2.

Martinez et al. (2007) detected also a binding of sticholysin I onto PC:Ch liposomes and argued against a unique role for SM in the pore forming capacity of this toxin [63]. Additionally, Mancheño et al. (2003) complexed sticholysin II with phosphatidylcholine for crystallographic analysis of the pore-forming process [64]. These results indicated a phospholipid-headgroup binding site and would also support the

inhibitory activity of PC liposomes against the haemolytic activity of the *A. aurita* toxin mixture as it was detected in the present study.

Moreover, a strong affinity to cholesterol has been detected as described for the Anthozoan lysine metridiolysin (*Metridium senile*) as it was found for the analyzed Scyphozoan lysins in the present SPR binding studies [13, 65].

The preference for cholesterol and sphingomyelin, measured utilizing a chip-based biomolecular-interaction technique for the first time, demonstrated the ability of jellyfish to synthesize a variety of toxins with different structural and functional characteristics in order to affect a wide range of possible target cells with different cell membrane compositions and outer cell surfaces.

In conclusion there were many advantages but also disadvantages of the molecular toxin-membrane interaction measurement in comparison to an in vitro haemolytic assay. Haemolysis has been a widely used procedure to assess and to characterize the activity of cytolysins. It was a relatively easy and sensitive assay for cell damaging toxins. However, the lack of standardization, the necessity to work with whole animal or human blood or freshly prepared erythrocytes, which loose their stability during storage, and the different susceptibilities of red blood cells from different species, which may require the replication of haemolytic assays were disadvantages of this test method [68]. By means of the results of this study it has been shown that alternative methods based on molecular interaction analysis were a useful tool to increase the specificity because the target structures are known and the handling of blood can be avoided. The EC50 and RU50 values were in the same order of magnitude indicating that the SPR studies were not remarkably more sensitive than the haemolytic assay. However, the sample consumption could be reduced both due to the micro-flow system which only requires small sample volumes and the reduced number of replicates needed thanks to the lower bias. The automatable Biacore® system allowed the SPR studies to be performed faster. Exhaustive assay preparations with

several steps are not necessary. Although a quick overview of the membrane binding was possible due to the real time observation, which is a significant advantage, the evaluation of the SPR sensograms was more time-consuming than the data analysis of the haemolytic assay.

The established lipid-protein interaction analysis based on the physical phenomenon of surface plasmon resonance is very useful to detect a membrane binding activity of jellyfish toxin mixtures with proven haemolytic activity and therefore to get insights in the mode of action. The analyzed jellyfish venom components did not express an exclusive specificity for a certain membrane lipid but a preference of cholesterol as well as sphingomyelin could be demonstrated for the two boreal Scyphozoan species *A. aurita* and *C.capillata*. The biomolecular assay was suited to distinguish between a lytic and a non-lytic protein fraction. Detecting only a reduced activity of an inactivated toxin mixture and not a complete loss of activity like in the haemolytic assay demonstrated that even when the first step in cell lysis occurred, the following steps like oligomerization, membrane-insertion and pore-formation did not necessarily function. This showed that the biomolecular interaction studies are more suited for a quick affinity screening in particular with toxin mixtures, but also to clarify modes of action e.g. the specificity and requirements of binding cites. To study the whole process including the membrane insertion and pore-formation, a cellular system seems to be more suitable.

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References

[1] R.J.C. Gilbert, Pore-forming toxins, Cell Mol Life Sci 59 (2002) 832-844.

[2] I. Iacovache, F.G. van der Goot, L. Pernot, Pore formation: An ancient yet complex form of attack, Biochim Biophys Acta - Biomembranes 1778 (2008) 1611-1623.

[3] Y. Shai, Mechanism of the binding, insertion and destabilization of phospholipid bilayer membranes by α-helical antimicrobial and cell non-selective membrane-lytic peptides, Biochim Biophys Acta - Biomembranes 1462 (1999) 55-70.

[4] M.W. Parker, S.C. Feil, Pore-forming protein toxins: from structure to function, Prog Biophys Mol Biol 88 (2005) 91-142.

[5] T. Turk, Cytolytic Toxins from Sea Anemones, J Toxicol Toxin Rev 10 (1991) 223-262.

[6] M. Torres, M.B. Aguilar, A. Falcon, L. Sanchez, F.F.Y. Radwan, J.W. Burnett, E.P. Heimer-de la Cotera, R.O. Arellano, Electrophysiological and hemolytic activity elicited by the venom of the jellyfish *Cassiopea xamachana*, Toxicon 39 (2001) 1297-1307.

[7] K.E. Jackson, T. Spielmann, E. Hanssen, A. Adisa, F. Separovic, M.W.A. Dixon, K.R. Trenholme, P.L. Hawthorne, D.L. Gardiner, T. Gilberger, L. Tilley, Selective permeabilization of the host cell membrane of *Plasmodium falciparum*-infected red blood cells with streptolysin O and equinatoxin II, Biochem J 403 (2007) 167-175.

[8] F.F.Y. Radwan, J.W. Burnett, D.A. Bloom, T. Coliano, M.E. Eldefrawi, H. Erderly, L. Aurelian, M. Torres, E.P. Heimer-de la Cotera, A comparison of the toxinological characteristics of two *Cassiopea* and *Aurelia* species, Toxicon 39 (2001) 245-257.

[9] K.O. Long, J.W. Burnett, Isolation, characterization, and comparison of hemolytic peptides in nematocyst venoms of two species of jellyfish (*Chrysaora*

quinquecirrha and *Cyanea capillata*), Comp Biochem Physiol B 94 (1989) 641-646.

[10] D. Suput, In vivo effects of cnidarian toxins and venoms, Toxicon 54 (2009) 1190-1200.

[11] C. Álvarez, J.M. Mancheño, D. Martínez, M. Tejuca, F. Pazos, M.E. Lanio, Sticholysins, two pore-forming toxins produced by the Caribbean Sea anemone *Stichodactyla helianthus*: Their interaction with membranes, Toxicon 54 (2009) 1135-1147.

[12] M.E. Lanio, V. Morera, C. Alvarez, M. Tejuca, T. Gomez, F. Pazos, V. Besada, D. Martinez, V. Huerta, G. Padron, M.d.l.A. Chavez, Purification and characterization of two hemolysins from *Stichodactyla helianthus*, Toxicon 39 (2001) 187-194.

[13] G. Anderluh, P. Macek, Cytolytic peptide and protein toxins from sea anemones (Anthozoa: Actiniaria), Toxicon 40 (2002) 111-124.

