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List of abbreviations

COOH (carboxyl), EDC (N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride), FLD (Fluorescent Detection), hIgG (human immunoglobulin G), PBS (Phosphate Buffer Saline), SEC (Size Exclusion Chromatography), Sulfo-NHS (N-Hydroxysulfosuccinimide sodium salt), TBS (Tris Buffer Saline), Ts (Tosyl),

Author contributions

Etienne Laborie: Investigation, writing original draft, validation, **Victor Le-Minh**: investigation, writing-review, **Thanh Duc Mai**: writing original draft, **Mehdi Ammar**: resources, supervision, **Myriam Taverna**: resources, writing-review, **Claire Smadja**: funding, project supervision, writing –original draft.

Conflicts of interest/Competing interests

The authors have no conflicts of interest to declare that are relevant to the content of this article.

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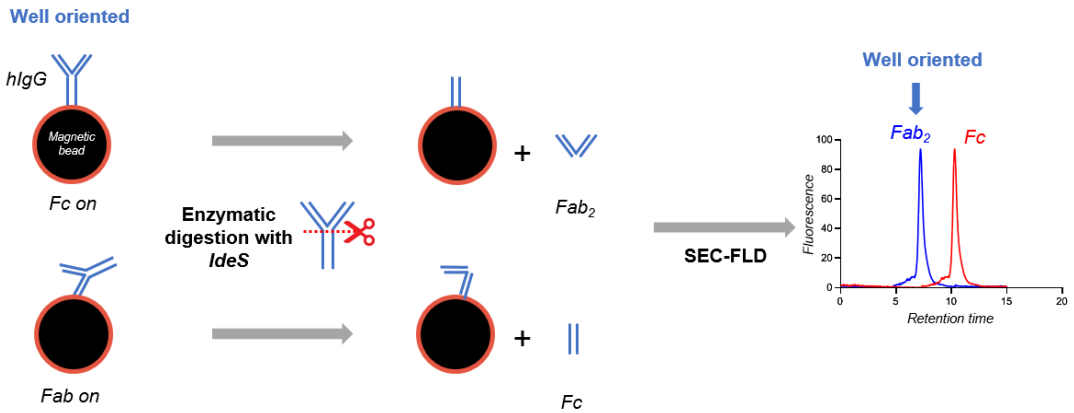
Graphical Abstract

A rapid and simple approach to evaluate orientation and density of antibodies immobilized on magnetic beads



Abstract

The use of magnetic beads bio-functionalized by antibodies (Ab), is constantly increasing with a wide range of biomedical applications. However, despite an urgent need for current methods to monitor Ab's grafting process and orientation, existing methods are still either cumbersome and/or limited.

In this work, we propose a new simple and rapid analytical approach to evaluate antibody orientation and density on magnetic beads. This approach relies on the cleavage by *IdeS*, a highly specific protease for human immunoglobulin G (hIgG), of immobilized antibodies. The $F(ab)_2$ and Fc fragments could be then accurately quantified by Size Exclusion Chromatography (SEC)- coupled to fluorescent detection (FLD) and the ratio of these fragments was used to give insight on the IgG orientation at the bead surface. Four different commercially available magnetic beads, bearing carboxyl groups, tosyl groups, streptavidin or protein G on their surface have been used in this study. Results obtained showed that this approach ensures reliable information on hIgG orientation and beads surface coverage. Protein G magnetic beads demonstrated an optimal orientation of antibodies for antigen capture (75% of accessible Fab_2 fragment) compared to tosylactivated, carboxylated and streptavidin ones. Capture efficiency of the different functionalized beads toward human TNF-α immunocapture, a biomarker of inflammation, has been also compared. Protein G beads provided a more efficient capture compared to other beads. In the future, this approach could be applied to any type of surface and beads to assess ~~the~~ hIgG coverage and orientation after any type of immobilization.

1. Introduction

Magnetic beads with their several advantages have found increasing applications in many fields including drug discovery, biomedicine, bioassays, diagnostics, genomics and proteomics [1, 2]. A typical application is peptides/proteins selective capture *via* magnetic separation using magnetic beads functionalized with a bio-receptor. Many recently developed diagnostic tools (e.g. lab-on-a-chip and biosensors) are based on bio-functionalized magnetic particles with antibodies [3]. For these purposes, several types of magnetic beads are currently available differing by their sizes, shape, surface chemistry and immobilization strategy that are crucial for antibodies grafting density and orientation. Preparation of magnetic nanoparticles with a stable surface remains of paramount importance. After the coating step bioreceptors such as antibodies will biofunctionalized the nanoparticles to target specific biomarkers for instance. A high antibody grafting density and a good orientation, which requires exposed and fully available $F(ab)_2$ fragments on bead surface, are highly desirable to ensure high loading/capture of target analytes during the immuno-precipitation/enrichment or bioassay processes.

The monitoring of surface antibody density and orientation is therefore critical not only for capture or recognition efficiency but also to produce batches of functionalized magnetic beads of equal performance and quality.

Conventional methods to assess the antibody immobilization on beads are based either on spectrophotometric or biophysical methods. Colorimetric assays (e.g. Lowry, Bradford and BCA assays) have been shown to provide only indirect determination of protein grafting efficiency as they are mainly based on the determination of non- immobilized proteins present in the supernatant [4][5]. In addition, they do not provide any information on antibodies orientation. Besides, many biases have been reported with these methods mainly related to possible background interferences due to nanoparticles themselves [6–8]. Atomic force

microscopy, surface plasmon resonance (SPR), spectroscopic ellipsometry and dual polarization interferometry [5, 9–12] can provide indirect insight on antibodies orientation mainly by measuring antibodies dimensions/thickness on surfaces or shift angle. Recently, Lämmerhofer *et al.* proposed an approach for quantification of the protein surface coverage on gold nanoparticles using high performance liquid chromatography (HPLC) [4, 13]. In this work, pepsin conjugated to gold nanoparticles is hydrolyzed (6N HCl). The released amino acids are tagged with a fluorescent dye and then analyzed by HPLC with fluorescence detection. The derivatized Glycine measurement is used to estimate the pepsin surface coverage on nanoparticles. Kozlowski *et al.* gave an estimate of surface coverage of dihydrofolate reductase (DHFR)-gold particle bioconjugates, using a combination of several techniques including SDS-PAGE, UV-vis spectrophotometry, dynamic light scattering and a fluorescence-based method [6]. These methodologies allowed a fine protein surface coverage characterization but required several steps and a combination of several techniques. In another study relying on streptavidin-magnetic beads, Gagey-Eilstein *et al*. communicated a chemiluminescent test to monitor the antibody grafting rate [14] and ~~by~~ sandwich-type immunoassay using magnetic beads grafted with antibodies have been also reported [15, 16]. All these methods, however, did not provide information on the antibody-grafting orientation nor on the functionality and ability of antibodies to capture the biological target.

