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1 **Immuno-capture of biomolecules on functionalized magnetic beads: from**
2 **characterization to fluorescent detection of a biomarker for Alzheimer's disease toward**
3 **high performance biosensing**

4

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20 **Keywords:** magnetic beads, density and orientation, antibody anti- amyloid beta, size exclusive
21 chromatogram, enzyme digestion, IdeZ.

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25

26 **Abstract**

27 It is reported herein a new approach to study the orientation and density of mouse antibody
28 grafting on magnetic beads, serving for immunoassays and biosensors with fluorescent
29 detection of biomolecules. This approach is based on selective enzymatic digestion of target
30 grafted antibodies at a specific site below the hinge position to provide F(ab')₂ and Fc
31 fragments, followed by separation and determination of these fragments with size exclusion
32 chromatography (SEC) coupled with fluorescence detection (FLD). The developed method was
33 applied for evaluation of immunoglobulin (IgG2a) grafting capacity on three different
34 biofunctionalized magnetic beads (i.e., Tosyl-activated, carboxylic, protein G). Tosyl-activated
35 and protein G beads at different optimal grafting IgG: bead ratios (i.e., 110 µg: 1000 µg and 240
36 µg: 1000 µg, respectively) exhibited superior grafting capacity than carboxylic counterparts.
37 Under the optimized conditions, more than 70 % of antibodies were grafted on tosyl-activated
38 and protein G beads in the right orientation. This approach was then demonstrated with different
39 commercially available antibodies specific to amyloid-beta peptide 1-42 (Aβ₁₋₄₂) for magneto-
40 immunoassays and fluorescent detection of this peptide that is an established biomarker for
41 molecular diagnosis of Alzheimer's disease.

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51 **1. Introduction**

52 Immuno-enrichment and immunoassays, relying on the capture of target peptides and proteins
53 via specific antibodies for their subsequent preconcentration and fluorescent detection, are the
54 gold techniques used in bioanalysis and biosensing. Among all solid supports for such
55 purpose, magnetic beads have gained great attention thanks to the ease of manipulation via an
56 external magnetic field and a variety of functionalities (commercially) available for magnetic
57 particles [1, 2]. The performance of immunocapture on magnetic beads nevertheless depends
58 significantly on density and orientation of the grafted antibodies in order to allow their
59 efficient and selective interaction with target molecules as well as avoid / minimize non-
60 specific adsorption [3-5]. Control and characterization of on-beads immobilized antibodies
61 are therefore of utmost importance to achieve the best immunocapture and immunoassay
62 performance. Different characterization strategies have been developed for such purpose,
63 notably spectrophotometric and colorimetric assays [6, 7]. Gagey-Eilstein et al.
64 communicated a chemiluminescent test to monitor the antibody grafting rate on streptavidin-
65 magnetic beads [8]. These methods nevertheless cannot provide any information on antibody
66 orientation and/or can induce measurement biases mainly related to possible background
67 interferences [9-11]. Other methods such as surface plasmon resonance (SPR), dual
68 polarization interferometry, spectroscopic ellipsometry and atomic force microscopy, can
69 provide insight on antibody orientation mainly by measuring antibody dimensions/thickness
70 on surfaces or shift angle [12-17]. Indirect methods for quantification of proteins on the
71 surface of nanoparticles (NPs) were as well proposed, relying on hydrolysis of grafted
72 proteins, followed by spectrophotometric or chromatographic quantification of primary
73 amines, amino acids or peptides in solution [18-20]. Our group recently proposed a new
74 simple and rapid analytical approach to evaluate human antibody orientation and density on
75 magnetic beads applied for immunocapture of a biomarker of inflammation (TNF- α) [21].

76 This approach relies on the cleavage of immobilized antibodies by IdeS, a highly specific
77 protease for human immunoglobulin G (hIgG). This approach using IdeS nevertheless is not
78 adapted to mouse IgG, which represents the majority of antibodies commercially available for
79 bioanalysis. Indeed, in the context of molecular diagnosis of Alzheimer's disease (AD), which
80 is the most prevalent neurodegenerative disease worldwide causing a huge burden to the
81 society and having no efficient AD treatment so far [22], mouse antibodies have been most
82 widely used for immunoassays of amyloid (A β) beta peptides (notably A β 1-42 and A β 1-40
83 which are established biomarkers for AD). No human antibodies recognizing specifically
84 these compounds have been available so far.

85
86 From this rationality, we report in this study the development of an analytical approach to
87 simultaneously characterize the density and orientation of grafted mouse antibody on
88 magnetic beads. This approach relies on selective enzymatic digestion of immobilized mouse
89 antibodies at one specific site below the hinge region using FabRICATOR Z (IdeZ) to give
90 F(ab')₂ and Fc fragments from mouse antibodies, followed by separation and determination
91 of these fragments with size exclusion chromatography (SEC) coupled to fluorescent
92 detection (FLD). Optimization of the SEC-FLD method was made to allow separation of the
93 F(ab')₂ and Fc fragments from residual antibodies and the enzyme, allowing non-bias
94 determination of the digestion efficiency via the F(ab')₂ / Fc ratios. The developed approach
95 was applied for three functionalized magnetic beads (i.e., Tosyl-activated, carboxylic, Pierce
96 Protein G) grafting with immunoglobulin (IgG2a) from mouse. Following the antibody-
97 grafting optimizations that can be monitored with our method, the activity of grafted
98 antibodies was confirmed via on-bead immunoassays with fluorescent detection of amyloid
99 beta 1-42 peptide (A β ₁₋₄₂) that is an established biomarker for AD molecular. The work was
100 extended to three antibodies specific to different epitopes of A β ₁₋₄₂ (i.e., NAB228 for N-

101 terminal, 12F4 for C-terminal, and 4G8 for 17-24 epitopes), serving for evaluation of the
102 performance of bead-based immunoassays of A β ₁₋₄₂.