[14] G. Anderluh, M. Dalla Serra, G. Viero, G. Guella, P. Macek, G. Menestrina, Pore formation by equinatoxin II, a eukaryotic protein toxin, occurs by induction of nonlamellar lipid structures, J Biol Chem 278 (2003) 45216-45223.

[15] G. Anderluh, P. Macek, Dissecting the Actinoporin Pore-Forming Mechanism, Structure 11 (2003) 1312-1313.

[16] G. Anderluh, A. Razpotnik, Z. Podlesek, P. Macek, F. Separovic, R.S. Norton, Interaction of the Eukaryotic Pore-forming Cytolysin Equinatoxin II with Model Membranes: 19F NMR Studies, J Mol Bio 347 (2005) 27-39.

[17] D. Suput, R. Fangez, M. Bunc, Cardiovascular effects of equinatoxin III from sea anemone *Actinia equina* (L.), Toxicon 39 (2001) 1421-1427.

[18] K. Crnigoj Kristan, G. Viero, M. Dalla Serra, P. Macek, G. Anderluh, Molecular mechanism of pore formation by actinoporins, Toxicon 54 (2009) 1125-1134.

[19] D. Sher, Y. Fishman, M.L. Zhang, M. Lebendiker, A. Gaathon, J.M. Mancheno, E. Zlotkin, Hydralysins, a new category of beta-pore-forming toxins in Cnidaria, J Biol Chem 280 (2005) 22847-22855.

[20] G. Anderluh, P. Macek, J. Lakey, Peeking into a secret world of pore-forming toxins: membrane binding processes studied by surface plasmon resonance, Toxicon 42 (2003) 225-228.

[21] F. Pazos, A. Valle, D. Martínez, A. Ramírez, L. Calderón, A. Pupo, M. Tejuca, V. Morera, J. Campos, R. Fando, F. Dyszy, S. Schreier, E. Horjales, C. Alvarez, M.E. Lanio, E. Lissi, Structural and functional characterization of a recombinant sticholysin I (rSt I) from the sea anemone *Stichodactyla helianthus*, Toxicon 48 (2006) 1083-1094.

[22] R. Karlsson, SPR for molecular interaction analysis: a review of emerging application areas, J Mol Recognit 17 (2004) 151-161.

[23] R.L. Rich, D.G. Myszka, Survey of the year 2007 commercial optical biosensor literature, J Mol Recognit 21 (2008) 355-400.

[24] H. Mozsolits, M.-I. Aguilar, Surface plasmon resonance spectroscopy: An emerging tool for the study of peptide-membrane interactions, J Pept Sci 66 (2002) 3-18.

[25] T. Lee, H. Mozsolits, M. Aguilar, Measurement of the affinity of melittin for zwitterionic and anionic membranes using immobilized lipid biosensors, J Pept Res 1001 (2001) 464-476.

[26] A. Lundquist, P. Wessman, A.R. Rennie, K. Edwards, Melittin-Lipid interaction: A comparative study using liposomes, micelles and bilayerdisks, Biochim Biophys Acta - Biomembranes 1778 (2008) 2210-2216.

[27] D. Vanhoye, F. Bruston, S. Al Amri, A. Ladram, M. Amiche, P. Nicolas, Membrane Association, Electrostatic Sequestration, and Cytotoxicity of Gly-Leu

Rich Peptide Orthologs with different Functions, Biochemistry 43 (2004) 8391-8409.

[28] C.L. Baird, E.S. Courtenay, D.G. Myszka, Surface plasmon resonance characterization of drug/liposome interactions, Anal Biochem 310 (2002) 93-99.

[29] Y.N. Abdiche, D.G. Myszka, Probing the mechanism of drug/lipid membrane interactions using Biacore, Anal Biochem 328 (2004) 233-243.

[30] N. Papo, S. Yechiel, Exploring Peptide Membrane Interaction Using Surface Plasmon Resonance: Differentiation between Pore Formation versus Membran Disruption by Lytic Peptides, Biochemistry 42 (2003) 458-466.

[31] M.A. Cooper, A.C. Try, J. Carroll, D.J. Ellar, D.H. Williams, Surface plasmon resonance analysis at a supported lipid monolayer, Biochim Biophys Acta - Biomembranes 1373 (1998) 101-111.

[32] E.-M. Erb, X. Chen, S. Allen, C.J. Roberts, S.J.B. Tendler, M.C. Davies, S. Forsen, Characterization of the Surfaces Generated by Liposome Binding to the Modified Dextran Matrix of a Surface Plasmon Resonance Sensor Chip, Anal Biochem 280 (2000) 29-35.

[33] G. Anderluh, M. Besenicar, A. Kladnik, J.H. Lakey, P. Macek, Properties of nonfused liposomes immobilized on an L1 Biacore chip and their permeabilization by a eukaryotic pore-forming toxin, Anal Biochem 344 (2005) 43-52.

[34] M. Besenicar, P. Macek, J.H. Lakey, G. Anderluh, Surface plasmon resonance in protein-membrane interactions, Chem Phys Lipids141 (2006) 169-178.

[35] H. Helmholz, C. Ruhnau, C. Schutt, A. Prange, Comparative study on the cell toxicity and enzymatic activity of two northern scyphozoan species *Cyanea capillata* (L.) and *Cyanea lamarckii* (Peron & Leslieur), Toxicon 50 (2007) 53-64.

[36] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal Biochem 72 (1976) 248-254.

[37] A. Emura, Y. Matsuyama, T. Oda, Evidence for the production of a novel proteinaceous hemolytic exotoxin by dinoflagellate *Alexandrium taylori*, Harmful Algae 3 (2004) 29-37.

[38] A. Yamaji, Y. Sekizawa, K. Emoto, H. Sakuraba, K. Inoue, H. Kobayashi, M. Umeda, Lysenin, a Novel Sphingomyelin-specific Binding Protein J. Biol. Chem. 273 (1998) 5300-5306.

[39] R.C. MacDonald, R.I. MacDonald, B.P.M. Menco, K. Takeshita, N.K. Subbarao, L.-r. Hu, Small-volume extrusion apparatus for preparation of large, unilamellar vesicles, Biochim Biophy Acta - Biomembranes 1061 (1991) 297-303.

[40] M.D. Serra, G. Menestrina, D. Nejat, Liposomes in the Study of Pore-Forming Toxins, Method Enzymol 372, Academic Press, (2003) 99-124.

