



HAL
open science

Analysis of the Ligand Recognition Specificities of Human Ficolins Using Surface Plasmon Resonance

Nicole M. Thielens, Evelyne Gout, Monique Lacroix, Jean-Baptiste Reiser, Christine Gaboriaud

► **To cite this version:**

Nicole M. Thielens, Evelyne Gout, Monique Lacroix, Jean-Baptiste Reiser, Christine Gaboriaud. Analysis of the Ligand Recognition Specificities of Human Ficolins Using Surface Plasmon Resonance. *Methods in Molecular Biology*, 2021, The Complement System. Innovative Diagnostic and Research Protocols, 2227, pp.205-226. 10.1007/978-1-0716-1016-9_19 . hal-03200980

HAL Id: hal-03200980

<https://hal.univ-grenoble-alpes.fr/hal-03200980v1>

Submitted on 19 Apr 2021

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

**Analysis of the ligand recognition specificities of human ficolins using
surface plasmon resonance**

**Nicole M. Thielens*, Evelyne Gout, Monique Lacroix, Jean-Baptiste Reiser, Christine
Gaboriaud**

Univ. Grenoble Alpes, CNRS, CEA, IBS, F-38000 Grenoble, France

*Corresponding author: nicole.thielens@ibs.fr

Running head: Surface plasmon resonance to study ficolin specificities

Abstract

Ficolins are innate immune recognition proteins involved in activation of the lectin complement pathway. These oligomeric lectin-like proteins are assembled from subunits consisting of a collagen-like triple helix and a trimeric fibrinogen-like recognition domain. In humans, three ficolins coexist: they differ in their ligand binding specificities, but share the capacity to associate with proteases through their collagen-like stalks and trigger complement activation. We describe methods to decipher the recognition specificities of ficolins, based on surface plasmon resonance, an optical technique allowing real-time and label-free monitoring of biomolecular interactions. This technique was mainly used to characterize and compare binding of the three recombinant full-length ficolins and of their isolated recognition domains to various immobilized BSA-glycoconjugates, acetylated BSA or biotinylated heparin. The avidity phenomenon that enhances the apparent affinity of interactions between oligomeric lectin-like proteins and the multivalent ligands is also discussed.

Key words: surface plasmon resonance, ficolins, molecular interactions; recognition specificity, neoglycoproteins; multivalency; avidity

1. Introduction

Ficolins are innate immune recognition proteins that circulate in association with serine proteases involved in activation of the lectin complement pathway (recently reviewed in [1,2]). These oligomeric proteins are assembled from trimeric subunits comprising an N-terminal collagen-like helix and C-terminal fibrinogen-like (FBG) recognition domains. Three ficolins have been identified in humans, ficolin-1 (formerly called M-ficolin), ficolin-2 (L-ficolin) and ficolin-3 (H-ficolin). Although the three ficolins have different binding specificities mediated by their FBG domains, a common hallmark is their capacity to trigger activation of complement proteases called mannose-binding lectin (MBL)-associated serine proteases (MASPs) that are associated to their collagen-like stalks. In the same way as other initiators of the lectin pathway, such as MBL [3,4] and collectin CL-LK [5], ficolins are able to sense both pathogen- and apoptotic cells-associated molecular patterns and to trigger immune effector mechanisms, including complement activation and enhancement of phagocytosis of their targets. In contrast to MBL and collectin CL-LK, ficolins do not possess a canonical C-type lectin carbohydrate recognition domain, but have a fibrinogen recognition domain with lectin-like properties [6]. In order to decipher the ligand binding specificities of ficolins, we have developed methods based on detection of real time interactions of recombinant ficolins with BSA glycoconjugates or glycosaminoglycans using surface plasmon resonance (SPR) (Biacore technology).

SPR is an optical technique that measures changes in the refractive index on the surface of a thin metal film, arising from concentration changes when molecules bind to or dissociate from the surface. Because mass changes are directly detected, this technique allows monitoring of biomolecular interactions without labelling of the interacting components. One of the interactants (called the ligand) is immobilized on the surface while the interacting partner (the analyte) is injected over the surface in a continuous buffer flow thanks to a microfluidic cartridge. The SPR response, recorded as resonance units (RU) is directly proportional to the

change in mass concentration close to the surface and the complex formation/dissociation is monitored in real time as an increase/decrease in the SPR signal. The sensor surface is composed of a glass plate covered by a thin gold film coated with a biocompatible matrix, in general carboxymethylated dextran in the case of Biacore technology (CM sensor chip). In a typical interaction experiment, one interactant is first immobilized to the dextran CM groups, either directly by covalent amine coupling (involving primary amine groups of lysine residues of proteins) or indirectly through its capture by a previously and covalently coupled molecule (e.g. streptavidin for capture of biotinylated ligands). The analyte is then passed over the surface (association phase of the interaction), followed by buffer (dissociation phase). A pulse injection of an appropriate regeneration solution (selected according to the nature of the interaction) finally allows full removal of the bound analyte without damaging the immobilized compound. Recording of binding curves (sensorgrams) with a series of analyte concentrations and global fitting of the curves to defined binding models allows determination of the kinetic parameters and apparent affinity of the interaction.

The use of SPR to study lectin-carbohydrate interaction has been described previously (for example see references [7,8]). Particular features are related to the fact that most lectins, including the lectin-like ficolins, are multivalent molecules, assembled from 4 to 6 trimeric subunits (i.e. 12 to 18 polypeptide chains) as observed by electron microscopy imaging of recombinant ficolins [9,10]. Each polypeptide chain contains a binding site in the fibrinogen-like moiety and, even if all binding sites may not be involved at the same time for steric hindrance reasons, in most cases simultaneous engagement of several binding sites will provide avidity, thereby enhancing the apparent affinity of the interaction. This observation is especially valid if the multivalent lectin/ficolin is used in fluid phase, mainly due to rebinding effects to the immobilized glycans during the dissociation phase [11,12]. In addition, the binding kinetics are markedly influenced by the density of immobilized ligands, as demonstrated in our previous

SPR study of the interaction between size-fractionated oligomers of recombinant MBL and surfaces coated with various densities of D-mannose-BSA (Man-BSA) [13].

Taking these limitations into account, we describe methods to compare the ligand binding specificity of the three ficolins using defined BSA glycoconjugate surfaces and recombinant full-length ficolins and their isolated recognition domains. We also provide assays to test the capacity of ficolins to interact with biotinylated heparin and the competitive effect of a sulfated compound for ficolin-2 binding. Finally, we report SPR analysis using the reverse configuration, i.e. immobilized ficolins and soluble modified BSA.

2. Materials

2.1. SPR consumables and buffers

All experiments were performed on a Biacore 3000 SPR system with versions 3.2 of Control and Evaluation softwares (GE Healthcare) (*see Note 1*).

1. Sensor chips CM5 for Biacore 3000 (GE Healthcare) (*see Note 2*).
2. Polypropylene (PP) vials (diameter 7 mm, 0.8 mL) and penetrable rubber cups (*see Note 3*).
3. Borosilicate glass vials (diameter 16 mm, 4 mL).
4. Amine coupling kit (GE Healthcare): 750 mg 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 115 mg N-hydroxysuccinimide (NHS), 10.5 mL 1.0 M ethanolamine-HCl pH 8.5 (*see Note 4*).
5. Immobilization buffers: 10 mM sodium acetate pH 4.0, 4.2 or 5.0, and 10 mM formate pH 3.0. Dissolve 0.41 g anhydrous sodium acetate or 0.34 g sodium formate in 450 mL distilled water. Adjust the pH to the desired value with acetic acid or formic acid, respectively, before adjusting the volume to 500 mL. Filter the solutions on a 0.22 μm filter and store at 4 °C (*see Note 5*).

