Minimizing the influence of fluorescent tags on the partition of IgG in PEG-salt aqueous two-phase systems towards miniaturized rapid screening applications

Mariana São Pedro[(1)]

[(1)]IBB – Institute for Bioengineering and Biosciences, Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal

Abstract

Aqueous two-phase extraction (ATPE) offers a significant potential for the selective separation of biopharmaceuticals from unclarified cell culture supernatants, as is the case of monoclonal antibodies. However, it is often difficult to predict the behaviour of the target molecule in each condition (e.g. tie-line length (TLL), pH, neutral salt concentration), making high-throughput screening tools highly valuable to perform an empirical optimization. Microfluidic ATPE screening devices coupled to fluorescence microscopy to continuously monitor IgG partition have been recently demonstrated. These systems provide short diffusion distances and rapid molecular partition without gravity settling, resorting to minimal reagent volumes. However, the influence of fluorescent tags on IgG partition must be carefully evaluated to assess the validity of extrapolating the results to the unlabelled molecule, a critical but currently unexplored effect. In this work, three fluorophores with different global charge and reactivity, namely BODIPY™ FL maleimide, Alexa Fluor® 430 and BODIPY™ FL NHS Ester, were selected to label IgG with different degrees of labelling. ATPE performed in microtubes or microchannels, SDS-PAGE electrophoresis and isoelectric focusing (IEF), were performed in order to characterize each labelled molecule relative to the native IgG. BODIPY™ FL maleimide, combined with tris(2-carboxyethyl)phosphine (TCEP) to generate free thiol groups without compromising the tertiary structure, was the most promising strategy. Using this fluorophore, the observed partition coefficient in a PEG 3350-phosphate system with and without the addition of NaCl, was comparable between the microtubes and microfluidic devices, and also comparable to those quantified for the unlabelled molecule using analytical protein G chromatography.

Keywords: Monoclonal antibodies, ATPE, downstream processing, high-throughput screening tool, fluorescent tags

1. Introduction

Antibodies are highly specific molecules which can recognize and eliminate pathogenic and disease antigens, playing an important role in medicine as well as in analytical biotechnology.[1] In the past few years, monoclonal antibodies (mAbs) have been playing an important role in several of the main advances in the treatment of several infectious diseases, cancers and autoimmune disorders. However, unlike other therapeutic products, mAbs are required at high purity and high doses (0.1 to 1.0 g) to achieve effective treatment, being administered in a chronic regime. Therefore, with concerns about a potential shortage of the manufacturing capacity in mind, important improvements in the upstream productivity have been achieved.[2,3] Advances in the downstream processing of antibodies are thus essential since the downstream processing can undercut some of the enhancements in cell line productivity, being even considered as the bottleneck in providing these therapeutics at reliable costs and at considerable amounts, accounting for 50-80% of total manufacturing cost.[1,3,4] Hence, a simple but efficient downstream purification strategy is demanded in order to reduce costs and make these biopharmaceuticals widely available to the general population.[2]

Aqueous two-phase extraction (ATPE) presents a great potential for selective separation of a wide range of biomolecules by exploring differences in molecular solubility in each of the two immiscible phases. This technique can be a potential alternative, reducing costs by simplifying the initial clarification and primary purification steps.[2,5] Aqueous two-phase systems (ATPSs) will provide a suitable environment to maintain biological activity and protein solubility, due to the high biocompatibility, high water content and low interfacial tension, minimising product degradation. By variating certain experimental conditions, such as pH, ionic strength, and polymer molecular weight, good resolution and yields can be obtained.[1] The use of aqueous two-phase extraction for the initial purification in the downstream processing of monoclonal antibodies has been reported in a few research studies, obtaining satisfactory recovery yields and protein purity.[3,6–8]

Aiming at effectively optimizing novel purification schemes involving ATPE, high-throughput screening tools can be highly valuable to optimize the separation of complex mixtures, particularly considering that many of the factors governing partitioning of analytes in ATPE are still poorly understood. Microfluidics is characterized by shorter reaction times due to shorter distances for diffusion, as compared to macroscale devices, with the use of low volumes of reagents and analytes and by having the potential of being low cost, disposable and portable.[5] Recently, several research studies have

been demonstrating the validation of microfluidic screening tools for the bioseparation of target molecules in ATPE[9–14], with Silva *et al*. designing a microfluidic device for the extraction of mAbs, showing that the reduction to the microscale did not greatly affect the antibody extraction yield when compared with macroscale results.[15]

Thus, an ATPE microfluidic screening tool was fabricated in order to explore the behaviour of the IgG molecule, resorting to fluorescence microscopy to continuously monitor and quantify the partition of this antibody. However, for the use of the fluorescence microscopy, the antibody IgG must be previously labelled with the proper fluorophore, without altering the antibody proprieties, so the results can be easily extrapolated to the unlabelled molecule. Therefore, the selection of the appropriate fluorophore is critical is crucial for obtaining unambiguous interpretations and remains currently unexplored. Numerous fluorophores are available to the IgG labelling, modifying several functional groups present in this protein, such as the primary amines present in the lysine residues (***Figure 1A***) or the thiols present in the cysteine residues (***Figure 1B***).

In this work, the fluorophores amine-reactive Alexa Fluor® 430 and BODIPY™ FL NHS ester, and the thiol-reactive BODIPY™ FL maleimide, presenting distinct global charge and reactivity, were accessed to label the IgG molecule. Different methods to proper label the molecule of interest were explored, resulting in a variety of degrees of labelling for each fluorescent dye. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing (IEF) were performed to characterize each labelled molecule relative to the native IgG. Resorting to fluorescence microscopy, aqueous two-phase extraction was performed in microtubes (mL-scale) or microchannels (nL-scale), using the obtained dye-protein conjugates, and the partition coefficient for each was calculated and compared to the partition coefficient of the non-labelled molecule, determined by analytical protein G.

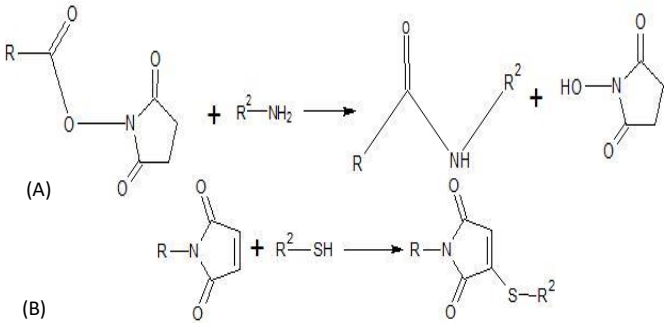

Figure 1 *- Standard reaction of:* ***(A)*** *succinimidyl ester with a primary amine, forming an amide bond; and* ***(B)*** *a maleimide with a thiol group, forming a stable thioether.*

2. Materials and methods

2.1. Chemicals

PEG polymers with molecular weights of 3350 Da were obtained from Sigma-Aldrich and dissolved in Milli-Q water to a stock solution with a concentration of 50%(w/w). The salts, sodium phosphate monobasic anhydrous and potassium phosphate dibasic, were both acquired from AppliChem PanReac and stock solutions of 40% (w/w) in Milli-Q water were prepared, where the acid form (NaH_2PO_4) is 0.43-fold the amount of the basic form (K_2HPO_4) of the salt in order to obtain a pH value of 7. Sodium chloride (NaCl) was purchased from AppliChem PanReac and dissolved in Milli-Q water to prepare a stock solution of 20%(w/w). The fluorophore Alexa Fluor® 430 was obtained from Life Technologies, whereas BODIPY™ FL maleimide and BODIPY™ FL NHS Ester were both purchased from Lumiprobe.