[41] M. Gheorghiu, A. Olaru, A. Tar, C. Polonschii, E. Gheorghiu, Sensing based on assessment of non-monotonous effect determined by target analyte: case study on pore-forming compounds, Biosens Bioelectron 24 (2009) 3517-3523.

[42] R.K. Tweten, Cholesterol-Dependent Cytolysins, a Family of Versatile Pore-Forming Toxins, Infect. Immun. 73 (2005) 6199-6209.

[43] S. Bhakdi, U. Weller, I. Walev, E. Martin, D. Jonas, M. Palmer, A guide to the use of pore-forming toxins for controlled permeabilization of cell membranes, Med Microbiol Immun 182 (1993) 167-175.

[44] S. Bhakdi, H. Bayley, A. Valeva, I. Walev, B. Walker, U. Weller, M. Kehoe, M. Palmer, Staphylococcal α-toxin, streptolysin-O, and *Escherichia coli* hemolysin: prototypes of pore-forming bacterial cytolysins, Arch Microbiol 165 (1996) 73-79.

[45] T. Wilkop, D.K. Xu, Q. Cheng, Surface plasmon resonance spectroscopic study on pore-forming behavior of streptolysin O on supported phospholipid bilayers, in: J.L. Thomas, K.L. Kiick, L.A. Gower (Eds.), Symposium on Materials Inspired by Biology held at the the MRS Spring Meeting, Materials Research Society, San Francisco, Ca, 2003, pp. 223-228.

[46] T. Wilkop, D. Xu, Q. Cheng, Characterization of Pore Formation by Streptolysin O on Supported Lipid Membranes by Impedance Spectroscopy and Surface Plasmon Resonance Spectroscopy, Langmuir 23 (2007) 1403-1409.

[47] S. Cocklin, M. Jost, N. Robertson, S. Weeks, H. Weber, E. Young, S. Seal, C. Zhang, E. Mosser, P. Loll, A. Saunders, R. Rest, I. Chaiken, Real-time monitoring of the membrane-binding and insertion properties of the cholesterol-dependent cytolysin anthrolysin O from *Bacillus anthracis*, J MolRecognit 19 (2006) 354-362.

[48] T.E.B. Keen, Interaction of the hemolysin of *Chironex fleckeri* tentacle extracts with lipid monolayers, Toxicon 11 (1973) 293-299.

[49] G. Rottini, L. Gusmani, E. Parovel, M. Avian, P. Patriarca, Purification and properties of a cytolytic toxin in venom of the jellyfish *Carybdea marsupialis*, Toxicon 33 (1995) 315-326.

[50] J.J. Chung, L.A. Ratnapala, I.M. Cooke, A.A. Yanagihara, Partial purification and characterization of a hemolysin (CAH1) from Hawaiian box jellyfish (*Carybdea alata*) venom, Toxicon 39 (2001) 981-990.

[51] P.M. Bailey, A.J. Bakker, J.E. Seymour, J.A. Wilce, A functional comparison of the venom of three Australian jellyfish--*Chironex fleckeri, Chiropsalmus* sp., and *Carybdea xaymacana*--on cytosolic Ca2+, haemolysis and *Artemia* sp. lethality, Toxicon 45 (2005) 233-242.

[52] J. Sanchez-Rodriguez, E. Torrens, L. Segura-Puertas, Partial purification and characterization of a novel neurotoxin and three cytolysins from box jellyfish (*Carybdea marsupialis*) nematocyst venom, Arch Toxicol 80 (2006) 163-168.

[53] L. Cariello, G. Romano, A. Spagnuolo, L. Zanetti, Isolation and partial characterization of Rhizolysin, a high molecular weight protein with hemolytic activity, from the jellyfish *Rhizostoma pulmo*, Toxicon 26 (1988) 1057-1065.

[54] N. Azila, Foot Kee Siao, I. Othman, Haemolytic, oedema and haemorrhage inducing activities of tentacular extract of the blubber jellyfish (*Catostylus mosaicus*), Comp Biochem Physiol C 99 (1991) 153-156.

[55] C. Kang, A. Munawir, M. Cha, E.-T. Sohn, H. Lee, J.-S. Kim, W.D. Yoon, D. Lim, E. Kim, Cytotoxicity and hemolytic activity of jellyfish *Nemopilema nomurai* (Scyphozoa: Rhizostomeae) venom, Comp Biochem Physiol C 150 (2009) 85-90.

[56] L. Segura-Puertas, G. Avila-Soria, J. Sanchez-Rodgriguez, M. Ramos-Aguilar, J.W. Burnett, Some toxinological Aspects of *Aurelia aurita* (Linne) from the mexican caribbean, J Venom Anim Toxins 8 (2002) 113-128.

[57] T.J. Nevalainen, H.J. Peuravuori, R.J. Quinn, L.E. Llewellyn, J.A.H. Benzie, P.J. Fenner, K.D. Winkel, Phospholipase A2 in Cnidaria, Comp BiochemPhysiol B 139 (2004) 731-735.

[58] K.O. Long-Rowe, J.W. Burnett, Characteristics of hyaluronidase and hemolytic activity in fishing tentacle nematocyst venom of *Chrysaora quinquecirrha*, Toxicon 32 (1994) 165-174.

[59] L. Gusmani, M. Avian, B. Galil, P. Patriarca, G. Rottini, Biologically active Polypeptides in the venom of the jellyfish *Rhopilema nomadica*, Toxicon 35 (1997) 637-648.

[60] A. Marino, R. Crupi, G. Rizzo, R. Morabito, G. Musci, G. La Spada, The unusual toxicity and stability properties of crude venom from isolated nematocysts of

Pelagia noctiluca (Cnidaria, Scyphozoa), Cell. Mol. Biol. 53 (2007) OL994-OL1002.

[61] B. Deuticke, Properties and Structural Basis of Simple Diffusion Pathways in the Erythocyte Membrane, Rev Physio Biochem P 78 (1977) 1-97.

[62] C. Alvarez-Valcarcel, M. Dalla Serra, C. Potrich, I. Bernhart, M. Tejuca, D. Martinez, F. Pazos, M.E. Lanio, G. Menestrina, Effects of Lipid Composition on Membrane Permeabilization by Sticholysin I and II, Two Cytolysins of the Sea Anemone *Stichodactyla helianthus*, Biophys. J. 80 (2001) 2761-2774.

