Towards the development of a surface plasmon resonance assay to evaluate the glycosylation pattern of monoclonal antibodies using the extracellular domains of CD16a and CD64

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We here report the production and purification of the extracellular domains of two Fcγ receptors, namely CD16a and CD64, by transient transfection in mammalian cells. The use of these two receptor ectodomains for the development of quantitative assays aiming at controlling the quality of monoclonal antibody production lots is then discussed. More specifically, the development of surface plasmon resonance-based biosensor assays for the evaluation of the glycosylation pattern and the aggregation state of monoclonal antibodies is presented. Our biosensor approach allows discriminating between antibodies harboring different galactosylation profiles as well as to detect low levels (i.e., less than 2%) of monoclonal antibody aggregates.

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**Abstract**

We here report the production and purification of the extracellular domains of two Fcγ receptors, namely CD16a and CD64, by transient transfection in mammalian cells. The use of these two receptor ectodomains for the development of quantitative assays aiming at controlling the quality of monoclonal antibody production lots is then discussed. More specifically, the development of surface plasmon resonance-based biosensor assays for the evaluation of the glycosylation pattern and the aggregation state of monoclonal antibodies is presented. Our biosensor approach allows discriminating between antibodies harboring different galactosylation profiles as well as to detect low levels (i.e., less than 2%) of monoclonal antibody aggregates.

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1. Introduction

Immunoglobulins are glycoproteins that are involved in the humoral response of the immune system, which enables the clearance of antigens (Saba et al., 2002). Among monoclonal antibodies (Mabs), immunoglobulins G (IgG) are the most widely used as therapeutic agents (Lim et al., 2008). IgGs are 150-kDa molecules composed of two heavy and light chains. When these Mabs target an antigen, an immune complex is formed; the latter may then be eliminated by the effectors functions of the immune system, such as the antibody- or the complement-dependent cellular cytotoxicity (ADCC or CDC) (Scallon et al., 2006). In order to fulfill this role at the molecular level, Mabs interact via their Fc region with various receptors present at the surface of leukocytes. Among these receptors are the Fcγ receptors which are transmembrane proteins composed of either two or three extracellular units. FcγRs are subdivided in three distinct types, based on their structural features and their interactions with human IgGs: the type I receptor (FcγRI or CD64) is unique, while different variants of the type II and type III receptors (FcγRII or CD32 and FcγRIII or CD16, respectively) have been identified (i.e., CD32a, CD32b, CD32c and CD16a, CD16b) (Powell and Hogarth, 2008). Immunoreceptor tyrosine-based...
inhibition and activation motifs (ITIM and ITAM, respectively), present in the intracellular portion of the FcγRs, are involved in signaling after receptor activation. Signaling through ITAM receptors (i.e., CD64, CD32a and CD16a) results in cell activation, while engagement of ITIM receptors (i.e., CD32b) is inhibitory (Male et al., 2007). CD32 and CD16 are considered to be low-affinity receptors for IgGs with $K_D$ of $10^{-10}$–$10^{-7}$ M (Lu et al., 2011; Radaev and Sun, 2002). In contrast, CD64 displays a higher affinity for the Fc region of the Mabs ($K_D$ of $10^{-8}$–$10^{-10}$ M), more likely due to the presence of three binding domains in its extracellular moiety (Lu et al., 2011). While CD16a and CD64 are known to bind to IgGs with a 1:1 stoichiometry (Kato et al., 2000; Pollastrini et al., 2011; Radaev and Sun, 2001), the stoichiometry of the CD32:IgG complex is still debated: results by Sondermann et al. (1999) have suggested a 2:1 stoichiometry using crystalllography, whereas evidences from other reports might be indicative of a 1:1 interaction (Kato et al., 2000; Pollastrini et al., 2011).

