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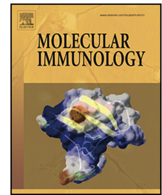

# Effect of protein aggregates on characterization of FcRn binding of Fc-fusion therapeutics

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ABSTRACT

Recycling of antibodies and Fc containing therapeutic proteins by the neonatal Fc receptor (FcRn) is known to prolong their persistence in the bloodstream. Fusion of Fc fragment of IgG1 to other proteins is one of the strategies to improve their pharmacokinetic properties. Accurate measurement of Fc–FcRn binding provides information about the strength of this interaction, which in most cases correlates with serum half-life of the protein. It can also offer insight into functional integrity of Fc region. We investigated FcRn binding activity of a large set of Fc-fusion samples after thermal stress by the method based on AlphaScreen technology. An unexpected significant increase in FcR binding was found to correlate with formation of aggregates in these samples. Monomer purified from a thermally-stressed sample had normal FcRn binding, confirming that its Fc portion was intact. Experiments with aggregates spiked into a sample with low initial aggregation level, demonstrated strong correlation between the level of aggregates and FcRn binding. This correlation varied significantly in different methods. By introducing modifications to the assay format we were able to minimize the effects of aggregated species on FcRn binding, which should prevent masking functional changes of Fc-fusion protein. Biolayer interferometry (BLI) was used as an alternative method to measure FcRn binding. Both optimized AlphaScreen- and BLI-based assays were sensitive to structural changes in Fc portion of the molecule, such as oxidation of methionines 252 and 428, and therefore suitable for characterization of FcRn binding.

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## 1. Introduction

The neonatal Fc receptor (FcRn) is responsible for extended half-life of IgG and Fc-conjugated proteins in serum. FcRn is also known to mediate bidirectional transcytosis of IgG across epithelial cells (Kuo and Aveson, 2011) and subcutaneous bioavailability (Deng et al., 2012).

The FcRn is a heterodimer that comprises a transmembrane α-chain in non-covalent association with a soluble β2-microglobulin (Simister and Mostov, 1989). FcRn binds to the $CH_2$–$CH_3$ regions of the Fc domain in the acidic environment of endosome (pH 6.0) and is recycled back into circulation where dissociation occurs at a neutral pH. This mechanism protects Fc-containing proteins from catabolism (Roopenian and Akilesh, 2007). Prolonged persistence of drugs in the body can have either positive or negative consequences for patients, and therefore it is important to characterize FcRn binding of Fc-containing biologics. It became increasingly clear that interplay between characteristics of the antibody (biophysical properties, antigen affinity, proteolytic stability, etc.) and its therapeutic target (soluble versus membrane-bound target, antigen load) can influence antibody clearance (Kuo and Aveson, 2011, Datta-Mannan and Wroblewski, 2014). However, affinity to FcRn remains one of the most important factors in prediction of antibody pharmacokinetics in vivo.

Recent studies demonstrated that affinities of the therapeutic proteins to FcRn measured in vitro correlated with their serum half-lives, with a few exceptions (Suzuki et al., 2010; Zalevsky et al., 2010; Dall'Acqua et al., 2006). Thus, valuable information can be

*Abbreviations:* BLI, biolayer interferometry; $CH_2$–$CH_3$, constant regions of heavy chains IgG; ELISA, enzyme-linked immunosorbent assay; FcRn, neonatal Fc receptor; FRET, fluorescence resonance energy transfer; HMW, high molecular weight aggregates; HPLC, high-pressure liquid chromatography; $k_a$, association constant; $k_{dis}$, dissociation constant; MES, *N*-morpholine ethanesulfonic acid; PBS, phosphate buffered saline; SEC, size exclusion chromatography; SPR, surface plasmon resonance; UV, ultraviolet light.

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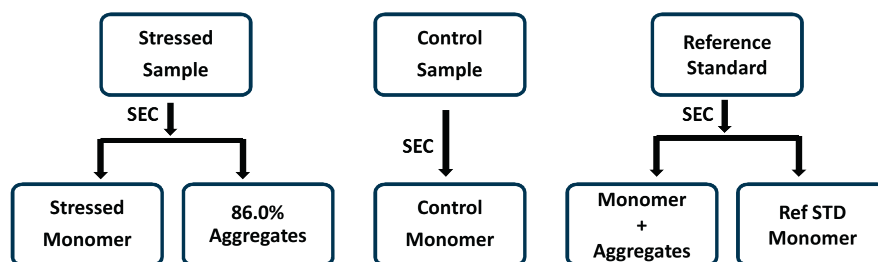


**Fig. 1.** Isolation scheme of Fc-fusion protein monomers from reference standard, control, and stressed sample. Monomers from reference standard, control sample, and stressed samples were purified by size exclusion chromatography (SEC).

obtained in in vitro assays, providing that they are set up correctly. Use of different methodologies for measuring Fc–FcRn interactions resulted in different affinity or relative potency values reported for the same Fc variant (Yeung et al., 2009; Mathur et al., 2013; Lu et al., 2011). This emphasizes the need for more thorough assay development and understanding. FcRn binding to Fc domain can be measured in cell-based assays, arguably more physiologically relevant (Mathur et al., 2013), and in cell-free assays using a wide variety of methods such as FRET, AlphaScreen®, ELISA, surface plasmon resonance (SPR), and biolayer interferometry (BLI) (Lu et al., 2011; Zhang et al., 2012; Arakawa et al., 2006; Wozniak-Knopp et al., 2012; Wu et al., 2015).