The reagents used for SDS-PAGE gels were ammonium persulphate (APS), N,N,N,N-tetramethylethylenediamine (TEMED), 30% acrylamide/bisacrylamide-solution, purchased from Sigma-Aldrich, and SDS micropellets (sodiumdodecyl sulphate) acquired from BIO-RAD. Coomassie brilliant blue R-250 was acquired from GE Healthcare, ethanol from Manuel Vieira & Companhia (Irmão) Sucessores Lda, acetic acid from Fisher Chemical, silver nitrate solution and formaldehyde solution from AppliChem PanReac, sodium thiosulfate and sodium carbonate from Sigma-Aldrich. For the isoelectric focusing, the kit and the marker used were obtained from GE Healthcare and trichloroacetic acid solution and glutaraldehyde required to develop the gel were purchased from Merck and Sigma-Aldrich, respectively.

2.2. Labelling of the antibodies with the fluorescent dyes

Human normal immunoglobulin, with the commercial name of Gammanorm®, was obtained from Octapharma (Lachen, Switzerland), with a concentration of 165 mg mL^{-1} and 95% IgG (59% IgG1; 36% IgG2; 4.9% IgG3; 0.5% IgG4 and at maximum 82.5 $\mu g.mL^{-1}$ of IgA).

The degree of labelling (DOL) of each protein-dye conjugate, that is, the number of dye molecules bonded per molecule of protein, was calculated according to Pinto *et al*.[16]

To prepare the IgG conjugated with Alexa Fluor® 430 with a degree of labelling of 7.6, the protocol from Pinto *et al*.[16] was followed, mixing a 4:1 molar ratio of reactive dye solution to antibody. To obtain conjugates with lower degrees of labelling, the same protocol was followed, using molar ratios of 2:1 and 1:1 of reactive dye solution to antibody, resulting DOL of 4.1 and 2.4, respectively.

To obtain the antibody conjugate with BODIPY™ FL NHS Ester, an identical protocol to Pinto *et al*. was followed, with the incubation step in the dark lasting for at least 4 hours. Additionally, to obtain a higher DL and to help to solubilize the dye, preventing the formation of aggregates, the addition of 10% of PEG 3350 in the bioconjugation step was also performed. Lastly, for the labelling with BODIPY™ FL maleimide, firstly, the Gammanorm® was diluted to a final concentration of 8.5 mg.mL^{-1} in PBS (Sigma-Aldrich), following with the mixture with the dye in a proportion of 20 mol to 1 mol of IgG. Then, the solution is incubated in the dark for 2 hours with mild agitation.

Finally, the washing of the solution with PBS is performed to discard any non-conjugated dye, in a series of 10 diafiltration steps using Amicon Ultra-0.5 centrifugal filter units (MWCO of 10 kDa), centrifuging at 14,000 $\times$ g for 5 mins. To generate free thiol groups without compromising the tertiary structure, an additional step was performed before the antibody-dye conjugation: the addition of the reducing agent tris(2-carboxyethyl)phosphine (TCEP) dissolved in PBS in a molar proportion of 0.5 to 1 mol of IgG, mixing in a vortex for 3 seconds.

2.3. Aqueous two-phase extraction studies

mL-scale experiments

ATPSs were prepared by weighting the corresponding stock solutions of the PEG 3350 along with the salts in order to achieve the desired final composition of each system. The ATPS had a total volume of 1.5 mL and were spiked with the biomolecule of interest, non-labelled and labelled IgG, in Eppendorf tubes, vortexed for 30 s and then centrifuged for 5 mins at 1400 $\times$ g, to separate the two phases. The top phase was then retrieved using a micropipette and the bottom phase with a syringe, each being placed in a new 1.5 mL Eppendorf, taking extra care not to disturb the interface. Samples from both phases were then taken for the determination of IgG concentration.

nL-scale experiments

As previously, ATPSs were prepared by weighing the corresponding stock solutions of PEG 3350 along with salt and NaCl, when required, with the total volume of 1.5 mL, followed by the vortex for 30 s and centrifugation for 5 mins at 1400 $\times$ g. After retrieving both phases, as mentioned above, to a new Eppendorf, the bottom phase was then spiked with the labelled antibody and the samples were taken for the determination of labelled IgG concentration.

For both experiments, the partition coefficient, K_P, was defined as the ratio of the IgG concentration in the PEG-rich phase, top phase, to that in the salt-rich phase, lower phase.

2.4. Microfluidic devices fabrication

Two microfluidic structures were used in this experimental work, a straight microchannel (100 µm high x 200 µm wide x 5 mm long) and a serpentine microchannel (20 µm high x 200 µm wide x 15 cm long) with two inlets and three outlets (50 µm wide), produced out of polydimethylsiloxane (PDMS). The fabrication of the hard mask of these two structures began with the design in the AutoCAD 2015 software, being subsequently transferred to a 1.5 µm thick layer of positive photoresist layer, spin-coated on top of an opaque 200 nm thick film of aluminium, previously deposited in a Nordiko 7000 magnetron sputtering system. The exposure of the photoresist was performed at 405 nm using direct write lithography (Heidelberg DWL II). After developing the photoresist, the unprotected aluminium was fully etched by wet etching using an aluminium etchant standard mix. The master mold and the respective PDMS structures were constructed according to Silva *et al*.[5]

2.5. Quantification of non-labelled IgG

The concentration of IgG was determined by affinity chromatography in Äkta™ Purifier 10 system from GE Healthcare (Uppsala, Sweden) equipped with an analytical POROS Protein G affinity column (2.1 x 30 mm) from Applied Biosystems (Foster City, CA, USA). The adsorption buffer was composed by 50 mM sodium phosphate buffer and 150 mM NaCl, at pH 7.4, whereas elution was carried out by decreasing the pH value to 2, with a buffer composed of 12 mM HCl and 150 mM NaCl. The samples containing the antibody were diluted 15 times in the adsorption buffer, before its injection in the column. Absorbance was monitored at 215 nm and a calibration curve obtained using Gammanorm® IgG, with a concentration range of 0.2 to 20 µg.mL^{-1}, was used to calculate the IgG concentration.