Herein we report the development of a method for quantification of antibody surface coverage and monitoring of antibody grafting orientation on magnetic beads functionalized with different functional groups or chemistries. Together with a recent publication by Shen *et al* [17], our study is one of the two pioneering works which shed light on both orientation and density of antibodies decorated on magnetic beads using chromatographic approaches. Shen *et al* proposed a method which used a proteolysis performed by trypsin-decorated magnetic beads to

quantify the number of antibodies attached to magnetic beads. LC-MS/MS of the released tryptic peptides allowed to quantify representative peptides of either the $F(ab)_2$ or Fc region of the antibody. However, it is important to note that the release of $F(ab)_2$ peptides from antibodies could arise from antibodies attached via one Fab fragment leading thereby to an overestimation of the number of well oriented antibodies.

Our work relies on the use of a selective proteolysis of $F(ab)_2$ regions of antibodies, using (*IdeS*) that cleaves antibodies with a unique degree of specificity in the hinge region leading to the release of Fc and $F(ab)_2$ fragments [18]. Our straightforward analytical approach relied on size exclusion liquid chromatography coupled to fluorescent detection (FLD) allowing $F(ab)_2$ and Fc separation according to their molecular weight (100 and 50 kDa respectively) [17]. $F(ab)_2$ and Fc will be clearly identified with this new approach. In our case the quantification of the ratio Fab / Fc is expected to be directly related to the orientation of immobilized antibodies. Indeed, after IdeS digestion, if the antibodies are grafted through their Fc fragment, $F(ab)_2$ is released whereas when they are immobilized via Fab fragment, Fc fragment is released. The relevance and utility of the developed approach was demonstrated with a high diagnostic-value application, by quantifying the human Tumor Necrosis Factor alpha (TNF-α), a pro-inflammatory cytokine, serving for screening of Major Depressive Disorders (MDD) and which is involved in many inflammatory disorders such as cytokine release syndrome observed in COVID 19 [19–25].

2. Experimental

2.1. Chemicals and reagents

IdeS FabRICATOR enzyme (5000 units) was purchased from Genovis AB (Lund, Sweden). HPLC-grade propan-2-ol and sodium di-hydrogen phosphate (NaH2PO4) were obtained from Fisher Scientific (Karlsruhe, Germany). Sodium chloride was provided from Euromedex (Souffelweyersheim, France). 32% sodium hydroxide was purchased from VWR SAS (Fontenay-sous-Bois, France). 85% phosphoric acid was obtained from Carlo Erba Reagents (Val-de-Reuil, France). EZ-Link Sulfo-NHS-LC-biotin kit and Dynabeads MyOne Carboxylated, Tosylactivated, Streptavidin T1, Pierce Protein G (1µm) magnetic beads and TNF alpha Human Uncoated ELISA Kit were purchased from Life Technologies SAS (Villebon-sur-Yvette, France). Human IgG from whole serum (10.2 mg/mL) and Amicon Ultra 0.5mL 50 kDa and 100 kDa filters for ultrafiltration were provided by Merck Millipore (Darmstadt, Germany). Phosphate buffer saline 10X (PBS), Tris-buffer saline 10X (TBS), boric acid, Tween 20, ammonium sulphate, N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and N-Hydroxysulfosuccinimide sodium salt (Sulfo-NHS) were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Apparatus and Material

Size exclusion liquid chromatography experiments were performed using an Agilent 1260 UHPLC- 1260 FLD detector (Xenon lamp) (Agilent Technologies, Santa Clara, CA, USA). An Agilent Bio SEC-3 column (3 µm particle size; 300Å pore structure; 300 mm length; 4.6 mm i.d.) was employed at temperature of 25 °C. The mobile phase (50 mM sodium phosphate, 150 mM NaCl and 10% isopropanol at pH 6.5) was isocratically pumped at 300 µL/min. Fluorescence detection (FLD) was performed at λ_{ex} = 280 nm and λ_{em} = 340 nm. Data acquisition and instrument control were performed by Chemstation (Agilent).

2.3. Methods

2.3.1: Preparation of bio-functionalized magnetic beads

Model human IgG (hIgG) were grafted on beads following commercial protocols for streptavidin and protein G beads. For beads tosylactivated (Ts) and COOH the protocols have been adapted from the supplier ones). IgG concentration in the grafting solution was kept constant at 0.1 mg/mL for accurate comparison of beads grafting efficiency.

2.3.1.1: Carboxylated magnetic beads

100 µL of Dynabeads MyOne Carboxylated (10 mg/mL) were added to 4 LoBind Eppendorf vials of 1.5 mL and washed with 1 mL of 20 mM NaOH overnight on a rotating wheel (Tube rotator SB3, Stuart, UK) at 20 rpm and 4°C. Beads were then concentrated using a magnet and washed 3 times with 500 µL of PBS. Then, beads were resuspended in 120 µL of human IgG solution at 0.66 mg/mL in PBS. 200 µL of EDC (10 mg/mL) and 200 µL of Sulfo-NHS (10 mg/mL) in PBS were added to the beads suspension. Finally, volume was adjusted to 800 µL with PBS, reaching a final concentration of 0.1 mg/mL of IgG for 1 mg of beads. Vials were then placed on a rotating wheel at 20 rpm overnight at 4°C for antibody grafting and beads were immobilized on a magnet and washed 2 times with 500 µL of a solution of PBS, Tween 20 (0.05%) (PBS-Tween) and one time with 500 µL of PBS. Washing solutions were kept at 4°C until analysis.