6. Running buffers for immobilization: 10 mM Hepes, 150 mM NaCl, pH 7.4, 0.005% Surfactant P20 (HBS-P, GE Healthcare) or HBS-P containing 3.4 mM EDTA (HBS-EP, GE Healthcare) (*see Note 6*).
7. Stock solution for conditioning the streptavidin surface: 2 M NaCl in 100 mM NaOH. Dissolve 1 g NaOH pellets and 29.2 g NaCl in 250 mL distilled water. Filter on a 0.22 μm filter and store at 4 °C.
8. Stock 0.4 M EDTA solution. Mix 58.44 g EDTA and 25 g of NaOH pellets in 500 mL distilled H₂O, which yields a 0.4 M solution with a pH of approximately 7.8. Filter on a 0.22 μm filter and store at 4 °C..
9. Running buffer for ficolin binding (HBS-CaP): 20 mM Hepes, 150 mM NaCl, CaCl₂ 1 mM, pH 7.4, 0.005% Surfactant P20 (*see Note 7*). Dissolve 2.38 g Hepes and 8.76 g NaCl, in 950 mL distilled water. Add 1 mL of a 1 M CaCl₂ stock solution (obtained by dissolving 14.7 g calcium chloride dihydrate in 100 mL distilled water) and adjust the pH to 7.4 before adjusting the volume to 1 L. Filter on a 0.22 μm filter and add 0.5 mL surfactant P20 (stock solution 10%, GE-Healthcare). The binding buffer with 1 mM EDTA is prepared in the same way, except that 2.5 mL of 0.4 M EDTA solution are added instead of CaCl₂. Store running buffers at 4 °C.
10. Regeneration solutions: 2 M NaCl (11.69 g in 100 mL distilled water); 1 M NaCl, 10 mM EDTA (5.84 g NaCl and 2.5 mL EDTA 0.4 M solution in 100 mL distilled water); 3 M MgCl₂ (36 g anhydrous MgSO₄ in 100 mL distilled water), 1 M Na₂SO₄ (14.2 g in 100 mL distilled water), 1 M Na₂CH₃COO, pH 7.2 (8.2 g in 100 mL distilled water with pH adjusted to 7.2); 0.3 M D-galactose (Gal) (2.7 g in 50 mL HBS-P); 0.3 M N-acetylgalactosamine (GalNAc) or 0.3 M N-acetylglucosamine (GlcNAc) (3.3 g in 50 mL HBS-P). All solutions are filtered on a 0.22 μm filter and stored at 4 °C.

11. Sodium dodecyl sulfate (SDS) 0.5% (BIAdesorb solution 1, GE Healthcare). This stock solution is stored at room temperature, but diluted solutions (prepared in distilled water) are stored at 4 °C.

2.2. Immobilized ligands

2.2.1 Protein ligands

- Bovine serum albumin (BSA) (Sigma-Aldrich).
- Acetylated BSA (Ac-BSA) (Sigma-Aldrich).
- BSA glycoconjugates, 14 atom spacer (Dextra Laboratories): Gal-BSA; GalNAc-BSA; GlcNAc-BSA (*see Note 8*).

Stock solutions at 1 mg/mL of BSA, Ac-BSA and BSA glycoconjugates in HBS-P (immobilization buffer) are prepared, aliquoted and stored at -20 °C.

- Streptavidin (SA) (Sigma-Aldrich). A stock solution at 2 mg/mL in HBS-P (immobilization buffer) is prepared, aliquoted and stored at -20 °C.

2.2.2. Sulfated ligands

- Biotinylated heparin (Heparin-biotin sodium salt, Sigma-Aldrich) (*see Note 9*).
- Sucrose octasulfate sodium salt (SOS) (US Biological). A 100 mM stock solution is prepared by dissolving 1.16 g in 10 mL HPS-P and stored at 4 °C. A 8 mM solution is prepared by diluting 80 µL of the stock solution in 1 mL HBS-P.

2.3. Soluble protein analytes

2.3.1. Recombinant ficolin-1

1. Full-length recombinant human ficolin-1 was produced in Schneider S2 Drosophila cells stably transfected with the pMT/Bip/V5-His A vector containing the cDNA of ficolin-1 and purified from the culture supernatants by affinity chromatography on a GlcNAc-agarose column (Sigma-Aldrich), as described by Gout et al [14]. The concentration of purified ficolin-1 was estimated using an absorbance coefficient ($A_{0.1\%, 1\text{ cm}}$) at 280 nm of 1.76 and a protomer

Mr value of 32,342, giving a Mr value of 388,000 for full-length ficolin-1, assuming assembly as tetramers of trimeric subunits.

2. A DNA segment encoding the C-terminal fibrinogen (FBG) domain of ficolin-1 (residues 80–297 of the mature human protein) was cloned in frame with the melittin signal peptide of the pNT-Bac baculovirus transfer vector [15] and the recombinant baculovirus was generated using the Bac-to-Bac™ system (Invitrogen). High Five cells were infected with the recombinant virus and the recombinant protein was purified from the culture supernatant by ion exchange chromatography on a Q-Sepharose Fast Flow column (GE Healthcare), as described by Garlatti et al [16]. The concentration of the purified trimeric ficolin-1 FBG domain was estimated using an absorbance coefficient ($A_{0.1\%, 1\text{ cm}}$) at 280 nm of 2.30 and a mass value of 73,650 Da.

2.3.2. Recombinant ficolin-2

1. Full-length recombinant human ficolin-2 was produced in CHO K1 cells stably transfected with the pcDNA3.1(+) vector containing the cDNA of ficolin-2 and purified from the culture supernatants by affinity chromatography on N-acetylcysteine-Sepharose as described previously [9,17]. The concentration of purified ficolin-2 was estimated using an absorbance coefficient ($A_{0.1\%, 1\text{ cm}}$) at 280 nm of 1.76 and a Mr value of 406,300, assuming that the proteins mainly associate as tetramers of trimers (protomer Mr value of 33,860).

2. Recombinant full-length ficolin-2 was produced in S2 insect cells (as described for ficolin-1) and purified in the same way as ficolin-2 produced in CHO cells.

3. The C-terminal FBG domain of ficolin-2 (residues 70–288 of the mature human protein) was produced in baculovirus-infected High Five cells as described for ficolin-1 FBG domain (*see Section 2.3.1*). The recombinant protein was purified from the culture supernatant by ion exchange chromatography on a Q-Sepharose Fast Flow column (GE Healthcare), as described by Garlatti et al [18]. The concentration of the purified trimeric ficolin-2 FBG domain was

estimated using an absorbance coefficient ($A_{0.1\%, 1 \text{ cm}}$) at 280 nm of 2.22 and a mass value of 78,000 Da.

2.3.3. Recombinant ficolin-3

1. Full-length recombinant human ficolin-3 was produced in CHO K1 cells stably transfected with the pcDNA3.1(+) vector containing the cDNA of ficolin-3 and purified from the culture supernatant by affinity chromatography on acetylated BSA-Sepharose as described by Jacquet et al. [19]. The concentration of the purified ficolin-3 was estimated using an absorbance coefficient ($A_{0.1\%, 1 \text{ cm}}$) at 280 nm of 1.94 and the molarity estimated using a Mr value of 396,000, assuming that the protein mainly associates as a tetramer of trimers (protomer Mr value of 33,000).

2. The C-terminal FBG domain of ficolin-3 (residues 58–276 of the mature human protein) was produced in baculovirus-infected High Five cells as described for ficolin-1 FBG domain (*see Section 2.3.1*). The recombinant protein was purified from the culture supernatant by ion exchange chromatography on a DEAE-cellulose DE52 column (Whatman) (*see Note 10*) and an S-Sepharose Fast Flow column (GE Healthcare), as described by Garlatti et al [18]. The concentration of the purified trimeric ficolin-3 FBG domain was estimated using an absorption coefficient ($A_{0.1\%, 1 \text{ cm}}$) at 280 nm of 2.36 and a mass value of 78,000 Da.

3. Methods

The SPR system preparation and routine maintenance are described in the Biacore 3000 instrument manual. The temperature is set at 25 °C.

3.1. Immobilization of BSA glycoconjugates

3.1.1. Immobilization of the BSA reference ligand

1. Dock a CM5 sensor chip and prime the system with the immobilization buffer (HBS-EP). Docking the sensor chip to the four open channels of the microfluidic cartridge surface defines

four independent flow cells (20 nL each) that are used for sample injection, either individually or serially.

