Optimizing the biological activity of Fab fragments by controlling their molecular orientation and spatial distribution across porous hydrogels

M. Ferrari, R. Barreto, E. Jackson, J.M. Guisan, F. Lopez-Gallego, L. Betancor

1. Introduction

An ample variety of versatile methodologies for protein immobilization has piled up in the literature which has provided a wide range of options for this purpose [1–3]. However, the choice of an optimal immobilization strategy depends on the final application of the immobilized proteins. This fact is particularly important in immuno conjugates, where the antibodies must be both optimally oriented and spatially distributed on the carrier to achieve maximum recognition of their corresponding antigens [4]. Scientists have tackled this challenge by customizing both the protein and the carrier surfaces in order to promote an optimal geometric and chemical congruence between the biomolecules and the solid materials [5,6].

The use of 3D porous beads containing suitably oriented antibodies with high biological activity unveils new opportunities in biosensing and chromatography. On one hand, this type of molecular sensing relies on particle volume rather than surface areas like for the typical ELISA methods. 3D molecular sensing has increased the volumetric sensitivity systems lowering the detection limits of the sensors. Moreover, by using transparent agarose beads, one could easily monitor and sort optical signal output on individual beads, enabling their integration to microfluidic devices with higher multiplex capacity [7]. This hypothesis has fuelled scientists and engineers in the last decade to improve the state-of-the-art immuno-sensing technology by developing innovative programmable bio-nano-chips based on antibodies immobilized on transparent porous beads [7]. This technology is rivalling traditional macroscopic sensing methods like ELISA at a lab-scale. Its application pursues opening new industrial horizons for point-of-care lateral-flow tests.

On the other hand, the use of porous micro beads increases the volumetric loads of antigens for immuno chromatography processes. Immuno conjugates on “3D-porous” beads bind higher amounts of antigens than immuno conjugates on 2D membranes because the surface-area-to-volume ratio is higher for 3D beads than for 2D membranes. Immuno chromatography is still one of the most powerful tools for selective purification of molecules [8,9], hence it is a motivation to develop novel chromatographic carriers that bind higher loads of antigens to readily purify and concentrate them in one-step. Recently, we have demonstrated

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ABSTRACT

Immobilization of antibodies (Ab) on hydrogels though long studied is still a challenge on account of the continuous development of new immune technologies. Enabling methodologies for antibody orientation, antibody stability and maximum recognition of their corresponding antigens is an object of intense study. Mini Ab such Fab fragments are less susceptible to conformational changes on surfaces, or unwanted reactivities compared to a whole Ab molecule. Herein, we have developed an immobilization protocol for a Fab anti epsilon toxin from Clostridium perfringens. Fine tuning of variables during immobilization showed a crucial role in the orientation and the spatial distribution of the Fab across the support surface. The final optimized immune-matrices demonstrated quantitative adsorption of antigen (1:1 molar ratio Fab to antigen) meaning that both the Fab biological activity was maintained after immobilization and an optimal orientation was achieved during the immobilization process. Immobilized Fab gained stability after immobilization as demonstrated by real time protein unfolding.

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how controlling the immobilization of anti-HRP immunoglobulin G (IgG) on agarose beads activated with both aldehydes and metal chelates (Ag-Cu2+/G) yields immuno conjugates with similar capacity to bind antigens as the soluble antibodies [10]. As this type of immobilization guarantees interaction to histidine clusters present in proteins, we were able to control the orientation of IgGs. Additionally, we obtained optimal spatial distribution of the antibodies by using a competitive agent. However, we only achieved low antibody loads per mass of the carrier.

Antigen binding fragments (Fabs) encompasses the antigen-binding domain of antibodies without the effector function fragment: the Fc part. This mini Ab has served in the past few years as a biological tool in biosensing as well as in immune chromatography [11,12]. The premise underneat their utilization is often that the complete Ab may be more susceptible to conformational changes on the surface, or unwanted reactivities mediated by their Fc part [13]. Herein, we present the immobilization of high loads of Fab fragments with good efficiency for antigen binding after careful optimization of immobilization conditions. To achieve this aim, a Fab that binds e-toxin from Clostridium perfringens, has been immobilized on porous agarose beads activated with both Cu2+-chelates and aldehyde groups (Ag-Cu2+/G); a strategy that has never been used with Fab fragments. We have determined and analyzed the immobilization parameters, the binding capacity and the stability of the resulting immune conjugates.