2.3.1.2: Tosylactivated magnetic beads

10 µL of Dynabeads MyOne Tosylactivated (100 mg/mL) were added to 4 LoBind Eppendorf vials of 1.5 mL and washed with 500µL of 0.1 M sodium borate buffer pH 9.5, gently vortexed and stirred at the same time using an Eppendorf Thermomixer for 15 min C at 25°C and 650 rpm. Beads were resuspended in 60 µL of hIgG solution at 0.66 mg/mL in PBS with 60 µL of 3M ammonium borate buffer pH 9.5 and 20 µL of 0.1M sodium borate buffer. Vials containing

beads were then incubated at 37°C and stirred at 650 rpm on a Thermomixer for 16h and then sonicated for 5 minutes to prevent their adsorption on the vial's walls. Beads are were immobilized on a magnet and washed 2 times with 500 µL of a solution of PBS-Tween, and one time with 500 µL of PBS. Washing solutions were kept at 4°C until their analysis.

2.3.1.3: Streptavidin magnetic beads

Biotinylation of human IgG was done using EZ-link sulfo-NHS-LC-biotin kit. A 10 mM solution of biotin was prepared by adding 224µL of ultrapure water to 1 mg of biotin. 6.66 µL of biotin solution was added to 1000 µL of hIgG solution (0.5 mg/mL) and incubated at 25°C for 30 minutes and stirred at 300 rpm on a Thermomixer at the same time. The solution was then ultrafiltrated on a 50 kDa filter cap at 4°C, 10 000 rpm for 4 minutes to get rid of biotin salts excess.

100µL of Dynabeads MyOne Streptavidin T1 (10 mg/mL) are added to 4 LoBind Eppendorf vials of 1.5 mL and washed with 1 mL of PBS-Tween solution. Beads were then immobilized on a magnet and washed 3 times with 500 µL of PBS-Tween. Beads were then resuspended in 40 µL of biotinylated hIgG solution at 0.5mg/mL and 160 µL of PBS. Vials are were then incubated at 25°C, 450 rpm for 30 minutes on a Thermomixer and beads are were immobilized on a magnet and supernatants are collected and kept at 4°C. Beads were washed 4 times with 500 µL of a solution of PBS Tween Washing solutions were kept at 4°C.

2.3.1.4: Protein G magnetic beads

100µL of Pierce Protein G Magnetic Beads (10 mg/mL) were added to 4 LoBind Eppendorf vials of 1.5 mL and washed with 500 µL of Tris-Tween solution and gently vortexed. The process was repeated one time with 1 mL of Tris-Tween. Beads were resuspended in 217 µL of human IgG solution at 0.23 mg/mL with 283 µL of Tris-Tween. Vials were then incubated at

25°C, 650 rpm for 1 hour on a Thermomixer and beads were immobilized on a magnet and supernatants were collected and kept in the fridge for analysis. Beads were then washed 2 times with 500 µL with Tris-Tween. Washing solutions were kept in the fridge for analysis.

2.3.2 Characterization of the hIgG grafting

2.3.2.1 Enzymatic digestion of grafted antibodies

For all digestions, 2µL aliquot of 100 U of *IdeS* FabRICATOR enzyme and 50 µL of phosphate buffer (100 mM, pH 7.0) were added to the sample, hIgG solution (60 µL) or hIgG grafted on magnetic beads (suspended) and incubated for 1h30 at 37°C 300 rpm on a Thermomixer. Beads suspensions were then placed on a magnet and supernatants were collected and analyzed.

2.3.2.2 Influence of beads grafting conditions on FLD signal for hIgG, F(ab)$_2$ and Fc fragments

To avoid any bias in the measurements, the influence of beads grafting protocols (medium, incubation steps) on hIgG FLD signals was investigated. Each sample was prepared in duplicate. hIgG samples at 0.1 mg/mL were incubated following conjugation protocols corresponding to tosylactivated carboxylic and protein G beads and then analyzed by SEC-FLD.

2.3.2.3 Calibration curves

hIgG

Calibration curve for whole hIgG was obtained with a 0.1 mg/mL hIgG solution in PBS. Dilution series of hIgG, ranging from 0.05 µg to 0.7 µg, have been injected analyzed to establish the calibration curve.

hIgG fragments

Calibration samples were made from IgG ~~stock~~ solution submitted to the different conjugation's protocols. hIgG solutions (0.23 mg/mL) were digested with 100 U of *IdeS* and analyzed ~~with~~ by SEC-FLD. Volumes of hIgG digests from 0.5 µL to 7 µL were analyzed and a calibration curve per conjugation protocol has been built for the two fragments ($F(ab)_2$ and Fc).

2.3.2.4: Quantitation of non-grafted hIgG in the supernatant

Antibodies remaining in the supernatant after the grafting step were first quantified. Then, washing solutions were concentrated by ultrafiltration on 100 kDa membrane. 500 µL of washing solution was added to the filter cap and centrifuge for 4 minutes at 10 000 rpm at 4°C on a Centrifuged MicroStar 17R (VWR). The process was repeated until all the solution was filtered. Retentates volumes were measured before SEC-FLD analysis.

2.3.3 Immuno-capture of TNF-α by biofunctionalized magnetic beads

1 mg of COOH, Ts and Protein G magnetic beads were grafted with anti-human-TNF-α monoclonal antibodies from the Invitrogen ELISA kit for human TNF-α following the procedure described previously. Five replicates of 100µg of grafted magnetic beads of each type were then incubated for 4 hours at 25°C with 5 ng of human TNF-α in 250µL of PBS (1X). Three blank samples were also prepared using grafted magnetic beads and PBS (1X). Beads were washed with 500µL of PBS-Tween for 10 minutes at 800 rpm on a Thermomixer. Then, heated 95°C for 5 minutes in 250µL of PBS (1X) for thermal elution of TNF-α. Eluates were diluted on PBS (1X, 10X, 100X) to match immunoassay detection range. Human TNF-α contents of non-diluted and diluted eluates were quantified using Invitrogen ELISA kit for human TNF-α using duplicates. Calibration curves were built by preparing serial dilution of human TNF-α solution at 500 pg/mL with and without thermal treatment at 95°C for 5 minutes to take into account the thermal effect on TNF-α.

