Magnetic precipitation: a new platform for protein purification

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Abstract

One of the trends in downstream processing comprises the use of "anything-but-chromatography" methods to overcome the current downfalls of standard packed-bed chromatography. Precipitation and magnetic separation are two techniques already proven to accomplish protein purification from complex media, yet never used in synergy. With the aim to capture antibodies directly from crude extracts, a new approach combining precipitation and magnetic separation was developed and named as affinity magnetic precipitation. A precipitation screening, based on the Hofmeister series, and a commercial precipitation kit were tested with affinity magnetic particles to assess the best condition for antibody capture from human serum plasma and clarified cell supernatant. The best conditions were obtained when using PEG3350 as precipitant at 4ºC for 1h, reaching 80% purity and 50% recovery of polyclonal antibodies from plasma, and 99% purity with 97% recovery yield of anti-TNFα mAb from cell supernatants. These results show that the synergetic use of precipitation and magnetic separation can represent an alternative for the efficient capture of antibodies.

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1 Introduction

Downstream processing of biological products usually comprises four main steps: recovery, isolation, capture and polishing, and accounts for up to 60-80% of the total manufacturing costs [1]. The purification of high added value biological drugs is mostly based on chromatographic methods [2,3]. Monoclonal antibodies (mAbs) and derived molecules are the most important players in the biopharmaceutical industry [4]. Albeit the advances in antibody engineering, antibody manufacturing still presents challenges. The improvement on upstream processes producing high volumes and product titers (up to g/l), has added several limitations to the overall purification process mostly due to the high matrix cost, diffusion limitations and limited binding capacity [5]. To overcome these problems, anything but chromatography (ABC) approaches emerged as promising alternatives. Although most ABC approaches are not new concepts, they are currently being re-adapted for the purification of biopharmaceuticals. The non-chromatographic methods used in ABC include membrane separation methods [6], aqueous two-phase systems [7–9], magnetic separation [10–12], precipitation and crystallization [5,13–15].

Precipitation is mainly used for the purification of low value products [16,17]. However, this method can cope with high titers and volumes produced upstream, since it scales-up with process volume, whereas chromatographic methods scale stoichiometrically with the reaction between molecules in solution [18]. Protein precipitation relies on the supersaturation of a homogenous protein solution. To exceed the proteins' saturation limit, changes in its environment must be introduced. Even though precipitation is a rather empirical methodology, several additives (e.g. salts or polymers) are known to help exceed a protein's solubilization limit. However, this effect is dependent on the target protein and on the salt or polymer concentration [19]. Protein precipitation by salt addition follows frequently the Hofmeister series [20], as it ranks the anions and cations according to their relative effect on protein solubility [21,22]. Kosmotropic agents are referred as "water structure makers" that promote the salting-out of proteins; chaotropic agents are defined as "water structure breakers" that promote the salting-in effect. On the other hand, polymers such as polyethylene glycol (PEG), with a molecular weight usually higher than 4000 Da, are often associated with protein precipitation. PEG captures water molecules from the proteins surface, enabling the establishment of protein-protein interactions that lead to protein precipitation [23–25]. Other parameters, mainly pH and temperature, also play a role in protein precipitation by interfering in inter-protein interactions. Precipitation methods have been attempted for mAbs purification, although many times the precipitated crude extract samples were previously conditioned, either purified by affinity chromatography or dialyzed in a more suitable buffer for precipitation. Nonetheless, recent studies show that precipitation can be used to purify mAbs that maintain structural and functional characteristics [14,26–28].

The magnetic separation of proteins, directly from crude extracts, using magnetic adsorbents, is another known ABC alternative [12,29,30]. Due to their superparamagnetic properties, iron oxide magnetic particles (MPs) can be used in viscous solutions to separate a target protein, presenting a high surface area to volume ratio. Given the virtual infinite surface modifications that can be made at the particles surface, different coatings (namely dextran) and functionalizations can be performed to

target a protein from a complex crude extract [30, 31]. From the different functionalization strategies, small synthetic affinity ligands, rationally designed towards a target protein, represent a low cost and viable option. For mAbs, the triazine synthetic ligand 22/8 was designed to capture antibodies from complex crude extracts [32], and its functionalization in MPs accomplished and optimized [10,30,33]. There are examples on the use of MPs alone (magnetic fishing) or in combination with other unit operations as aqueous two-phase systems [9,34,35].

Precipitation and magnetic separation were never explored in synergy for antibody purification from complex media. The potential to use a hybrid system based on precipitation and magnetic fishing with affinity towards protein purification was explored here. Such an approach enables the selective capture and concentration of antibodies in a single step.

2 Materials and methods

2.1 Materials

All used chemicals were at least 98% pure of analytical or HPLC grade.

The 96-well UV-star® half area microplates from Greiner (Kremsmünster, Austria) and 96-well transparent microplates flat-bottom from Sarstedt (Nümbrecht, Germany).