2. Use flow cell 1 for immobilization of unmodified BSA to serve as a reference cell (*see Note 11*). Set the flow rate at 10 $\mu\text{L}/\text{min}$.
3. Activate the surface by injecting 80 μL (8 min) of a 1:1 mixture of 0.4 M EDC and 0.1 M NHS, using the *Quickinject* command. An increase of the baseline level of 200 to 300 RU is observed at the end of injection.
4. *Quickinject* 20 μL of BSA diluted at 10 $\mu\text{g}/\text{mL}$ in 10 mM sodium acetate pH 4.0. The injection may be stopped or new injection cycles performed to reach the desired immobilization level.
5. *Quickinject* 80 μL of 1 M ethanolamine, pH 8.5 to block the unreacted NHS-activated carboxylic groups of the dextran.
6. Determine the amount of immobilized ligand as the difference in the baseline level before activation and after blocking of the surface. Injection of 20 μL BSA at 10 $\mu\text{g}/\text{mL}$ is expected to yield an immobilization level of 4,000 RU.

3.1.2. Immobilization of BSA glycoconjugates and acetylated BSA

1. BSA glycoconjugates are immobilized in the same way as BSA, by repeating **steps 3-6** of **Section 3.1.1** for each of the three individual other flow cells. The immobilization levels of BSA glycoconjugates should be kept comparable to that of BSA on the reference cell. Injection of 40 μL GlcNAc-BSA or GalNAc-BSA at 10 $\mu\text{g}/\text{mL}$ and of 50 μL Gal-BSA at 10 $\mu\text{g}/\text{mL}$ is expected to yield immobilization levels ranging from 3,500 to 4,500 RU.
2. Immobilization of Ac-BSA is performed in the same way, except that the ligand is diluted at 25 $\mu\text{g}/\text{mL}$ in 10 mM formate pH 3.0 and that 150 μL diluted Ac-BSA are injected (*see Note 12*). Under these conditions, an immobilization level of 750-900 RU of immobilized Ac-BSA

is obtained. The corresponding BSA reference surface is obtained by injecting 7 μL BSA diluted at 10 $\mu\text{g}/\text{mL}$ in 10 mM formate pH 3.0.

3.2. Immobilization of heparin

3.2.1. Immobilization of streptavidin

1. Dock a CM5 sensor chip and prime the system with HBS-P.
2. Use flow cells 1 and 2 for simultaneous immobilization of streptavidin. Set the flow rate at 5 $\mu\text{L}/\text{min}$.
3. Activate both surfaces by injecting 50 μL (10 min) of a 1:1 mixture of 0.4 M EDC and 0.1 M NHS. An increase of the baseline level of about 150 RU is observed at the end of injection.
4. Inject 50 μL of SA diluted at 200 $\mu\text{g}/\text{mL}$ in 10 mM sodium acetate pH 4.2.
5. Inject 50 μL of 1 M ethanolamine, pH 8.5 to block the unreacted NHS-activated carboxylic groups of the dextran.

A level of 3,000-3,500 RU of immobilized SA is expected (*see Note 13*).

3.2.2. Immobilization of biotinylated heparin

1. Prime the system again with HBS-P and set the flow rate at 10 $\mu\text{L}/\text{min}$. Select flow cell 2 (flow cell 1 with immobilized SA will serve as the reference surface).
2. Condition the SA surface with three consecutive 10 μL injections of 1 M NaCl in 50 mM NaOH (obtained by diluting the stock solution 2-times with distilled water).
3. Inject 20 μL of biotinylated heparin diluted at 10 $\mu\text{g}/\text{mL}$ in HBS-P.
4. Wash needle and sample loop using 50% isopropanol in 1 M NaCl and 50 mM NaOH (obtained by diluting the stock solution 2-times with isopropanol) after ligand injection.

An immobilization level of 150-200 RU heparin is expected (*see Note 14*).

3.3. Immobilization of the three ficolins

1. Dock a CM5 sensor chip and prime the system with HBS-EP.
2. Immobilize BSA on flow cell 1 as described in **Section 3.1.1**. Injection of 40 μl BSA diluted at 25 $\mu\text{g}/\text{mL}$ in sodium acetate pH 4.0 results in about 11,000 RU immobilized.
3. The three ficolins are immobilized in the same way as BSA, by repeating **steps 3-6** of **Section 3.1.1** for each of the three individual other flow cells. Dilutions are performed in 10 mM sodium acetate, pH 5.0 (*see Note 15*). Injection of 40 μL ficolin-1 at 22 $\mu\text{g}/\text{mL}$, 120 μL ficolin-2 at 30 $\mu\text{g}/\text{mL}$ and of 30 μl ficolin-3 at 30 $\mu\text{g}/\text{mL}$ is expected to yield immobilization levels of 11,300 RU for ficolin-1 and about 20,000 RU for ficolin-2 and ficolin-3.

3.4. Characterization of the binding specificity of recombinant human ficolins

The binding properties of ficolins and the calcium-dependence of the interactions have been studied by comparing the signals obtained at identical ficolin concentrations on a set of different immobilized BSA-conjugates. An example is presented with GlcNAc-, Gal- and GalNAc-BSA.

1. BSA, GlcNAc-BSA, Gal-BSA and GalNAc-BSA are immobilized on a CM5 sensor chip as described in **Section 3.1.2**).
2. Prime the system in HBS-P containing 1 mM CaCl_2 or 1 mM EDTA.
3. Inject 60 μL of the ficolin sample at a flow rate of 20 $\mu\text{L}/\text{min}$ followed by a 3-min dissociation phase with running buffer using the *Kinject* command over the four flow cells, selecting flow cell 1 with immobilized BSA as the reference cell for automatic subtraction of the blank signal.
4. Regenerate the surface by a 30-s injection of a regeneration solution. Depending on the nature of the interaction, the regeneration solution may consist in 0.3 M sugar solution (Gal, GalNAc or GlcNAc), 1 M NaCl and 10 mM EDTA, or 1 M sodium acetate, pH 7.2. If necessary, the injections of the regeneration solutions can be combined and/or repeated until the signal returns to the baseline level (before injection).

5. Use the BIAevaluation software to process the data by overlaying the binding curves, zeroing the baseline response and aligning the injection start. As shown in Fig. 1, clear differences are observed in the binding properties of the three ficolins. Ficolin-1 binds to BSA acetylated glycoconjugates (GlcNAc- and GalNAc-BSA) in a Ca^{2+} -dependent manner (as exemplified for Gal-NAc-BSA), but not to Gal-BSA (Fig. 1A). Ficolin-2 binds to GlcNAc-BSA, Gal-BSA, independently of calcium, but not to GalNAc-BSA (Fig. 1B). Ficolin-3 binds to Gal-BSA and the interaction is maintained, although slightly diminished, in the presence of EDTA (Fig. 1C) (*see Note 16* for use of a similar protocol with mutated ficolins).

3.5. Kinetic analysis of ficolins binding to immobilized Ac-BSA

3.5.1. Generation of the binding data

The ligand immobilization level for kinetic analysis should be as low as possible (< 800 RU in our case) to avoid mass transfer limitations, occurring when the diffusion of the analyte from the bulk solution to the interaction surface is slower than the rate of binding to the ligand. It can be tested by analyzing the effect of different buffer flow rates on analyte binding rates and selecting conditions for which no dependency on the flow rate is observed.

Samples for kinetic and affinity analysis should be as pure and homogeneous as possible and matched in buffer composition with the running buffer. Dialysis of the proteins in running buffer (without P20) prior to analysis is recommended, which allows subsequent use of the dialysis buffer for sample dilution and as running buffer for the binding experiments, after filtering and addition of 0.005% surfactant P20. The molar concentrations of the analytes should be determined accurately since these values are used for calculation of the association binding constants. The range of analyte concentrations for kinetic and affinity determination is dependent on the affinity constant (K_D) for analyte binding to the ligand. It should ideally cover a 0.1-10 x K_D range, the highest concentrations should preferably reach steady state during the

injection, and most or all sensorgrams should exhibit significant curvature in both association and dissociation phases. However, this is not always possible with the lectin-glycoconjugates interactions that exhibit apparent high affinities due to the avidity phenomenon (*see Introduction*).