3. Results and Discussion

The first part of the study was dedicated to the development of an analytical method to evaluate the grafting efficiency on four types of beads. Two main criteria have been considered: number of antibodies grafted on the bead's surface and their orientation. For this purpose, we specifically cleaved ~~human~~ hIgG grafted on the beads surface under the hinge region with an enzyme, *IdeS*, and analyzed the digestion products by SEC-FLD analysis. If the hIgG is immobilized *via* its Fc region, the $F(ab)_2$ fragment will be released in the supernatant whereas for immobilization through $F(ab)_2$ fragment, Fc fragment will be obtained.

3.1. Influence of the grafting conditions on native and digested human IgG fluorescent detection

We first developed a SEC-Fluorescent detection (FLD) method to quantify hIgG in solution. Analysis of human IgG in solution (0.1 mg/mL) showed two peaks (Figure 1A): the first one corresponds to a dimeric form of hIgG (~8.2 min) and the second to the monomeric hIgG (~9.2 min). As the first peak area was very small compared to ~~that of~~ the 2nd one (peak area ratio: ~8), dimeric hIgG was not considered for hIgG calibration. Repeatability and intermediate precision of SEC-FLD method have been evaluated. The intra (n=3) and inter-day (n=3) RSD of the retention times (Tr) were less than 0.1%. Resolution and limit of quantification were also evaluated at 1.3 and 160 nM respectively (see Supplementary Material, method validation chapter).

Considering the antibodies grafting density, it is important to note that for each type of beads, the grafting protocol differs in terms of buffers (i.e. pH and ionic strength) thermal and/or agitation that may impact protein conformation and thereby fluorescent intensities measured. Therefore, hIgG free in solution (0.1 mg/mL) were submitted to experimental conditions

mimicking the grafting protocols applied to COOH and Ts beads. The agitation used for hIgG grafting on COOH beads (rotating wheel at 4 °C overnight) led to a slight decrease of the FLD signal (-5,2 %) compared to the reference sample of hIgG (PBS at 4 °C). The FLD signal further decrease (19%) when EDC and Sulfo-NHS were added in the reaction medium. To explain this decrease, we hypothesized that the temperature (4 °C) as well as EDC and Sulfo-NHS could increase the intra amino acids interactions favoring thereby conformation where fluorescent amino acids are hindered. For Ts beads, an increase of the FLD signal (+9,6 %) was observed with the Ts grafting protocol (stirring at 37 °C during 16h). This signal is increased by 18 % when Ts conjugation protocol is performed in borate buffer pH 9.5 (Figure 1B). The increase of the fluorescent signal could be related to a partial denaturation of the hIgG. Several studies have demonstrated that thermally induced unfolding of proteins led to the exposure of the fluorescent amino previously buried (e.g. tryptophan) increasing thereby the fluorescent signal [26]. The same type of experiments has been performed for hIgG cleaved by IdeS. Digested IgG (0.22 mg/mL) has been first analyzed with SEC-FLD, showing two peaks: 1st peak (~9.99 min) corresponding to Fab_2 fragment of 100 kDa; 2nd peak (~10,98 min) to Fc one (50 kDa) (Figure 2). Four calibration curves of hIgG cleaved by *IdeS* into Fab_2 (~100 kDa) and Fc (~50 kDa) fragments have been performed under the grafting conditions selected for the 4 types of beads (figure 3). A variation has been observed for Fc peaks: a 25% decrease of the curve's slope for COOH beads conditions compared to Ts beads which could lead to underestimate an unfavorable orientation (see Figure 3A and B). Therefore, we can conclude from these results that calibration curves of hIgG and its fragments have to be established for each type of beads studied (i.e. COOH, Ts, streptavidin, protein G) to evaluate accurately the antibodies grafting density and orientation.

3.2. Antibodies grafting density and orientation on magnetic microbeads

We then evaluated the grafting density and hIgG orientation on the four types of beads: carboxylated, tosylated, protein G and streptavidin. For this purpose, magnetic beads bearing hIgG have been digested by *IdeS* and the fragments released analyzed by SEC-FLD.

3.2.1. F(ab)2 and Fc quantitation.

We first verified that *IdeS* did not overlap with peaks corresponding to hIgG fragments in our experimental conditions by analysing *IdeS* (5 U) as a control. Indeed, molecular weight of Ides is approximately 37 kDa wich might interfere with hIgG fragments detection. For *IdeS,* two peaks were observed at 10.08 min (dimer) and 11.0 min (monomer) with retention times close to $F(ab)_2$ and Fc ones which could lead to overestimate peak areas related to Fc and, into a lesser extent, to Fab_2 (see Figure S1 in Electronic Supplementary Material). Therefore, to accurately quantify the amount of $F(ab)_2$ and Fc released after the digestion of hIgG grafted on magnetic beads, a calibration curve for *IdeS* (from 1 U to 14 U) was drawn using different injection volumes (see Fig S2, Electronic Supplementary Material) and was used to evaluate ~~of~~ *IdeS* contribution in Fab_2 and Fc peak areas values. Therefore, peak areas of $F(ab)_2$ and Fc fragments were corrected with *IdeS* calibration in the whole study allowing a more accurate quantification (see Table S1, Supplementary material).