The importance of antibody glycosylation upon interactions with FcγRs has been intensively studied over the last decade (Houde et al., 2010; Jefferis, 2005; Okazaki et al., 2004; Shibata-Koyama et al., 2009; Spearman et al., 2011). The glycosylation of the IgGs on Asn297 confers a stable conformation to their Fc region, which enables the effector functions of the immune system. Furthermore, the absence of glycosylation on this specific residue is known to abrogate the antibody interactions with the FcγRs (Ghirlando et al., 1999; Krapp et al., 2003; Walker et al., 1989). Many studies have shown that the absence of core fucose in the Fc glycan of Mabs increases their affinity for CD16a, without however influencing Mab interactions with CD64 (Ferrara et al., 2011; Houde et al., 2010; Scallon et al., 2006; Shields et al., 2002). The same observation has been made in the presence of bisecting GlcNAc, as the latter likely reduces the amount of fucosylation (Umana et al., 1999). Highly sialylated Mabs were shown to harbor a reduced ADCC activity (Scallon et al., 2007), whereas Mabs bearing increased levels of galactosylation have been reported to bind to CD16a with higher affinity (Houde et al., 2010).

Altogether, these reports have highlighted the importance of IgG glycosylation upon binding to their receptors, subsequent ADCC and CDC, and ultimately their therapeutic effect. The latter may thus be improved by producing IgGs with specific glycan profiles. On that note, recent work has shown that a glycoform with no fucose has a 53-fold higher binding capacity to the receptor that triggers its therapeutic activity. This enhancement of ADCC allows this glycoform to be effective at lower doses (Shinkawa et al., 2003). The glycosylation profile of a recombinant Mab is dependent on a number of parameters that include the profile of the glycosylating enzymes in the producing cell line, the medium composition, the cell culture conditions as well as the method of downstream processing. In a context where new therapeutic antibodies need to be on the market rapidly while the quality and integrity of the product need to be verified from batch to batch, it is essential to develop routine assays to evaluate Mab glycosylation profile as well as Mab aggregation state. In that endeavor, the design of robust assays combining surface plasmon resonance (SPR)-based biosensors with the use of FcγR ectodomains may be an interesting avenue as both Mab aggregation (Luo et al., 2009) and glycosylation (Ferrara et al., 2011) have been reported to affect Mab interactions with their receptor ectodomains when monitored by SPR biosensing. In this manuscript, we first report the production and purification of two FcγR ectodomains, i.e. those of CD16a and CD64, by transient transfection of mammalian cells in vitro. We then describe and discuss their potential use to develop an SPR assay aiming at assessing IgG glycosylation pattern and presence of aggregation.

## 2. Materials and methods

### 2.1. Production of the extracellular domains of FcγRs

#### 2.1.1. Plasmids and DNA

Codon-optimized (human codon usage) cDNA encoding the CD16a (F158) variant (GENE ID: 2214 FCGR3A; amino acids 1–193) or CD64 (GENE ID: 2209 FCGR1A; amino acids 34–302 with signal peptide MWQLLLPTALLLVSAGMRT) were cloned into pT5 vector. Both constructs contain a H10G C-terminal tag preceded by a TEV cleavage site (ENLYFQGTTGSCHHHHHHHHHHHHHHG) to facilitate their purification. The pTTo-GFP plasmid has been described elsewhere (Durocher et al., 2002). The pTT2-AKTDD plasmid is derived from pTT vector and encodes constitutively active bovine AKT (Alessi et al., 1996).

#### 2.1.2. CD16a[F-158]

The human embryonic kidney 293 cell line, stably expressing a truncated EBNA1 protein (HEK293-6E), was cultured in suspension in shake flasks (120 rpm) in 500 mL of FreeStyle™ F17 medium (Life Technologies, Burlington, ON) supplemented with 4 mM of glutamine, 25 mg/mL of genetin and 0.1% (v/v) of pluronic acid in a humidified incubator at 37 °C with 5% CO2. The cells were transfected at a density of 1.6–2.0 $\times 10^6$ cells/mL (Raymond et al., 2011). A total of 500 μg of plasmid (25% pT5-CD16aTevHis, 5% pTTo-GFP and 70% ssDNA) was diluted into 25 mL of F17 medium prior to the addition of 1.5 mg of linear 25-kDa polyethylenimine (LPEI; Polysciences, Warrington, PA), was diluted into 25 mL of F17 medium to reach a final concentration of 0.5% (w/v). The supernatant was harvested 5 dpt and clarified by centrifugation at 3000 x g for 20 min.