[63] D. Martínez, A. Otero, C. Alvarez, F. Pazos, M. Tejuca, M. Eliana Lanio, I. Gutiérrez-Aguirre, A. Barlic, I. Iloro, J. Luis Arrondo, J.M. González-Mañas, E. Lissi, Effect of sphingomyelin and cholesterol on the interaction of St II with lipidic interfaces, Toxicon 49 (2007) 68-81.

[64] J.M. Mancheño, J. MartIn-Benito, M. MartInez-Ripoll, J.G. Gavilanes, J.A. Hermoso, Crystal and Electron Microscopy Structures of Sticholysin II Actinoporin Reveal Insights into the Mechanism of Membrane Pore Formation, Structure 11 (2003) 1319-1328.

[65] A.W. Bernheimer, Lois S. Avigad, A cholesterol-inhibitable cytolytic from the sea anemone *Metridium senile*, Biochim Biophys Acta 541 (1978) 96-106.

List of tables and figures

SPR sensograms to demonstrate the concentration-dependent binding of streptolysin O (10; 40; 60; 100 and 150 nM SLO) onto (A) Ch:PC 10:1 bulk correction with Ch:PC 1:10 as reference cell (B) Ch:SM 10:1 bulk correction with Ch:SM 1:10 as reference cell

Figure 4:

Size exclusion chromatograms of the purification of *Aurelia aurita* (Aaur) and *Cyanea capillata* (Ccap) venom by Sephadex G75 (solvent 10 mM ammonium acetate buffer pH 5.5; flow 1 mL/min)

Figure 5:

Interaction of *Aurelia aurita* lysine with A/B Ch:PC 10:1 and C/D Ch:PC 1:10, A/C concentration series of an exemplary run B/D Response vs. concentration plots symbols represented the results of the replicate runs; line represented the overall fit

Figure 6:

Interaction of *Cyanea capillata* lysine with A/B Ch:SM 1:10 and C/D Ch:PC 1:10, A/C concentration series of an exemplary run B/D Response vs. concentration plots symbols represented the results of the replicate runs; line represented the overall fit

Figure 7:

Sensogram demonstrating the negligible interaction of a non-haemolytic fraction of the *Aurelia aurita* venom with pure SM and Ch:PC 10:1, respectively at 5 and 50 µg/mL

Table 1:

	A. aurita lysine EC50 (µg/mL)	C. capillata lysine prep1 EC50 (µg/mL)	C. capillata lysine prep2 EC50 (µg/mL)
sheep	35.3	43.1	n.c.
rabbit	13.5	8.8	n.c.

n.c. not calculated because >> 50 µg/mL

Prep 1 fresh

Prep 2 stored at -80°C; 24 month

Table 2:

Lipid	A. aurita lysine RU50 (µg/mL)	C. capillata lysine prep1 RU50 (µg/mL)	C. capillata lysine prep2 RU50 (µg/mL)
Ch	26.1 (25.5 - 26.7)	23.2 (21.8 – 24.7)	22.2 (21.1 - 23.0)
SM	27.1 (25.4 - 29.0)	21.0 (18.9 – 23.5)	22.5 (20.7 - 24.5)
PC	29.9 (29.7 - 30.2)	31.0 (26.8 – 33.9)	27.8 (26.5 - 29.7)
Ch:SM 10:1	12.4 (11.0 - 13-3)	24.6 (19.4 - 29.0)	26.0 (22.3 - 30.7)
Ch: PC 10:1	13.6 (12.4 - 14.2)	28.2 (25.1 - 31.9)	29.3 (23.4 - 35.6)
Ch:SM 1:10	17.3 (14.0 - 20.7)	25.7 (20.6 – 30.5)	26.6 (22.6 - 30.7)
Ch:PC 1:10	23.4 (20.0 - 26.3)	34.6 (32.5 -37.6)	35.4 (33.7 - 37.9)