2.6. Quantification of labelled IgG

mL-scale experiments

Each PEG and salt-rich phase was continuously flowed in the straight microchannel using a syringe pump (New Era Pump Systems, Inc., NE-1000 model) at a flow rate of 1 µL.min^{-1}. The fluorescence was measured using the Leica (Solms, Germany) DMLP fluorescence microscope coupled to a CCD color camera XC30, Olympus (Shinjuku, Tokyo, JP). A band pass filter for excitation between 450 and 490 nm and long pass emission above 515 nm was used. Polyethylene tubing was acquired from Instech Solomon and 1 mL syringes were purchased from CODAN (Lensahn, Germany). This structure was used to obtain similar fluorescence acquisition conditions between both mL and nL-scale and the images were obtained with several exposure times, ranging from 250 ms to 1 s. A fluorescence micrograph in the middle of the channel was taken

and the corresponding fluorescence values were measured with ImageJ software from the National Institutes of Health (Bethesda, MD, USA) and subtracted the background value. This background corresponds to the signal intensity outside the microtube.

nL-scale experiments

Both the PEG phase and the salt phase containing the labelled IgG antibody were loaded in each of the two inlets of the microfluidic structure at a flow rate of 0.2-0.3 μL.min^{-1} and 1 μL.min^{-1}, respectively, using two distinct syringe pumps (New Era Pump Systems, Inc., NE-1000 model and Kd Scientific, Legato® 100 model). The location of the interphases and the IgG partitioning phenomena were observed using the aforementioned microscope and filter. Several fluorescence images were obtained through the microfluidic channel, with several different exposure times, ranging from 250 ms to 1 s, taking into consideration to coincide the exposure time used in the mL-scale for a given dye, enabling the tracking of the labelled IgG partition from one phase to the other. The analysis of the images was also made with ImageJ software, subtracting the background value, with a fluorescence micrograph at the end of the microchannel being used to determine the partition coefficient, K_P.

2.7. Determination of the molecular weight

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed to evaluate any molecular weight alterations which may occur after each labelling process. All analysed samples, with a concentration of 1 mg.mL^{-1}, were prepared, in a final volume of 50 μL, by adding 20 μL of each sample, 5 μL of Milli-Q water and 25 μL of sample buffer (62.5 mM Tris-HCl, pH 6.2, 2% SDS, 0.01% bromophenol blue and 10% glycerol), followed by boiling for 10 minutes at 100°C. A 20 μL of each sample and 5 μL of molecular marker (Precision Plus Protein™ Dual Color Standards, Bio-Rad) were applied to 12% acrylamide gels, prepared with a 40% acrylamide/bisacrylamide stock solution. The gels were run at 110 mV, for about 120 minutes, with a running buffer composed of 25 mM Tris-HCl, 192 mM of glycine and 0.1%(w/v) SDS, with pH 8.3. After, for the detection of the protein bands, the gels were stained with Coomassie Brilliant Blue R-250 for one hour and distained successive washes with a solution of 30% ethanol, 10% acetic acid. Whenever the intensity of the bands was too low, the gels were subsequently stained with silver nitrate.

2.8. Determination of the isoelectric point

The isoelectric point (pI) of the different conjugates was assessed through the technique IEF, using a PhastGel® IEF 3-9 with 50 x 46 x 0.45 mm. PhastGel® IEF media are precast homogeneous polyacrylamide gels (5%T, 3%C) containing 2-6% Pharmalyte® as carrier ampholytes. The IEF separation was performed in a multi-tasking PhastSystem™ from Pharmacia. As for pI marker, it was used a broad pI kit (pH 3-10) (GE Healthcare). The IEF gels were then silver stained, by incubation with a series of different solutions.

3. Results

3.1. Quantification of non-labelled IgG

Firstly, two systems composed of 12% PEG 3350 and 12% of phosphate with and without the addition of the neutral salt NaCl were used to study the partition behaviour of the non-labelled IgG and the obtained results for the K_p value are represented in ***Table 1,*** using protein G chromatography resorting to Äkta™ Purifier 10 system to quantify the IgG concentration in each phase.

Table 1 *- The partition coefficient, K_P, for the non-labelled IgG in the system 12/12 PEG 3350/salt, with and without the addition of 7.5% of NaCl (IgG concentration of 30 μg.mL^{-1}), quantified by protein G chromatography in Äkta™ Purifier 10 system.*

K_P of non-labelled IgG	
wo/ NaCl	0.73 ± 0.01
w/ NaCl	2.30 ± 1.20

Without NaCl, the IgG molecule partitions preferentially to the salt-rich phase (K_P < 1), and with NaCl, it partitions to the PEG-rich phase (K_P > 1). Knowing the partition behaviour of the non-labelled IgG, it is possible to test the results obtained using each of the systems above when labelling the IgG molecules with different fluorophores and conjugation chemistries, aiming at achieving labelling conditions which allow an extrapolation of the results to the unlabelled molecule.

3.2. Labelling protocol and determination of the degree of labelling

For this experimental work, two different fluorophores and two different conjugation chemistries were selected to label the IgG molecule, Alexa Fluor® 430 NHS ester, BODIPY FL™ NHS ester and BODIPY FL™ maleimide, each providing different characteristics namely the (1) labelling mechanisms, (2) global bioconjugate charge, (3) water solubility and (4) quantum yields.

Regarding the Alexa Fluor® 430 NHS ester, this dye presents good water solubility and a global mononegative charge[18], which makes the bioconjugation step simpler. However, since the IgG molecule has approximately 30 lysine residues that can be modified, the degree of modification must be

controlled by adjusting the molar ratio (100:1, 50:1 and 25:1 of reactive dye solution to IgG to achieve DOL values of 7.6, 4.1 and 2.4, respectively).

For the BODIPY FL™ NHS ester, although the conjugation principle is the same as for the Alexa Fluor® 430 dye, the bioconjugation step was not so straightforward due to the high hydrophobicity and neutral charge of the BODIPY molecules.[19] To minimize this problem, a low-molecular weight PEG (3350 Da) was added in solution to help solubilize the dye and prevent the formation of intermolecular interactions and aggregates.

Lastly, the labelling with the BODIPY FL™ maleimide was more challenging. Although the IgG molecule has cysteines disulphide bonds connecting the heavy and the light chains, these are well protected within the structure and are typically not available for conjugation. The addition of TCEP will selective reduce some or all of the disulphide groups in a protein and make them more available for the labelling.[17]

In ***Table 2***, the different degree of labelling (DOL) calculated according to Pinto *et al*.[16] for each of the three fluorophores used are shown.

Table 2 *- Summary of the several fluorophores used for the labelling of the IgG molecule and the respective DOL obtained.*

	DOL
IgG labelled with BODIPY FL™ maleimide without using TCEP	0.6
IgG labelled with BODIPY FL™ maleimide using TCEP	0.5
IgG labelled with Alexa Fluor 430 NHS ester	7.6
	4.1
	2.4
IgG labelled with BODIPY FL™ NHS Ester	2.6
	1.4

3.3. Determination of the molecular weight of the labelled IgG

The next step was to evaluate if the binding of the different fluorophores would not change the conformation and structure of the IgG molecule. Thus, an SDS-PAGE electrophoresis was performed, as it will separate the charged molecules by their molecular masses in an electric field. Denaturation by heating (100°C for 10 minutes) was required in the samples to break the hydrogen bonds and disrupt secondary and tertiary structure. Even though this technique is not ideal to study the molecular weight of a protein, it served as a preliminary assay and it allowed to take a few conclusions. The obtained gel is shown in ***Figure 2***.