103

104 **2. Experimental**

105 ***2.1. Chemicals, reagents and samples***

106 2-(Cyclohexylamino)ethanesulfonic acid (CHES), Tris(hydroxymethyl)aminomethane
107 (TRIS), sodium dodecyl sulphate (SDS), sodium hydroxide (NaOH), potassium hydroxide
108 (KCl), acid boric (H₃BO₃), sodium dihydrophosphate (NaH₂PO₄), disodium hydrophosphate
109 (Na₂HPO₄), Tween 20, ammonium sulfate ((NH₄)₂SO₄), EDC (N-(3-Dimethylaminopropyl)-
110 N'-ethylcarbodiimide hydrochloride), S-NHS (N-Hydroxysulfosuccinimide sodium salt),
111 mouse IgG2a (2 mg/ml) (mIgG), phosphate buffer saline 10X (PBS), Tris buffer saline 10X
112 (TBS), ammonium hydroxide (NH₄OH), human IgG from whole serum (hIgG at 10 mg/ml),
113 as well as different anti-Alzheimer antibodies (1 mg/ml), including NAB228 and 6E10-HRP
114 were all provided by Sigma Aldrich (St. Louis, MO, United States). Other antibodies 12F4-
115 HRP, 12F4, and 4G8 were obtained from Biologend (San Diego, California, United State).
116 HPLC grade isopropanol (IPA), methanol and acetonitrile were purchased from VWR
117 (Pennsylvania, US). FabRICATOR enzyme (Ides) and FabRICATOR Z enzyme (Idez) were
118 obtained from Genovis (Lund, Sweden). Amyloid beta peptide Ab 1-42 (A β ₁₋₄₂) was
119 purchased from Eurogentec (Seraing, Belgium). FP-488 NHS ester solution was purchased
120 from Interchim (Montluçon, France). Pierce Protein G (ProG), Tosyl-activated (Ts) and
121 Carboxylated (COOH) magnetic beads, QuantaRed Enhanced Chemifluorescent HRP kit
122 were provided by Thermo Fisher (Massachusetts, United States). All buffers were prepared
123 with deionized water and were filtered through a 0.22 μ m membranes (Pall Corporation, New
124 York, USA) prior to use. For mass spectrometry experiments, acetonitrile and formic acid
125 were purchase from Sigma Aldrich.

126

127 **2.2. Apparatus and Material**

128 Deionized water used in all experiments was purified using a Direct-Q3 UV purification system
129 (Millipore, Milford, MA, USA). Conductivity and pH values of buffer solutions and samples
130 were measured by a Seven Compact pH meter (Mettler Toledo, Schwerzenbach, Switzerland).
131 SEC-FID experiments were carried out using a 1260 Agilent UPLC, and a 1260 fluorescent
132 detector ($\lambda_{\text{excitation}}$: 280 nm, $\lambda_{\text{emission}}$: 345 nm) (Agilent Technologies, Santa Clara, CA, USA).
133 Two columns, including Agilent AdvanceBio SEC (2.7 μ m particle size, 130A° pore size, i.d
134 7.8 , length 300 mm) and BioSEC-3 (3 μ m particle size, 300A° pore size, i.d 4,6 mm, length
135 300 mm) were employed for SEC-FLD at temperature of 25 °C. Fluorescence detection with
136 the excitation and emission wavelengths of 530 nm and 582 nm respectively were carried out
137 with a FP-750 Spectrofluorometer from Jasco (Lisses, France) using a 40 μ L quartz cuvette
138 (Starna Scientific, Essex, England). Mass spectrometry measurements were performed on a
139 QToF Synapt G2 Si (Waters, Manchester) equipped with ion mobility. Parameters were
140 optimized to obtain the best signal. In brief, capillary voltage was set to 3.5 kV, source
141 temperature to 40°C, the cone voltage and the offset were fixed at 50 V, désolvation
142 temperature was 450 °C, and nebulizer gas flow to 500 L/hr. Calibration was performed on the
143 500 to 5000 m/z range using TFANa. Error on the measurement was 0.2 ppm.

144

145 **2.3. Methods**

146 **2.3.1. Antibody digestion in solution**

147 IdeS FabRICATOR enzyme 5000 U and IdeZ FabRICATOR Z enzyme 2000 U were
148 dissolved in 100 μ L and 50 μ L miliQ water, respectively, then aliquoted to 2 μ L and stored at
149 -20°C. For IgG digestion, 2 μ L IdeS enzyme (100UI) was mixed with 50 μ L of 100 mM
150 phosphate buffer (pH 7.0) whereas 2 μ L IdeZ enzyme (80UI) was added to 50 μ L of 10mM

151 phosphate buffer and 10 mM sodium chloride (pH 6.5). hIgG and 6E10 were diluted in PBS
152 to 50 μ L at concentration of 0,1 mg/ml and then digested with IdeS at 37⁰C on a thermo-
153 mixer at 650 rounds per minute (rpm) for 90 minutes. NAB228 and 6E10 (0,1 mg/ml) were
154 prepared and digested with IdeZ under the same conditions for 120 minutes.