2.2 Biological material

Pure polyclonal antibodies (pAbs) (product name: Gammanorm ®, from Octapharma (Lachen, Switzerland)) and pAbs from human serum plasma (Sigma-Aldrich) were used for screening of precipitation conditions and capture from complex media, respectively. Clarified cell culture supernatant from CHO cell lines producing anti-TNFα or anti-HER2 monoclonal antibodies (mAbs) were used for screening and capture of mAbs by precipitation.

2.2.1 Anti-TNFα monoclonal antibody and anti-HER2 monoclonal antibody production

The anti-tumor necrosis factor alpha (anti-TNFα) mAb and anti-human epidermal growth factor receptor 2 (anti-HER2) mAb were produced in FreeStyle™ 293-F cells (Thermo Fisher Scientific, Waltham, Massachusetts, USA) transfected using polyethylenimine (PEI) (Polysciences, Warrington, Pennsylvania). The details about the biological materials used can be found in the Supporting Information (SI 1).

2.3 Production and characterization of magnetic particles

Iron oxide magnetic particles were synthesized by the co-precipitation method as described by [30]. After synthesis, magnetic particles coated with dextran (MP-Dextran) were further functionalized with synthetic triazine ligand 22/8 (MP-22/8) as described by [30] (SI 2, Figure SI 1A).

All particles were characterized by Fourier transform infrared (FT-IR), dynamic light scattering (DLS), zeta potential and transmission electron microscopy (TEM). DLS and zeta potential (0.05 mg/ml

solution in MilliQ and precipitant solution (20% PEG3350)) were measured using Dynamic Zetasizer Nano ZS from Malvern instruments (Figure SI 1B and C). FT-IR spectra were recorded on a Spectrum Two™ spectrometer from Perkin Elmer. MP samples were prepared drying the samples overnight at 60ºC, then grounding and mixing with KBr (1:100) and finally pressing in a hydraulic press, up to 10 tones force (Figure SI 1D). Analytical TEM was done in a Hitachi 8100 microscope with ThermoNoran light elements EDS detector and digital image acquisition without staining (Figure SI 1E).

2.4 Antibody purification

2.4.1 Monoclonal antibodies precipitation assays

Different precipitation conditions (SI 3, Table SI 1) based on the Hofmeister series were screened for antibody precipitation using pure human pAb at 2 and 20 mg/ml, and human serum plasma at 0.5 mg/ml of total protein. Simultaneously, crystallization experiments using mAbs were carried out at the High Throughput Crystallization Laboratory (EMBL Grenoble), using the following crystallization screens PEGs-I (from Qiagen), PACT *premier*™ HT-96 (from Molecular Dimensions), JCSG-plus™ HT-96 (from Molecular Dimensions) and Wizard Classic 1 & 2 (from Rigaku). From the conditions tested, two additional precipitation conditions for antibody precipitation were unveiled – 20% PEG3350 and 2.5 M NaCl (Table SI 2). The precipitation assays were carried out in 2 ml tubes placed vertically in a tube holder at 20ºC for 1h with 200 RPM orbital shaking (20 mm agitator shaking diameter (KS 4000 i control, from IKA)) with a 1:1 crude extract to precipitant volumetric ratio, at a 1 ml scale. The separation of supernatant and precipitate was performed by centrifugation (5000 *xg*, 15min at 15ºC). The supernatant was removed, and the precipitate solubilized in 10 mM sodium phosphate, 150 mM sodium chloride at pH 7.4 (PBS). Protein quantification of pAb supernatant and precipitate for the different precipitant conditions were performed by measuring absorbance at 280 nm in UV-star® half area microplates from Greiner. The pAb precipitation yield was calculated as in Equation 1:

$$\text{Precipitation yield (\%)} = = \frac{\mu g \text{ pAb precipitated}}{\mu g \text{ pAb loaded}} \times 100 \qquad \text{Equation 1}$$

For plasma samples, total protein quantification was performed using bicinchoninic acid (BCA) assay reagent (Sigma-Aldrich). Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed to evaluate the samples Immunoglobulin (Ig) recovery and purity. Samples were applied in a 12.5% acrylamide gel and migrated at 90 mV. Images were acquired with Gel Doc™ XR+ System with Image Lab™ software from Bio-Rad and analyzed with ImageJ software. The total protein precipitation yield was calculated as in Equation 2:

$$\text{Precipitation yield (\%)} = = \frac{\mu g \text{ total protein precipitated}}{\mu g \text{ total protein loaded}} \times 100 \qquad \text{Equation 2}$$