1. BSA and Ac-BSA are immobilized as described in **Section 3.1.2**.
2. Prime the system in binding buffer HBS-P containing 1 mM CaCl₂.
3. Prepare 5 to 6 serial dilutions from a stock ficolin solution in binding buffer.
4. It is recommended to use an automatic procedure (by creating a method file through the Biacore Control software) to inject all sample dilutions under the same conditions to facilitate further evaluation and determination of binding parameters.

Sixty 60 μ L of each dilution are injected at a flow rate of 20 μ L/min followed by a 3-min dissociation phase using the *Kinject* command over both flow cells, selecting flow cell 1 with immobilized BSA as the reference cell. The surface is regenerated by two 30-s injections of 1 M sodium acetate, pH 7.2 between injections. An injection of buffer only under the same conditions (zero concentration sample) is included at the beginning and the end of the series.

3.5.2. Evaluation of the binding data

The binding data are evaluated using the BIAevaluation software (*see Note 17* for the T200 SPR system)

1. Open overlaid plots of the processed data (*see Section 3.3.1, Step 5*) from one set of analyte concentrations.
2. Subtract the signal recorded for zero concentration sample from all curves (double referencing).
3. Select global fitting of the data (*Fit kinetics simultaneous k_a/k_d*)
4. Adjust the injection start and stop markers if needed and select the data in the association and dissociation phases to be used in the fitting.

5. Enter the analyte concentrations for each curve, choose the 1:1 (Langmuir) binding model and press *Fit*.

The k_a and k_d values are extracted from global fitting of the binding data to the selected model and the apparent equilibrium constant (K_D) is calculated from the ratio of the association and dissociation rate constants (*see Note 18*).

The experimental binding curves and fits for binding of the three ficolins to immobilized Ac-BSA are presented in Fig. 2. The kinetic rate constants and apparent equilibrium dissociation constants (K_D) (reported in **Table 1**) are in the same sub-nM range for the three ficolins, which reflects high apparent affinity for Ac-BSA [9]. However some differences can be noted in the association rate constants, which are slightly higher for ficolin-1 and ficolin-3 than for ficolin-2, corresponding to a slower association for ficolin-2. The dissociation rate constants are also higher for ficolin-1 and ficolin-3, corresponding to a slightly faster dissociation of the complex with Ac-BSA by comparison with ficolin-2. These differences are reflected by the shapes of the association and dissociation curves observed in Fig. 2A-C.

As mentioned previously, interaction of oligomeric ficolins with multivalent BSA conjugates is inherently complex and likely influenced by the avidity phenomenon. However, fitting our binding data using a simple 1:1 binding model yielded satisfactory Chi2 values (below 8, Table 1). The Chi2 value measures the average squared residual (difference between the experimental data and the fitted curve) and represents the mean square of the signal noise if the model fits the experimental data correctly. The Chi2 value might be reduced in some cases by setting the R_{max} parameter (analyte binding capacity of the surface) as a local parameter. This may be justified only if there is a reason to suspect that the ligand activity varies between cycles (i.e. if regeneration is not complete). However, the kinetic parameters should be similar using fitting with either global or local R_{max} .

3.6. Influence of the oligomerization state of ficolins on their binding properties

3.6.1. Binding properties of the isolated FBG domains of ficolins

SPR analysis was used to analyze the ligand binding capacity of the recombinant FBG domains of ficolins on immobilized acetylated BSA.

1. BSA (670 RU) and Ac-BSA (720 RU) are immobilized as described in **Section 3.1.2**.
2. The protocol described in **Section 3.3, steps 2-5** is used for injection of 0.37 μ M of ficolin-1, -2 and -3 FBG domains over immobilized Ac-BSA and BSA.

The binding curves (Fig. 3A) show that the FBG domains behave differently from the full-length proteins regarding the interaction with Ac-BSA. Indeed, binding is observed only for the FBG domain of ficolin-2 and it is inhibited in the presence of EDTA. Remarkably, the shape of the ficolin-2 binding curve is different from that obtained for full-length ficolin-2 (Fig. 2B), with faster association and dissociation phases, resulting from the lack of avidity provided by the oligomeric state of ficolin-2.

3.6.2. Binding properties of recombinant ficolin-2 produced in mammalian and insect cells

1. BSA (850 RU) and Ac-BSA (700 RU) are immobilized as described in **Section 3.1.2**.
2. The protocol described in **Section 3.3, steps 2-5** is used for injection of 1 μ g/mL of full-length recombinant ficolin-2, produced either in CHO-K1 or S2 cells, over immobilized Ac-BSA and BSA.

The binding curves (Fig. 3B) show that ficolin-2 produced in S2 cells behaves very similarly to the FBG domain of ficolin-2 (Fig. 3A), indicating that the insect cells are not able to produce correctly oligomerized ficolin-2. The production of recombinant ficolin-2 species is likely limited to the trimeric subunits that do not further assemble into tetramers in S2 cells, indicating that SPR can be used as a valuable tool for quality control of recombinant protein material in this case.

3.7. Heparin binding properties of ficolins

3.7.1. Binding of the three ficolins to heparin and kinetic analysis for ficolin-2

1. Streptavidin is immobilized on flow cells 1 and 2 and biotinylated heparin is captured on streptavidin (Flow cell 2) as described in **Section 3.2**.
2. Prime the system in HBS-P.
3. Inject 60 μL of each ficolin (1.7 $\mu\text{g}/\text{mL}$) at a flow rate of 20 $\mu\text{L}/\text{min}$ followed by a 3-min dissociation phase with running buffer using the *Kinject* command over both flow cells, selecting flow cell 1 with immobilized SA as the reference cell.
4. Regenerate the surface by a 30-s injection of 1 M NaCl (*see Note 19*).
5. Use the BIAevaluation software to process the data by overlaying the binding curves, zeroing the baseline response and aligning the injection start. As shown in Fig. 4A, clear differences are observed in the heparin binding properties of the three ficolins. Ficolin-2 binds to heparin whereas no interaction is observed for ficolin-1 and ficolin-3.
6. A series of ficolin-2 dilutions in HBS-P are injected over heparin using the protocol described in **Section 3.4.1, steps 2-4**, except that the surface is regenerated with 1 M NaCl between injections.
7. The binding data are evaluated as described in **Section 3.4.2, steps 1-5**.

The experimental binding curves and the fits to a 1:1 Langmuir model for binding of ficolin-2 to immobilized heparin are presented in Fig. 4B. The ficolin-2- heparin interaction exhibits a high apparent affinity ($K_D = 2.1 \times 10^{-10}$ M), with an association rate constant (k_a) of 1.35×10^7 $\text{M}^{-1}\text{s}^{-1}$ and a dissociation rate constant (k_d) of $3.18 \times 10^{-3}\text{s}^{-1}$. However, as mentioned previously, this value cannot be taken as an absolute value since it will depend on the heparin density on the chip (a Chi2 of 11 was obtained).

3.7.2. Competition of sucrose octasulfate (SOS) for ficolin-2 binding to heparin

1. Streptavidin is immobilized on flow cells 1 and 2 and biotinylated heparin is captured on streptavidin (Flow cell 2) as described in **Section 3.2**.
2. Prime the system in HBS-P.
3. Preincubate ficolin-2 (0.82 $\mu\text{g}/\text{mL}$, 2 nM) with serial dilutions (0.25-8 mM) of SOS in HBS-P for 15 min at room temperature. Prepare the same series of SOS dilutions with no ficolin added.
4. Inject 60 μL of each sample at a flow rate of 20 $\mu\text{L}/\text{min}$ followed by a 3-min dissociation phase with running buffer using the *Kinject* command over both flow cells, selecting flow cell 1 with immobilized SA as the reference cell.
5. Regenerate the surface by a 30-s injection of 1 M NaCl.
6. Use the BIAevaluation software to process the data by overlaying the binding curves, zeroing the baseline response and aligning the injection start. The signal recorded for injection of the competitor alone is subtracted from the signal of the corresponding competitor concentration with ficolin-2. As shown in Fig. 5A, SOS competes for ficolin-2 binding to heparin.
7. Plotting bound ficolin-2 versus SOS concentration (Fig. 5B) allows estimation of a half maximal inhibitory concentration (IC₅₀) value of 1.5 mM.