155

156 **2.3.2. Antibody grafting on magnetic beads**

157 *Protein G magnetic bead*

158 50 μ L of ProG magnetic beads (10 mg/mL) was added to 2 mL LoBind Eppendorf vials and
159 gently vortexed with 500 μ L of Tris solution containing 0.05 % v/v of Tween 20. The
160 supernatant was removed, and the beads were washed one more time with 1 mL of Tris-
161 Tween 20 (0.05%). A magnet was employed to retain magnetic beads during removal or
162 addition of a suspension solution. After removal of the supernatant, a predefined volume (0-5-
163 15-22,5-45-60-75-90 μ L) of NAB228 at 2 mg/mL was subsequently added to beads. The Tris-
164 Tween 20 (0.05%) solution was then added to 500 μ L. The mixtures were then incubated at
165 25^oC, 650 rpm for 1 hour on a Thermomixer. The protocol was also carried out with 100 μ L
166 ProG (10 mg/mL) and 4 batches of NAB228 (0-10-55-120 μ L at 2 mg/mL). After incubation,
167 the supernatants were collected. The beads were then washed twice with 500 μ L of Tris-
168 Tween 20 (0.05%) solution. The washing solutions were collected for subsequent analyses.

169

170 *Tosyl-activated magnetic bead*

171 10 μ L of Dynabeads MyOne Tosylactivated (100 mg/mL) was added to 2mL LoBind
172 Eppendorf vials and washed with 500 μ L of 0.1 M sodium borate buffer (pH 9.5) using a
173 Thermomixer for 15 min C at 25^oC and 650 rpm. Pre-defined volumes (0-10-55-90-120 μ L)
174 of NAB228 solution (2 mg/mL) were added to beads. 60 μ L of 3M ammonium sulphate
175 buffer (pH 9.5) and 20 μ L of 0.1M sodium borate buffer were then added. The mixtures were

176 then incubated at 37 °C on a Thermomixer (650 rpm) for 16h. The supernatants were
177 collected with the help of a magnet. The beads were washed twice with 500 µL of PBS-
178 Tween 20 (0.05%) and once with 500 µL of PBS. The washing solutions were also collected
179 for subsequent analyses by SEC-FLD.

180

181 *Carboxylated magnetic bead*

182 100 µL of a Dynabeads MyOne carboxylic acid suspension (10 mg / mL) were rinsed twice
183 with 1 mL PBS after removing the suspending solution. 500 µL of EDC solution (10 mg / mL
184 inPBS) and 500 µL of S-NHS solution (10 mg / mL in PBS 1X) were then added into the
185 washed beads, followed by the addition of NAB228 2 mg/ml (10 µL or 55 µL). The mixture
186 was incubated for 3 hours under 650 rpm at room temperature (RT). The supernatant was
187 collected. The antibody-bound magnetic beads were subsequently washed twice with 1 mL of
188 PBS, followed by an incubation at RT with 1mL of 50 mM Tris-HCl (pH 7.4) for 15 min in
189 order to quench the non-reacted activated carboxylic acid groups. The antibody-bound
190 magnetic beads were then washed 3 times with 1 mL of PBS - 0.1 % Tween-20. A magnet
191 was employed to retain magnetic beads during removal or addition of a suspension solution.

192

193 **2.3.3. Digestion of NAB228 grafted on magnetic beads by IdeZ**

194 100 µL of enzyme Idez (3 UI) was used for 1 µg NAB228. The enzyme solutions were added
195 directly to grafted and washed magnetic beads and incubated for 3h at 37 °C on a
196 Thermomixer at 650 rpm. Further optimization of the enzymatic digestion procedure can be
197 referred to section 3.1.

198

199 **2.3.4. SEC-FLD conditions**

200 The SEC-FLD analyses of digested samples were conducted on two Agilent columns:
201 BioSEC-3 (3 μ m particle size, 300 \AA , i.d 4,6 mm, length 300 mm) and AdvanceBio SEC
202 (2.7 μ m particle size, 130 \AA pore size, i.d 7.8, length 300 mm). Both columns were
203 equilibrated with the mobile phase for 5 column volumes prior to analysis; and flushed with a
204 mixture of miliQ water (80%) and MeOH (20%) for 15 column volumes after each analysis.
205 The flow rate was fixed at 0,3 ml/min for both columns. For each analysis, 2 μ L of sample
206 was injected, and analyzed with the phosphate buffer 50 mM that contains 10 % IPA and 150
207 mM KCl (pH 6,8).

208

209 Calibration curves were made using native NAB228 prepared in different matrices and under
210 different conditions that were used for on-bead antibody grafting protocols. The calibration
211 curve served for the study on Tosyl-activated beads was made with NAB228 (at six points
212 from 0,05 to 0.3 μ g) diluted in ammonium sulphate 3 M and sodium borate buffer 0,1 M at
213 pH 9.5 and incubated on Thermomixer at 37 $^{\circ}$ C during 16h. The one used for the study on
214 proG beads was done with NAB228 (6 concentration points) prepared in Tris-Tween 20
215 (0.05%) and incubated at 25 $^{\circ}$ C for 1h on Thermomixer. The non-grafted condition was used
216 as the reference with six points of native mouse IgG2a prepared in PBS. Three SEC-FLD
217 analyses were implemented for each concentration. The respective calibration curve for each
218 bead type was used to determine the non-grafted NAB228 amount in supernatant and washing
219 solutions. The successfully grafted NAB228 was estimated from the difference between the
220 initial antibody concentration and the recovered ones in the supernatant and washing
221 solutions.

222

223 ***2.3.5. Immunoassay of A β 1-42 peptide***

224 A volume of 50 μ L A β 1-42 was incubated with 200 μ g magnetic beads coated with the desired
225 antibodies (12F4, 4G8 or NAB228) and 5 μ L 6E10- HRP or 12F4- HRP antibodies at
226 concentration of 0.04 μ g/ml in PBS 1X on a mixer at RT for 1 hour. The beads were then
227 washed 3 times (10 min each time on a mixer) with 400 μ L PBS 1X/ 0.1 % BSA (m/v)/ 0.1 %
228 Tween- 20 (v/v). Then, 100 μ L QuantaRed solution was added to the washed beads. The
229 incubation was carried out over 7 min on shaking, followed by addition of 10 μ L QuantaRed
230 Stop solution. The colour intensity of the achieved solution was measured with the excitation
231 and emission wavelengths of 530 nm and 582 nm, respectively.