2.4.2 Affinity magnetic precipitation

The four best performing precipitant conditions - 300 mM $LiSO_4$, 300 mM $CaCl_2$, 20% PEG3350 and 2.5 M NaCl - were tested for the affinity magnetic precipitation of antibodies having 0.5 mg/ml MP-Dextran (control) or MP-22/8. Affinity magnetic precipitation was carried out at a 1 ml scale with a 1:1 crude extract to precipitant volumetric ratio. Prior to incubation with the crude extract, all MP solutions were sonicated and homogenized for 10 minutes. The crude extract and magnetic precipitant incubation were optimized in terms of orbital shaking (200 *vs* 0 RPM), temperature (4º *vs* 20ºC) and time (1h *vs* 24h). The best incubation parameters were 200 RPM at 4ºC for 1h. Crude extract and magnetic precipitant were incubated, and the MPs separated by the application of an external magnetic field for 15 min, generating the magnetic supernatant and magnetic precipitate. The magnetic supernatant was further centrifuged (5000 *xg*, 15min at 15ºC), the supernatant harvested, and the precipitate resuspended in PBS and the bound proteins eluted. MPs were recovered from solution by the application of an external magnetic field for 15 minutes and the eluted proteins were harvested in the soluble fraction. The magnetic precipitation yield was calculated as in Equation 6.3:

$$\text{Magnetic precipitation yield (\%)} = = \frac{\mu g \text{ total protein recovered}}{\mu g \text{ total protein loaded}} \times 100 \qquad \text{Equation 3}$$

This pipeline was performed for antibody capture from human serum plasma (0.5 mg/ml total protein), anti-TNFα monoclonal antibody and anti-HER2 monoclonal antibody from clarified cell culture supernatant (10 mg/ml total protein), the assays were performed in triplicates for each precipitation condition. As controls, the magnetic precipitation was performed with PBS as precipitant with and without MP. Total protein quantification was performed using BCA assay reagent (Sigma-Aldrich) (SI 4). SDS–PAGE was performed to evaluate samples' purity and composition (SI 5). The antibody recovery yield was calculated by densitometry as in Equation 4 and purity as in Equation 5:

$$\text{Antibody recovery yield (\%)} = = \frac{\text{Area of heavy and light chains of antibody recovered}}{\text{Area of heavy and light chains of antibody loaded}} \times 100 \qquad \text{Equation 4}$$

$$\text{Antibody purity (\%)} = = \frac{\text{Area of heavy and light chains of antibody recovered}}{\text{Area total of protein recovered}} \times 100 \qquad \text{Equation 5}$$

High performance size-exclusion chromatography was carried out to assess IgG aggregation and HPLC purity resulting from the precipitation strategy developed for antibody purification (SI 6).

2.4.3 Assessing mAb activity by Indirect enzyme-linked immunosorbent assay – Anti-TNFα mAbs

To evaluate the biological activity of anti-TNFα monoclonal antibodies after purification by precipitation, an indirect enzyme-linked immunosorbent assay (ELISA) was performed, 96-well half-area microplates (Corning Costar, USA) were coated with human TNFα (200 ng/well) in PBS and

incubated overnight at 4ºC. The plate was then aspirated and washed once with 150 µl PBST (PBS with 0.05% (v/v) Tween® 20). Nonspecific binding sites were blocked with 3% (w/v) BSA in PBST for 1h at 37°C. Afterwards, the plate was washed as previously above. 50 µl of the purified anti-TNFα mAb samples (0.1 µl/ml) and calibration curve (Infliximab, Remicade® 10 mg/ml at 0; 0.01; 0.03; 0.05; 0.1; 0.2; 0.3 µg/ml in 1% (w/v) BSA in PBST) were added and incubated for 1h at 24ºC. Subsequently, the plate was washed 3 times as previously described. Detection was performed with the addition of 50 µl of secondary antibody anti-Human Kappa Light Chain-horseradish peroxidase antibody from goat (RRID: AB_ 2535630, Invitrogen) diluted 1:50,000 (0.02 ng/ml) in 1% (w/v) BSA in PBST. The secondary antibody was incubated for 30 min at 24ºC, and the plate was washed 3 times as previously described. The reaction was developed with 50 µl TMB (3,3',5,5'-Tetramethylbenzidine, Merck, Germany) substrate for 30 min at 24ºC in the dark and stopped with 2 M H_2SO_4 (Merck) for 30 min at 24ºC in the dark. Absorbances were registered at 450 nm, on Model 680 microplate reader (Bio-Rad, USA).

2.4.4 Assessing mAb activity by Indirect flow cytometry – Anti-HER2 mAbs

The activity of anti-HER2 monoclonal antibodies after purification through precipitation was determined by flow cytometry analysis. Cells were harvested with dissociation buffer (0.6 nM EDTA in PBS) and centrifuged at 300 x*g* for 5 min and resuspended in ice cold PBS with 3% (w/v) BSA (Sigma) to a final concentration of 1 x 10^6 cells/ml. Cell suspension with 1 x 10^5 cells were incubated with 0.5 µg of anti-HER2 mAbs samples for 30 min at 4ºC. Afterwards, the cells were washed twice by centrifugation at 300 x*g* for 5 min and resuspended in ice cold PBS with 3% (w/v) BSA. The detection was performed with addition of the secondary antibody Goat anti-Human IgG (H+L) Cross-Adsorbed Secondary Antibody conjugated with FITC (RRDI: AB_2535589, Invitrogen), diluted to a final concentration of 1:400. The secondary antibody was incubated for 30 min at 4ºC in the dark, the cells were then washed by centrifugation as previously described and 10 µl of propidium iodide (10 µg/ml, Sigma) added to the stained cells. Assessment of antibody binding were performed using Guava easyCyte™ (Merck Millipore). Flow cytometry data was analysed with FlowJo software (TreeStar).