#### 2.1.3. CD64

The Chinese hamster ovary cell line expressing a truncated EBNA1 protein (CHO-3E7) (Raymond et al., 2012), was cultured in the same medium as for HEK293-6E cell line but without genetin supplementation. Transfections were performed at a cell density of 2 $\times 10^6$ cells/mL. A total of 375 μg of plasmids containing 50% pTT5-CD64a, 15% pTT5-AKTDD (v-akt murine thymoma viral oncogene homolog 1 with T308D and S473D mutations), 5% pTTo-GFP and 30% ssDNA was added to 25 mL of F17 while 2.62 mg of polyethylenimine max (PEImax; Polysciences, Warrington, PA) was diluted into 25 mL of F17 medium to reach a final concentration of 1.5 μM of linear 25-kDa polyethylenimine (L-PEI; Polysciences, Warrington, PA). The plasmids and L-PEI were mixed, vortexed and incubated for 3 min at room temperature (RT), before addition to the cells. One day post-transfection (dpt), TN1 peptone stock solution (20% w/v) was added to the cell suspension in order to reach a final concentration of 0.5% (w/v). The supernatant was harvested 5 dpt and clarified by centrifugation at 3000 x g for 20 min.
the temperature was shifted to 32 °C to enhance protein production (Furukawa and Ohsuye, 1998; Sunley et al., 2008). The supernatant was harvested at 12 dpt as described before.

2.1.4. Purification
Both receptors were purified by adapting the protocol described by Tom et al. (2008). Polyacrylamide gel electrophoresis (PAGE) was performed as described in previous works by Boucher et al. (2008). Purified receptors were quantified by absorbance at 280 nm using a Nanodrop™ spectrophotometer (Thermo Fisher Scientific, Madison, WI).

2.2. IgG production

2.2.1. Antibodies
Trastuzumab (TZM), a humanized mouse IgG1, was selected as our reference antibody. The non-glycosylated TZM (TZM NG) corresponded to a TZM mutant where the Fc N-glycosylation site was abolished by substituting the Asparagine 297 by a Glutamine.

2.2.2. Production
TZM and TZM NG were produced by transient co-expression of the heavy and light chains in CHO-3E7 (Raymond et al., in preparation). TZM was enriched in galactose (TZM-gal+) by the additional co-expression of the human beta 1,4-galactosyltransferase (Raymond et al., 2012). TZM was enriched in galactose (TZM-gal+) prior to purification on a 0.5-mL Econo-Pac® 10DG column (Bio-Rad, Mississauga, ON) equilibrated in PBS. The column was washed with PBS and IgGs were eluted with 100 mM citrate buffer at pH 3.6. The fractions containing the IgG were pooled and the citrate buffer was exchanged against PBS with an Econo-Pac® 10DG column (Bio-Rad, Mississauga, ON). Purified Mabs were sterile-filtered, aliquoted and stored at −80 °C for TZM and TZM-gal, 7 dpt at a viability >80% for TZM and TZM-gal, 2 dpt at a viability >65% for TZM NG. The supernatants were collected and loaded onto a 4-mL MabSelect SuRe column (GE Healthcare, Mississauga, ON) equilibrated in PBS. The column was washed with PBS and IgGs were eluted with 100 mM citrate buffer at pH 3.6. The fractions containing the IgG were pooled and the citrate buffer was exchanged against PBS with an Econo-Pac® 10DG column (Bio-Rad, Mississauga, ON). Purified Mabs were sterile-filtered, aliquoted and stored at −80 °C. TZM-gal+ was concentrated on an Amicon Ultra-4 30K centrifugal filter unit (Millipore, Mississauga, ON) and incubated with neuraminidase (MP Biomedicals, Solon, OH) in 250 mM phosphate buffer at pH 5 overnight at 37 °C to remove sialic acid (TZM-gal) prior to purification on a 0.5-mL MabSelect SuRe column. The elution buffer was exchanged against water on an Amicon Ultra-4 30K centrifugal filter unit. Purified Mabs were quantified by absorbance at 280 nm using a Nanodrop™ spectrophotometer.

2.3. Aggregate separation
FcγRs and mAbs were purified by size-exclusion chromatography (SEC) using a Superdex200 column (GE Healthcare, Baie d’Urfe, Canada) to remove aggregates. The column was equilibrated with HBS-N (GE Healthcare) and 1 mL of sample was loaded onto the column at 1 mL/min. HBS-N buffer was used to elute the proteins. The aggregate-free Mabs as well as aggregate fractions were individually pooled and quantified by absorbance at 280 nm using a spectrophotometer (Unico, Dayton, NJ).