232

233 **2.3.6. MS measurements of A β 1-42 peptide**

234 Five lots of A β 1-42 peptide stock solution at 2 mg/ml were prepared by balancing and
235 dissolving in corresponding volume of DMSO. The Eppendorf vials were vortexed gently with
236 hand. Then, the stock solutions were diluted in NH₄OH 0.16% to obtain the samples at a
237 concentration of 5 μ M. These samples were injected directly into MS to verify the quality of
238 A β 1-42. For MS conditions see the section 2.2 above.

239

240 **3. Results and Discussion**

241 **3.1. Enzymatic digestion of grafted antibodies and analysis of released fragments**

242 Our approach (see Fig.1) to characterize the performance of on-bead antibody grafting relies
243 on the selective enzymatic digestion of the grafted antibody below the hinge position to
244 release (Fab')₂ and Fc fragments. The analysis of the remaining antibody as well as these
245 released fragments, which are typical for an antibody, can then provide insight in the density
246 and the orientation of the grafted antibodies thanks to the ratio of F(ab')₂ 's signal to Fc one.
247 Unlike chemically mediated or trypsin-based methods, our approach is softer and more
248 selective, and can provide both information on antibody density and orientation. The

249 challenges here were to find an appropriate enzyme that works on the target mouse antibodies
250 and develop the subsequent separation strategy for determination of the resulting fragments.
251 In our case, SEC which is frequently used for separation of proteins and peptides was chosen
252 for analysis of F(ab')₂ and Fc fragments. The obtained F(ab')₂ and Fc fragments and residue
253 IgG possess respective molecular weights of about 100, 50 and 150 kDa. Phase mobile
254 optimization was first carried out to separate these target fragments from the residual
255 antibodies in the solution (Fig. 2). Among two salts commonly used in SEC buffers, KCl
256 offered more symmetric and less tailing peaks than NaCl (Fig 2 A-B vs Fig 2 C-D). Indeed,
257 KCl was found stronger than NaCl in suppression of the secondary interaction between target
258 molecules and the stationary phase, probably due to the larger size and higher interactivity of
259 K⁺ cation [23]. It was observed from Fig 2 C vs Fig. 2 D that the column of a smaller pore
260 size (130A^o) provides better resolution for separation of F(ab')₂ and Fc. Moreover, thanks to
261 these optimized conditions, the peak of IdeZ enzyme was well separated from the Fc fragment
262 (see Figs. 3 and 4), which was not the case in the previous work using IdeS [21]. This would
263 help avoid the bias in quantification of the digested fragments.

264

265 The optimized SEC-FLD conditions were then used to monitor the IgG digestion
266 optimization. FabRICATOR IdeS, which was found to offer fast reaction on hIgG, and was
267 found to work well in our previous work on hIgG [21] was tested as the reference. By
268 comparing the peak of IgG before and after digestion in Figs. 3A-B, one can see that almost
269 80% of hIgG was digested with IdeS without optimization, whereas only 10% of mouse IgG1
270 could be digested under the same conditions. Indeed, IdeS can digest only antibodies having
271 CPPCPPELLG/GPSVF sequence at hinge position that is typical for human, rabbit and
272 sheep IgG. This enzyme nevertheless is less favorable for mouse IgG, which represents the
273 majority of antibodies commercially available for bioanalysis and is the most widely used one

274 for immunoassays of A β peptides. To overcome this problem, FabRICATOR Z IdeZ was
275 selected for mouse IgG. This kind of enzyme shows its specificity on mouse IgG2a or IgG3,
276 having CPAPNLLG/ GPSVF sequence at the hinge site. As can be seen in Fig 3C, more than
277 60% of NAB228 antibody (mouse IgG2a type) was digested in the first trial, which provided
278 encouraging results. IdeZ enzyme was therefore chosen for further optimization of mouse
279 IgG2a digestion. A two-level-three-factors (3^2) $\frac{1}{2}$ fraction experiment was designed to
280 optimize the digestion protocol using Minitab statistical software 17. The three factors
281 covered (i) enzyme unit for 1 μ g IgG digestion (1 - 3 UI), (ii) incubation time (2 to 4 hour) and
282 (iii) temperature (low 30°C and high 44°C). The response to be evaluated was the digestion
283 efficiency estimated via F(ab')₂ : Fc peak-area-ratio. A Pareto chart was used to determine the
284 optimal conditions for complete digestion of IgG while using the lowest enzyme unit as
285 possible. Among the investigated factors, the one with the highest impact was found for the
286 added enzyme unit. As can be seen from Fig. 4, with 3 U of enzyme IdeZ for 1 μ g IgG (in an
287 incubation over 3 hours at RT), quasi-complete digestion (98%) of IgG in solution, calculated
288 according to peak areas of initial IgG and residual one after digestion, could be achieved.
289 Compared to the digestion performance obtained with the commercial protocol for IdeZ (see
290 Figs. 2 C-D where there was much residual IgG after the digestion), that achieved with our
291 optimized conditions is superior with almost no trace of IgG left (Fig. 4).