3.8. Binding of acetylated BSA to immobilized ficolins

1. The three ficolins are immobilized as described in **Section 3.3**.
2. Prime the system in HBS-P containing 1 mM CaCl₂ or 1 mM EDTA.
3. Inject 60 μL of Ac-BSA (8.3 $\mu\text{g}/\text{mL}$, 12.5 nM) at a flow rate of 20 $\mu\text{L}/\text{min}$ followed by a 3-min dissociation phase with running buffer using the *Kinject* command over the four flow cells, selecting flow cell 1 with immobilized BSA as the reference cell.
4. Regenerate the surface by two 30-s injection of 1 M sodium acetate pH 7.2.

5. Use the BIAevaluation software to process the data by overlaying the binding curves, zeroing the baseline response and aligning the injection start. As shown in Fig. 6A, Ac-BSA binds to the three ficolins. Binding is inhibited by EDTA, as shown for ficolin-3. The lower level of fiicolin-1 binding, compared to ficolin-2 and ficolin-3 arises from the lower level of immobilized ficolin-1 (11,000 RU versus 20,000 RU for immobilized ficolin-2 and ficolin-3).
6. A series of Ac-BSA dilutions in HBS-CaP are injected over ficolin-3 using the protocol described in **Section 3.4.1, steps 2-4**, except that the surface is regenerated with by two 30-s injection of 1 M sodium acetate pH 7.2 between injections.
7. The binding data are evaluated as described in **Section 3.4.2, steps 1-5**.

The experimental binding curves and the fits to a 1:1 Langmuir model for binding of Ac-BSA to immobilized ficolin-3 are presented in Fig. 6B. The Ac-BSA-ficolin-3 interaction exhibits a high apparent affinity ($K_D = 1.85 \times 10^{-9}$ M), with an association rate constant (k_a) of 4.95×10^5 $M^{-1}s^{-1}$ and a dissociation rate constant (k_d) of 9.17×10^{-4} s^{-1} . However, as mentioned previously, this value cannot be taken as an absolute value because of the possibility of an avidity phenomenon, even if a satisfactory Chi2 value (1.55) is obtained. By comparison with the values obtained in the reverse configuration (i.e. using immobilized Ac-BSA and soluble ficolin-3) (Table 1), the dissociation rate constant is in the same range whereas the association rate constant is 10-times smaller (*see Note 20* for other uses of this sensor chip).

4. Notes

1. The Biacore 3000 instrument is now discontinued and can be replaced by the Biacore T200 SPR system (GE Healthcare). Special requirements for the T200 system are indicated in the following notes when needed.
2. For Biacore T200 system, use Series S CM5 sensor chips
3. For Biacore T200 system, use ventilated caps

4. The EDC (0.4 M) and NHS (0.1 M) solutions are prepared by adding 10 mL filtered (0.22 μm) deionized water to each of the vials of the kit. The solutions are stored in 100 μL -aliquots in 0.4 mL vials at $-20\text{ }^{\circ}\text{C}$. The ethanolamine solution is stored at $4\text{ }^{\circ}\text{C}$.
5. Ready-to-used 10 mM sodium acetate solutions at pH 4.0, 4.5, 5.0 and 5.5 can be purchased from GE Healthcare.
6. The P20 detergent (non-ionic surfactant Polysorbate 20) (10% aqueous solution, GE Healthcare) can be replaced by Tween 20. For Biacore T200 system, use 0.05% P20 or Tween 20. For the T200 system, 10 x buffers (HBS-P+ and HBS-EP+) can be purchased from GE Healthcare.
7. Whereas the running buffer for immobilization should not contain amines, it is possible to use Tris-HCl or triethanolamine-HCl buffers for the binding experiments.
8. The average sugar residues per molecule (provided by Dextra Laboratories) are the following: Gal-BSA, 30; GalNAc-BSA, 28; GlcNAc-BSA, 39. The number of acetyl groups per Ac-BSA molecule is not provided by Sigma-Aldrich.
9. The commercial biotinylated heparin solution should be dialyzed (Spectrapor membrane MWcut-off 6,000 to 8,000) against HBS buffer to get rid of residual contamination with free biotin.
10. The DE52 resin from Whatman has been discontinued. A possibility would be to replace it by the DEAE cellulose resin from Santa Cruz Biotechnology or DEAE-Sepharcel from GE Healthcare, although we have not verified it.
11. The signal recorded over flow cell 1 will be subtracted from that on the other flow cells with immobilized ligands. This allows to correct for bulk response changes arising from differences in refractive index between samples and running buffer and provides a control for non-specific binding of the sample with the sensor surface.

12. Acetylation of BSA lowers its pI and it is necessary that the pH of the diluted ligand solution is below the ligand pI for efficient preconcentration before coupling. Indeed, the ligand has to bear a positive charge to be attracted by the negatively charged CM dextran matrix. However, the dextran surface carries a net negative charge only when the pH is above 3.5, which explains why the immobilization level is always lower for Ac-BSA than for the other glycoconjugates.

13. CM5 sensor chips with pre-immobilized SA may be purchased from GE Healthcare (SA sensor chips)

14. If a lower level of immobilized heparin is targeted, heparin capture can be performed in a running buffer containing 300 mM instead of 150 mM NaCl.

15. It is important to dilute the ficolins in 10 mM sodium acetate pH 5.0 for immobilization of ficolins. Use of a solution at a pH of 4.5 was found to affect the binding properties of immobilized ficolin-2 and ficolin-3.

16. A similar protocol has been used to show the crucial role played by specific amino acid residues in the FBG domains of ficolins using site-directed mutagenesis of the full-length ficolins. In this respect, the ficolin-1 Tyr²⁷¹Phe and ficolin-2 Arg¹³²Ala mutations abolished the respective ligand binding capacities of both ficolins to acetylated ligands [14,17]. A similar protocol also allowed to show that mutations of a conserved lysine residue in the collagen-like regions of ficolin-2 and ficolin-3 had no impact on the ligand binding capacities of their FBG domains [9].

17. Performing kinetic analyses is facilitated using the Biacore T200 system. Indeed, the T200 control software proposes the use of customizable methods to include the characteristics of the interactants (concentrations, molecular mass). At the end of the method, fitting of the binding curves to the model is done automatically and automated fit-quality assessments facilitate interpretation of the data. The potential mass transfer contribution is automatically included in the interaction models proposed.

18. If the binding signal reaches an equilibrium value at the end of each injection, it is possible to determine the K_D from steady state analysis, which should be comparable to that calculated from the k_a/k_d ratio.
19. If the regeneration is not complete with 1 M NaCl or 1 M Na₂SO₄, a solution containing SDS (up to 0.1%) can be used without damaging the surface.
20. A sensor chip with immobilized ficolins can also be used to analyze the ficolin binding properties of MBL-associated serine proteases [9].

Acknowledgements

This work used the platforms of the Grenoble ERIC-Instruct Center (ISBG; UMS 3518 CNRS-CEA-UGA-EMBL) with support from FRISBI (ANR-10-INSB-05-02) and GRAL (ANR-10-LABX-49-01) within the Grenoble Partnership for Structural Biology (PSB). We thank I. Bally for access to the SPR facility. Parts of the described work were supported by the Agence Nationale de la Recherche (grants ANR-05-MIIM-023-01 and ANR-09-PIRI-0021).