3.2.2. Grafting density and hIgG orientation on the different types of beads

To estimate the percentage of hIgG grafted (%), washing fractions and supernatants were analyzed and Equation 1 was used to estimate this value:

$$\%_{grafted} = \frac{m_i - (m_{washing} + m_{supernatant})}{m_i} \qquad Eq.1$$

where m_i is the initial quantity of hIgG per mg of beads before grafting, $m_{washing}$ and $m_{supernatant}$ are the quantity of hIgG found after the grafting step in washing solutions and supernatants

358 respectively. A calibration curve of non-digested hIgG in solution in PBS was performed to
359 quantify remaining hIgG in the supernatant and washing solution (see Figure S2 in
360 Supplementary Material).
361 From the percentage values evaluated for the different beads (Figure 4A), we can conclude that
362 protein G beads can graft more IgG than other beads (95.1% of injected IgG are grafted), with
363 COOH beads being the less efficient with a lower density of IgG grafted on their surface
364 (73.8%).
365 The number of hIgG per bead has been also evaluated by considering the average mass of an
366 hIgG (150 kDa), the bead's density (1.8 g/cm^3 for Ts, COOH and streptavidin beads, 2.0 g/cm^3
367 for protein G beads) and average diameter of the beads (1 µm), using the following equation:

368 $$Number\ of\ IgG\ molecules\ per\ bead = \frac{\%_{grafted} \times m_i}{m_{IgG}} \times \frac{m_{bead}}{1\ mg\ of\ beads} \quad \text{(Eq.2)}$$

369 where $\%_{grafted}$ is the proportion of grafted hIgG on the beads, m_i the initial mass (µg) of hIgG
370 incubated with 1 mg of beads for grafting, m_{IgG} the mass (µg) of one hIgG molecule, m_{bead} the
371 mass (µg) of the corresponding bead.
372 According to Figure 4B, protein G beads grafted a high number of hIgG (~70 000 molecules
373 per bead), followed by Ts beads (~56 000 IgG molecules per bead), streptavidin beads (~25 000
374 molecules) and finally COOH ones (~12 000). We can note that despite a higher quantity of
375 hIgG employed for carboxylic beads (79 µg) compared to protein G (49.91 µg) the amount of
376 hIgG grafted is better for proteins G beads. The same observation can be made for Ts beads,
377 where better grafting compared to carboxylic beads is observed despite the smaller
378 concentration of hIgG used (39,6 µg). These results suggest that the grafting efficiency is not
379 only related to the amount of hIgG in the immobilization solution but also to the surface
380 chemistry.
381

The hIgG orientation has been evaluated by *IdeS* digestion and the analysis of released fragments. Figure 5A displays the different chromatograms obtained for COOH, Ts, streptavidin and protein G beads. The peak areas and notably Fab_2/Fc ratio highlighted the differences between beads in terms of number of hIgG grafted and hIgG orientations. From Figure 5A, we have observed that protein G beads lead to a larger peak of $F(ab)_2$ compared to other beads. The orientation ratio (Fab_2/(Fab_2+Fc) showed that protein G beads also exhibit the best orientation (~73.8% of antibodies grafted by their Fc fragment) (Figure 5B) compared to Ts (31.3%), streptavidin (9.9%) and carboxylic beads (5.2%). We can conclude from these results that protein G led not only to more grafted antibodies but also to a higher proportion of well orientated immobilized hIgG. This could be due to the surface chemistry of carboxylic and Ts beads that led to a random immobilization of hIgG via amino (carboxylic and Ts) and sulfhydryl groups (Ts). Therefore, only a fraction of the $F(ab)_2$ binding sites will be potentially available for biorecognition. The better orientation ratio observed for Ts compared to carboxylic beads could be related to the hydrophobic surface of tosyl beads favoring hIgG orientation through the more hydrophobic Fc region. In contrast, for protein G, immobilization through Fc fragment allow a better orientation with a higher proportion of F(ab)2 available.

IdeS digestion efficiency on the magnetic beads

To ensure that the better peak area ratio Fab_2/Fc observed in Protein G was related to a better orientation and not to a higher enzymatic digestion efficiency, *IdeS* digestion yield has been estimated (Equation 3).

$$\%_{digested} = \frac{m_{digested}}{m_i - (m_{washing} + m_{supernatant})} \qquad Eq.3$$

Digestion efficiency $\%_{digested}$ introduced in Eq.3 is directly correlated to biologically available hIgG, where m_i is the initial quantity of hIgG per mg of beads before grafting, $m_{washing}$

406 and $m_{supernatant}$ are the quantity of hIgG found in washing liquids and supernatants and $m_{digested}$
407 is the quantity of hIgG found in the digestate after IdeS digestion.

408 Indeed, we considered that *IdeS* cannot fully digest every hIgG molecule grafted on the
409 bead, which means that this method could give an underestimated quantification of hIgG
410 available to *IdeS* molecules. Being a relatively large molecule (~37kDa), *IdeS* may not access
411 easily to the hinge region of some hIgG molecules within the antibody layers (or multilayers),
412 resulting in a partial proteolysis yield. Assuming that these unavailable hIgG molecules would
413 not be available for other reactions with biomolecules (such as antigen recognition and capture),
414 we can suppose that the digestion yield also reflects the antigen capture potential of the beads.
415 The results showed that *IdeS* digestion yield is higher for tosylactivated beads (46.3%) than
416 protein G (34,2%) and streptavidin (37,5%). Unexpectedly, we found a very low digestion yield
417 for COOH beads (5.6%), as depicted in Figure 6.
418 The lower digestion yield for protein G beads compared to the tosylactivated beads is probably
419 related to the higher grafting density leading thereby to a steric hindrance detrimental for *IdeS*
420 biological activity. Considering the better ratio $Fab_2/(Fab_2+Fc)$ and the lower digestion yield of
421 IdeS for protein G beads, these results confirm the better orientation of hIgG on this kind of
422 beads.

423 Therefore, the overall results showed that protein G beads exhibit the best orientation
424 ratio as well as the best grafting density. This could be explained by their ability to capture hIgG
425 through Fc fragment, leading thereby to a more favorable orientation [27]. In contrast, hIgG are
426 randomly immobilized by covalent bonding for COOH and Ts beads and via Biotin interactions
427 (non-covalent) for Streptavidin beads [28]. In addition, Streptavidin beads rely on a
428 biotinylation procedure prior to antibody grafting on beads. This additional step may decrease
429 the efficiency of the whole conjugation procedure as biotinylation will not be total. Finally,
430 protein G beads offer the best results in terms of antibody orientation and density on the surface.