3 Results and discussion

3.1 Screening conditions for antibody precipitation

Salts have a major effect in protein solubility and propensity to precipitate. The Hofmeister series ranks the salt anions and cations according to their kosmotropic and chaotropic character, translating into a salting-out or salting-in effect on proteins, respectively. An initial precipitation screen inspired in the Hofmeister series was designed for the precipitation of pure human polyclonal antibody (pAb) at 2 and 20 mg/ml (Figure 1A). All tested precipitation conditions lead to pAb precipitation, however the majority did exhibit a very low precipitation yield (<20%). In addition, when the precipitant

concentration was constant, a 10-fold increase in protein concentration did not reflect a 10-fold increase in the precipitation yield. The combination of these results may indicate that the precipitant concentration is lower for the protein to precipitate in the set conditions. Nonetheless, and since protein-protein interactions also play a major role in protein precipitation, the same rationale was applied to human serum plasma, with a total protein concentration of 0.5 mg/ml, with the aim to precipitate and capture the pAb. Two additional precipitation conditions (unveiled by a high throughput precipitation screening) were tested – 20% (w/v) PEG3350 and 2.5 M NaCl (Figure 1B). Of the 21 precipitation conditions tested, 5 presented a precipitation yield higher than 40%, namely 300 mM $LiSO_4$; 300 mM $CaCl_2$; 300 mM $CaCl_2$, pH7; 20% (w/v) PEG3350 and 2.5 M NaCl (Figure 1B). However, none of the different conditions enabled a selective precipitation of the pAb (Figure 1C).

3.2 Affinity magnetic precipitation – pAb capture from human serum plasma

To provide antibody selectivity, magnetic particles functionalized with a triazine-base ligand, known to bind antibodies (ligand 22/8) [32], were used as additives in the precipitant solution. The orbital shaking, temperature and incubation time of crude extract and precipitant in presence of affinity magnetic particles (MP-22/8) were optimized (Figure 2) using human serum plasma with a total protein concentration of 0.5 mg/ml. The orbital shaking (200 *vs* 0 RPM) had a notorious effect in the precipitation yield, however, depending on the precipitant, the orbital shaking had a positive, negative or neutral effect. For 300 mM $LiSO_4$ and $CaCl_2$, the lack of orbital shaking had a negative effect in the precipitation yield, whereas for 2.5 M NaCl the lack of orbital shaking had a positive effect in the precipitation yield. For 20% (w/v) PEG3350 the precipitation yield was not affected by the orbital shaking (Figure 2B). The orbital shaking at 200 RPM does improve the homogeneous dispersion of MPs in solution, nevertheless both 2.5 M NaCl and 20% (w/v) PEG3350 precipitation conditions were unveiled in static crystallization assays. In the case of 20% (w/v) PEG3350, the orbital shaking, or lack of it, did not present any difference in the final precipitation yield, mainly due to the MPs stability in this solution. On the other hand, in 2.5 M NaCl the same did not occur, the orbital shaking led to a more stable condition maintaining the protein in solution, hence a lower precipitation yield (Figure 2B).

Protein-protein interactions mainly occur by weak interactions such as hydrophobic interactions, hydrogen bonds, salt bridges or van der Waals forces [37]. Since precipitation is dependent on protein-protein interactions, and being these interactions temperature-dependent, the precipitation yield was assessed at 4ºC and 20ºC (Figure 2C), at high salt concentration or in the presence of hygroscopic species, such as PEG. At lower temperatures, hydrogen bonds and salt bridges are weakened [38], contributing to the destabilization of proteins in solution. This explains the results obtained, since protein precipitation with 20% (w/v) PEG3350 and 2.5 M NaCl showed a greater increase in the magnetic precipitation yield from 20ºC to 4ºC. On the other hand, the change in temperature did not have a major influence in protein magnetic precipitation yield with 300 mM of $LiSO_4$ and $CaCl_2$. At last, the incubation time was optimized, and two time points were checked – 1 h and 24 h. In all conditions, without exception, the 24h incubation led to a lower magnetic precipitation yield (Figure 2D). This phenomenon can be due to the equilibrium that is established between protein in solution and protein precipitated. Additionally, the lower precipitation yield was not proportional to a

higher antibody purity and capture. In this way, the best incubation condition was 200 RPM at 4ºC for 1 h. For the tested precipitants, the best performer was 20% (w/v) PEG3350, as it was the condition with higher pAb content in the elution fraction. Since precipitant concentration does play a major role, three different concentrations of $LiSO_4$, $CaCl_2$ (100 mM; 200 mM and 300 mM), PEG3350 (10%; 20% and 30% (w/v)) and NaCl (1.5 M, 2.5 M and 3.5 M) were tested in the presence of MP-22/8 (Figure 3A), MP-Dextran (Figure 3B) and in the absence of MPs (Figure 3C). PEG3350 showed to be the best precipitant condition with higher antibody recovery and purity (Figure 3).