5. References

1. Endo Y, Matsushita M, Fujita T (2015) New insights into the role of ficolins in the lectin pathway of innate immunity. *Int Rev Cell Mol Biol* 316:49-110
2. Garred P, Genster N, Pilely K, Bayarri-Olmos R, Rosbjerg A, Ma YJ, Skjoedt MO (2016) A journey through the lectin pathway of complement-MBL and beyond. *Immunol Rev* 274 (1):74-97
3. Matsushita M, Fujita T (1992) Activation of the classical complement pathway by mannose-binding protein in association with a novel C1s-like serine protease. *J Exp Med* 176 (6):1497-1502
4. Thiel S, Vorup-Jensen T, Stover CM, Schwaeble W, Laursen SB, Poulsen K, Willis AC, Eggleton P, Hansen S, Holmskov U, Reid KB, Jensenius JC (1997) A second serine protease

- associated with mannan-binding lectin that activates complement. *Nature* 386 (6624):506-510
5. Henriksen ML, Brandt J, Andrieu JP, Nielsen C, Jensen PH, Holmskov U, Jorgensen TJ, Palarasah Y, Thielens NM, Hansen S (2013) Heteromeric complexes of native collectin kidney 1 and collectin liver 1 are found in the circulation with MASPs and activate the complement system. *J Immunol* 191 (12):6117-6127
 6. Ichijo H, Hellman U, Wernstedt C, Gonez LJ, Claesson-Welsh L, Heldin CH, Miyazono K (1993) Molecular cloning and characterization of ficolin, a multimeric protein with fibrinogen- and collagen-like domains. *J Biol Chem* 268 (19):14505-14513
 7. Duverger E, Lamerant-Fayel N, Frison N, Monsigny M (2010) Carbohydrate-lectin interactions assayed by SPR. *Methods Mol Biol* 627:157-178
 8. Shinohara Y, Furukawa J (2014) Surface plasmon resonance as a tool to characterize lectin-carbohydrate interactions. *Methods Mol Biol* 1200:185-205
 9. Lacroix M, Dumestre-Perard C, Schoehn G, Houen G, Cesbron JY, Arlaud GJ, Thielens NM (2009) Residue Lys57 in the collagen-like region of human L-ficolin and its counterpart Lys47 in H-ficolin play a key role in the interaction with the mannan-binding lectin-associated serine proteases and the collectin receptor calreticulin. *J Immunol* 182 (1):456-465
 10. Gout E, Moriscot C, Doni A, Dumestre-Perard C, Lacroix M, Perard J, Schoehn G, Mantovani A, Arlaud GJ, Thielens NM (2011) M-ficolin interacts with the long pentraxin PTX3: a novel case of cross-talk between soluble pattern-recognition molecules. *J Immunol* 186 (10):5815-5822
 11. Monsigny M, Mayer R, Roche AC (2000) Sugar-lectin interactions: sugar clusters, lectin multivalency and avidity. *Carbohydr Lett* 4 (1):35-52
 12. Shinohara Y, Hasegawa Y, Kaku H, Shibuya N (1997) Elucidation of the mechanism enhancing the avidity of lectin with oligosaccharides on the solid phase surface. *Glycobiology* 7 (8):1201-1208
 13. Gjelstrup LC, Kaspersen JD, Behrens MA, Pedersen JS, Thiel S, Kingshott P, Oliveira CL, Thielens NM, Vorup-Jensen T (2012) The role of nanometer-scaled ligand patterns in polyvalent binding by large mannan-binding lectin oligomers. *J Immunol* 188 (3):1292-1306
 14. Gout E, Garlatti V, Smith DF, Lacroix M, Dumestre-Perard C, Lunardi T, Martin L, Cesbron JY, Arlaud GJ, Gaboriaud C, Thielens NM (2010) Carbohydrate recognition properties of human ficolins: glycan array screening reveals the sialic acid binding specificity of M-ficolin. *J Biol Chem* 285 (9):6612-6622

15. Rossi V, Bally I, Thielens NM, Esser AF, Arlaud GJ (1998) Baculovirus-mediated expression of truncated modular fragments from the catalytic region of human complement serine protease C1s. Evidence for the involvement of both complement control protein modules in the recognition of the C4 protein substrate. *J Biol Chem* 273 (2):1232-1239
16. Garlatti V, Martin L, Gout E, Reiser JB, Fujita T, Arlaud GJ, Thielens NM, Gaboriaud C (2007) Structural basis for innate immune sensing by M-ficolin and its control by a pH-dependent conformational switch. *J Biol Chem* 282 (49):35814-35820
17. Laffly E, Lacroix M, Martin L, Vassal-Stermann E, Thielens NM, Gaboriaud C (2014) Human ficolin-2 recognition versatility extended: an update on the binding of ficolin-2 to sulfated/phosphated carbohydrates. *FEBS Lett* 588 (24):4694-4700
18. Garlatti V, Belloy N, Martin L, Lacroix M, Matsushita M, Endo Y, Fujita T, Fontecilla-Camps JC, Arlaud GJ, Thielens NM, Gaboriaud C (2007) Structural insights into the innate immune recognition specificities of L- and H-ficolins. *EMBO J* 26 (2):623-633
19. Jacquet M, Lacroix M, Ancelet S, Gout E, Gaboriaud C, Thielens NM, Rossi V (2013) Deciphering complement receptor type 1 interactions with recognition proteins of the lectin complement pathway. *J Immunol* 190 (7):3721-3731

Table 1

Kinetic and dissociation constants for binding of human ficolins to immobilized Ac-BSA

	k_a ($M^{-1} s^{-1}$)	k_d (s^{-1})	K_D (M)	R_{max} (RU)	Chi2
Ficolin-1	3.97×10^6	1.33×10^{-3}	3.34×10^{-10}	493	7.7
Ficolin-2	2.26×10^6	3.10×10^{-4}	1.37×10^{-10}	488	1.49
Ficolin-3	5.15×10^6	1.54×10^{-3}	2.99×10^{-10}	257	1.84

Figure legends

Fig 1 Analysis of the interaction of the three recombinant human ficolins with different immobilized BSA glycoconjugates. Sixty μ L of 1.6 nM ficolin-1 (**A**), 10 nM ficolin-2 (**B**) and 10 nM ficolin-3 (**C**) were injected over GlcNAc-, Gal- and GalNAc-BSA (3,500-4,000 RU) in HBS-CaP at a flow rate of 20 μ L/min in the presence of 1 mM $CaCl_2$ (solid lines) or 1 mM

EDTA (dashed lines). The binding signals shown were obtained by automatic subtraction of the signal over the BSA reference surface. Adapted from Gout et al [14].

Fig. 2 Kinetic analysis of binding to immobilized Ac-BSA. Sixty μL of ficolin-1 (**A**), ficolin-2 (**B**) and ficolin-3 (**C**) at the indicated concentrations were injected over the same surfaces in HBS-CaP at a flow rate of 20 $\mu\text{L}/\text{min}$. The binding signals shown were obtained by subtracting the signal over the BSA reference surface and further subtraction of buffer blanks. Fits are shown as red lines and were obtained by global fitting of the data using a 1:1 Langmuir binding model.

Fig. 3 Influence of the oligomerization state of ficolins on their binding properties. (**A**) Sixty μL of the FBG domain of ficolin-1, -2 and -3 (460 nM) were injected over Ac-BSA (720 RU) in HBS-P at a flow rate of 20 $\mu\text{L}/\text{min}$ in the presence of 1 mM CaCl_2 (solid lines) or 1 mM EDTA for ficolin-2 FBG domain (dashed lines). (**B**) Sixty μL of 1 $\mu\text{g}/\text{mL}$ of recombinant ficolin-2, produced either in S2 (solid line) or CHO-K1 (dashed line) cells, were injected over immobilized Ac-BSA in HBS-CaP at a flow rate of 20 $\mu\text{L}/\text{min}$. The different oligomerization states of the ficolin-2 samples are represented schematically. The binding signals shown in (**A**, **B**) were obtained by automatic subtraction of the signal over the BSA reference surface.

Fig. 4 Heparin binding properties of ficolins. (**A**) Sixty μL of ficolin-1, ficolin-2 and ficolin-3 (1.7 $\mu\text{g}/\text{mL}$) were injected over biotinylated heparin in HBS-P at a flow rate of 20 $\mu\text{L}/\text{min}$. The binding signals shown were obtained by automatic subtraction of the signal over the SA reference surface. (**B**) Kinetic analysis of ficolin-2 binding to immobilized heparin. Sixty μL of ficolin-1 at the indicated concentrations were injected over the heparin in HBS-P at a flow rate of 20 $\mu\text{L}/\text{min}$. The binding signals shown were obtained by subtracting the signal over the SA

reference surface and further subtraction of buffer blanks. Fits are shown as red lines and were obtained by global fitting of the data using a 1:1 Langmuir binding model. Panel A is adapted from Gout et al [14].