Moreover, they are particularly easy to graft with ~1h30 to complete the whole procedure compared to 18h for Ts and 30h for COOH beads, which makes them the best candidate for antibody grafting and immunocapture.

Concerning ProtA/ProtG beads, these results are in agreement with *Shen et al* [17] in terms of antibody coverage and orientation ratio. In contrast, for carboxylated beads, a high difference is observed with our study. This discrepancy could be related to the digestion tool employed. Indeed, with trypsin digestion, employed by *Shen et al*., the peptides released from the F(ab)2 region, detected and quantified by LC-MS, could arise from antibodies attached on beads via one Fab fragment. This could lead therefore to an overestimation of the orientation ratio for COOH beads where antibodies are randomly immobilized. On Prot G/A beads, ~~where~~ antibodies are immobilized via Fc fragments and thereby well oriented. Our method combining IdeS digestion with SEC analysis offers size discrimination making it possible to retrieve more information to identify the fragment released (i.e. Fc (50 kDa) vs F(ab)2 (100 kDa).

We have therefore developed a direct accurate and rapid quantification method of bioavailable hIgG on magnetic beads. Our study takes into account beads grafting conditions and *IdeS* contribution effect on FLD signal and absorption on beads for a more accurate quantification. Finally, we compared the performance of COOH, Ts and protein G beads for capturing TNF-α, a cytokine involved in Major Depressive Disorders (MDD) and other inflammatory-related pathologies. Streptavidin beads were not considered in the study as they did not provide added value compared to carboxylated and tosylactivated beads.

3.3. Application for immunocapture of TNF-α: towards quantification of biomarkers in biological fluids

To illustrate the performance of the previously studied magnetic beads, COOH, Ts and protein G beads were grafted with monoclonal anti-human-TNF-α antibodies following the

procedure previously described. 5 ng of human TNF-α were then incubated for 4 hours at 25 °C with 100 µg of grafted magnetic beads of each type. Elution of antigens was done using thermal elution in PBS at 95°C for 5 minutes in order to maximize the amount of antigen release and minimize protein denaturation [29]. Eluates were then prepared in three dilutions: non diluted, ten and one hundredfold diluted to ensure that recovered antigen concentration matched the immunoassay's linear range. Quantification was carried out using an ELISA kit for human TNF-α on microplate by absorbance spectrometry. A calibration curve was built using human TNF-α standard solution. The standard was also treated at 95°C for 5 minutes in order to reflect possible conformational changes of TNF-α that would affect immunoassay detection. As shown in Figure S3, thermal treatment influenced calibration curve slope with a 40% decrease in slope after thermal treatment. Quantification of eluates from the beads was therefore performed using the thermal treatment calibration curve. Results are shown in Table 1.

For 5.0 ng of human TNF-α incubated with 100µg of beads, 67.6% of TNF-α was recovered with protein G beads, but only 6.6% and 2.8% for COOH and Ts beads respectively. These results support the high antibody grafting efficiency of protein G beads in terms of both antibody coverage and orientation. For COOH and Ts beads, they suggest a very low capture ability of these beads for this application probably due to poor orientation and/or antibody density. However, it should be noted that thermal elution does not necessarily elute all the captured antigens. Nevertheless, it is still the mildest procedure for antigen elution without causing full protein denaturation (from acidic media) that would render accurate quantification with ELISA impossible. These results are in agreement with our SEC FLD study highlighting the better grafting and orientation of hIgG on protein G magnetic beads. They are therefore promising candidates for sensitive and specific detection of biomarkers like TNF-α and could be extended to other cytokines or proteins.

4. Conclusion

In the present study, we present an original analytical method for the evaluation of human IgG grafting efficiency on 4 types of commercial magnetic beads for immunocapture applications. This method relies on an innovative combination of a highly specific digestion of hIgG by IdeS together with the high resolution of the fragments' analysis by SEC, offering an efficient determination of antibodies orientation. This work offers a comprehensive approach of the influence of grafting conditions, *IdeS* and bead's surface chemistry on fluorescent signal detection. All results support the high efficiency of protein G magnetic beads for a high grafting yield of human IgG (~70 000 IgG molecules per bead) and an optimal orientation of antibodies for antigen capture (75% of Fab_2 fragment free). Moreover, protein G beads ease-of-use in terms of grafting conditions and time make them a powerful and convenient tool for biosensing and lab-on-a-chip applications. These findings have been illustrated with a high-value application for the immunocapture and elution of human TNF-α at 20 ng/mL, towards the monitoring of inflammatory-related pathologies. This work will be extended to other cytokines. Finally, this method can be more generally used to assess the hIgG coverage and orientation on any type of surface, relying on the high specificity and efficiency of the *IdeS* digestion of hIgG.

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Figures captions

Fig. 1 Analysis of hIgG (0.1 mg/mL) by SEC-FLD: (A) control sample (human IgG in PBS at 4°C), (B) Influence of bead grafting conditions on FLD signal of human IgG. COOH conditions are agitation at 20 rpm on rotating wheel at 4°C overnight in PBS, with 10 mg/mL of EDC and Sulfo-NHS. Ts conditions are agitation a 650 rpm on Thermomixer at 37°C for 16h with borate and ammonium buffer pH 9.5.
Mobile phase was 50 mM sodium phosphate, 150 mM NaCl and 10% isopropanol pH 6.5. Column: BioSEC-3, (4.6 mm × 300 mm, 300 Å, particle diameter 3 µm); Flow rate: 300 µL/min; FLD: λex=280 nm, λem=340 nm

Fig. 2 SEC-FLD analysis of human IgG at 0.22 mg/mL in PBS after IdeS digestion. Experimental conditions as mentioned in Figure 1

Fig. 3 Calibration curves corresponding to grafting conditions, A) Fab_2 fragment, B) Fc fragment. Experimental conditions as mentioned in Figure 1

Fig. 4 hIgG immobilization study on beads: (A) grafting efficiency, (B) number of hIgG molecules per bead