292

293 **3.2. Optimization of the density and orientation of antibodies grafted on magnetic beads**

294 The optimized digestion conditions (i.e., incubation with 3 UI IdeZ over 3h at 37 °C) and the
295 optimized SEC-FLD ones (i.e., column of 130 Å pore-size, phosphate buffer containing 150
296 mM KCl) were employed to release and determine the F(ab')₂ and Fc fragments from on-
297 bead grafted NAB228 antibody (mouse IgG). Quantification of grafted NAB228 was made
298 with following the formula (1), respectively:

299
$$m_{\text{grafted}} = m_{\text{initial}} - (m_{\text{supernatant}} + m_{\text{washing}}) \quad (1)$$

300 *where*

301 *m_{initial}, m_{grafted}, m_{supernatant} and m_{washing} are the initial quantity of antibodies, the*
302 *quantity of antibodies grafted on 1000 μg beads, and those of remaining antibodies in*
303 *the supernatant and washing solutions, respectively.*

304

305 The peak ratios for F(ab')₂/Fc fragments released from NAB228 antibodies grafted on Tosyl-
306 activated, ProG and carboxylated magnetic beads were determined and compared (Fig. 5).

307 With the same quantity of antibodies available for grafting, proG and tosyl-activated beads
308 allowed higher amount of grafted NAB228, whereas very little trace of grafted antibodies
309 could be found with carboxylated ones. As shown in Fig 5A and B, proG and tosyl-activated
310 beads would require different initial antibody amount to achieve the best F(ab')₂/ Fc ratio.

311 The best grafted antibody density and orientation was achieved with 110 μg antibodies per 1
312 mg bead for tosyl-activated beads (at F(ab') / Fc of 2.9), whereas for proG beads the best
313 F(ab')₂ / Fc ratio of 2.5 was achieved at a higher quantity of 240 μg antibodies per 1 mg
314 beads (Fig. 5C). This can be explained by the fact that tosyl-activated beads with a covalent
315 group (H₃CC₆H₄SO₂) has more affinity to Fc fragment. Note that further addition of
316 antibodies led to less grafting performance on tosyl-activated beads (Fig. 5C), probably due
317 the abundant presence of PBS used for antibody suspension, which is the blocking solution
318 and not favorable for tosylated groups on magnetic beads, according to the instructions in the
319 commercial protocol. In the case of proG, at low IgG quantity (< 50 μg), the non-covalent
320 linkage is more effective towards F(ab')₂, leading to less-oriented antibody grafting (with
321 decreased F(ab')₂ / Fc peak ratio). At higher IgG quantity, the tendency was reversed, leading
322 to an improved in F(ab')₂/Fc peak ratio for ProG beads. This important observation indicates
323 that different magnetic beads would require different antibody quantities to reach the

324 maximum density and best orientation. Under our best grafting conditions, 74.4 % of
325 antibodies grafted on tosyl-activated beads were at the right orientation, calculated from the
326 $(F(ab')_2 / (Fc + F(ab')_2))$ peak ratio. This was 71.9 % for the case of ProG beads. Using the
327 formula (1), the maximum grafted quantity of NAB228 antibodies on ProG and tosyl-
328 activated beads were estimated to be 43 μ g and 35 μ g per 1000 μ g beads, respectively. Note
329 that different conditions (i.e., the matrices, the temperatures and incubation times) were used
330 for grafting antibodies on different beads. To avoid bias in the quantification of grafted
331 antibodies, different calibration curves were made for different bead types, using NAB228
332 prepared in respective grafting matrices (see Fig. S1 in the supporting information ESI). Note
333 also that the peak of IdeZ was observed together with those of $F(ab')_2$ and Fc fragments after
334 the digestion (Fig. 5). The peaks of IdeZ in the supernatants after incubation with different
335 beads were lower than that of IdeZ in the initial one, which was due to some absorption of
336 IdeZ on to ProG and tosyl-activated beads during the incubation for digestion of grafted IgG,
337 as demonstrated in Fig. S2 in the ESI. The peak of residual IdeZ, if not well separated from
338 those of target $F(ab')_2$ and Fc, could induce some interference to these target peaks, and
339 therefore confirm again the need to have an improved SEC-FLD method in our case to
340 separate IdeZ from the target fragments.

341

342 **3.3. Immunoassays with fluorescent detection of A β 1-42 on magnetic beads**

343 To validate the aforementioned optimization, the tosyl-activated and proG beads grafted with
344 NAB228 (an anti A β 1-42 antibody specific at N-terminal of this peptide) at their optimal
345 concentrations were employed to carry out immunoassays of A β 1-42 (Fig. 6). Since A β 1-42
346 peptide is prone to aggregation [22], the good quality of each batch was first confirmed with
347 MS (see Fig. S3 in the ESI). A β 1-42 solutions were prepared freshly from the confirmed
348 batch just before each immunoassay series. Detection in this case was made with another anti

349 A β 1-42 antibody specific at C-terminal of this peptide, which is bound with HRP to trigger
350 subsequently the enzymatic reaction with Quanta Red substrate followed by fluorescent
351 detection (Fig. 6A). NAB228 grafted on ProG showed higher fluorescent signals than those
352 obtained with tosyl-activated beads (Fig. 6B). Further results on the immunoassays of A β 1-42
353 can be seen in Fig. S4 in the ESI. This observation was in good accordance with the results
354 indicating that more antibodies were grafted on ProG beads than on tosyl-activated ones, with
355 equivalent antibody grafting orientation (see section 3.2 above).

356

357 Employing the antibody / bead ratios optimized for NAB228, the test on immunocapture of
358 A β 1-42 was also extended to other antibodies specific to this peptide, i.e., 12F4 specific to N-
359 terminal and 4G8 specific to 17-24 epitopes (Fig. S5 in the ESI). An antibody specific to N-
360 terminal of A β 1-42 (6E10-HRP) was employed in this case for detection of the captured
361 peptide. Among three capture antibodies, NAB228 exhibited the best on-bead capture
362 performance, regardless of the bead type used (tosyl-activated or ProG). The signals obtained
363 with 12F4 and 4G8 were relatively low for both types of beads. The limit of detection was 10
364 nM for NAB228 and 20 nM for other antibodies. This implies that for each antibody, re-
365 optimization of the antibody : bead ratio would be needed. And the optimal ratio achieved for
366 1 specific antibody (NAB228 in our case) cannot be generic to other ones. This remark is
367 important, because so far one tends to follow the same *antibody : bead* ratio according to the
368 established grafting protocol for different antibodies, which may lead to non-optimal
369 immunocapture performance.