2.4. Glycosylation analysis

2.4.1. Trypsin digestion
IgG samples (ca. 0.1 mg) were dissolved in 25 mM ammonium bicarbonate (100 μL) and digested with trypsin (5 μg; Sigma, St. Louis, MO) at 37 °C for 16 h. The digests were fractionated on an HPLC Waters system using a Vydac 218 TP54 Protein&Peptide C18 analytical column (300-Å pore size, 0.46 × 25 cm, Separation Group, Hesperia, CA, USA). Solvent A was 5% ACN in water with 0.1% TFA and solvent B was 90% ACN with 0.1% TFA. An elution gradient was applied from 10 to 70% ACN over 60 min. UV detection was performed at 245 nm. All fractions were collected manually, and then completely dried.

2.4.2. N-Glycan isolation from intact glycoprotein
Each sample solution (100 μL; 50 μg of glycoprotein) was treated with PNGaseF (2 μL, 2U, Roche). After incubation at 37 °C for 18 h digested mixtures were purified on a Carb-CleanTM cartridges (Phenomenex, Torrance, CA) according to the protocol supplied by manufacturer.

2.4.3. Matrix-assisted laser desorption ionization mass spectrometric (MALDI-MS) analysis
Trypsin digested fractions were reconstituted in 7 μL of deionized water and 1 μL was spotted onto a partially dried matrix of 2,5-dihydroxybenzoic acid. Glycan fractions were spotted onto the matrix solution consisting of 2-aza-2-thiothymine/phenylhydrazine hydrochloride predeposited on the target and labeled with phenylhydrazine (PNH) (Lattova et al., 2010). MALDI-TOF/TOF-MS analysis was carried out in the reflectron positive or negative ion modes (UltraflexXtremeTM, Bruker, Billerica). Individual parent ions were manually selected for MS/MS experiments.

2.5. SPR experiments

The SPR experiments were performed using Biacore 3000 and T100 instruments (GE Healthcare) at a flow rate of 50 μL/min at 25 °C on CM5 sensor chips using HBS-EP 1X, pH 7.4 (GE Healthcare) as running buffer. Kinetic analysis (global fit) was performed with the BIAevaluation v.4.1.1 or Biacore T100 Evaluation softwares.

2.5.1. CD16a surface
CD16a was covalently bound to the sensorchip by means of a standard amine coupling kit (GE Healthcare, 250 nM of CD16a injected for 1 min at 10 μL/min at pH 4.5) following the manufacturer’s recommendations in order to reach a final response of 2000 RU. The reference surface was generated following the same protocol except for the injection of CD16a. TZM binding was monitored by injecting TZM solutions (diluted in HBS-EP buffer, GE Healthcare) on both surfaces for 2 min, followed by HBS-EP buffer injection (2 min) to monitor receptor/IgG dissociation. The concentrations of TZM were varied between 1 and 1000 nM. Data were double-referenced prior analysis (Myszka, 1999).

2.5.2. Anti-histidine surface
As an alternative to covalent coupling, receptors were also stably captured at the surface of the biosensor by the means
of an anti-histidine antibody (His Capture Kit, GE Healthcare) that had been covalently bound to the surface as recommended by the manufacturer (approximately 13,000 RU). CD16a or CD64 were injected (0.3 μg/mL and 0.2 μg/mL, respectively) over the anti-histidine antibody surface for 1 min (125 RU and 78 RU, respectively). No receptor was injected over the reference surface. Mabs solutions were then injected over captured CD16a (1–1000 nM, 2 min), CD64 (0.1–300 nM, 1 min) and control surfaces. The receptor/IgG dissociation was monitored by injecting running buffer for 130 s or 740 s for CD16a or CD64, respectively. Surface regeneration (dissociation of free receptors and receptor/IgG complexes) was done by injecting glycine buffer (10 mM, pH 1.5, 1 min). Data were double-referenced prior analysis (Myszka, 1999).