3.4. Determination of the isoelectric point of the labelled IgG

Isoelectric focusing was the following technique used to characterize the labelled IgG since it allows the separation of different molecules according to their isoelectric point (pI). The resulting gels for the three different fluorescent tags, with a diversity of degrees of labelling, are shown in ***Figure 3***. The antibody used, Gammanorm IgG, is a mixture of different subclasses of IgG (59% IgG1; 36% IgG2; 4.9% IgG3; 0.5% IgG4), and, even though these are more than 90% identical on the amino acid sequence level, each subclass has a unique profile with different

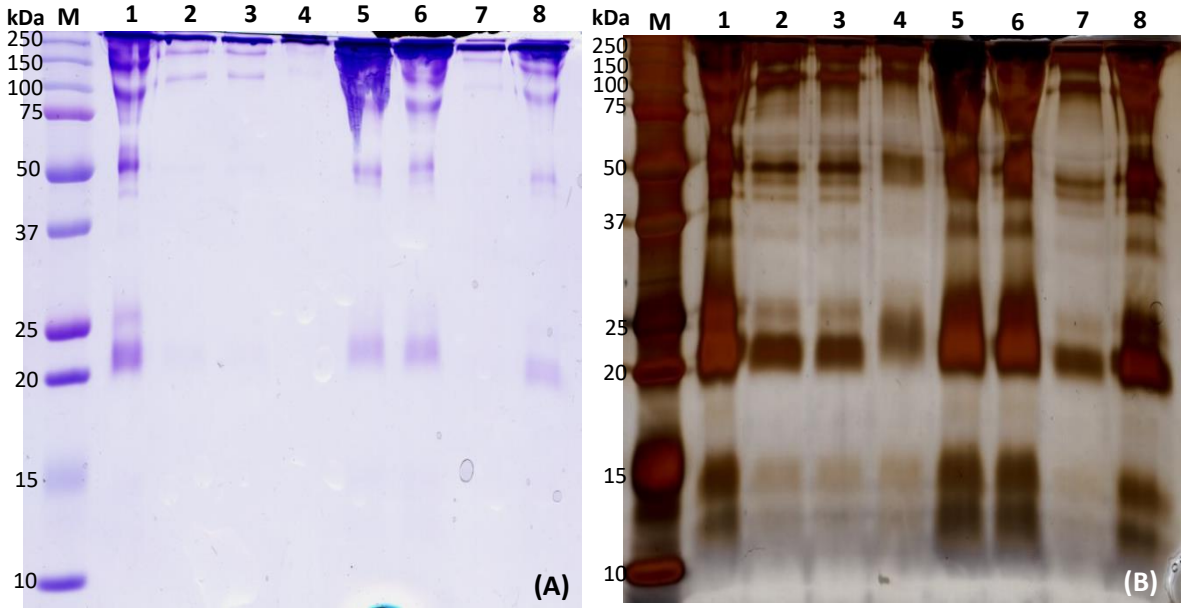

Figure 2 *- Determination of the molecular weight of the different IgG labelled with the three fluorophores and with different degrees of labelling, through SDS-PAGE electrophoresis without denaturating conditions, with (A) staining with Coomassie protocol and (B) staining with silver stain protocol. M: Protein molecular weight marker; 1: IgG non-labelled; 2: IgG-BODIPY ™ FL maleimide using TCEP, DOL 0.5; 3: IgG-BODIPY ™ FL maleimide without using TCEP, DOL 0.6; 4: IgG-Alexa Fluor® 430, DOL 7.6; 5: IgG-Alexa Fluor® 430, DOL 4.1; 6: IgG-Alexa Fluor® 430, DOL 2.4; 7: IgG-BODIPY ™ FL NHS Ester, DOL 2.6; and 8: IgG-BODIPY ™ FL NHS Ester, DOL 1.4.*

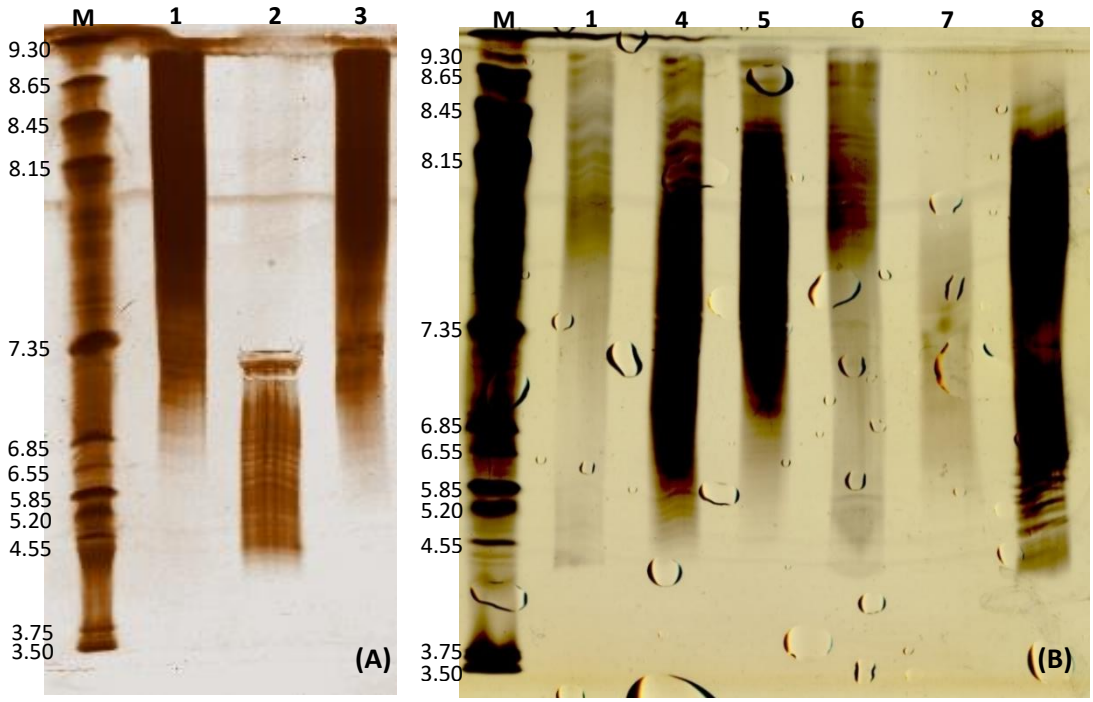

Figure 3 *- Determination of the pI of the different IgG molecules labelled with the three fluorophores and with different degrees of labelling, via isoelectric focusing (IEF). M: Protein molecular weight marker; 1: non-labelled IgG; 2: IgG-Alexa Fluor® 430, DOL 7.6; 3: IgG-BODIPY ™ FL maleimide without using TCEP, DOL 0.6; 4: IgG-Alexa Fluor® 430 NHS ester, DOL 4.1; 5: IgG-Alexa Fluor® 430 NHS ester, DOL 2.4; 6: IgG-BODIPY ™ FL maleimide using TCEP, DOL 0.5; 7: IgG-BODIPY ™ FL NHS Ester, DOL 2.6; and 8: IgG-BODIPY ™ FL NHS Ester, DOL 1.4. The concentration of IgG used in (A) was 1 mg.mL^{-1} and in (B) 0.5 mg.mL^{-1}.*

molecular weights and pIs. For this reason, it is not possible to determine a well-defined value for the non-labelled antibodies, but instead a band ranging from pH values of 6.5 up to 9. Therefore, at physiologic pH values (the pH used in the further ATPE studies), IgG will have mostly a positive overall charge.

3.5. Aqueous two-phase extraction using microtubes (mL-scale) and a microfluidic device (nL-scale)

Afterwards, the first experiments of ATPE at mL-scale were performed, with the labelled IgG concentration from each phase collected from a ATPS and later being quantified using a straight microchannel, with 100 µm high, 200 µm wide and 5 mm of length, resorting to a fluorescence microscope. Each phase was continuously flowed into the microstructure and a fluorescence micrograph was taken in the middle of the channel, followed by quantification of the corresponding fluorescence intensity values. An example of these fluorescence micrographs, for the IgG labelled with the fluorophore BODIPY™ FL maleimide using TCEP, is shown in ***Figure 4***.