Mean (Min - Max), Ch; SM; PC n=4; lipid mixtures n=3

Prep 1 fresh

Prep 2 stored at -80°C; 24 month

Figure 1

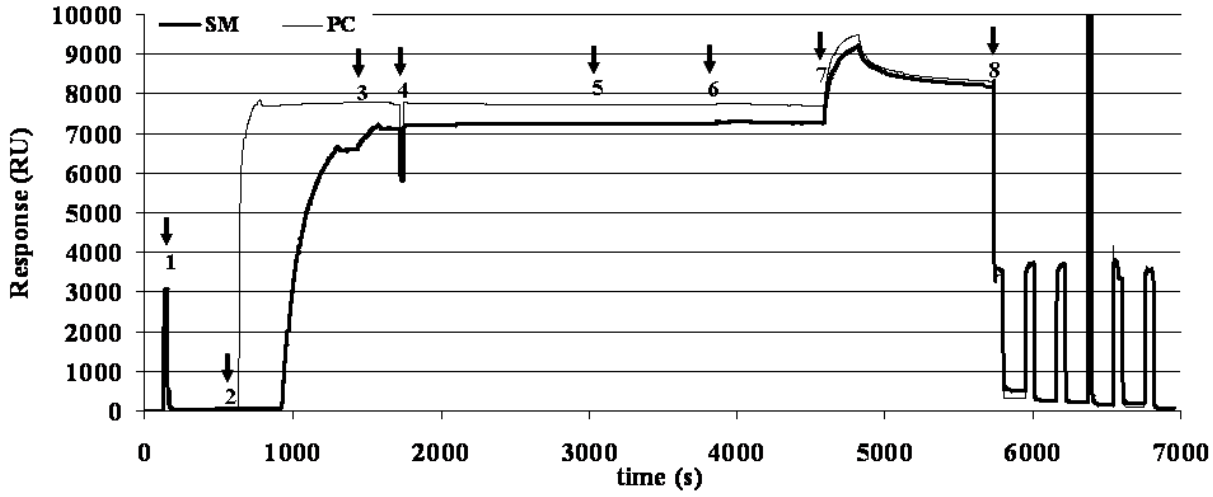
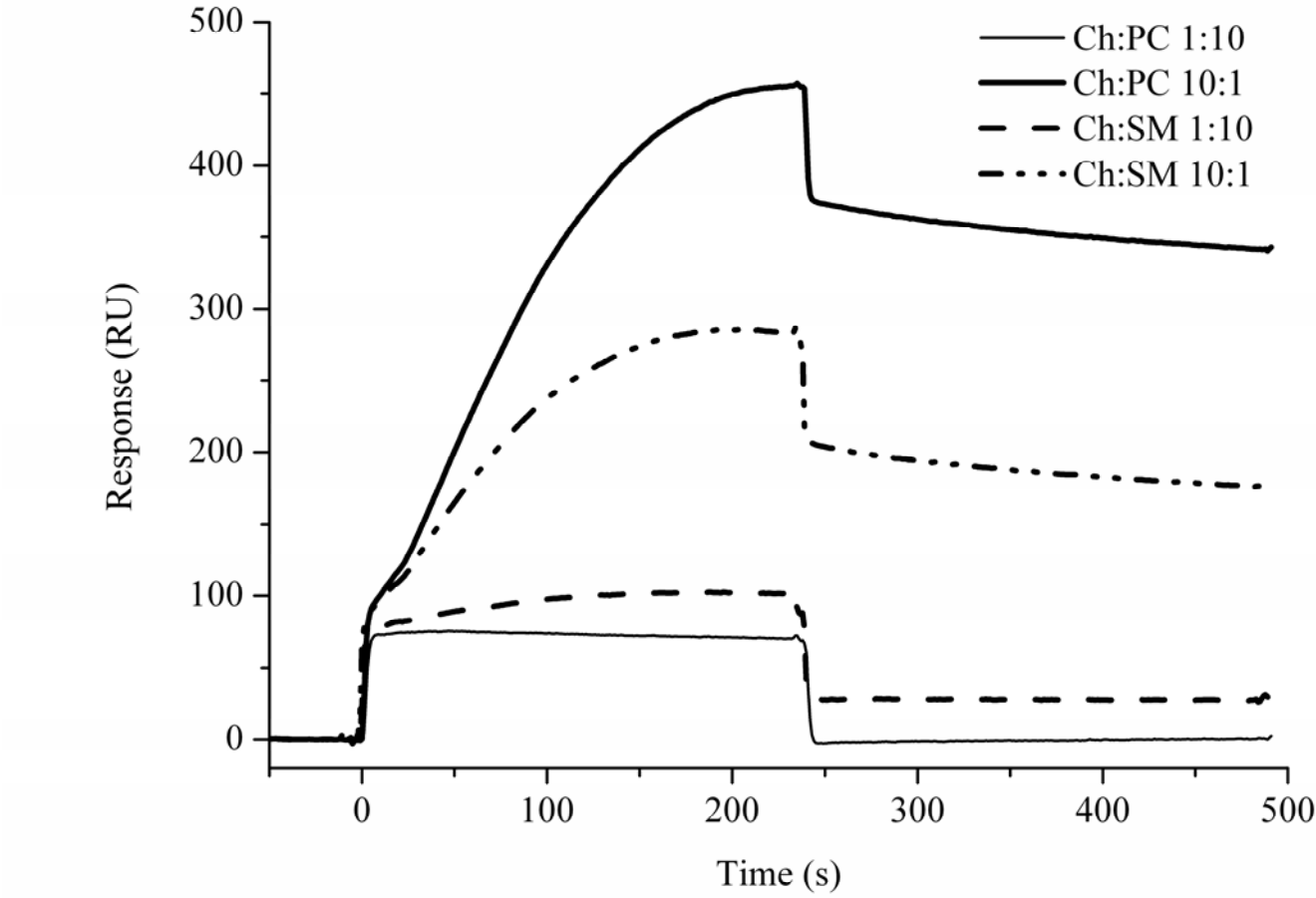

SM
PC
Response (RU)
time (s)
1
2
3
4
5
6
7
8
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
1000
2000
3000
4000
5000
6000
7000

Figure 2


Ch:PC 1:10
Ch:PC 10:1
Ch:SM 1:10
Ch:SM 10:1
Response (RU)
Time (s)
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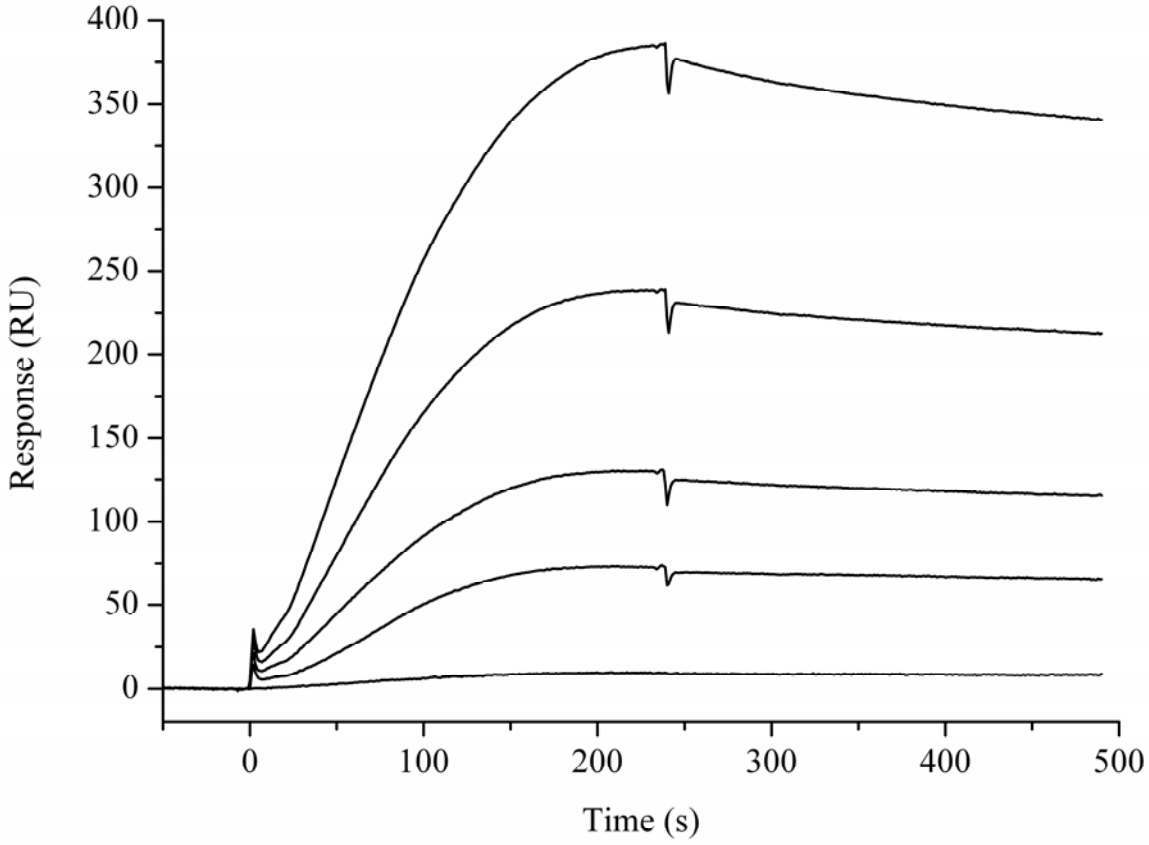
100
200
300
400
500