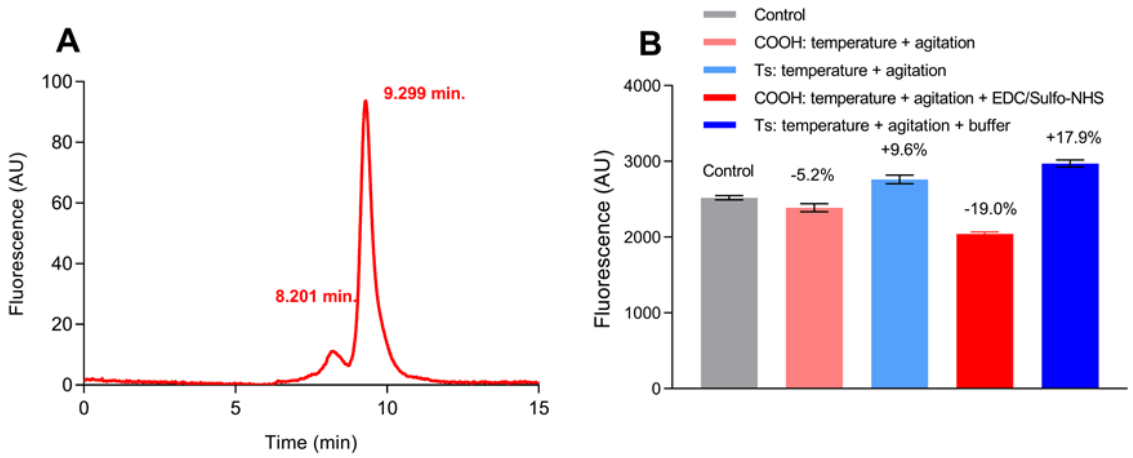
Fig. 5 IdeS digestion of Immobilized hIgG: **A)** SEC – FLD Analysis,
B) Evaluation of the percentage of hIgG orientated with $F(ab)_2$ free on the beads surface. Experimental conditions as Figure 1

Fig. 6 Comparison of IdeS digestion efficiency for COOH, Ts, Streptavidin and Protein G magnetic beads

626 **Table 1** Comparison of beads efficiency for immunocapture of TNF-α (n=5 replicates per

627 bead)

628



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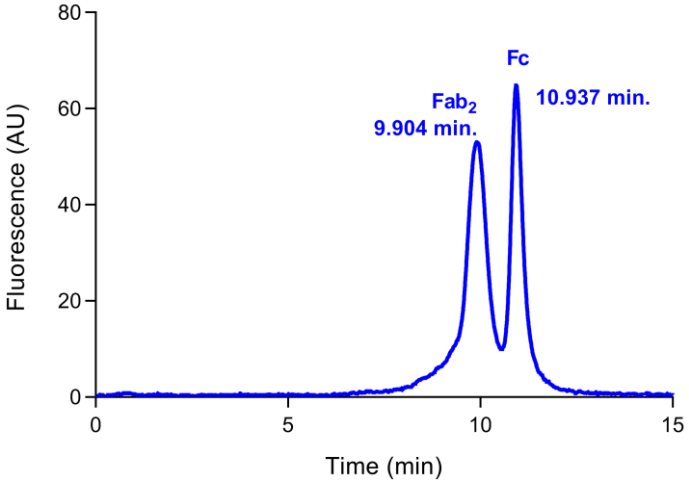

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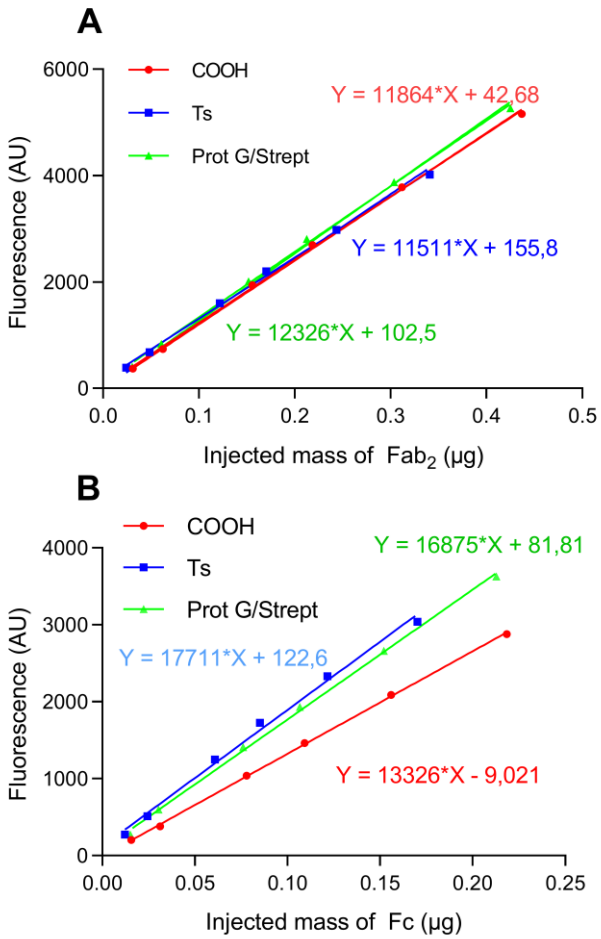

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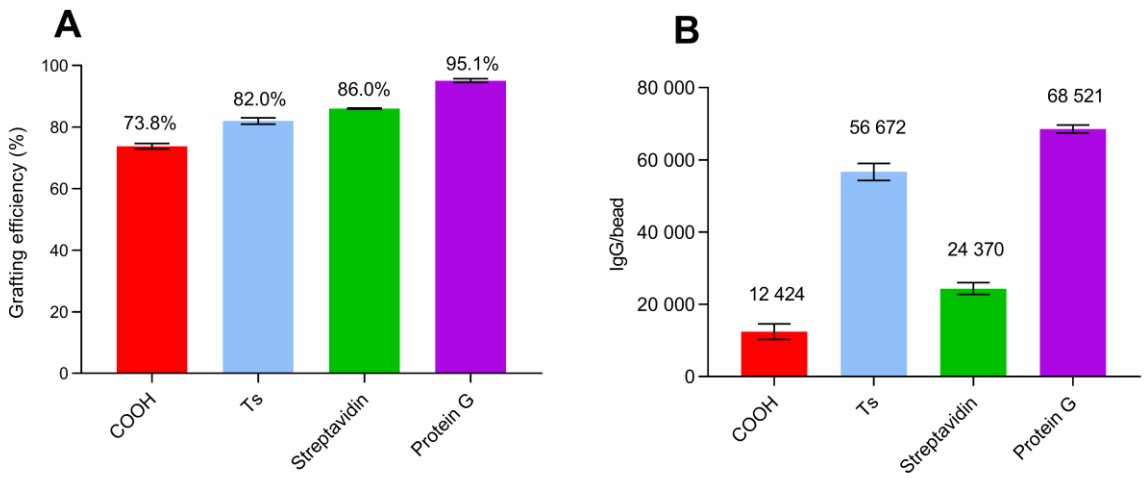