Finally, the aqueous two-phase extraction was performed at the nL-scale, using a serpentine microfluidic structure with two inlets and three outlet

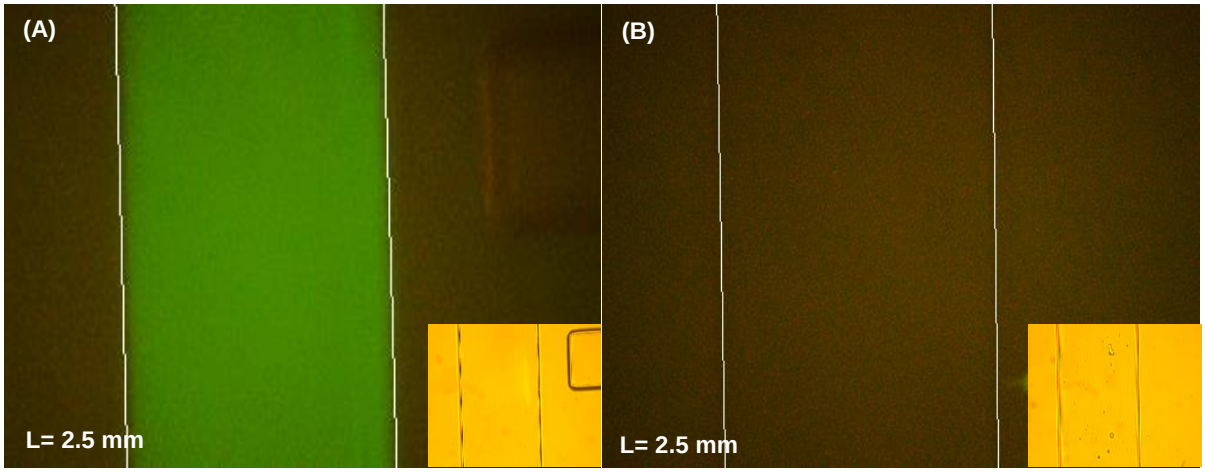

Figure 4 *- Fluorescence micrographs captured in the middle of the straight microchannel, for the PEG-rich phase (A) and the salt-rich phase (B) of the system 12/12 PEG 3350/salt with the addition of NaCl, with IgG labelled with BODIPY™ FL maleimide using TCEP, DOL 0.5, at a concentration of 20 µg.mL^{-1}, with an exposure time of 1000 ms and gain of 0 dB. The corresponding bright field images are also presented as an inset.*

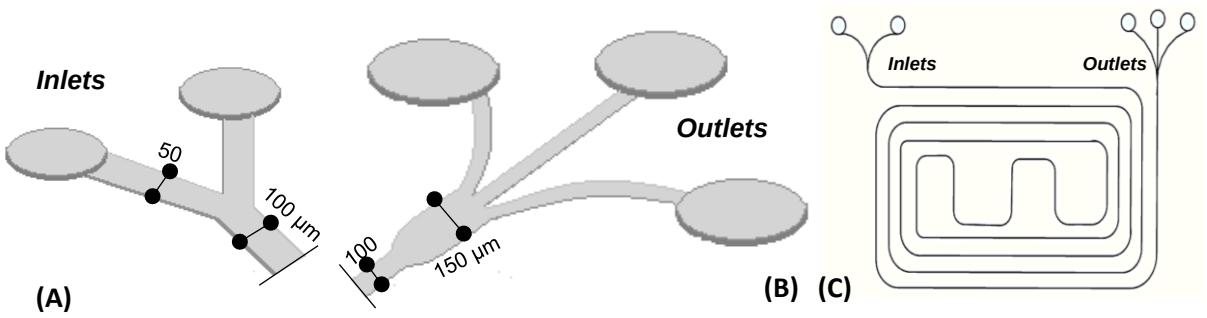

Figure 5 *- (A) Schematics of the two inlets of the microfluidic structure which converge to a main 15 cm long separation channel. (B) Schematics of the three outlets of the microfluidic structure. (C) Top view of the microfluidic structure design used for the molecular partition experiments in the miniaturized ATPE. The height of the microchannel is 20 µm high throughout the entire structure.*

channels (***Figure 5***), resorting to a fluorescence microscope to continuously monitor the labelled IgG concentration in the PEG and salt-rich phases. The small cross-section dimensions of the 15 cm long separation microchannel will provide fast diffusion times across the channel width, creating a continuous partition under laminar flow conditions and allowing for both phases to flow parallel to each other along the serpentine channel, designed to increase the microchannel length while keeping a small footprint. The salt-rich phase was spiked with the labelled IgG antibody and both phases were loaded to the two inlets of the microfluidic structure. The flow rates for each phase were carefully selected to create a stable interface between both immiscible phases throughout the entire length of the microfluidic channel: 0.2 $\mu L.min^{-1}$ for the PEG-rich phase and 1 $\mu L.min^{-1}$ for the salt rich phase.[15] In order to enable the tracking of the labelled IgG partition from one phase to the other, several fluorescence micrographs were acquired throughout the length of microfluidic channel. An example of these micrographs, for the conjugate IgG-BODIPY™ FL maleimide labelled without using TCEP, is presented in ***Figure 6***. To calculate the partition coefficient, the image captured at the end of the microchannel was used (***Figure 6C***), measuring the fluorescence value in each phase and subtracting to the background value. The results obtained for all the conjugates studied will be subsequently shown.

Amine-reactive Alexa Fluor® 430 and BODIPY™ FL NHS ester

Regarding the Alexa Fluor® 430 NHS Ester, a significant aggregation and precipitation effect could be observed when performing these studies. Therefore, the concentration of the dye-protein conjugates had to be reduced when compared to the BODIPY™ FL dyes: 15 $\mu g.mL^{-1}$ for the mL-scale and 25 $\mu g.mL^{-1}$ for the nL- scale, to avoid this aggregation effect. This aggregation might be due to the labelling of the primary amines of the IgG molecule by the Alexa Fluor® 430 generating a significantly more negatively charged dye-protein conjugate relative to the remaining fluorophores under study (***Figure 3***). This shift in pI makes the protein more neutral under the pH conditions (physiological conditions) used in the tested ATPE. When this happens, the proteins will possess both positively and negatively charged groups and the anisotropic charge distribution on the protein surface gives rise to dipoles, making protein–protein interactions, such as aggregation, highly energetically favourable.[20]

