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Purification of Small Batches of Biotinylated Antibodies by Immobilized Metal Affinity Chromatography for Ligand Binding Method Development

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ABSTRACT

Biotinylated-antibodies are one type of conjugated critical reagent commonly used in Ligand Binding Assays (LBAs). The antibody degree of biotinylation affects the outcomes in assay functional performance. Usually, the amount of antibodies available for preparation of critical reagents is very limited and therefore alternative purification processes of smaller batches became more attractive. Immobilized Metal Affinity Chromatography (IMAC), is a well-known method for purification of proteins with histidine-clusters, such as the Fc domain of immunoglobulin-type antibodies. Here we discuss the feasibility to conjugate and characterize smaller amounts of humanized monoclonal antibodies for screening purposes in LBAs. Our data shows that as little as 0.25mg of protein are efficiently purified using IMAC in a time effective manner. Initial steps of LBAs development should consider the screening of optimal degree of labelling and therefore the testing of batches conjugated at different degrees of labelling purified by IMAC is a straightforward approach.

Introduction

Critical reagents are essential tools in Ligand Binding Assays (LBAs). They can range from peptides to larger molecules such as antibodies, which may be conjugated [1]. Several types of conjugation tags are available depending on the assay purpose and chemistry of conjugation. Among them, biotin is a versatile and popular label that binds streptavidin (or avidin) with high affinity [2]. For instance, biotinylated Monoclonal Antibodies (mAbs) are widely used for the development of LBAs [3]. Characterization of conjugated critical reagents is a hot topic in the bioanalytical industry [4,5]. Bioanalytical industry leaders have recently released guidance on this subject and agree that thorough characterization of critical reagents at the beginning of a drug candidate's life cycle is vital for the long-term success of that program [6]. The expected level of characterization includes, but is not limited to, analysis of biophysical properties, including the protein concentration, purity, DoL, and analysis of functional properties, such as orthogonal methods that relate to the LBA purpose.

Recently, more attention has been drawn to what should be the optimal Degree of Labelling (DoL), i.e., the average number of labels coupled to a protein molecule, for each assay. As an example, in the context of an anti-drug antibody LBA, by optimizing the biotin DoL, the authors reported an improvement on the functional assay performance of ~60% [7]. Another interesting finding was that lower and higher DoLs presented a decrease in functional activity, suggesting that a balance between under and over labelling, must exist. In a different study (LBA to detect neutralizing antibodies), the authors observed a significant degree of biotin-drug conjugate leaching was affecting the report of false positive and false negative results [8]. Furthermore, optimizing the antibody DoL mitigated that effect. After the antibody biotinylation

process, a buffer exchange step is generally required to remove the excess of unconjugated biotin, which might affect the assay performance [7]. Common techniques include desalting columns, size exclusion chromatography, ultra-filtration, tangential flow filtration or dialysis, however larger amounts of proteins are generally required. Taking into account that these antibodies might be scarce and expensive, alternative purification techniques that allow preparation of smaller amounts should be considered.

Immobilized Metal Affinity Chromatography (IMAC) is widely used for purification of proteins with histidine tags or clusters [9]. Among the later, several classes of antibodies have been purified using IMAC, including IgG1 type mAbs [10]. IMAC exploits the binding affinity of his-tagged proteins to positively charged metal ions (Zn, Cu, Ni, Co) [11]. The histidine rich region of both the conjugated and unconjugated proteins binds to a volume of metal beads in a positively charged resin and can be eluted by excess of imidazole [12]. Here we extensively purified smaller batches of conjugated mAbs by IMAC. These molecules include three humanized monoclonal antibodies utilized in immunotherapy: Trastuzumab, Rituxumab and Ofatumumab [13]. Each mAb was biotinylated at different molar ratios and further characterized for protein concentration, purity and degree of labelling. This strategy allows easy generation of a toolset of conjugated critical reagents with small amount of starting material, allowing the scale up of conjugations and lower processing times.