Figure 3a


Response (RU)
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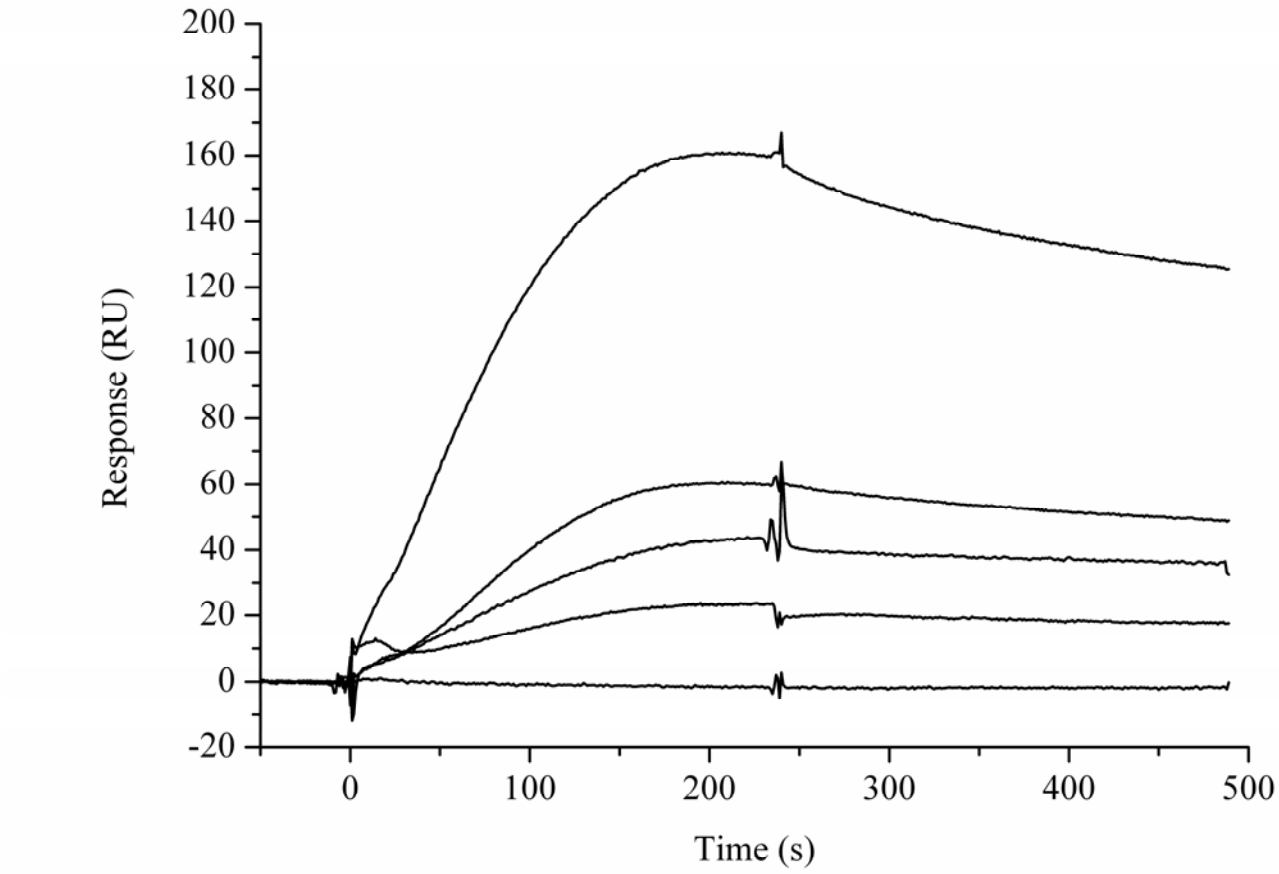
300
250
200
150
100
50
0
0
100
200
300
400
500
Time (s)

Figure 3b


Response (RU)
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180
160
140
120
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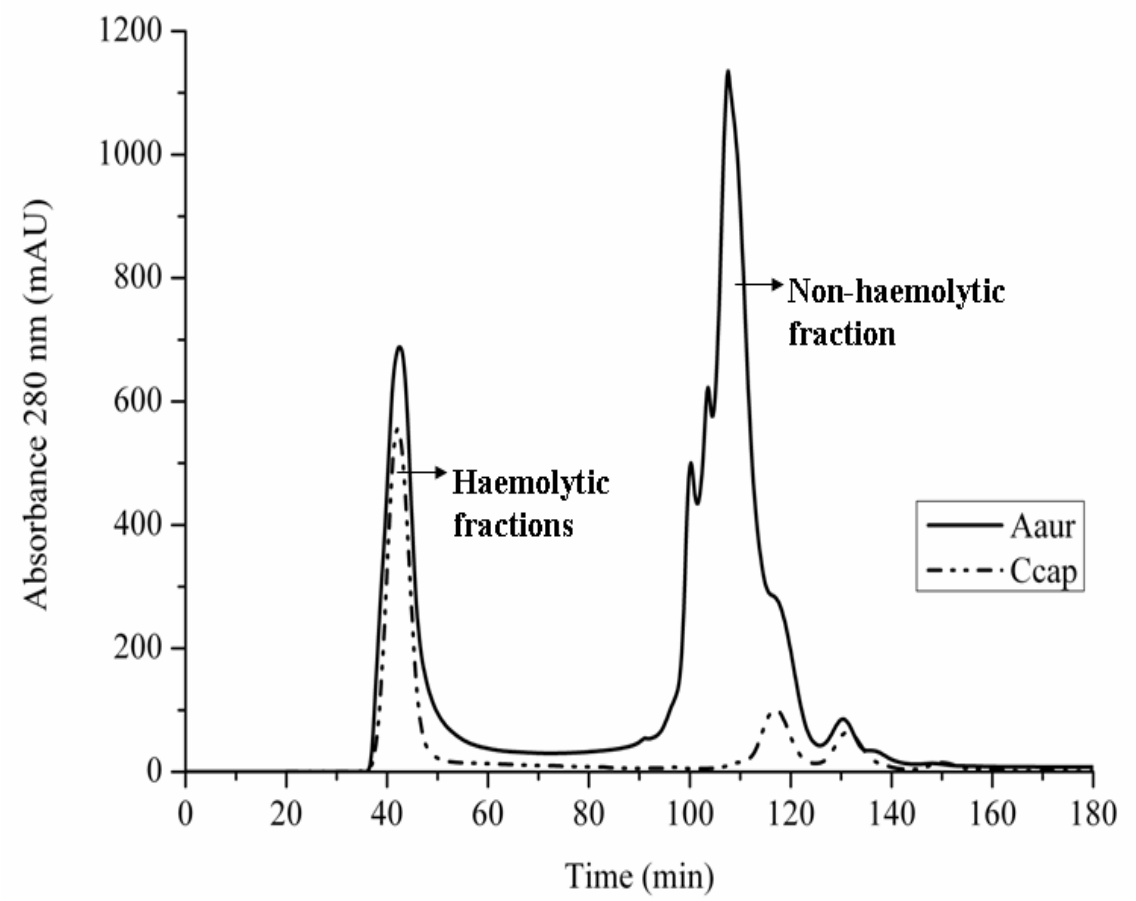
80
60
40
20
0
-20
0
100
200
300
400
500
Time (s)