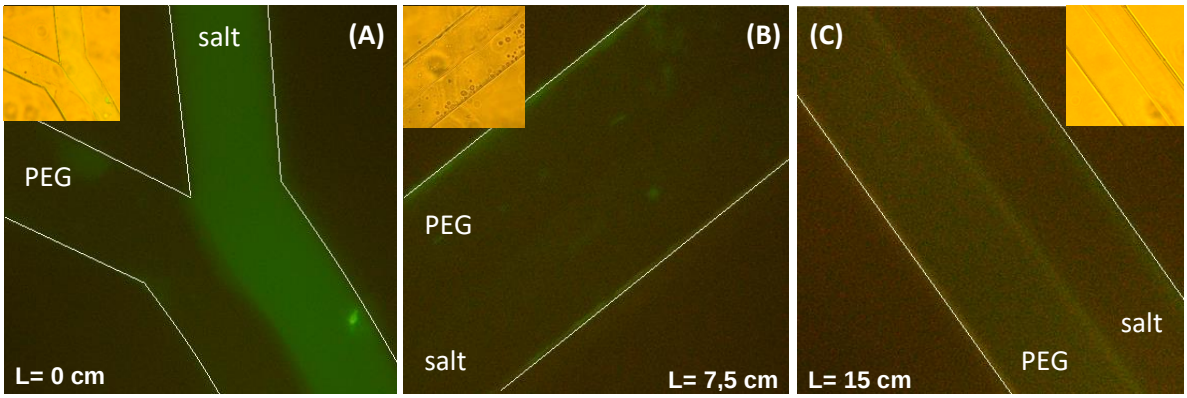

Figure 6 *- Examples of the fluorescence micrographs obtained throughout the microfluidic channel, with the system 12/12 PEG 3350/salt without the addition of NaCl, with the salt-rich phase spiked with IgG labelled with BODIPY™ FL maleimide without using TCEP, DOL 0.6, at a concentration of 50 $\mu g.mL^{-1}$ (the exposure time of 1 s and gain of 0 dB): (A) at the beginning of the channel, length 0 cm; (B) in the middle of the channel, length of 7,5 cm; and (C) at the end of the channel, length 15 cm. The corresponding bright field images are also presented as an inset.*

The results obtained for both quantification methods for both fluorophores are represented in ***Figure 7***. A major shift can be observed for the partition coefficient values obtained for the mL-scale and the nL-scale: for example, the IgG labelled with the dye Alexa Fluor® 430, DOL 2.4, the protein-dye conjugate remains almost completely in the salt-rich phase, with no diffusion occurring to the PEG-rich phase and resulting in a K_P < 1, which fully contradicts the results obtained at the mL-scale, where the obtained K_P > 1, indicating that the IgG molecule partitions to the PEG-rich phase.

Thiol-reactive BODIPY™ FL maleimide

Although the conjugates having identical DOL labelling, the achieved K_P values are significantly different, as can be observed in ***Figure 8***. A possible explanation can be the presence of free fluorophore, since without using TCEP, a lower number of free sulfhydryl groups are available for dye conjugation.[17] Therefore, to investigate the partition of this free fluorophore, a system of the 15/15 PEG 1500/salt and 15/15 PEG 8000/salt without NaCl were performed with only the addition of the unconjugated dye and it was observed for both systems that this molecule solely partitions to the PEG-rich phase, probably due to this phase being more hydrophobic, favouring the partition of hydrophobic molecules towards this phase. Additionally, for a lower molecular weight molecule, the excluded volume effect will not be present.[21] To confirm the presence of free fluorophore in the dye-protein conjugate labelled without using TCEP, protein G chromatography was performed for both labelled IgG, with and without the use of TCEP, as it is more sensitive and reproductive technique, where all the interfering components that might exist will be present in the flow-through, not binding to the column, and the IgG molecule will be the only component binding specifically to the column, selectively eluted later, with the results also being represented in ***Figure 8***.

4. Discussion

Regarding the amine-reactive fluorophore, Alexa Fluor® 430 and BODIPY™ FL NHS ester, no alterations in the molecular weight of dye-IgG conjugates were shown, except for the Alexa Fluor® 430 with a degree of labelling of 7.6, due to a high level of molecules of the fluorescent tag binding to the IgG and the superior molecular weight of this dye (***Figure 2***). However, for the isoelectric point of the labelled IgG (***Figure 3***), major differences were observed: the lysine residues, which are a positively charged group, confer an overall positive protein at a physiologic pH, but the labelling neutralizes these residues and the overall charged of the protein will diminish. Additionally, the Alexa Fluor® 430 dye is negatively charged[18], adding an even more negative charge to the overall IgG protein. The degree of labelling of the conjugate was also proven to be crucial to the antibody proprieties, with the isoelectric point and molecular weight dramatically changing with the increasing number of dye molecules attached to the IgG structure. Therefore, when labelling the

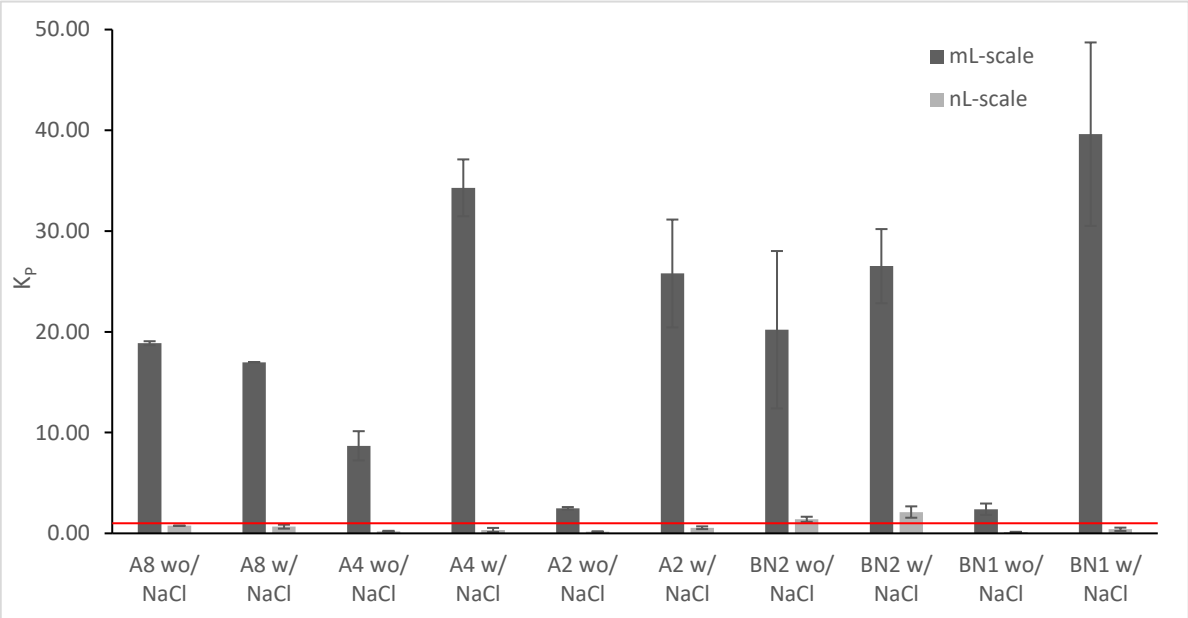

Figure 7 - *Graphic representation of the partition coefficient, K_P, of the IgG-Alexa Fluor® 430 NHS ester, DOL 7.6 (A8), the IgG-Alexa Fluor® 430 NHS ester, DOL 4.1 (A4), the IgG-Alexa Fluor® 430 NHS ester, DOL 2.4 (A2), the IgG-BODIPY ™ FL NHS Ester, DOL 2.6 (BN2), and the IgG-BODIPY ™ FL NHS Ester, DOL 1.4 (BN1), in the 12/12 PEG 3350/salt system, with and without the addition of NaCl, using fluorescence microscopy quantification methods: in a straight microchannel, mL-scale (20 µg.mL^{-1}), and in a microfluidic device, nL-scale (50 µg.mL^{-1}). The line, K_P=1, delimitates the IgG partition behaviour: when K_P<1, the partition is favoured to the salt-rich phase and when K_P>1, the partition is favoured to the PEG-rich phase. All the K_P values achieved are the average of two experiments and the error bars represent ±SD.*