370

371 **4. Conclusions**

372 We developed successfully an enzyme-based approach for evaluation at same time the density
373 and orientation of mouse IgG_{2a} immobilized on three different magnetic beads. Using this

374 approach to follow the optimization of antibody grafting conditions, more than 70 % of
375 antibodies could be grafted on tosyl-activated and protein G beads in the right orientation. The
376 developed method was applied for monitoring the density and orientation of anti- A β 1-12
377 antibodies on different magnetic bead supports, serving for immunoassays and fluorescent
378 detection of this amyloid-beta peptide. The antibody NAB228 grafted on ProG beads
379 exhibited the best performance, with the detection limit of 10 nM achieved for A β 1-12. The
380 strategy using IdeZ enzyme to check the density and orientation of mouse antibodies on
381 magnetic beads provides us a powerful tool for optimization and improvement of the
382 performance of immunoassays and biosensing of target biomolecules on magnetic beads.
383 Other biosensing applications targeting different biomarkers using this enzyme-based method
384 are envisaged to further demonstrate the significance of our work.

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387 **Acknowledgement**

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389 France’ (for PhD scholarship of N.V.T. Nguyen).

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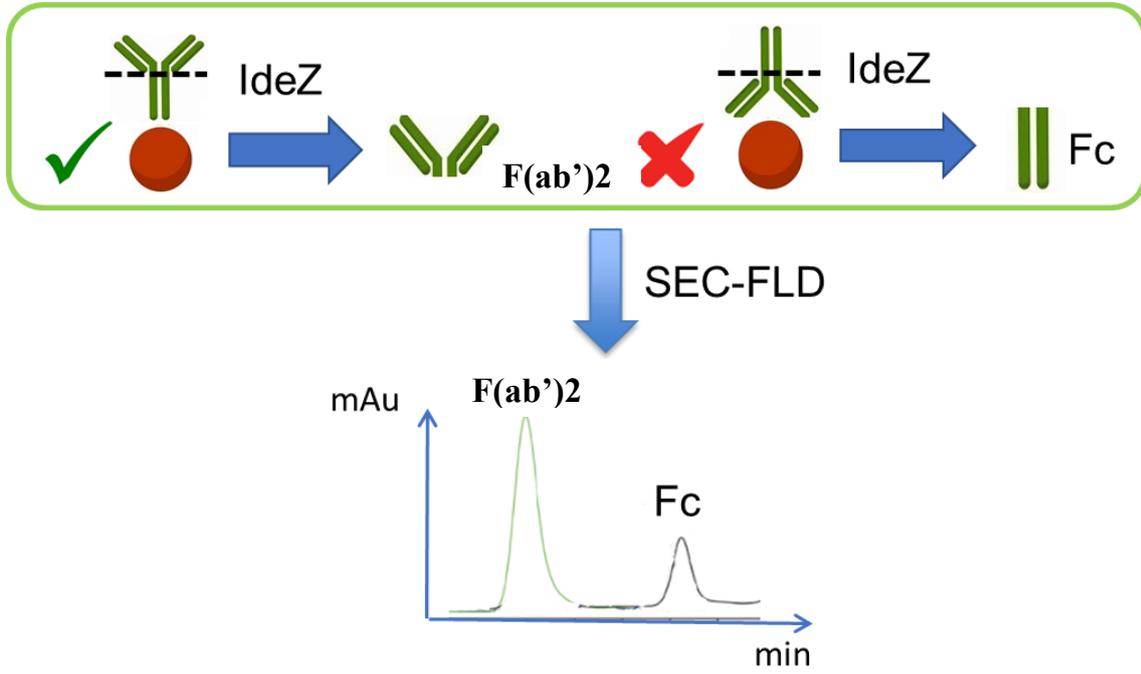
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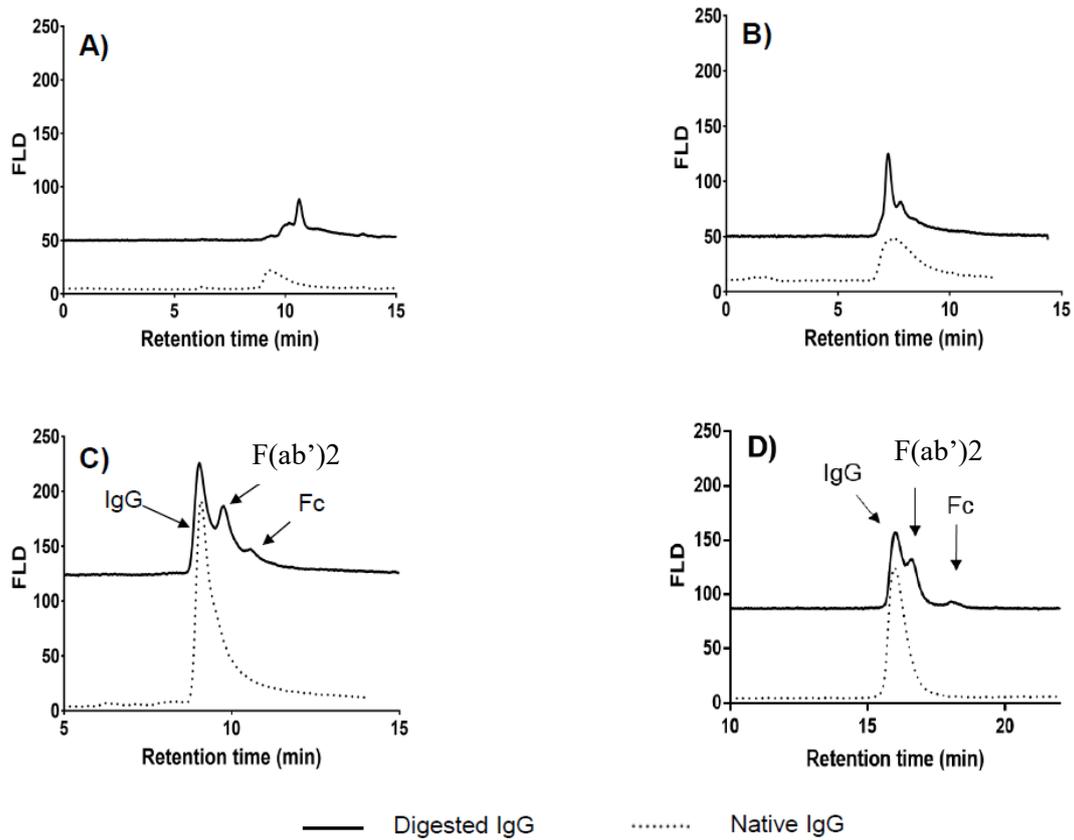
399 **Figure captions:**