Results

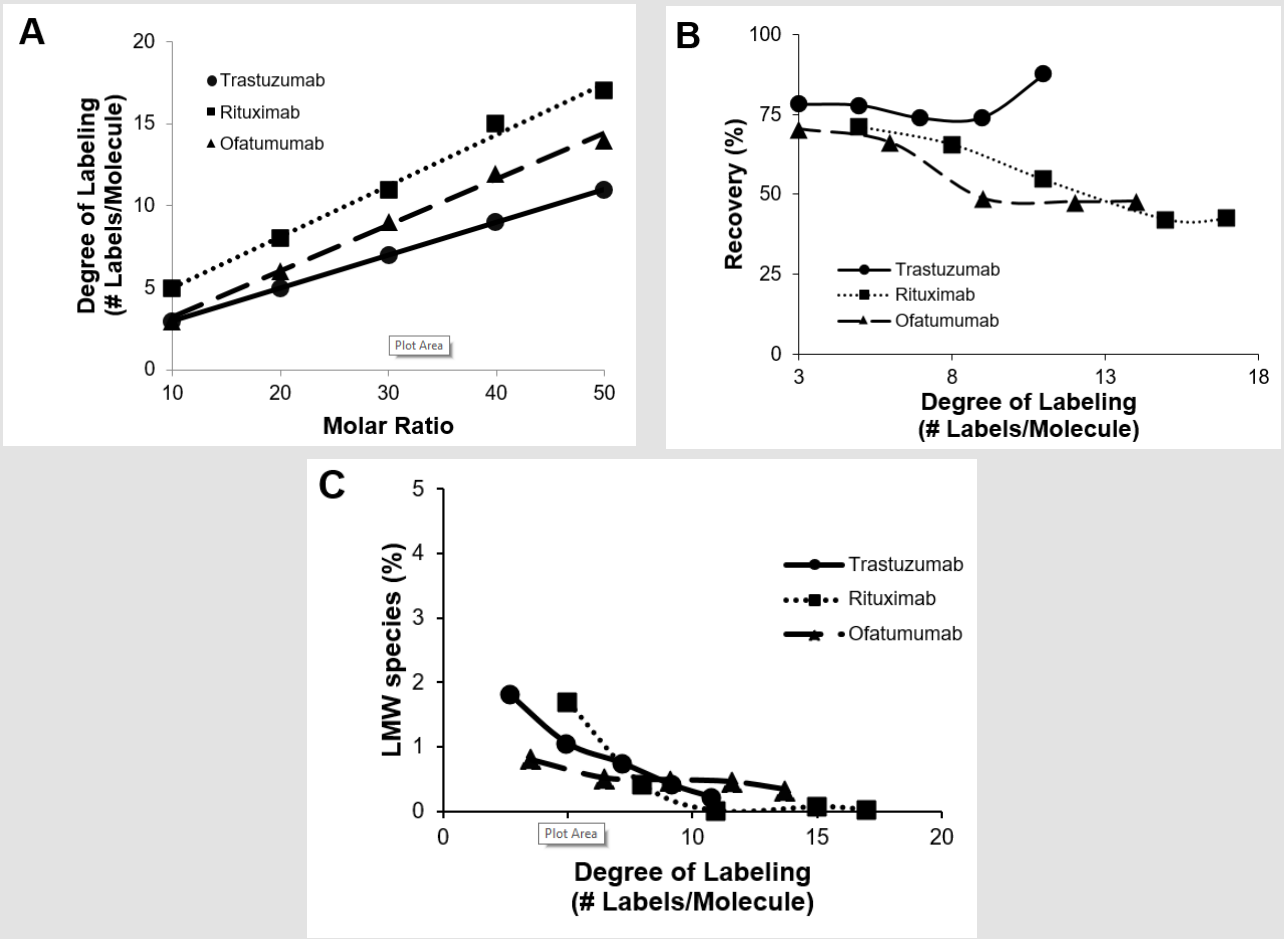

Figure 1: Biophysical properties of three biotinylated humanized mAbs (Trastuzumab, Rituximab and Ofatumumab) purified by IMAC.

a) Correlation between conjugation challenge ratio and incorporation ratio (determined by HRMS).