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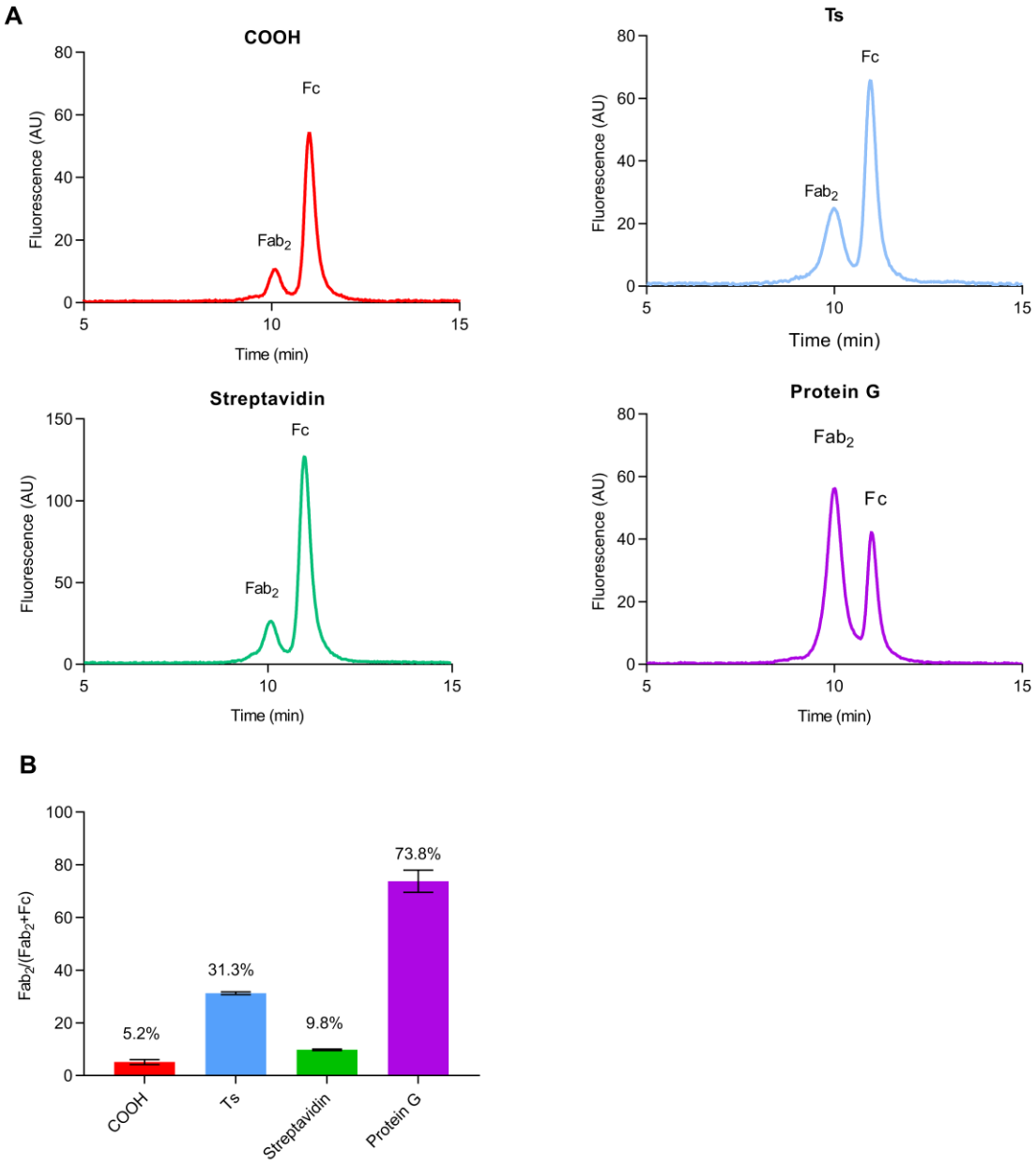

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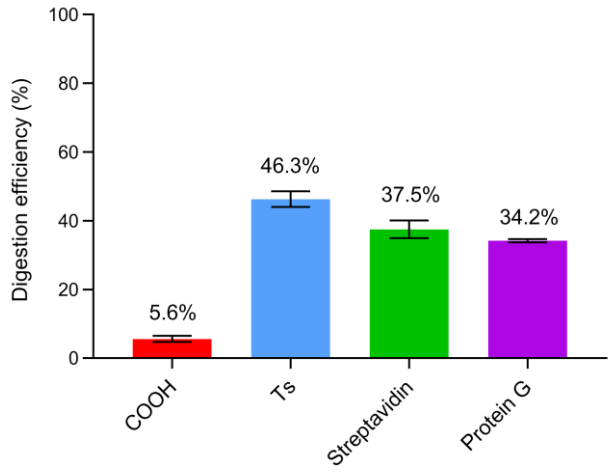
673



674

675 **Fig. 6** Comparison of IdeS digestion efficiency for COOH, Ts, Streptavidin and Protein G
676 magnetic beads
677

	COOH beads	**Ts beads**	**Protein G beads**
TNF-α recovered after thermal elution (% RSD) (ng/100 µg of beads)	0.33 (15%)	0.14 (13%)	3.38 (17%)

678

679 **Table 1** Comparison of beads efficiency for immunocapture of TNF-α (n=5 replicates per
680 bead)
681