Fig. 5 Competition of sucrose-octasulfate (SOS) for ficolin-2 binding to immobilized heparin. (A) Sixty μL ficolin-2 (2 nM) were injected over the heparin sensor chip in HBS-P at 20 $\mu\text{L}/\text{min}$ in the presence of the indicated concentrations of SOS. The binding signals shown were obtained after automatic subtraction of the signal over the SA reference surface and further subtraction of the signals of the corresponding SOS concentration without ficolin-2. (B) The signals obtained at the end of the injection in the presence of SOS are expressed as percentages of the signal obtained in its absence (considered as 100%). The IC₅₀ value is calculated from the plot of bound ficolin-2 versus SOS concentration. Reproduced with proper permission obtained from John Wiley and Sons as published in Laffly et al [17].

Fig. 6 Analysis of the interaction of acetylated BSA with the three human ficolins. (A) Sixty μL of Ac-BSA (12.5 nM) were injected over immobilized ficolin-1 (11,000 RU), ficolin-2 (20,000 RU) and ficolin-3 (19,800 RU) in HBS-P at a flow rate of 20 $\mu\text{L}/\text{min}$ in the presence of 1 mM CaCl_2 (solid lines) or 1 mM EDTA (dashed lines). The binding signals shown were obtained by automatic subtraction of the signal over the BSA reference surface. (B) Sixty μL of Ac-BSA at the indicated concentrations were injected over immobilized ficolin-3 (19,800 RU) in HBS-CaP at a flow rate of 20 $\mu\text{L}/\text{min}$. The binding signals shown were obtained by subtracting the signal over the reference surface and further subtraction of buffer blanks. Fits are shown as red lines and were obtained by global fitting of the data using a 1:1 Langmuir binding model.