3. Results

3.1. Production of the extracellular domains of FcγRs

Plasmids corresponding to the extracellular portion of CD16a and CD64, each of them being tagged with ten histidine residues at its C-terminus, were used to produce the extracellular portions of these Fcγ receptors by transient transfection. CHO cells were selected for the production of CD64 as very low yields were obtained with our HEK293 cell line (data not shown). Both receptors were then purified by immobilized metal affinity chromatography (IMAC). Non-reducing PAGE gels were run with samples corresponding to each purification steps (Fig. 1): the lanes corresponding to CD16a and CD64 pools both featured a smear that more likely corresponded to receptor aggregation (Note that molecular weights were calculated to be around 25 kDa and 36 kDa for CD16a and CD64, without taking glycosylation into account). The protein at 40 kDa (Fig. 1B, indicated by the arrow) more likely corresponded to a minute fraction of non-glycosylated CD64 as this receptor is known to be highly glycosylated (Lu et al., 2011). The yields were 34 mg and 3 mg of purified CD16a and CD64 per liter of medium, respectively.

3.2. Aggregate removal

Receptor extracellular portions and Mabs were then purified by SEC to remove soluble protein aggregates from the purified material (Fig. 2) in order to ease subsequent SPR data interpretation. The integration of the chromatogram peak indicated that approximately 20% of CD16a, 50% of CD64 and 10% of the Mabs were aggregated prior SEC purification. These values were consistent with results derived from analytical ultracentrifugation analysis of each sample prior SEC (data not shown).

3.3. Kinetic experiments

Since our goal was to develop a high-throughput and versatile SPR assay based on the interaction between Fcγ receptors binding to various antibodies, we chose to immobilize the extracellular portion of the receptors onto the SPR biosensor surface. Two approaches were tested. First, the receptor was covalently bound by amine coupling on the carboxymethyl dextran sensor surface. The second approach relied on a sensor surface-bound anti-histidine antibody to capture the His-tagged receptor extracellular portions in an oriented fashion. Real-time monitoring of the interaction between CD16a and TZM was performed using a concentration range of 0–1000 nM of injected TZM, in duplicates (Fig. 3). For both experiments, the baseline was stable before the injection of the analyte, and, at the end of the dissociation, the signal corresponding to the accumulated IgG went back to 0 response units (RU). Moreover, results from both assays were reproducible (replicate injections were almost superimposed). At the end of the injection phase, a downward slope was observed at high concentration of analyte.

![Fig. 1](image_url). Coomassie staining of non-reducing PAGE gels corresponding to the production of the extracellular portion of His-tagged CD16a (A) and CD64 (B). 75 μL of sample corresponding to each purification step were loaded while 3 μg (A) and 2.5 μg (B) of both elution and PBS pool aliquots were loaded onto the non-reducing gel.
with the anti-histidine capture method (Fig. 3B1). Such a decrease was not observed with the covalently bound CD16a surface (Fig. 3A1). The sensorgrams were globally analyzed using a simple model (Langmuir interaction) (Fig. 3A2, B2) or a heterogeneous ligand model (Fig. 3A3, B3). The latter was chosen to better describe complex kinetics more likely emanating from the presence of distinct receptor populations at the sensor surface due to non-oriented coupling procedure of our first experimental strategy. The related kinetic parameters are given in Table 1. For each assay, the apparent thermodynamic dissociation constants were also calculated from the plateau values observed at the end of each injection, assuming a Langmuirian interaction (Table 1).

To study the interaction between CD64 and TZM, SPR assays were carried out using the anti-histidine capture method (Fig. 4, Table 2). TZM was injected at concentrations ranging from 0 to 300 nM. The baseline was stable before the injection of the analyte and the experiments reproducible, as one can judge from the sensorgrams resulting from duplicate injections. However, at 100 and 300 nM, the response signal went below the baseline during the dissociation phase. The set of sensorgrams was globally fit using a simple kinetic model in order to get a gross approximation of the kinetic parameters of the interaction (Fig. 4, Table 2). The amine-coupling strategy was also tried with CD64, but, as we were unable to regenerate the surface in between Mab injections, the approach was thus abandoned (data not shown).