Although the MPs were functionalized with a synthetic affinity ligand with a K_a of 7.7×10^5 M^{-1} in 50 mM phosphate buffer at pH 8 [30], the affinity between two molecules is regulated by the interactions established between them on defined solution conditions, such as ionic strength or pH. The 22/8 triazine affinity ligand interacts with the Fab and Fc domains of IgG molecule but with higher affinity towards the Fc [39]. Nonetheless, affinity interactions are a combination of hydrophobic, H-bonding and electrostatic interactions, strongly dependent on the pH and ionic strength [39]. All precipitant solutions presented a pH range between 5 and 8, except $LiSO_4$ that had a pH range between 3 and 4. Previous theoretical and experimental data suggest that pH plays a role on the interaction between 22/8 and IgG, but also the matrix used to attach the ligand. In this work, the elution from MP-22/8 was performed with PBS at pH 7.4, suggesting that the pH did not have a major contribution in the present situation, but the ionic strength of the precipitant solutions did, not only due to the elution buffer but also due to the best performing precipitant – 20% (w/v) PEG3350 – that presented the lower ionic strength. PEG is a polymer known to attract water molecules from the protein's surface increasing its interprotein interactions, leading to protein precipitation. Furthermore, in solution, PEG presents a amphiphilic character, exhibiting hydrophilic and hydrophobic characteristics, although neglectable PEG-protein interaction can induce protein conformational changes leading to the exposure of the protein hydrophobic residues that may interact with other proteins, PEG molecules or small molecules [40].

The increasing PEG3350 concentration (from 10% to 30% (w/v)) led to a higher pAb recovery and purity in the presence of MP-22/8 as additives, reaching 80% purity and 50% recovery, whereas when MP-Dextran was used as additive, 43% pAb was recovered with 23% purity. When No MPs were added, similar results were obtained with 47% recovery and 32% purity (Figure 4A and B). In the absence of PEG, having only PBS in solution, no pAb was recovered in the elution fraction (Figure 4A and B). Without the presence of PEG, having only PBS in solution, no pAb was recovered in the elution fraction (Figure 4A and B). The low recovery yields obtained were due to the co-elution of human serum albumin (HSA), the most abundant protein in human serum plasma. Even though most HSA did not precipitate or bind to the MPs (Figure 4C), a portion of this protein still precipitated and co-eluted with pAb. Nevertheless, at concentrations higher than 20% (w/v) PEG3350, pAb was only recovered in the elution fraction (Figure 4C)

3.3 Affinity magnetic precipitation – mAb capture from CHO clarified supernatant

Affinity magnetic precipitation was tested for the capture of two monoclonal antibodies (mAbs) from clarified cell supernatants, both IgG1– anti-TNFα and anti-HER2 – using PEG3350 as precipitant. The incubation step was performed with 200 RPM at 4ºC for 1h with the respective precipitant solution. Control experiments included an assay with MP-Dextran and without MPs using PBS at pH 7.4 as a precipitant control. MP-Dextran were used as control since they are an intermediate product of the MP-22/8 synthesis and the use of high salt solution (> 0.25 M) and PEG may lead to non-specific interactions between the medium proteins and the solid matrix [41].

For anti-TNFα mAb capture, three different PEG3350 concentrations were tested – 10%, 20% and 30% (w/v) – to assess IgG recovery and purity in the elution fraction (Figure 5). The maximum recovery was observed with 20% (w/v) PEG3350. For 10% (w/v) PEG3350, approximately 10% of the mAb did not precipitate onto the magnetic particles and leaked in the supernatant fraction. On the other hand, with 30% (w/v) PEG3350, 20% of the mAb precipitated without binding to MP-22/8 (Figure 5C). Although pure, the recovery of this fraction comprises an additional centrifugation step in the downstream pipeline of anti-TNFα mAb capture. In this way, the best recovery and purity yields were obtained with 20% (w/v) PEG3350 using MP-22/8, reaching a 97% recovery and 99% purity, as opposed to 31% and 52% recovery from MP-Dextran and no MP, respectively, and 10% recovery using PBS (Figure 5).

For anti-HER2 mAb, only the best precipitant solution was tested – 20% PEG3350 – with 200 RPM at 4ºC for 1h and keeping the same MPs controls: MP-Dextran and no MP (Figure 6). The best results were obtained using MP-22/8 with 100% recovery and 63% purity, whereas for MP-Dextran 89% recovery and 55% purity was obtained. When no MPs were used, the recovery yield was 87% with 49% purity.

The two mAbs tested were produced in two different types of clarified CHO cell supernatant. While anti-TNFα mAb was produced in a serum free medium, anti-HER2 mAb was produced in a serum containing medium, making this a less pure medium with lower IgG to total protein ratio. Since protein concentration is one of the factors for protein precipitation – the higher the target protein concentration, the higher the precipitation yield – the ratio IgG to total protein should be as high as possible. On the other hand, if the ratio is low, multiple rounds of non-denaturing precipitation can be performed until higher purities are obtained.

The presence of aggregates and biological activity were evaluated after antibodies capture by affinity magnetic precipitation. Three different techniques were used: (i) HPLC size-exclusion for both mAbs; (ii) indirect enzyme-linked immunosorbent assay (ELISA) for anti-TNFα mAbs and (iii) indirect flow cytometry for anti-HER2 mAbs.