2. Materials and methods

WHO International Standard for C. perfringens epsilon antitoxin, was from National Institute for Biological Standards and Control (NIBSC), Potters Bar, UK. Toxin epsilon was donated by Laboratorios Santa Elena (Montevideo, Uruguay). Sodium periodate was from Merck (Darmstadt, Germany). Fluorescine isothiocyanate (FITC) and β-mercaptoethanol, were supplied by Sigma Chem. Co. (St. Louis, U.S.A.). Coomassie (Bradford) protein assay kit was purchased from Thermo Fisher Scientific Inc. (Rockford, U.S.A.). LMW-SDS Marker Kit (14–97 kDa) was from Amersham (Buckinghamshire, UK). Acrylamid/Bis 30% solution was supplied by BioRad (Hercules, U.S.A.). Pre-cast gels from 4 to 12%, pre-stained protein marker kit and 5–6650 SYPRO orange were from Life Technologies, Thermo Fisher Scientific (UK). Agarose (4% cross-linked beads) was supplied by ABT technologies (Madrid, Spain). Agarose 4BCL activated with glyoxyl groups (Ag–G) was prepared as described by Guisán [14]. All other reagents were of analytical grade.

2.1. Protein determination assay

Protein concentration was determined by Bradford reagent using the protocol recommended by the manufacturer, using bovine serum albumin (BSA) as standard [15].

2.2. Electrophoretic analysis

For solid samples 15 mg were mixed with 150 μL of 5 × sample buffer (Tris–HCl 625 mM pH 6.8, 2% sodium–dodecyl sulfate (SDS), 5% β-mercaptoethanol, 10% glycerol and 0.005% bromophenol blue, or a commercial sample buffer). For liquid samples, 20 μL were mixed with 5 μL of 5 × sample buffer. All the samples were boiled for 5 min under reducing conditions. Non-covalently attached Fab molecules are released from the support after this treatment. 15 μL of the supernatants from these preparations were analyzed by SDS-PAGE (10% or 4–12% pre-cast gels from Life Technologies). The gels were stained using either Cooomassie blue or Silver staining protocol.

2.3. Synthesis of heterofunctional supports (Ag–Cu2+/G)

The supports used in this work are based on agarose 4BCL activated with epoxide groups. Briefly, an agarose–epoxide support was synthesized: 10 g agarose 4 BCL was resuspended in a mix containing 44 ml of water, 16 ml of acetone, 3.3 g of NaOH, 0.2 g of NaBH4 and 11 ml of epichlorohydrin. Then, the suspension was gently stirred for 16 h at room temperature and afterward it was washed with an excess of water. The support was then modified with a solution of 0.5 M iminodiacetic acid adjusted at pH 11.0 in a ratio 1/10 (w/v), at 25 °C. It was gently stirred for 24 h, and then it was washed with distilled water. Then, the support was oxidized with sodium periodate for 2 h and finally washed with an excess of water. Finally, the oxidized support was incubated for 1 h in a ratio 1/10 (w/v) with a 30 mg mL−1 solution of the salt containing the desired divalent cation. After this, the support was washed with abundant distilled water. The resulting heterofunctional supports were named as Ag–Cu2+/G. Glyoxyl and metal chelate groups were quantified as reported elsewhere [16].