3.4. Effect of TZM aggregation on kinetics

The impact of two types of Mab aggregates upon binding kinetics to CD16a was then assayed with the anti-His capture assay. The aggregates were collected during the SEC final
purification step for TZM and pooled into two distinct fractions: high (i.e., corresponding to the peak being closest to that of the monomeric Mab) and very high molecular weight (corresponding to higher molecular weight fractions), now noted HMW and VHMW, respectively. Monomeric TZM was injected at a fixed concentration (1000 nM) to which increasing amounts of aggregates had been added, i.e., from 0 to 50% (w/w) (Fig. 5). In order to evaluate the binding contribution of monomers vs aggregates, the corrected SPR signals at 170 s in Fig. 5A and B were used since it had been observed that TZM injected over CD16a was completely dissociated at 170 s (Figs. 3B1 and 5A, B). Histograms showing accumulated TZM aggregates at 170 s (RU) for the different percentages of HMW and VHMW, are shown in Fig. 5C. A close-up view of the 0–1.85% (w/w) range is also provided on Fig. 5D. Since the noise of the SPR biosensor has been determined to be around 1 RU, a threshold of 3 RUs was used to evaluate the limit of detection for the aggregates. The presence of 1.85% of HMW or, alternatively, 0.62% of VHMW TZM could be detected with our captured CD16a surface. Note that the 170-s time point was chosen in this manuscript since we repeatedly observed total dissociation of monomeric TZM at that time point for more than 10 independent experiments; this time point could however be shifted towards higher values (e.g., 200 s) to increase the robustness of the assay (i.e., make sure that all monomeric TZM has been eluted) since the apparent dissociation rates of monomeric and aggregated antibodies are extremely dissimilar.

3.5. Glycosylation analysis of IgGs — effect of the glycosylation pattern of IgGs on kinetics

Mass spectrometric analysis of the glycan pools obtained from individual TZM samples confirmed the differences in N-glycan profiles. The dominant oligosaccharide peak derived from the TZM model was observed at m/z 1575.61 and

### Table 1

<table>
<thead>
<tr>
<th>Purification step for TZM (Fig. 3).</th>
<th>Covalent immobilization of CD16a</th>
<th>CD16a captured by anti-histidine</th>
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<tr>
<td></td>
<td>Langmuir</td>
<td>Heterogeneous ligand</td>
</tr>
<tr>
<td></td>
<td>Langmuir</td>
<td>Heterogeneous ligand</td>
</tr>
<tr>
<td>$k_{a1}$ (M$^{-1}$ s$^{-1}$)</td>
<td>$(4.38 \pm 0.02) \times 10^4$</td>
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<td>$k_d$ (s$^{-1}$)</td>
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<td>$K_D$ (M)</td>
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<td>$1.42 \times 10^{-10}$</td>
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<td>$k_{a2}$ (M$^{-1}$ s$^{-1}$)</td>
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<td>$R_{max2}$ (RU)</td>
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<td>$R_{max2}$ (%)</td>
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<td>$K_D$ (M) equilibrium</td>
<td>$1.59 \times 10^{-6}$</td>
<td>$29.2 \times 10^{-6}$</td>
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![Fig. 4. Sensorograms corresponding to the interactions between injected TZM (0; 0.1; 0.3; 1; 3; 10; 30; 100; 300 nM), and CD164 that had been captured via its His-tag by a covalently bound anti-histidine antibody (A). The kinetics were fitted using the Langmuir model (B).](image-url)
its fragmentation pattern corresponded to the biantennary core-fucosylated structure with zero galactose (Fig. 6A). A glycan displaying galactose on both antennae (m/z 1899.73) was observed with the highest intensity in the engineered TZM-gal sample (Fig. 6B). The same differences in the abundances of galactosylated and non-galactosylated glycans were observed in the spectra recorded from the glycopeptides resulting from trypsin digested samples. Most of these glycopeptides were consistent with the peptide sequence EEQYNSTYR (m/z 1189.51) with glycosylation site at the Asp297. As expected, no glycan or glycopeptide were detected in the positive or negative ion modes when TZM NG sample was treated and analyzed under the same conditions (i.e., non-glycosylated; data not shown).

The SPR assay relying on the capture of CD16a by means of an anti-His antibody at the surface of our biosensor (Fig. 3B) was then evaluated for its ability to evaluate the impact of TZM glycosylation upon CD16a binding with a single Mab injection. 1000 nM of TZM, TZM-gal and TZM NG were injected in duplicates (Fig. 7). As expected, no interaction was observed between CD16a and TZM NG. For TZM and TZM-gal, the responses at the end of the injection phase were normalized to 100 RU in order to compare their dissociation profiles. Complete dissociation from CD16a was observed at 170 and 240 s for TZM and TZM-gal, respectively (Fig. 7).