Size exclusion chromatography was performed in order to evaluate if IgG aggregates were present. The magnetic precipitation of anti-TNFα mAbs in the presence of MP-22/8 did not show aggregates formation, presenting 98% HPLC-purity across all PEG3350 concentrations. The same behaviour was observed in the presence of MP-dextran, presenting 97% HPLC-purity. When no MPs were present,

high HPLC-purities of 98% were only possible with 20% and 30% (w/v) PEG3350. For anti-HER2 mAb, magnetic precipitation with MP-22/8 showed the presence of possible mAb aggregates, representing 30%, being the mAb monomer present with 66% HPLC-purity. On the other hand, when MP-dextran was present, the formation of aggregates was reduced to 3% and the mAb monomer HPLC-purity increased to 84%.

The biological activity of purified anti-TNFα mAb was evaluated by indirect ELISA (Figure 7A). The elution fractions from the magnetic precipitation using PEG 3350 as precipitant at different concentrations in the presence of either 22/8 or dextran-coated MPs were analyzed, as well as the eluted fractions of mAb precipitation without the presence of MPs. Magnetic precipitation - MPs in the presence of PEG3350 as precipitant - presented higher mAb activity when compared to magnetic fishing - MPs in the presence of PBS (Figure 7A) - indicating that PEG can stabilize the mAb along the precipitation steps and does not diminish nor interfere with the mAb biological activity. In the conditions tested, mAb activity varied depending on the MP present. Nonetheless, amongst the different PEG 3350, 10% (w/v) showed to be the best precipitant concentration with both MPs. In the presence of MP-22/8 with 10% (w/v) PEG3350 a 60% anti-TNFα mAb activity was observed. However, the highest mAb activity was achieved in the presence of MP-dextran with 10% (w/v) PEG3350, achieving 84% activity (Figure 7A).

For anti-HER2 mAb, since its antigen is the transmembrane protein HER2, its activity was evaluated by indirect flow cytometry (Figure 7B). Anti-HER2 mAb magnetic precipitation was evaluated in the presence of MP-22/8, MP-dextran and absence of MP having as precipitant 20% (w/v) PEG3350. For the flow cytometry analyses SKBR3 cells were used to evaluate the activity, whereas HeLa cells were used as a negative control. Overall, PEG3350 precipitation did not affect mAb activity, except when MP-dextran was present for the magnetic precipitation. A summary of mAb yield, purity and activity is reported in Table 1.

4 Concluding remarks

The viability of using affinity magnetic precipitation for antibody capture from human plasma and clarified cell supernatant was for the first time demonstrated. Three different antibodies sources were tested: (i) pAbs from human serum plasma, (ii) anti-TNFα mAb and (iii) anti-HER2 mAb from clarified CHO cell supernatant. The study comprised the discovery of the best precipitant and precipitation conditions using MPs functionalized with a small affinity ligand previously discovered to have affinity towards antibodies [32]. The addition of MPs was demonstrated to be crucial to achieve higher antibody purity and recovery. Nonetheless, the optimization of the precipitant and precipitation conditions also revealed to be essential to minimize antibody loss and maximize its purity. The best affinity magnetic precipitation condition was using PEG3350 as precipitant with varying concentrations of 20% and 30% (w/v) for 1h at 4ºC with 200 RPM orbital shaking. For pAbs capture from human serum plasma it was possible to reach 80% purity and 50% recovery with 30% (w/v) PEG3350. For both mAbs, the best result was accomplished with 20% (w/v) PEG3350 reaching a 97% recovery and

99% purity for anti-TNFα mAb and 100% recovery and 63% purity for anti-HER2 mAb. The elution was always performed using PBS at pH 7.4. As such, it is possible not only to do antibody capture but also to condition and concentrate in one single step. As a standard capture for mAbs, Protein A chromatography presents a high recovery yield and purity, usually above 95%. However, contrary to Protein A chromatography where a harsh pH denaturing elution condition is used [3], the combination of the precipitant condition, with PEG, along with the elution condition with PBS makes this a biocompatible and mild capture process for mAbs purification [40,41]. The elution fraction from Protein A chromatography is many times directly used as the source for a viral inactivation step due to its low pH, but with no control regarding the pH lowering rate or mAb concentration that may lead to protein aggregation and precipitation [42]. The method proposed in this work still makes possible downstream viral inactivation steps but using controlled decreasing rates of pH in the eluted fraction and management according to protein concentration.

In this way, comparing a precipitation assay with the current Protein A chromatography capture step, the lower costs associated (15-140 €/L precipitant *vs* 5,000-14,000 €/L Protein A resin) [5], the scalability of the precipitation process already implemented in the downstream pipeline of biopharmaceuticals [43,44], and the potential scale and implementation of magnetic fishing processes under cGMP equipment [45, 46], makes affinity magnetic precipitation a potential new platform for the capture of high added value proteins directly from the complex cell supernatant.