2.4. Bioconjugate preparation: Fab immobilization on Ag–Cu2+/G

Immobilizations were carried out incubating under gentle stirring 0.1 g of support with 1 ml Fab solution (0.6–1.2 mg mL−1) in phosphate buffered saline (PBS) pH 7.4 at 24 °C for variable times (15 min to 24 h). After immobilization the preparation was filtered under vacuum and the supernatant was analyzed for protein content and in some experiments by SDS-PAGE. To promote the covalent binding of the Fab to the support, the bioconjugate was incubated for 2 h at 24 °C in 0.1 mM sodium bicarbonate buffer pH 10.0. In some cases the Cu2+ of the support was removed by filtering the mixture and incubating it with 0.5 M EDTA solution at pH 10.0 for 30 min. The bioconjugates (with or without Cu2+) were reduced with 1 mg mL−1 NaBH4 solution in 150 mM sodium bicarbonate buffer at pH 8.5 for 30 min (R 1:10 w/v) and then washed thoroughly with distilled water. To demonstrate the effect of imidazole concentration on the immobilization of Fabs on Ag-Cu2+/G, immobilization was performed using PBS containing different imidazole concentrations (0, 1, 5, 10, 20, 50 mM). Fast immobilized bioconjugates were prepared without imidazole. Slow immobilized bioconjugates were prepared using 5 mM imidazole during immobilization. For the gradual immobilization protocol, immobilization started using PBS containing 10 mM imidazole. After 1 h at 24 °C, PBS was added to the support/supernatant mixture to dilute 1/2 the imidazole concentration. This procedure was repeated until a final imidazole concentration of 0.6 mM. Samples were then withdrawn after each incubation and analyzed for protein content and by SDS-PAGE.

For analysis with CLSM, the same immobilization procedure was carried out but utilizing Fab previously labelled with FITC.

2.5. Fluorescein modification of Fab fragments

0.5 mg mL−1 antibody solution is dialyzed against a sodium bicarbonate solution 0.1 M pH 9.5. 10 mg mL−1 solution of fluorescein isothiocyanate (FITC) in DMSO was prepared. Then the antibody and the FITC solutions were mixed in a molar ratio 1:20 (antibody:FITC). The mixed solution was incubated for 1 h at room temperature under gentle shaking in the dark. Subsequently, the excess of FITC was removed by purification of the labelled antibody using Q-Sepharose. In this purification step, 1 g of wet Sepharose Q was incubated with 10 ml labelling solution for 1 h under gentle stirring. Then the labelled antibody was eluted with sodium phosphate 25 mM pH 7.0 and 0.5 M NaCl.
2.6. Confocal laser scanning microscopy

Each labelled bioconjugate after the immobilization process was resuspended in their corresponding immobilization buffer in a ratio 1:10 (w/v), 30 μL of such suspension was analyzed by CLSM. Confocal images were acquired using an inverted Leica TCS SP5 laser confocal microscope with a 10× plan–apochromatic objective. Sequential scanning mode was used to avoid crosstalk between channels. All images shown correspond to a single confocal section. Images were processed with the LAS AF Leica application suite and Adobe Photoshop CS2 (Adobe Systems Inc.)

2.7. Stability of immobilized Fab fragments

To determine the stability of the antibody on the agarose particles a protein denaturation temperature of material assay was performed by using a real time PCR machine (Rotor gene, QIAGEN). SYPRO Orange fluorescent probe (Invitrogen) was used to monitor the denaturation of the antibody bound to the support. To perform this test, 2.5 μL of SYPRO orange (1/40 dilution) in 25 mM phosphate buffer pH 7.0 were mixed with (A) 5 μL of bioconjugate (Fab bound to Ag-Cu2+/G) (0.1 g/mL), (B) 5 μL sample of the soluble antibody plus 5 μL of antibody free Ag-Cu2+/G, (C) 5 μL of soluble Fab, (D) 5 μL of 25 mM phosphate buffer pH 7.0. All volumes were adjusted to a final volume of 20 μL. The samples were subjected to a melting test with a temperature gradient ranging from 35 to 100 °C. In this test each degree was incremented at a rate of 1 min per degree. The probe was excited at 470 nm and emission was detected at 610 nm. The protein denaturation was revealed by the increase in absorbance in fluorescence emission. For best viewing results slopes were plotted as derived by the computer software of Rotor Gene Q.