4. Discussion

4.1. SPR Assay Development

SPR has been used in many studies in order to evaluate the kinetic constants of IgG/FcγR complexes. Among these studies, either receptors or antibodies have been immobilized, in an oriented manner or not, on the biosensor surface. Additionally, the variety of setups—especially in terms of orientation of the partners, flow rates and ligand densities—yielded discrepancies between the values reported in the literature. We here produced CD16a and CD64 using mammalian expression systems in order to get appropriate glycosylation pattern for these receptor extracellular soluble moieties, while receptor aggregates were removed by SEC before developing our biosensor assay (Figs. 1 and 2). Great care was also taken off to optimize SPR experiments: high flow rate (50 μL/min) and

<table>
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<td>$k_a$ $(M^{-1} s^{-1})$</td>
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<tr>
<td>$k_d$ $(s^{-1})$</td>
</tr>
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<td>$K_D$ (M)</td>
</tr>
<tr>
<td>$R_{max}$ (RU)</td>
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Table 2

Kinetics parameters for anti-His captured CD64 interacting with TZM (Fig. 4).

Fig. 5. Effect of TZM aggregation on its kinetics of dissociation from CD16a. Sensorgrams corresponding to the injection of 1000 nM monomeric TZM in the presence of 0–50% of HMW and VHMW aggregates over captured CD16a are shown in Panels (A) and (B), respectively (The two bottom curves on each panel correspond to aggregate-free TZM injections). Remaining amount of TZM at 170 s, time at which aggregate-free TZM was observed to be completely eluted, is shown in Panel C. For low percentage of aggregated TZM, a close-up of Panel C is given (D). Any response above the dash line at 3 RU is considered to be different from the non-aggregated pool of TZM (D).
Fig. 6. MALDI-TOF/TOF mass spectra recorded in the reflectron positive ion mode for N-glycan pools obtained from MAbs: TZM (A) and TZM-gal (B). Glycans are labeled with PHN (+90.05) and detected as MNa+. Proposed structures are deduced from MS/MS fragmentation patterns and from the data obtained before and after exoglycosidase digestion with the β-galactosidase. Symbols: red triangle (fucose), blue square (N-acetyl-glucosamine), green circle (mannose) and yellow circle (galactose).

Fig. 7. Sensorgrams corresponding to the injection of TZM (black), TZM NG (light gray), TZM-gal (dark gray) at 1000 nM over CD16a using the anti-histidine capture method, in duplicates.
low receptor densities were used to avoid any mass transport/rebinding artifact that might have hampered subsequent data analysis (Myszka, 1999). At last, the impact of receptor orientation at the biosensor surface was also addressed by comparing covalent (random) coupling to His-tag mediated stable capture (Fig. 3, Table 1). For CD16a, our experimental results unambiguously demonstrated that the type of immobilization (random versus oriented) of this ectodomain greatly influenced its binding to TZM. Indeed, random covalent coupling lead to complex kinetics, more likely resulting from the presence of multiple receptor populations at the biosensor surface (those were induced by the amine coupling procedure) whereas data corresponding to TZM binding to captured His-tagged CD16a were fit with a 1:1 interaction model with equivalent residual profiles as those corresponding to more complex kinetic model (Fig. 3). Altogether, the apparent dissociation constant related to TZM interactions with CD16a, as determined with both approaches (random coupling/His tag capture), either derived from the plateau values or the ratio of the kinetics rates determined by global fit, is approximately 1 μM (Table 1). Both apparent kinetic and thermodynamic constants we derived are in excellent agreement with those reported in the literature by others research groups (Lu et al., 2011). The fine characterization of the interactions between CD64 and TZM was unsuccessful with a covalent coupling procedure approach (CD64 immobilization): complexes between CD64 and injected TZM were extremely stable and all our attempts to regenerate the surface negatively impacted CD64 bioactivity (data not shown). When Mabs were injected over an anti-His surface on which CD64 had been captured, the analysis of the collected data with a simple model (Fig. 4) yielded a $K_D$ of $5.1 \times 10^{-8}$ M (Table 2), a value being in agreement with the literature (Lu et al., 2011). However, concentrations of Mabs of 100 nM or higher resulted in a net response signal being negative after 600 s. As non-specific adsorption of the Mab on the mock surface or misalignment of the curves during the double-referencing procedure have been ruled out, such a behavior may be due to the fact that, upon TZM binding, the stability of the His-tagged CD64/anti-His antibody is lowered (possibly due to a conformational change occurring within the His-tagged CD64/Mab complex that may in turn affect the anti-His antibody/His tag interaction). Therefore, the receptor partly dissociates from the anti-histidine upon TZM binding, which in turn provokes this higher-than-expected mass detachment from the biosensor surface. A better characterization of the CD64/Mab interactions will thus require to capture CD64 in a stable and oriented fashion via a different tag system.