400 Fig. 1. Proposed strategy for characterization of density and orientation of antibodies
401 grafted on magnetic beads.



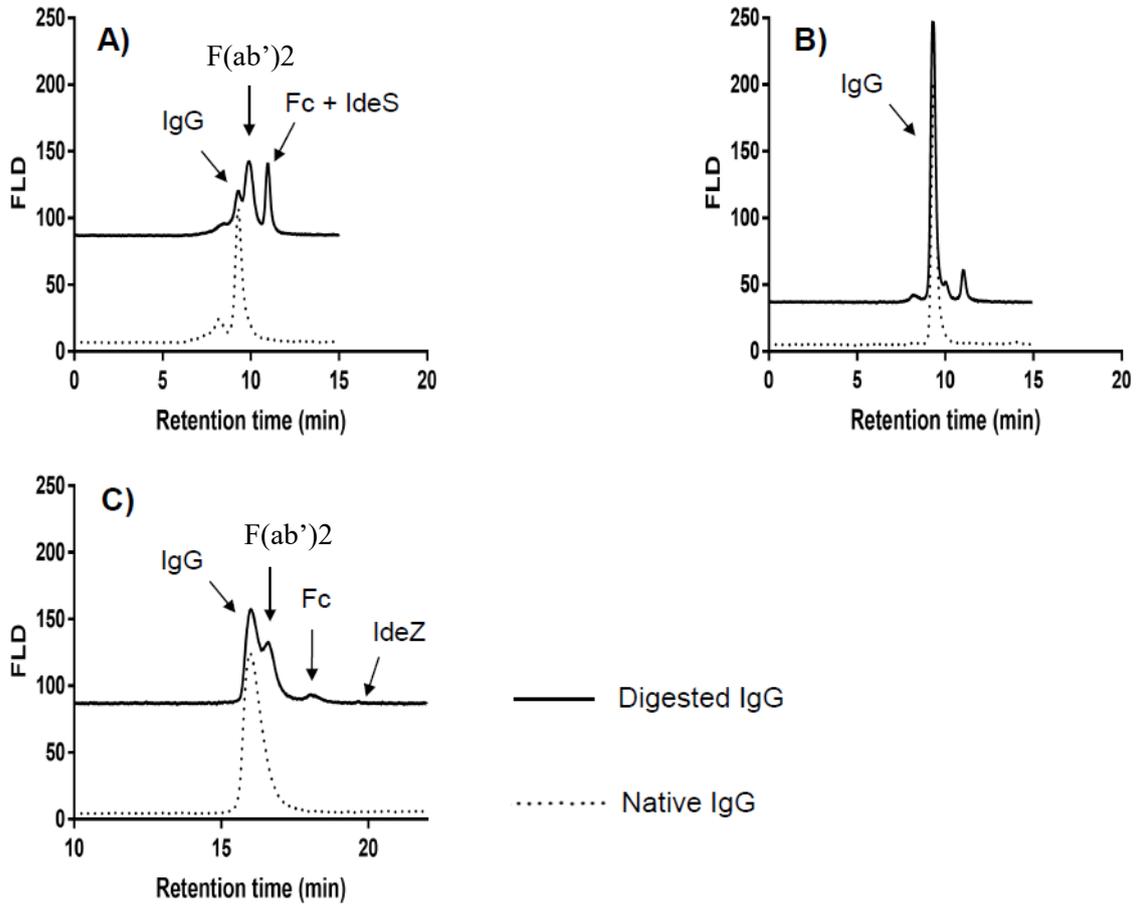
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416 Fig. 2. Analysis of IdeZ-digested NAB228 by SEC- FLD using a column having
417 dimensions of 4.6 x 300mm, particle size of 3 μ m; pore size of 300 \AA and the
418 phosphate buffer containing A) 150 mM NaCl, C) 150 mM KCl; or a column
419 having dimensions of 7.8 x 300mm, particle size of 2.7 μ m, pore size of 130 \AA and
420 the phosphate buffer containing B) 150 mM NaCl, and D) 150 mM KCl.



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430 Fig. 3. Digestion of 0,1 mg/ml antibodies in PBS: A) human IgG by IdeS; B) Mouse IgG1
431 (6E10) by IdeS; C) Mouse IgG2a (NAB228) by IdeZ. SEC- FLD conditions:
432 column having dimensions of 7.8 x 300mm, particle size of 2.7 μ m, pore size of
433 130Å and the phosphate buffer containing 150 mM KCl.