2.8. Purification of epsilon toxin with optimized affinity support (Fab on Ag-Cu2+/G)

For the purification of ε-toxin, 0.15 g of bioconjugated support were incubated at 24 °C with 300 μL of filtered cultured of C. perfringens. After 1 h, the support was filtered and washed twice with a PBS 0.5 M NaCl solution for 30 min under gentle stirring. Epsilon toxin was eluted incubating the support with 300 μL of 0.1 M glycine buffer pH 2.5 or pH 1.5. The pH of the samples was modified to pH 7.0 using 2 M tris for protein quantification and analysis by SDS-PAGE.

3. Results and discussion

3.1. Covalent immobilization of Fab fragments on agarose-based heterofunctional carriers

Heterofunctional carriers have been previously used to improve or optimize the stability, orientation and distribution of the resulting immobilized proteins [17,18,16]. Agarose beads activated with both aldehyde groups (also named glyoxyl) and Cu2+-chelates (Ag-Cu2+/G) have been reported to immobilize IgGs through a two-step mechanism [10]. The first step is a rapid affinity interaction between the histidine groups and metal cations on the antibody and support surfaces, respectively. In the second step, the glyoxyl groups are able to react with ε-NH2 groups from the lysine residues on the already immobilized antibody surface. The bonds formed are reversible Schiff’s bases that needed to be reduced for covalent linkage. This conjugation chemistry facilitates the preparation of carriers with oriented and multi-point attached antibodies throughout the histidine patches [10]. Surface densities of each reactive group could determine the immobilization performance.

Fig. 1. Optimal incubation time for Fab immobilization. SDS-PAGE (10%) of applied sample of Fab in the experiment (line 1, 12 mg g⁻¹ of support), supernatant of the immobilization after 15 min of incubation (line 2), after 30 min (line 3) after 45 min (line 4), after 60 min (line 5), after 150 min (line 6). Released Fabs from immobilized preparations incubated solely at pH 7.0 (line 7). Released Fabs from immobilized preparations after incubation at pH 10.0 and reduction with NaBH4 (line 8).

High density of Cu2+-chelates would allow for rapid immobilization in the first step, whereas, the high density of aldehydes groups (G) would promote intense covalent attachment in the second step that increases protein rigidity and, consequently, protein stability. We have immobilized a Fab fragment (Fabs) that binds ε-toxin from C. perfringens on Ag-Cu2+/G. Changes in the concentration of chelating groups (imidodiacetic acid) during support synthesis allowed for the preparation of agarose carriers with different ratios of Cu2+/G groups (data not shown).

Low chelates densities (24 μmol of Cu2+/g) were enough to quantitatively immobilize the Fab fragments (data not shown). Therefore, agarose beads activated with low densities of chelates groups were selected for further experiments because they contained higher densities of aldehyde groups that facilitated in a more intense covalent and irreversible attachments between the protein fragments and the solid surface. SDS-PAGE analysis of Fabs immobilized on heterofunctional supports demonstrated that the interaction between the biomolecules and the solid surface occurred rapidly and no further protein was immobilized after 30 min (Fig. 1). Moreover, Fab fragments were desorbed from solid supports under drastic conditions when they were not covalently linked (Fig. 1, line 7). On the contrary, Fab fragments remained irreversibly attached to the solid surface of Ag-Cu2+/G after incubation at pH 10.0 and reduction with NaBH4. However, partial desorption of some Fabs subunits was noticed after covalent attachment (Fig. 1, line 8). This result demonstrated that among the possible orientations of Fab immobilization on the support (Scheme 1) there was a preferential orientation of Fabs on the support surface as only the lower molecular weight Fab subunit was released. Moreover, it is possible to conclude that a heterogeneous population of Fabs is immobilized on the matrix as some of the Fab fragments were linked to the support by the two subunits while others were attached by only one. These results were in concordance with other works wherein the utility of heterofunctional surfaces to orient and multipoint covalently immobilized proteins was due to the cooperative action of the different reactive groups on the surface carrier [19].