Fig. 1

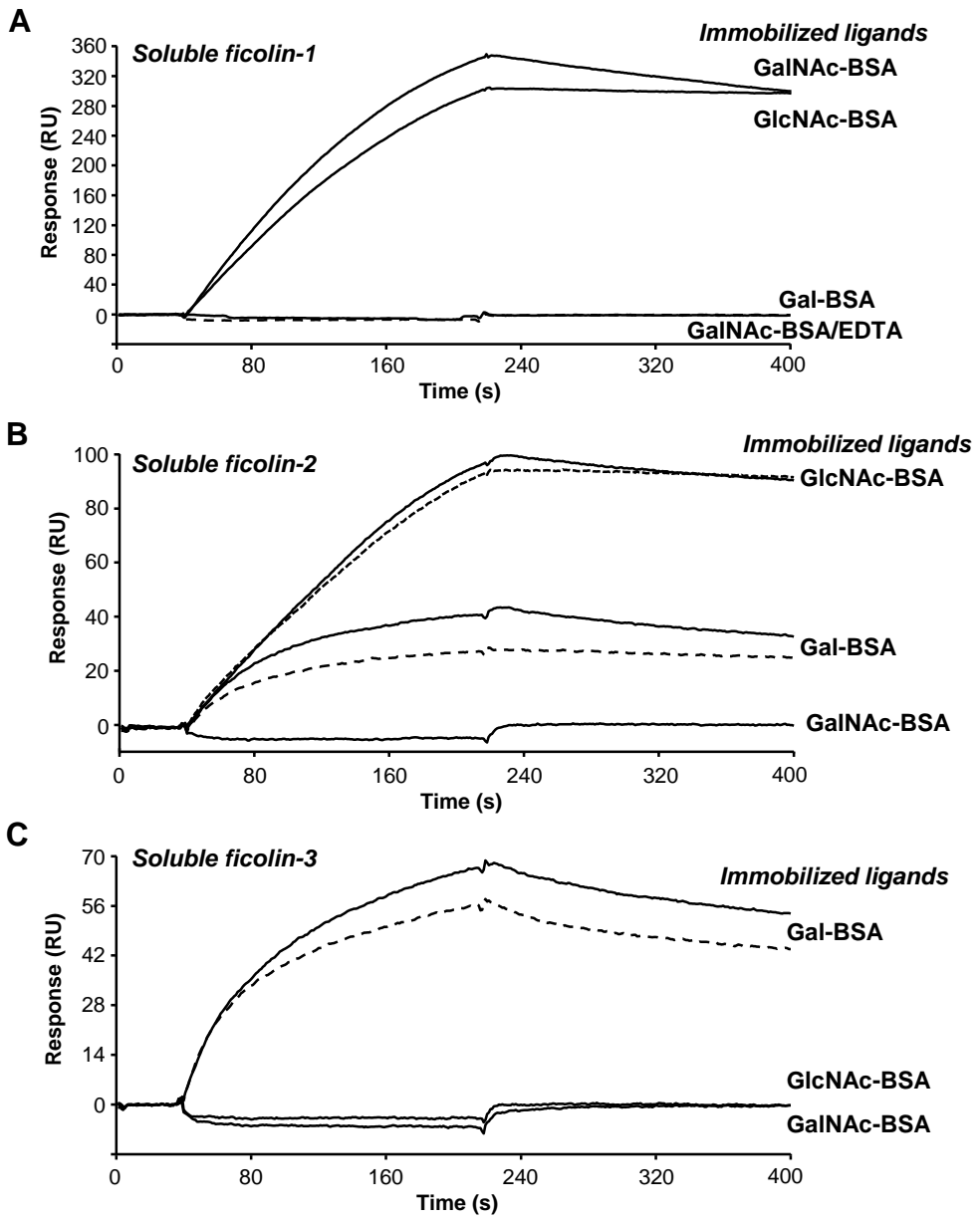


Fig. 2

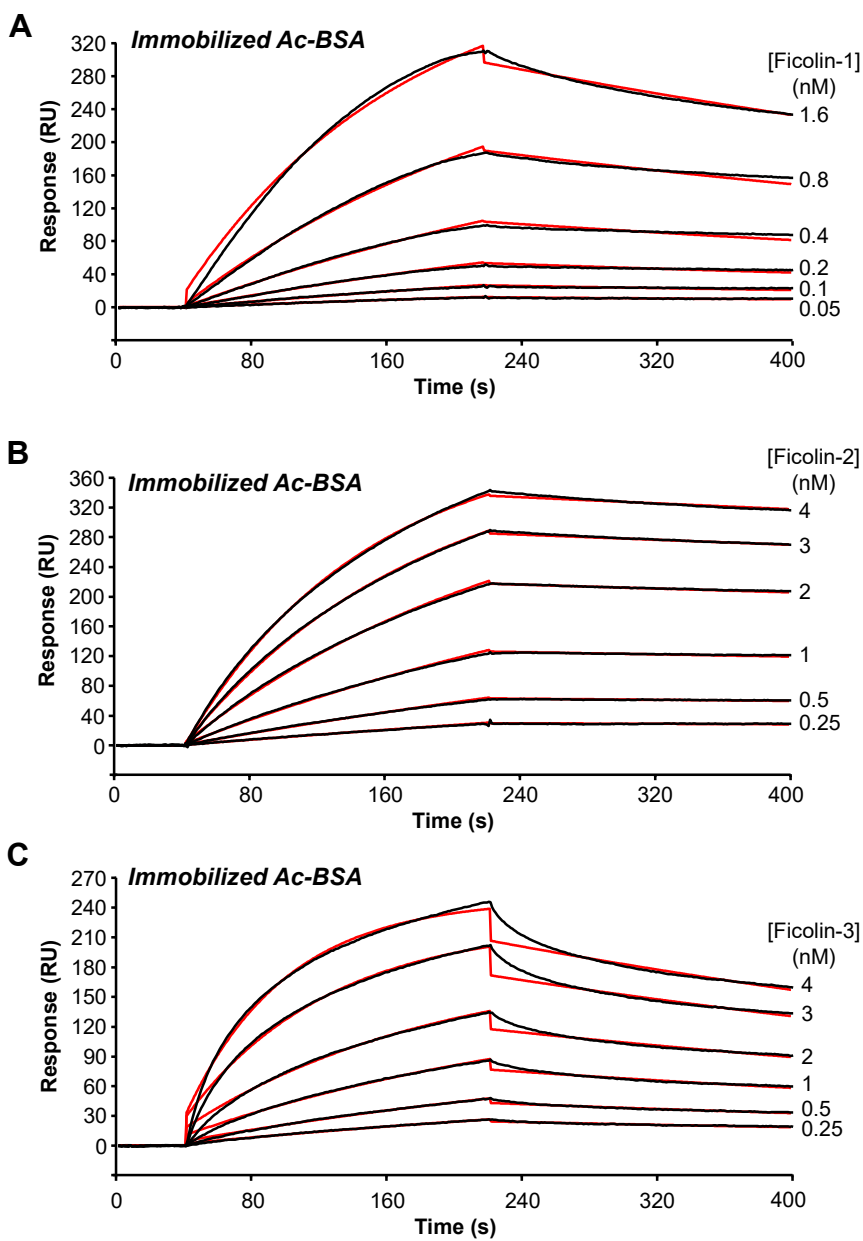


Fig. 3

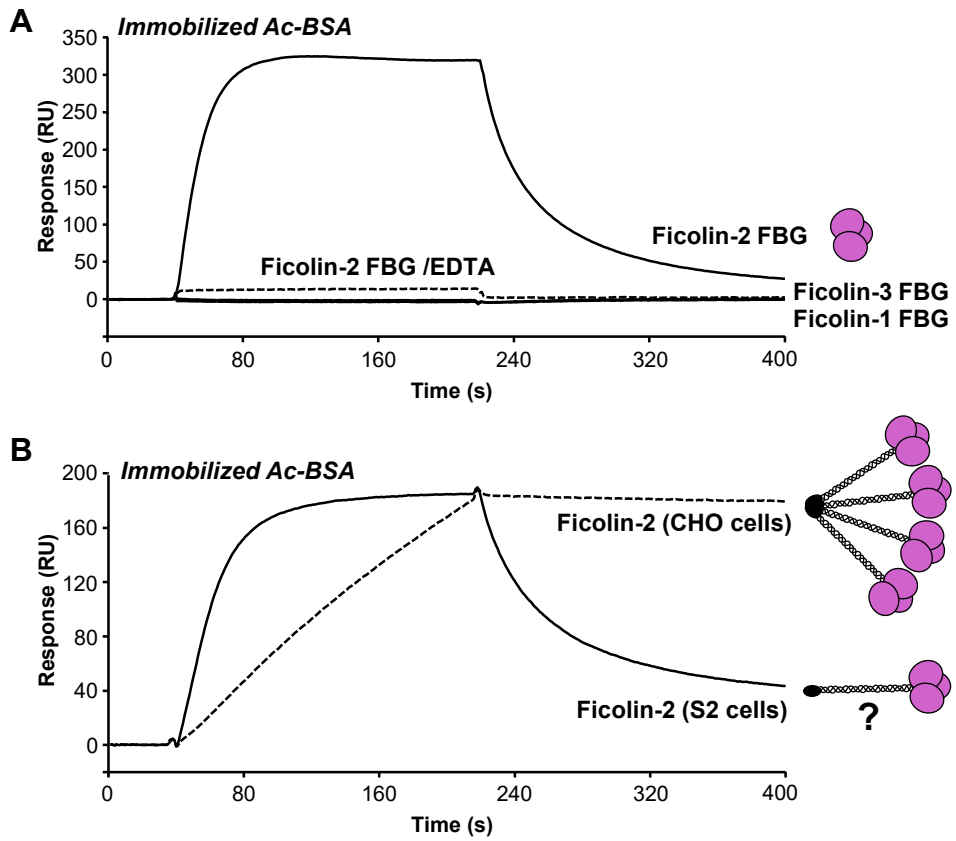


Fig. 4

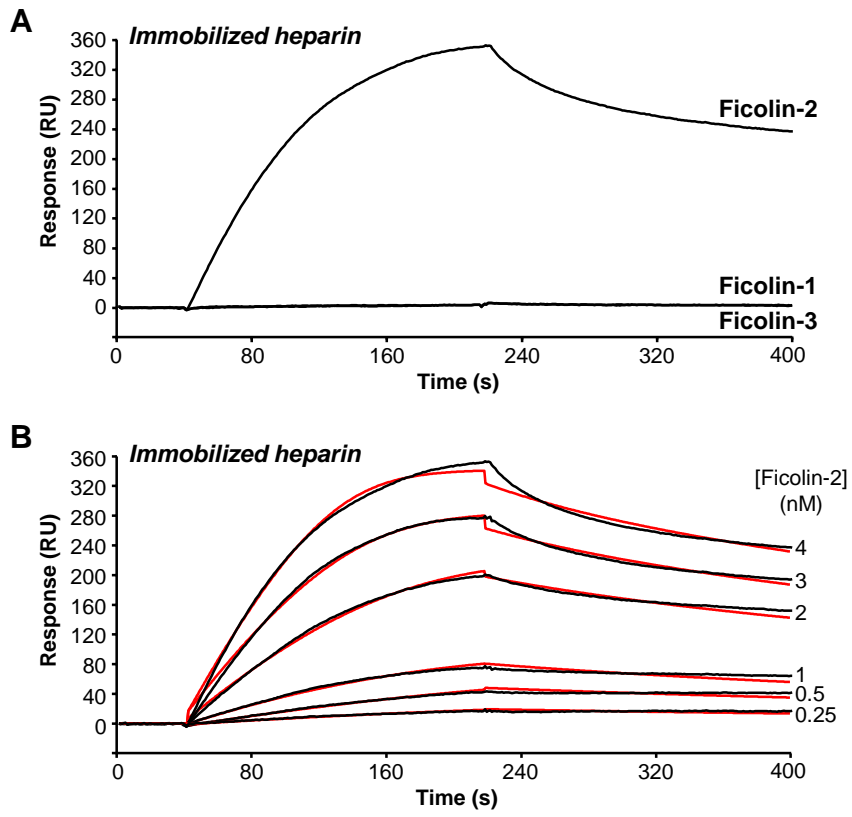


Fig. 5

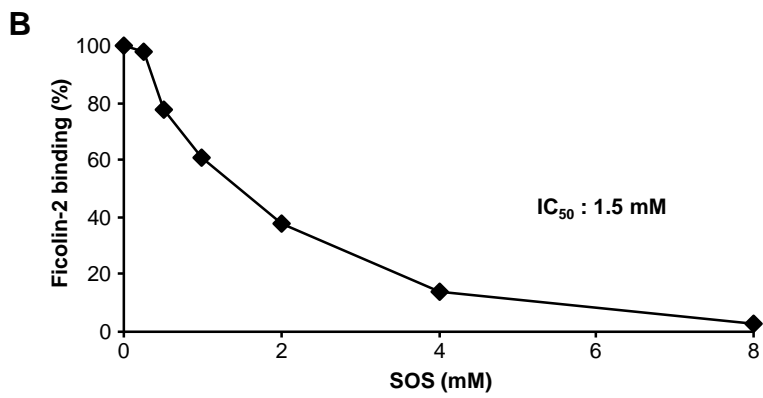
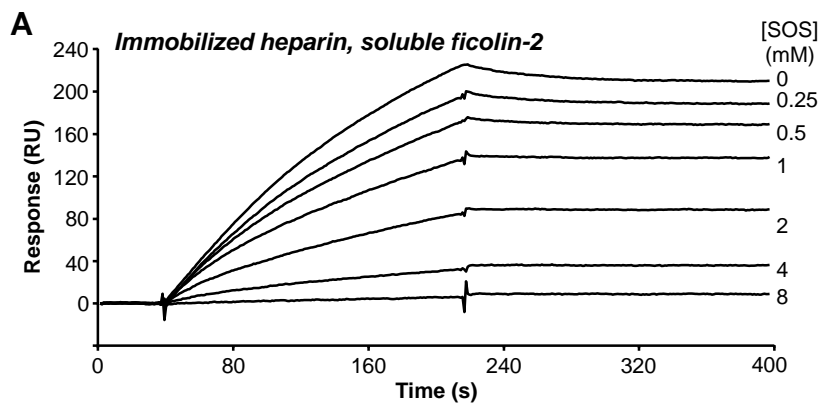


Fig. 6