Even when the same technology such as SPR is used, different assay formats, parameters and conditions can lead to significant discrepancies in measured values (Abdiche et al., 2015; Neuber et al., 2014). More than ten-fold differences in binding affinities have been reported for FcRn–IgG interaction depending on whether FcRn (FcRn down format) or IgG (antibody down format) was coupled to the chip (reviewed in Datta-Mannan and Wroblewski, 2014). FcRn down assay format is believed to be more suitable for dissecting the role of IgG affinity versus avidity for FcRn and the contribution of these parameters to antibody clearance (Datta-Mannan and Wroblewski, 2014; Abdiche et al., 2015). Employment of diverse technologies with their specific limitations and advantages further contributes to variability in the in vitro measurements. Even transferring the assay from SPR to BLI platform is not as straightforward due to different FcRn mobilization levels on SPR chip and BLI biosensor tip (Neuber et al., 2014). Cell-based assays add another level of complexity, since they measure FcRn/IgG interactions at the cellular surface where steric and charge distribution properties are significantly different from those in the assay with purified components (Datta-Mannan and Wroblewski, 2014). Regardless of the method used for measuring FcRn–IgG interactions it is important to design assay format which minimize assay artifacts and produces consistent results. Moreover, in order for the method to serve as an analytical assay for lot characterization, it has to be specific, sensitive, accurate, and precise. For Fc stability assessment it also should be able to detect changes in binding activity in the presence of degradation products and/or aggregates.

Aggregates of antibodies and Fc-fusions are known to affect protein–protein binding (Mathur et al., 2013; Lu et al., 2011; Wu et al., 2015). The magnitude of these effects varies for different products and assays, possibly depending on the nature of aggregates and the assay design. Aggregation can be induced by high and low temperatures, changes in pH, UV exposure, and presence of a small amount of contaminant, such as damaged form of the protein product itself, host cell proteins, leachates or silica particles (Zhang et al., 2012; Arakawa et al., 2006). Different stresses stimulate distinct mechanisms of aggregation and produce different forms of aggregates, from soluble oligomers to high molecular weight irreversible complexes (Zhang et al., 2012; Arakawa et al., 2006). The nature of the aggregates in combination with the binding assay methodology can determine whether accuracy of the measurement is going to be affected. Therefore, it is important to understand what level of aggregates can be tolerated in particular binding assay for a specific product.

Here we investigated binding of a recombinant Fc-fusion protein, which contains Fc domain of a human IgG1, to FcRn. In this study we show a clear correlation between levels of aggregates present in Fc-fusion protein samples and FcRn binding values measured using two different formats of AlphaScreen technology. We also demonstrate that biolayer interferometry technology can be used as an alternative method for the assessment of FcRn binding activity in samples containing aggregates.

## 2. Material and methods

### 2.1. Sample peparation

#### 2.1.1. Preparation of thermally stressed and control Fc-fusion protein samples

1) Fc-fusion protein samples were stressed either by incubation at 40 °C for 3 or 6 months, or for 3 months at 25 °C.
2) Fc-fusion protein reference standard was incubated at 25 °C for 22 months to produce stressed aggregated sample, while control sample was stored at 5 °C for the same period of time.

#### 2.1.2. Isolation of monomers of Fc-fusion protein samples

Monomers from reference standard, control sample, and stressed samples were purified by size exclusion chromatography (SEC) using a Waters Alliance HPLC system (Milford, MA). As handling control, reference standard was injected multiple times, and collected as monomer plus aggregates using the same chromatographic conditions. Fig. 1 shows the preparation scheme.

#### 2.1.3. Preparation of Fc-fusion protein samples with aggregates

High molecular weight impurity fraction was collected from stressed Fc-fusion protein reference standard by size exclusion chromatography. This material containing 86.0% of aggregates (including dimers) was spiked into control sample to achieve target aggregate levels of 2.5, 4.0, 5.0, 7.5 and 10% as shown in Fig. 2.

#### 2.1.4. Size exclusion chromatography (SEC)

The SEC procedure was performed as follows, for analytical chromatography, typically 15 μg of stressed Fc-fusion protein was injected into a Waters Alliance HPLC system and separated using a TSKgel G3000SWxl column (Tosoh Bioscience #08541; 5 μm particle size), 7.8 mm ID X 30 cm length, at ambient temperature, and eluted with SEC buffer 100 mM sodium phosphate, 100 mM sodium chloride, pH 6.5 at flow rate 0.5 mL/min. The run time was 38 min and UV detection at 214 nm was used to monitor the elution profile.

For chromatography used in the collection work, approximately 300 μg of stressed Fc-fusion protein was injected multiple times

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