Figure 4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1000
800
600
400
200
0
0
20
40
60
80
100
120
140
160
180
Time (min)
Haemolytic fractions
Non-haemolytic fraction
Aaur
Ccap

Figure 5

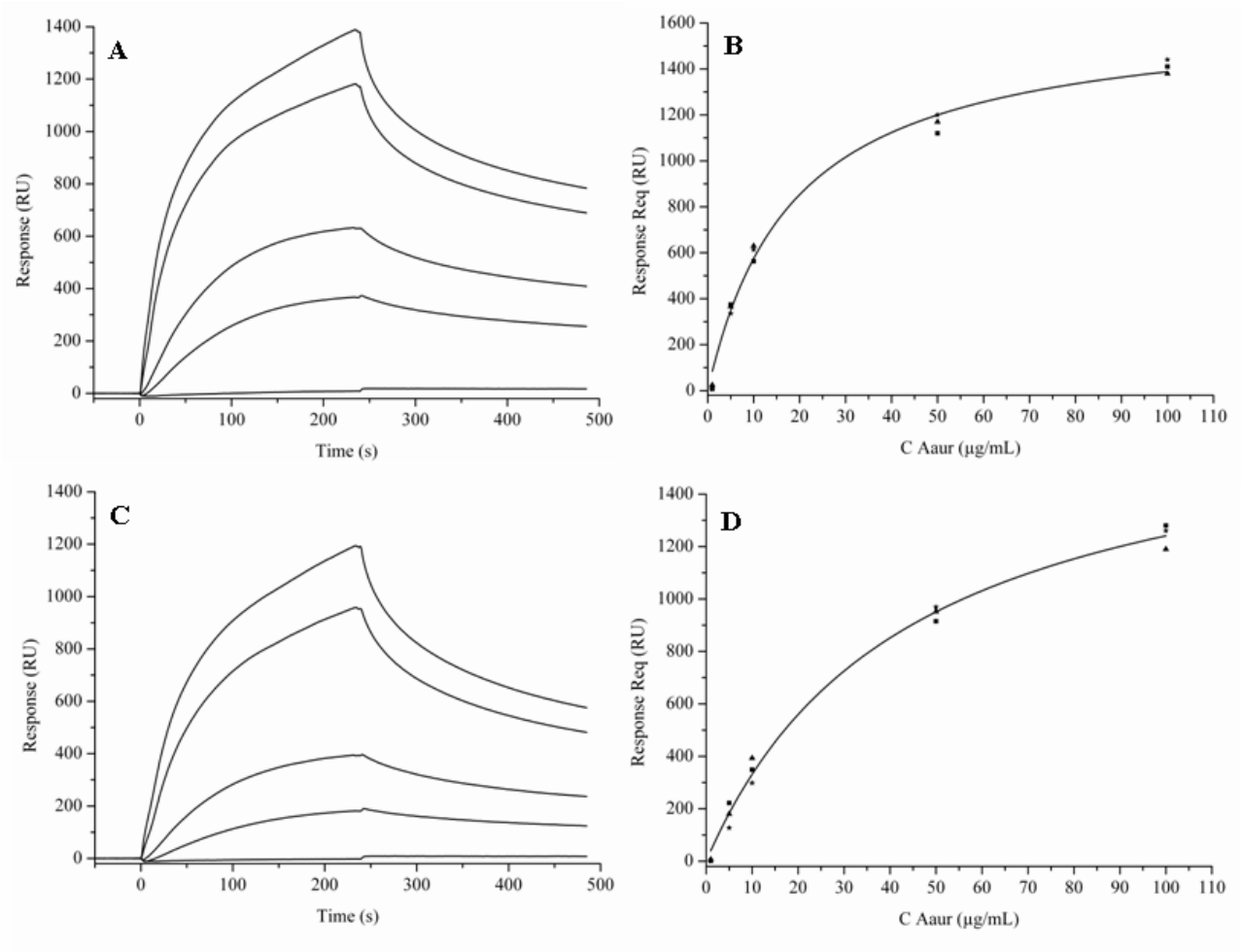

A
Response (RU)
Time (s)
B
Response Req (RU)
C Aaur (µg/mL)
C
Response (RU)
Time (s)
D
Response Req (RU)
C Aaur (µg/mL)