3.2. Fab distribution across porous hydrogel immobilization on Ag-Cu2+/G

Considering the lack of structural information for our biomolecule of interest, further experiments with regard to
immobilization kinetics and protein localization will provide valuable data towards a rational design for the immobilization process. Studies that seek to optimize uniform distribution on surface biomolecule binding are crucial for applied purposes. For example, high local densities of immobilized Ab reduces biomolecule binding efficiencies, whereas, insufficient surface coverage of ligand and can cause unspecific adsorption of non-target molecules or surface induced denaturation of biomolecules [20,21]. Recently, it has been demonstrated that protein distribution on porous surfaces can be easily controlled by varying the immobilization rate [10]. The use of competing agents during affinity interactions influences the binding equilibrium of proteins to carriers. In this regard, we have immobilized Fabs on Ag-Cu2+/G under different imidazole concentrations. Fig. 2 shows that higher the imidazole concentration, lower was the immobilization yield of Fabs on this type of carrier. Expectedly, immobilization was minimal (<10%) using 50 mM imidazole. Using 10–20 mM imidazole, immobilization yield reached 20% which did not change over the time. In presence of 0–5 mM imidazole, Ag-Cu2+/G immobilized more than 80% of the Fab fragments. Under low imidazole concentration, the coordination chemistry between the histidine residues on the protein and metal chelates on the carrier were maximized. Immobilization in presence of imidazole may force the interaction of the biomolecule with the support surface through histidine rich areas, minimizing unspecific interactions. In the light of these results, we suggest that Fab fragment used herein may contain one or several histidine clusters as high immobilization percentages obtained using 5 mM imidazole. Such imidazole concentration might be enough to affect the immobilization rate and consequently the protein distribution across the porous surface. In fact, as previously discussed, it has been demonstrated that a slow immobilization distributes biomolecules more uniformly than rapid immobilization. Nonetheless, protein loads were low due to imidazole competence [10]. We therefore designed a gradual immobilization protocol aiming at high load, uniformly distributed and active Fab-support preparations. Fast and slow immobilization protocols were also tested as previous studies had been conducted with whole Ab molecules as opposed to the Fabs used in this study.

We therefore designed three different protocols for the immobilization of Fabs on Ag-Cu2+/G: (1) a fast immobilization in which we performed the experiment without imidazole for a rapid interaction of the protein with the support; (2) a slow immobilization protocol in which we added 10 mM imidazole to decelerate the immobilization rate; (3) a gradual protocol in which we started the experiment with 10 mM imidazole and progressively decreased the imidazole concentration by dilution. With the last strategy we observed how the Fabs gradually bound to the support while the imidazole concentration diminished in the bulk solution (Fig. 3). We observed similar results by analysing the supernatants by SDS-PAGE (data not shown).

**Fig. 2.** Effect of imidazole concentration on the immobilization of Fabs on Ag-Cu2+/G. Immobilization was monitored after 1 h (black) and 24 h (grey).

**Fig. 3.** Gradual immobilization of Fabs on Ag-Cu2+/G. Immobilization yield regarding to the offered protein concentration (bars) after gradually diluting the initial sample from 1 to 16 times (black circles). The dilution decreased the imidazole concentration in the immobilization solution thus increasing the effective immobilization yield.
The localization of the proteins throughout the carrier particles was determined by immobilizing Fab fragments labelled with fluorescein and analysing the resulting immobilized preparation was carried out with confocal laser scanning microscopy (CLSM) (Fig. 4) (Scheme 2). In the absence of imidazole, Fab fragments were mainly located on the outer surface of the agarose beads (Fig. 4B), while in the presence of 10 mM imidazole, Fab fragments were uniformly distributed across the whole particle (Fig. 4C).

Gradual immobilization enabled not only the uniform distribution of the Fabs across the porous surface (Fig. 4D) but also allowed immobilization of two-fold higher protein load as compared to the slow protocol using 10 mM imidazole. We have demonstrated that gradual immobilization promotes the uniform distribution of the Fab fragments and a steady loading of the protein onto the carrier. At 10 mM imidazole, immobilization rate is dramatically reduced, being even lower than the Fab fragment diffusion rate, driving the Fab fragments to diffuse across the whole particle. Once the Fabs were within the particle the gradual reduction of imidazole concentration allowed an interaction with the support without the precluding of the protein entrance generated by rapid protein immobilization in the vicinity of the carrier pores as seen in the fast protocol (Fig. 4B). By a rational design of the immobilization protocol we have been able to overcome the most important issue when uniformly distributing proteins: the low protein load (Scheme 2).