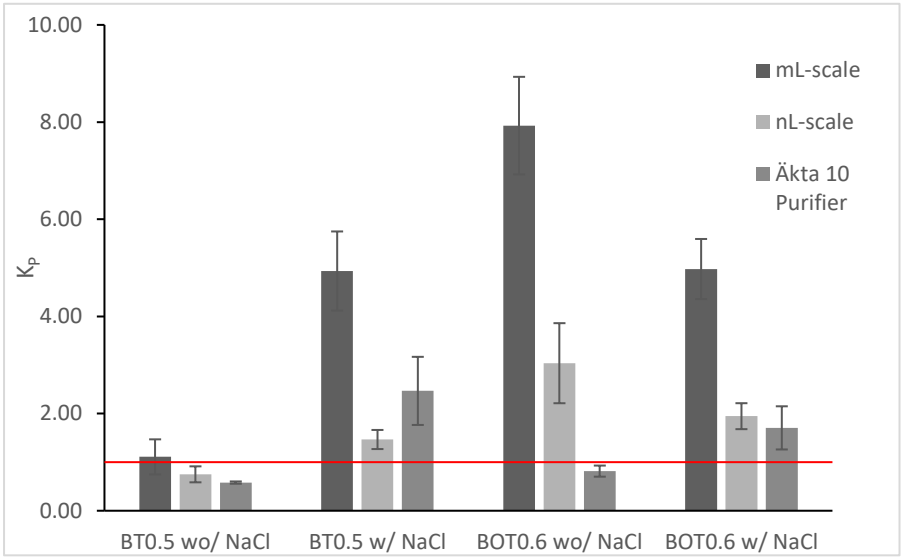

Figure 8 *- Graphic representation of the partition coefficient, K_P, of the IgG-BODIPY™ FL maleimide using TCEP, DOL 0.5 (BT0.5) and the IgG-BODIPY™ FL maleimide without using TCEP, DOL 0.6 (BT0.6), in the 12/12 PEG 3350/salt system, with and without the addition of NaCl, using different quantification methods, namely mL-scale ATPE followed by fluorescence microscopy in a straight microchannel (20 µg.mL^{-1}), ATPE in a microfluidic device (50 µg.mL^{-1}) and protein G chromatography in Äkta™ Purifier 10 system (30 µg.mL^{-1}). The line, K_P=1, delimitates the IgG partition behaviour: when K_P<1, the partition is favoured to the salt-rich phase and when K_P>1, the partition is favoured to the PEG-rich phase. All the K_P values achieved are the average of two experiments and the error bars represent ±SD.*

primary amines, the degree of labelling should be preferentially one molecule of dye for one molecule of protein, in order to minimize the decrease the negative effects of this type of labelling. Hence, the labelling of the primary amines of the IgG will produce in a more negative dye-protein conjugate, decreasing the isoelectric point and overall charge, which will subsequently result in the formation of aggregates at a neutral pH. Whereas, in the mL-scale (system spiked with the protein-dye conjugate), the partition arises from the convective mixing of the solution, resulting in the aggregates partition to the PEG-rich phase, in the nL-scale (spiking of the labelled IgG in the salt-rich phase), due to the general absence of turbulence, there is no convective mixing of the solution and molecular transport processes perpendicularly to the flow direction occurring by diffusion.[22] The formation of the aggregates will then reduce the diffusion coefficient of the IgG molecule and the antibody might not have time to diffuse from one phase to another in the length of the separation microchannel, which can explain the considerable differences obtained for the partition coefficient between the mL and nL-scale (***Figure 7***). Therefore, to provide sufficient residence time for the protein-dye conjugate, at the selected flow rates, a longer separation channel might be constructed to complete the extraction to the PEG-rich phase[15,22], as expected from the results achieved in the mL-scale.

Concerning the thiol-reactive BODIPY™ FL maleimide, it was established as the more promising fluorophore since the antibody properties, the molecular weight and the isoelectric point, do not change with the labelling (***Figure 2*** and ***3***). The addition of TCEP was also proven to be essential to this type of labelling in the IgG molecule, generating the free thiol group necessary, prior to conjugation, to the maleimide label, without disrupting the ternary structure. Without the addition of this reductant, the antibody will have a low concentration of free thiol groups available for the labelling, not allowing the fluorescent tag to bind, and, since the BODIPY™ FL dye is water insoluble[17], the unconjugated dye will not be completely eliminated. This free dye will have a singular partition behaviour, partitioning to the PEG-rich phase, which will disturb any fluorescence measures, creating dubious and biased K_P values (***Figure 8***). The partition coefficients obtained for the IgG labelled with BODIPY™ FL maleimide using TCEP were comparable to those calculated for the non-labelled molecule.

5. Conclusions and Future Work

The selection of the proper fluorophore to label proteins, such as antibodies, was proven to be a critical step for the development of miniaturized high-throughput screening tools applied in the context of ATPE optimization, allowing to accurately extrapolate the measurements to the native non-labelled molecule. The ideal fluorophore to label the IgG molecule should not overload the protein, generating a degree of labelling preferentially lower than one, and must present sufficient water solubility to prevent the formation aggregates. Thus, the labelling of the IgG molecule with the BODIPY™ FL maleimide, when using TCEP in the bioconjugation step, was the most promising labelling strategy found since it does not compromise the antibody properties, with the resulting conjugate having a low degree of labelling (less than 1) and the partition coefficients were comparable to those measured using the non-labelled molecule.

Since the molecular partitioning between both phases in ATPE is highly dependent on the surface properties of the proteins[21], such as its hydrophobicity and charge, properties which can potentially be affected by the fluorescent label, it is critical to evaluate the structural integrity of the protein. Here, SDS-PAGE and isoelectric focusing were used to characterize the conjugates, however, additional structural studies, based on nuclear magnetic

resonance (NMR), X-ray diffraction, circular dichroism or fourier-transform infrared spectroscopy (FTIR), may also be performed to provide complementary information. It should also be highlighted that this experimental work was performed by labelling Gammanorm IgG, a mixture of different subclasses of IgG and each subclass has a unique profile with different molecular weights and pIs, instead of monoclonal antibodies. Hence, further validation of these results applied to different monoclonal antibodies with defined pIs is required.

Although microfluidics combined with fluorescence microscopy allows the continuous monitoring and quantification of the partition of the IgG molecule in ATPE, with all its intrinsic advantages, this experimental work demonstrated that the appropriate selection of fluorophore in this context is an extremely challenging and critical task. Hence, the development of microfluidic aqueous two-phase extraction screening devices without resorting to fluorescence microscopy would be highly advantageous, where the formation of the two immiscible phases and stabilization of the interphase could be automatically controlled by optical microscopy[23] and the collection of each phase through the three outlet could be coupled to a secondary detection strategy such as on-chip analytical protein G chromatography or an immunoassay. On the other hand, to still benefit from an on-line reading of partition throughout the separation channel, the incorporation of biosensors in microfluidic devices has been recently explored[24,25], including the integration of microfluidic UV absorbance detectors[26], which could be incorporated as a dense miniaturized array along the separation column and/or aligned with the outlets of the device.