Figure 6

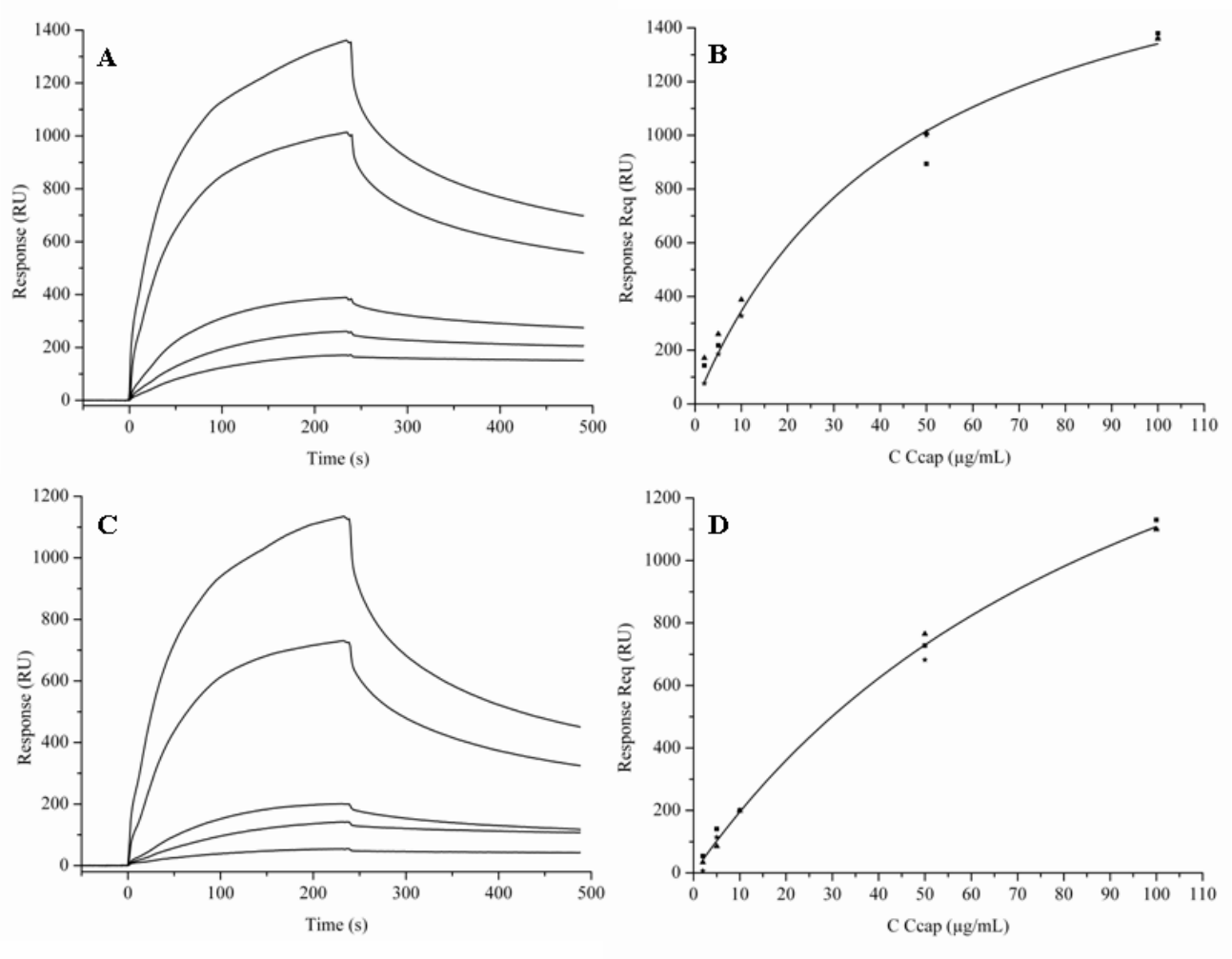
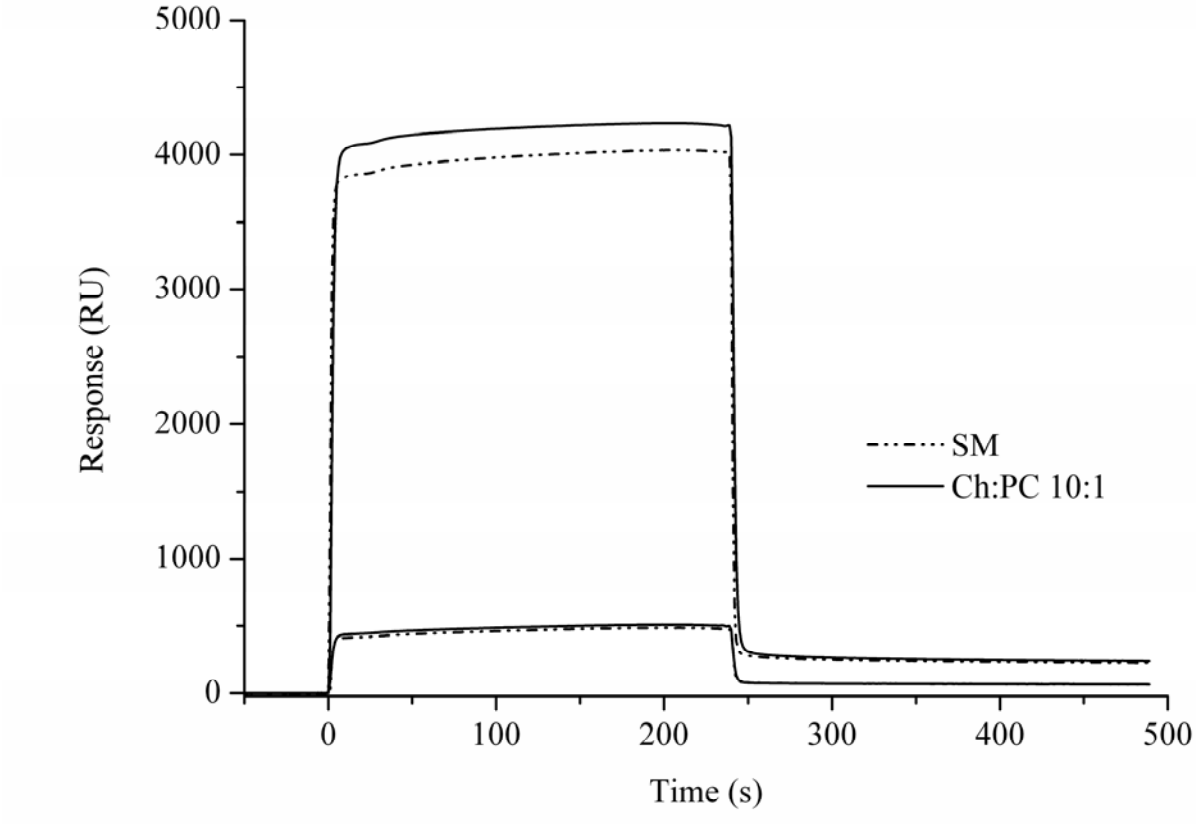

A
Response (RU)
Time (s)
B
Response Req (RU)
C Ccap (μg/mL)
C
Response (RU)
Time (s)
D
Response Req (RU)
C Ccap (μg/mL)

Figure 7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