Table 1
Protein bound to Ag-Cu2+/G and Fab-Ag-Cu2+/G with different protocols.

<table>
<thead>
<tr>
<th></th>
<th>Fast (mg_protein/kg_carrier)</th>
<th>Gradual (mg_protein/kg_carrier)</th>
<th>Slow (mg_protein/kg_carrier)</th>
</tr>
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<tbody>
<tr>
<td>Bound Fab</td>
<td>7.63 ± 0.07</td>
<td>6.58 ± 0.09</td>
<td>3.23 ± 0.01</td>
</tr>
<tr>
<td>Bound toxin</td>
<td>2.41 ± 0.03</td>
<td>9.02 ± 0.03</td>
<td>3.7 ± 0.07</td>
</tr>
</tbody>
</table>

3.3. Characterization of Fab-Ag-Cu2+/G bioconjugates

The biological activity of the bioconjugates was analyzed by incubating them with the pure ε-toxin from C. perfringens. We observed that the gradual immobilization protocol had the best performance in antigen binding.

Gradually immobilized bioconjugates captured a much higher amount of toxin per gram of the carrier than both bioconjugates when slowly and rapidly immobilized (Table 1). Therefore, the uniform distribution of high loads of Fab fragments resulted in optimal immobilized preparations able to capture high amounts of antigen.

We then evaluated the unspecific interaction of the optimized carrier towards proteins from an extracellular sample of a C. perfringens culture containing the ε-toxin. Table 2 shows that the non-bioconjugated agarose beads activated with only Cu2+ chelates unspecifically adsorbed proteins. However, when aldehydes were the only groups present on the support surface, unspecific
protein adsorption was rather reduced. Therefore, to assure that toxin was selectively captured by antigen–antibody interaction rather than by unspecific interactions, the copper ions were removed by EDTA after Fab fragments were covalently attached, optimally oriented and uniformly distributed. These results were similar to those found for the immobilization of whole IgGs on the same type of supports [10]. Hence, removing of metal ions makes an inert surface that negligibly binds protein in an unspecific manner.

Solid supports conjugated with antibodies or Fab fragments have interesting applications as chromatography resin and biosensors. Stability of the immobilized antibody impacts on the performance and utility life of both chromatographic resins and immune sensors. Assessment of the stability of an immobilized antibody may be difficult, lengthy and may require large amounts of protein. In an attempt to study the stability of the immobilized Fab produced herein, we have used a recently developed method for studying protein denaturation [22]. A fluorescent probe capable of binding to hydrophobic sites in proteins is used to evaluate the kinetics of the immobilized Fab fragment unfolding. As the hydrophobic areas are mostly concentrated in the inner parts of proteins, the increase in fluorescence emissions reveals exposure of these areas to the milieu, and therefore it is an indicator of the denaturing state. The inflection point resulted from plotting the fluorescence versus the temperature is depicted as a minimum negative peak when the first derivative of fluorescence intensity was plotted. Values obtained at minimum peaks indicated a 50% denaturation of protein (D50) (Fig. 5). Analysis of fluorescence intensity as a function of temperature revealed that the soluble Fab fragment had a D50 at 76 °C, whereas, the immobilized sample had two minimum peaks at 78 °C and 84 °C (Fig. 5). Mixtures of soluble Fab fragments and inert agarose beads where the protein is not bonded to the agarose produced no shifts in the D50. This means that any change in the minimum peaks observed with the immobilized is consequence of the interaction between the Fab fragments and the support.

The shifts in D50 observed for the immobilized samples could be explained by a stabilizing effect produced by the oriented and multipoint covalent immobilization. Asfabs may have either one or two polypeptide chains covalently bound to the support, different denaturing kinetics might be expected. The minimum peak observed at 78 °C for immobilized samples could therefore be attributed to those polypeptide chains that are bound to the support but not covalently attached to the solid surface. Stability in this case is closer to that observed in the soluble sample. On the other hand, the peak at 84 °C denotes stabilization. These two peaks could be explained by a heterogeneous population of immobilized Fabs on the support: there might be antibodies that have both polypeptide chains covalently bound to the support and others that are attached through only one, showing different denaturing kinetics of Fab polypeptide chains. This hypothesis is supported by the release of some Fab subunits which was confirmed by SDS-PAGE (Fig. 1). These results highlight the relevance of orientation during immobilization. Further studies should focus on favouring the homogeneous immobilization of all Fab fragments through their most stabilizing orientation.