Based on the levels of captured CD16a and CD64 (125 and 75 RUs, respectively), the theoretical $R_{\text{max}}$ values corresponding to the interactions of these captured receptors with TZM were calculated assuming a 1:1 stoichiometry for these interactions. Those are approximately 500 and 220 RUs for CD16a and CD64, respectively, while $R_{\text{max}}$ values determined by globally fitting were approximately equal to 55 RUs for both CD16a and CD64 surfaces (Table 1). These discrepancies are unlikely to be due to the fact that only low proportions of both receptor ectodomains preparations are biologically active. Indeed, additional SPR experiments in which TZM had been covalently coupled to the sensorchip and CD16a had been injected gave a similar apparent $K_D$ value (1 μM, data not shown) to that reported with His-tag captured CD16a (Table 1) — such would not have been the case if the values of the concentrations of injected CD16a that we used for the calculations had been significantly different from those of bioactive CD16a. These differences between calculated and theoretical $R_{\text{max}}$ may be due to steric hindrance as TZM is significantly bigger than both ectodomains. Alternatively, one may not exclude the fact that a significant proportion of the receptor ectodomains that were captured may be interacting already with the Fc portion of the surrounding anti-His antibodies (these interactions may be weak in solution but favored significantly as the local densities of both anti-His antibody and captured receptor ectodomain are high within the biosensor matrix). Once again, an alternate capture approach for the receptor ectodomains, which would not rely on antibody, may thus improve our assay in the future.

4.2. Impact of aggregation and glycosylation upon TZM/CD16a kinetics

The influence of the aggregation of IgGs upon their kinetics of interaction with the FcγRs receptor within a SPR
assay has already been discussed through the literature (Li et al., 2007; Luo et al., 2009). However, to our knowledge, there are currently no studies dealing with the limit of detection of aggregation by SPR and whether both partners (i.e. receptor and antibody) need to be aggregate-free when carrying out an experiment to get accurate kinetics. In our hands the presence of aggregates in the CD16a pool did not influence the kinetics as similar sensorgrams to those related to monomeric CD16a were obtained with IMAC purified CD16a that had not been SEC purified (Fig. A1). In stark contrast, the presence of aggregates within the TZM pool greatly influenced the kinetics of binding to CD16a (Fig. 5), thus confirming that size exclusion chromatography is necessary for the Mab samples, prior performing an accurate kinetic characterization. Since as few as 1.85% of analyte aggregate was detectable, the use of SPR biosensors combined to captured CD16a may thus be a good alternative to analytical ultracentrifugation in order to evaluate rapidly the presence of aggregates within a given sample. The limit of detection of such a SPR assay could be significantly improved by increasing the amount of captured receptor on the sensor chip (thus increasing the signal-to-noise ratio).

The anti-His capture strategy that we developed for CD16a was then proven to be efficient to discriminate between various patterns of glycosylation of the same antibody (Fig. 6) based on antibody dissociation from CD16a (Fig. 7). More precisely, non-glycosylated TZM did not bind to CD16a, in excellent agreement with the literature (Chirlando et al., 1999; Krapp et al., 2003; Walker et al., 1989), while a hyper-galactosylated (mostly G2F) version of TZM (Fig. 6) formed a more stable complex with CD16a when compared to our model TZM (Fig. 7). This observation is consistent with previous results from Houde et al. (2010) when using a competitive binding assay.

5. Conclusion

The extracellular portions of both CD16a and CD64 were produced by transient transfection in mammalian cells in order to develop a surface-based assay based on the real-time monitoring interactions with Mabs. Altogether, our results demonstrate that the oriented capture of CD16a at the biosensor surface is promising for the set up of an assay aiming at a) detecting the presence of aggregates within the injected solution of antibody while b) assessing the glycosylation profile of the antibody. Since the evaluation of both the aggregation state and the glycosylation profile of a given antibody sample only requires a single injection, an SPR assay based on captured CD16a may thus be implemented as a high-throughput routine assay in a Mab screening platform.

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