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6 References

[1] Somasundaram, B., Pleitt, K., Shave, E., Baker, K., Lua, L.H.L., Progression of continuous downstream processing of monoclonal antibodies: Current trends and challenges. *Biotechnol. Bioeng.* 2018, *115,* 2893–2907.

[2] Rathore, A.S., Kumar, D., Kateja, N., Recent developments in chromatographic purification of biopharmaceuticals. *Biotechnol. Lett.* 2018 *40,* 895–905.

[3] Ramos-de-la-Peña, A.M., González-Valdez, J., Aguilar, O., Protein A chromatography: Challenges and progress in the purification of monoclonal antibodies. *J. Sep. Sci.* 2019, *42,* 1816–1827.

[4] Grilo, A.L., Mantalaris, A., The Increasingly Human and Profitable Monoclonal Antibody Market. *Trends Biotechnol.* 2019, *37*, 9–16.

[5] dos Santos, R., Carvalho, A.L., Roque, A.C.A., Renaissance of protein crystallization and precipitation in biopharmaceuticals purification. *Biotechnol. Adv.* 2017, *35*, 41–50.

[6] Schuster, C., Matzinger, J., Jungbauer, A., Micro-Phase Separation within Epoxy Resin Yields Ultrathin Mesoporous Membranes with Increased Scalability by Conversion from Spin- to Dip-Coating Process. *Macromol. Mater. Eng.* 2019, *304,* 1900321.

[7] Jacinto, M.J., Soares, R.R.G., Azevedo, A.M., Chu, V. et al., Optimization and miniaturization of aqueous two phase systems for the purification of recombinant human immunodeficiency virus-like particles from a CHO cell supernatant. *Sep. Purif. Technol.* 2015, *154*, 27–35.

[8] São Pedro, M.N., Azevedo, A.M., Aires-Barros, M.R., Soares, R.R.G., Minimizing the Influence of Fluorescent Tags on IgG Partition in PEG–Salt Aqueous Two-Phase Systems for Rapid Screening Applications. *Biotechnol. J.* 2019, *14*, 1800640.

[9] Dhadge, V.L., Rosa, S.A.S.L., Azevedo, A., Aires-Barros, R., Roque, A.C.A., Magnetic aqueous two phase fishing: A hybrid process technology for antibody purification. *J. Chromatogr. A.* 2014,*1339*, 59–64.

[10] Batalha, I.L., Hussain, A., Roque, A.C.A., Gum Arabic coated magnetic nanoparticles with affinity ligands specific for antibodies. *J. Mol. Recognit.* 2010, *23,* 462–471.

[11] Borlido, L., Azevedo, A.M., Roque, A.C.A., Aires-Barros, M.R., Potential of boronic acid functionalized magnetic particles in the adsorption of human antibodies under mammalian cell culture conditions. *J. Chromatogr. A.* 2011, *1218*, 7821–7827.

[12] dos Santos, R., Fernandes, C.S.M., Ottengy, S., Viecinski, A.C. et al., Affitins for protein purification by affinity magnetic fishing. *J. Chromatogr. A.* 2016, *1457*, 50–58.

[13] Hammerschmidt, N., Hobiger, S., Jungbauer, A., Continuous polyethylene glycol precipitation of recombinant antibodies: Sequential precipitation and resolubilization. *Process Biochem.* 2015, *51*, 325–332.

[14] Sommer, R., Satzer, P., Tscheliessnig, A., Schulz, H. et al., Combined polyethylene glycol and CaCl2 precipitation for the capture and purification of recombinant antibodies. *Process Biochem.* 2014, *49*, 2001–2009.

[15] Trapp, A., Faude, A., Hörold, N., Schubert, S. et al., Multiple functions of caprylic acid-induced

impurity precipitation for process intensification in monoclonal antibody purification. *J. Biotechnol.* 2018, *279*, 13–21.

[16] Wang, D., Ye, Y., Liu, H., Ma, H. et al., Effect of alkaline precipitation on Cr species of Cr(III)-bearing complexes typically used in the tannery industry. *Chemosphere.* 2018, *193*, 42–49.

[17] Zhang, Y., Hu, Y., Wang, L., Sun, W., Systematic review of lithium extraction from salt-lake brines via precipitation approaches. *Miner. Eng.* 2019, *139*, 105868.

[18] Oelmeier, S.A., Ladd-Effio, C., Hubbuch, J., Alternative separation steps for monoclonal antibody purification: Combination of centrifugal partitioning chromatography and precipitation. *J. Chromatogr. A*. 2013, *1319*, 118–126.

[19] McPherson, A., Gavira, J.A., Introduction to protein crystallization. *Acta Crystallogr. Sect. F:Structural Biol. Commun.* 2014, *70*, 2–20.

[20] Collins, K.D., Ions from the Hofmeister series and osmolytes: Effects on proteins in solution and in the crystallization process. *Methods.* 2004, *34,* 300–311.

[21] Zhang, Y., Cremer, P.S., Interactions between macromolecules and ions: the Hofmeister series. *Curr. Opin. Chem. Biol*. 2006, *10*, 658–663.

[22] Zhang, Y., Cremer, P.S.,Chemistry of Hofmeister anions and osmolytes. *Annu. Rev. Phys. Chem.* 2010, *61*, 63–83.