References

1. Azevedo, A., Rosa, P., Ferreira, I., Aires-Barros, M., **Optimisation of aqueous two-phase extraction of human antibodies**, *Journal of Biotechnology,* Vol. 132, pp. 209–217, 2007.
2. Azevedo, A., Rosa, P., Ferreira, I., de Vries, J., Visser, T., Aires-Barros, M., **Downstream processing of human antibodies integrating an extraction capture step and cation exchange chromatography**, *Journal of Chromatography B,* Vol. 877, pp. 50–58, 2009.
3. Rosa, P., Azevedo, A., Sommerfeld, S., Mutter, M., Aires-Barros, M., Bäcker, W., **Application of aqueous two-phase systems to antibody purification: A multi-stage approach**, *Journal of Biotechnology, Vol.* **139,** pp. 306–313, 2009.
4. Roque, A., Silva, C., Taipa, M., **Affinity-based methodologies and ligands for antibody purification: Advances and perspectives**, *Journal of Chromatography A,* Vol. 1160, pp. 44–55, 2007.
5. Silva, D., Azevedo, A., Fernandes, P., Chu, V., Conde, J., Aires-Barros, M., **Determination of partition coefficients of biomolecules in a microfluidic aqueous two phase system platform using fluorescence microscopy**, *Journal of Chromatography A,* Vol. 1487, pp. 242–247, 2017.
6. Sulk, B., Birkenmeicr, G., Kopperschäger, G., **Application of phase partitioning and thiophilic adsorption chromatography to the purification of monoclonal antibodies from cell culture fluid,** *Journal of Immunological Methods,* Vol. 149, pp. 165–171, 1992.
7. Azevedo, A.,Rosa, P., Ferreira, I., Aires-Barros, M., **Integrated process for the purification of antibodies combining aqueous two-phase extraction, hydrophobic interaction chromatography and size-exclusion chromatography**, *Journal of Chromatography A,* Vol. 1213, pp. 154–161, 2008.
8. Rosa, P., Azevedo, A., Aires-Barros, M., **Application of central composite design to the optimisation of aqueous two-phase extraction of human antibodies**, *Journal of Chromatography A,* Vol. 1141**,** pp, 50–60, 2007.
9. Nam, K., Chang, W., Hong, H., Lim, S., Kim, D., Koo, Y., **Continuous-flow fractionation of animal cells in microfluidic device using aqueous two-phase extraction**, *Biomedical Microdevices,* Vol. 7, pp. 189–195, 2005.
10. Tsukamoto, M., Taira, S., Yamamura, S., Morita, Y., Nagatani, N., Takamura, Y., Tamiya, E., **Cell separation by an aqueous two-phase system in a microfluidic device**, *Analyst, Vol.* 134, pp. 1994–1998, 2009.
11. Huh, Y., Jeong, C., Chang, H., Lee, S., Hong, W., Park, T., **Rapid separation of bacteriorhodopsin using a laminar-flow extraction system in a microfluidic device**, *Biomicrofluidics,* Vol. 4, pp. 1–10, 2010.
12. Hu, R., Feng, X., Chen, P., Fu, M., Chen, H., Guo, L., Liu, B., **Rapid, highly efficient extraction and purification of membrane proteins using a microfluidic continuous-flow based aqueous two-phase system**, *Journal of Chromatography A*, Vol. 1218, pp. 171–177, 2011.
13. Soares, R., Novo, P., Azevedo, A., Fernandes, P., Aires-Barros, M., Chu, V., Conde, J.**, On-chip sample preparation and analyte quantification using a microfluidic aqueous two-phase extraction coupled with an immunoassay**, *Lab on a Chip,* Vol. 14, pp. 4284–4294, 2014.
14. Jacinto, M. J., Soares, R., Azevedo, A., Chu, V., Tover, A., Conde, J., Aires-Barros, M., O**ptimization and miniaturization of aqueous two phase systems for the purification of recombinant human immunodeficiency virus-like particles from a CHO cell supernatant,** *Separation and Purification Technology,* Vol. 154, pp. 27–35, 2015.
15. Silva, D., Azevedo, A., Fernandes, P., Chu, V., Conde, J., Aires-Barros, M., **Design of a microfluidic platform for monoclonal antibody extraction using an aqueous two-phase system,** *Journal of Chromatography A,* Vol. 1249, pp. 1–7, 2012.
16. Pinto, I., Caneira, C., Soares, R., Madaboosi, N., Aires-Barros, M., Conde, J., Azevedo, A., Chu, V., **The application of microbeads to microfluidic systems for enhanced detection and purification of biomolecules**, *Methods,* Vol. 116, pp. 112–124, 2017.
17. Hermanson, G., **Bioconjugate Techniques**, *Academic Press*, 2nd Edition, 2008.
18. Thermo Fisher Scienctific, **The Molecular Probes Handbook: a Guide to Fluorescent Probes and Labeling Technologies**, 2010.
19. Vu, T., Dvorko,M., Schmidt, E., Audibert, J., Retailleau, P., Trofimov, B., Pansu, R., Clavier, G., Méallet-Renault, R., **Understanding the spectroscopic properties and aggregation process of a new emitting boron dipyrromethene (BODIPY)**, *The Journal of Physical Chemistry* C, Vol. 117, pp. 5373–5385, 2013.
20. Chi, E., Krishnan, S., Randolph, T., Carpenter, J., **Physical stability of proteins in aqueous solution: Mechanism and driving forces in nonnative protein aggregation**, *Pharmaceutical Research*, Vol. 20, no. 9, pp. 1325- 1336, 2003.
21. Asenjo, J., Andrews, B., **Aqueous two-phase systems for protein separation: A perspective**, *Journal of Chromatography A*, Vol. 1218, pp. 8826–8835, 2011.
22. Soares, R., Silva, D., Fernandes, P., Azevedo, A., Chu, V., Conde, J., Aires-Barros, M., **Miniaturization of aqueous two-phase extraction for biological applications: From micro-tubes to microchannels,** *Biotechnology Journal,* Vol. 11, pp. 1498–1512, 2016.
23. Silva, D., Azevedo, A., Fernandes, P., Chu, V., Conde, J., Aires-Barros, M., **Determination of aqueous two phase system binodal curves using a microfluidic device**, *Journal of Chromatography A,* Vol. 1370, pp. 115–120, 2014.
24. Soares, R., Santos, D., Chu, V., Azevedo, A., Aires-Barros, M., Conde, J., **A point-of-use microfluidic device with integrated photodetector array for immunoassay multiplexing: Detection of a panel of mycotoxins in multiple samples**, *Biosensors and Bioelectronic*, Vol. 87, pp. 823–831, 2017.
25. Pinto, I., Santos, D., Caneira, C., Soares, R., Azevedo, A., Chu, V., Conde, J., **Optical biosensing in microfluidics using nanoporous microbeads and amorphous silicon thin-film photodiodes: Quantitative analysis of molecular recognition and signal transduction**, *Journal of Micromechanics and Microengineering*, Vol. 28, 2018.
26. Zhu, L., Lee, C., DeVoe, D., **Integrated microfluidic UV absorbance detector with attomol-level sensitivity for BSA**, *Lab on a Chip,* Vol. 6, pp. 115–120, 2005.