b) Protein recovery of the biotinylated molecules after IMAC purification.

c) Purity of LMW species determined by analytical SEC

A toolset of conjugated critical reagents was prepared to be used for method development purposes in LBAs. Here we conjugated three different humanized mAbs: Trastuzumab, Rituximab and Ofatumumab with biotin. These molecules were conjugated at distinct molar ratios of biotin/mAb (10:1, 20:1, 30:1, 40:1 and 50:1). Each reaction took place in a vial containing 0.25mg of protein with the respective amount of biotin, for 1 hour and subsequently quenched with tris buffer. After conjugation,

all proteins were purified to remove the excess of unconjugated biotin using high-performance nickel-IMAC resin as described in the methods section. The DoL (number of labels per molecule) was assessed by high-resolution mass spectrometry (HRMS). As expected, increasing amounts of biotin in the reaction led to increasing amounts of biotin labels incorporated to each mAb (Figure 1A). Under the conditions used, the rate of incorporation fell between 0.2 to 0.31 labels incorporated / molar ratio / hour. Due to the versatility of the method, each set of mAbs was purified in parallel. The overall IMAC purification process took about one hour. After elution with an imidazole containing solution, the protein concentration was determined and the respective protein recovery calculated (Figure 1B).

The overall recovery of all five Trastuzumab-biotinylated molecules was around or over 75%. Both Rituximab and Ofatumumab conjugated molecules had higher recoveries (~75%) at lower degrees of labeling and lower recoveries (~50%) at lower degrees of labelling (>8 biotin labels / molecule). This data suggests that not all conjugated mAbs have the same affinity to the nickel coated resin due to factors such as accessibility of the histidine cluster or different degrees of glycosylation. The effectiveness of this method in removing the excess of non-conjugated biotin from each sample was also assessed. To that end, we tested each sample for purity using analytical Size Exclusion Chromatography (SEC). All samples had less than 2% of non-conjugated biotin (Figure 1C). Note that no high molecular weight aggregation was observed for any of the samples (data not shown). For a general method development strategy, the 2-to-3 different reagents conjugated at different degrees of labeling should be sufficient to better define the optimal critical reagent for the assay. We used 0.25mg of protein / conjugation and were able to recover sufficient reagent to perform full biophysical characterization.

Methods

Biotinylation – The mAbs were conjugated with EZ-Link™ NHS-LC-LC-Biotin (Thermo Scientific) as per vendor instructions. Briefly, 0.25 mg was incubated with the respective amounts of biotin at the different molar ratios. The reactions were carried out for one hour at room temperature and stopped with Tris-HCl (7.4). IMAC-Conjugated proteins (0.25 mg) were allowed to bind HisPur™ Ni-NTA Superflow Agarose (Thermo Scientific). Briefly, the Ni-NTA agarose was equilibrated with Phosphate Buffered Saline (PBS) in mini spin columns. The conjugated proteins were then incubated with the agarose at RT. After three washing steps with PBS, the conjugated critical reagent was eluted with imidazole buffer in PBS (7.4). and stored at 4ºC until further biophysical characterization. Protein concentration- Total protein concentration was assessed using the colorimetric bicinchoninic acid assay (Thermo Scientific). Bovine Gamma Globulin was used as standard protein. Protein Purity-Purity was assessed by analytical size exclusion chromatography as described in Rocha et al [7]. Degree of Labelling- The degree of labelling (or incorporation ratio) was determined by HRMS as previously described [7].

Conclusion

Development and optimization of bioanalytical methods is an essential phase of any clinical sample analysis program. One of the initial steps involves identifying, titrating and optimizing the critical reagent conditions in the assay. Since biophysical and functional aspects can dictate the performance of a given conjugated critical reagent, it is important to perform systematic upfront characterization. Therefore, preparing a toolset of critical reagents conjugated at different DoLs might be beneficial for proper method development. Here we demonstrate that IMAC is a powerful protein purification technique that allows purification of minimal biotinylated mAb amount. The amount of protein recovered was sufficient for downstream characterization. It is also important to highlight that this is a very time and cost effective workflow.

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