Table 2

Unspecific adsorption of proteins from an extracellular sample of a Clostridium perfringens culture containing epsilon toxin on Ag-Cu2+/G.

<table>
<thead>
<tr>
<th>Cu2+</th>
<th>Aldehydes</th>
<th>Protein bound mg g⁻¹</th>
</tr>
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<tbody>
<tr>
<td>−</td>
<td>−</td>
<td>0.020 ± 0.03</td>
</tr>
<tr>
<td>+</td>
<td>−</td>
<td>0.159 ± 0.01</td>
</tr>
<tr>
<td>−</td>
<td>+</td>
<td>0.033 ± 0.01</td>
</tr>
<tr>
<td>+</td>
<td>+</td>
<td>0.146 ± 0.02</td>
</tr>
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(*): Presence of 24 μmol of Cu2+ or 45 μmoles of aldehydes per g of agarose. (-): Absence of correspondent functional group.

Fig. 6. SDS-PAGE analysis of toxin purification. SDS-PAGE (4–12%) of applied sample of epsilon toxin (line 1), supernatant of the epsilon toxin adsorption to the anti-toxin antibody immobilized on Ag-IDA/G (line 2), washings (line 3) and (line 4), elution of epsilon toxin at pH 2.5 (line 5) and at pH 1.5 (line 6). Second elution at pH 1.5 (line 7).
3.4. Immunochromatography of α-toxin from C. perfringens by using optimal bioconjugates

The optimal bioconjugates bearing Fab fragments were tested in a chromatography experiment in batch with an excess of an extracellular sample of a *C. perfringens* culture containing the α-toxin (Fig. 6). The bioconjugates successfully proved their selective interaction with the target toxin and were revealed by elution through shifting the pH to the pI of the toxin, pH 5.4. In this experiment an optimal immune conjugate that contained 39 ± 1 μmoles of Fab fragment per g of the carrier was able to capture 43 ± 3 μmoles of toxin per g of the carrier. This demonstrated that all the immobilized antibodies although in different orientation, preserved their biological activity upon immobilization.

4. Conclusion

The control of both orientation and spatial distribution of Fab fragments across porous agarose beads activated with both aldehydes and copper-chelates groups provided immune conjugates with high protein loads and high antigen binding efficiency. In this work, we have developed a novel immobilization protocol to achieve high protein loads of Fab fragments uniformly distributed across the carrier’s porous structure. This was possible because the Fab fragments were gradually immobilized in the presence of decreasing imidazole concentrations. The optimal immune conjugate was able to bind to higher concentrations of antigen per mass, increasing the global binding capacity of the immuno complex. Agarose matrix is a naturally occurring hydrophilic hydrogel and is an ideal carrier for protein immobilization due to its high versatility to be activated with a plethora of functional groups. We have utilized this porous matrix to orient and spatially distribute Fab fragments throughout a 3D-microstructure. The immuno complexes have been tested for binding of extracellular sample of a *C. perfringens* culture containing the α-toxin; such immuno complexes are able to efficiently and selectively bind to the corresponding toxin. Therefore, the methodology proposed envisions the fabrication of immuno-beads for cost-efficient of both sensing and chromatography technologies. Using this bead approach we may circumvent the fundamental signalling and capacity limitations of flat surfaces to enable modular and multiplexed applications [23]. Moreover, these beads conjugate with antibody fragments that can be readily integrated into microfluidic systems to fabricate novel point of care devices by using lateral flow test and to purify target antigens by flow chromatography. Hence, controlling the immobilization of either antibodies or antibody fragments onto porous beads can unveil new opportunities for innovative applications in biosensing and chromatography.

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