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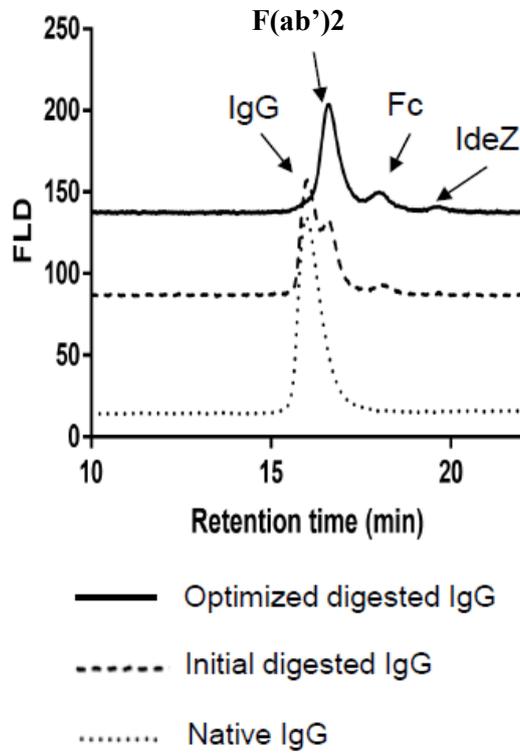
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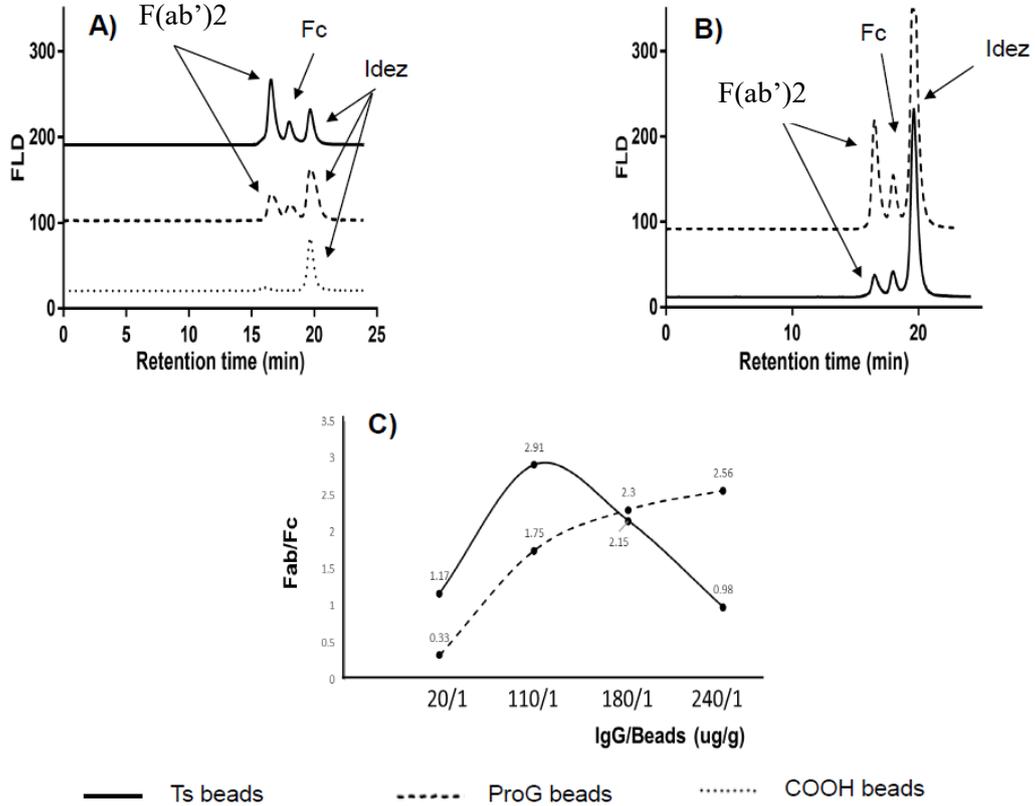
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442 Fig. 4. Digestion of NAB228 antibodies (0.1 mg/ml IgG2a) in PBS by IdeZ under different
443 digestion conditions. SEC-FLD conditions as in Fig. 3



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458 Fig. 5. Digestion of NAB228 antibodies grafted on different magnetic beads (*i.e.*,
459 carboxylated, tosylactivated, protein G using IgG/ beads ($\mu\text{g}/\text{mg}$) ratio of A) 110:
460 1; B) 240:1. C) Dependence of F(ab')₂ :Fc peak ratios on IgG quantity for on-bead
461 grafting. SEC-FLD conditions as in Fig. 3



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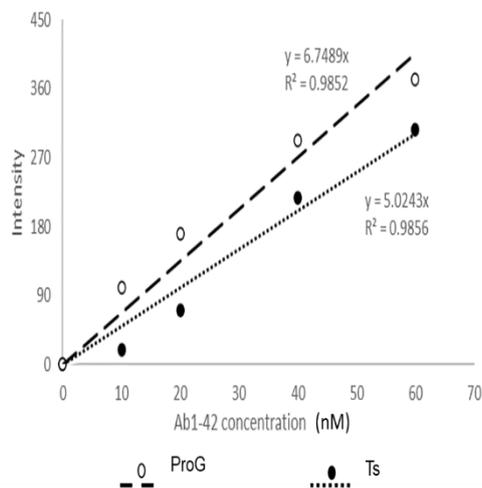
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471 Fig. 6. A) Schematic of the immunoassay with fluorescent detection of Ab1-42 on different
472 magnetic beads grafted with NAB288. Detection was carried out using ELISA with
473 12F4 HRP as the detection antibody. See section 2.3 for ELISA protocol. B)
474 Calibration curves for Ab1-42 made with NAB288 grafted on ProG and
475 Tosylactivated beads

476 A)



479 B)



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