[23] McPherson, A., Crystallization of proteins from polyethylene glycol. *J. Biol. Chem.* 1976, *251*, 6300–6303.

[24] Atha, D.H., Ingham, K.C., Mechanism of precipitation of proteins by polyethylene glycol. Analysis in terms of excluded volume. *J. Biol. Chem.* 1981, *256*, 12108–12117.

[25] Bolen, D.W., Effects of naturally occurring osmolytes on protein stability and solubility: Issues important in protein crystallization. *Methods.* 2004, *34*, 312–322.

[26] Zang, Y., Kammerer, B., Eisenkolb, M., Lohr, K. et al., Towards protein crystallization as a process step in downstream processing of therapeutic antibodies: Screening and optimization at microbatch scale. *PLoS One.* 2011, *6*, 3–10.

[27] Smejkal, B., Agrawal, N.J., Helk, B., Schulz ,H. et al., Fast and scalable purification of a therapeutic full-length antibody based on process crystallization. *Biotechnol. Bioeng.* 2013, *110*, 2452–2461.

[28] Sommer, R., Tscheliessnig, A., Satzer, P., Schulz, H. et al., Capture and intermediate purification of recombinant antibodies with combined precipitation methods. *Biochem. Eng. J.* 2015, *93*, 200–211.

[29] Ditsch, A., Yin, J., Laibinis, P.E., Wang, D.I.C. et al., Ion-exchange purification of proteins using magnetic nanoclusters. *Biotechnol. Prog.* 2006, *22*, 1153–1162.

[30] Santana, S.D.F., Dhadge, V.L., Roque, A.C.A., Dextran-coated magnetic supports modified with a biomimetic ligand for IgG purification. *ACS Appl. Mater. Interfaces.* 2012, *4*, 5907–5914.

[31] Dias, A.M.G.C., Hussain, A., Marcos, A.S., Roque, A.C.A., A biotechnological perspective on the application of iron oxide magnetic colloids modified with polysaccharides. *Biotechnol. Adv.* 2011, *29*, 142–155.

[32] Teng, S.F., Sproule, K., Hussain, A., Lowe, C.R., A strategy for the generation of biomimetic ligands for affinity chromatography. Combinatorial synthesis and biological evaluation of an IgG binding ligand. *J. Mol. Recognit.* 1999, *12*, 67–75.

[33] Barroso, T., Casimiro, T., Ferraria, A.M., Mattioli, F. et al., Hybrid monoliths for magnetically-driven protein separations. *Adv. Funct. Mater.* 2014, *24*, 4528–4541.

[34] Jones, S., Thornton, J.M., Principles of protein-protein interactions. *Proc. Natl. Acad. Sci.* 1996, *93*, 13–20.

[36] Mizan, T.I., Savage, P.E., Ziff, R.M., Temperature dependence of hydrogen bonding in supercritical water. *J. Phys. Chem.* 1996, *100*, 103–408.

[37] Branco, R.J.F., Dias, A.M.G.C., Roque, A.C.A., Understanding the molecular recognition between antibody fragments and protein A biomimetic ligand. *J. Chromatogr. A.* 2012, *1244*, 106–115.

[38] Wu, J., Zhao, C., Lin, W., Hu, R. et al., Binding characteristics between polyethylene glycol (PEG) and proteins in aqueous solution. *J. Mater. Chem. B.* 2014, *2*, 2983-2992.

[39] Thompson, R.W., Latypov, R.F., Wang, Y., Lomakin, A. et al., Evaluation of effects of pH and ionic strength on colloidal stability of IgG solutions by PEG-induced liquid-liquid phase separation. *J. Chem. Phys.* 2016, *145*, 185101.

[40] Liu, X.Y., Nothias, J.-M., Scavone, A., Garfinkel, M., Millis, J. M. Biocompatibility investigation of polyethylene glycol and alginate-poly-l-lysine for islet encapsulation. ASAIO J. 2010, *56*, 241–245.

[41] Bjugstad, K.B., Lampe, K., Kern, D.S., Mahoney, M. Biocompatibility of poly(ethylene glycol)-based hydrogels in the brain: An analysis of the glial response across space and time. J Biomed Mater Res A. 2010, *95A*, 79–91

[42] Mazzer A., Perraud X., Halley J., O'Hara J., Bracewell D. Protein A chromatography increases monoclonal antibody aggregation rate during subsequent low pH virus inactivation hold. *J. Chromatogr. A.* 2015, *1415*, 83-90

[43] Jackson, R., Processor the crystallization of the ammonium and alkali metal salts in insulin. US Patent 3719655, 1969.

[44] Alstine, J.V., Berg, M., Kjorning, J.,Shanagar, J., Plasma protein fractionation by sequential polyacid precipitation. US Patent 2014/0343253 A1, 2014.

[45] Ebeler, M., Pilgram, F., Wolz, K., Grim, G., Franzreb, M. Magnetic separation on a new level: Characterization and performance prediction of a cGMP compliant ”Rotor-Stator” high-gradient magnetic separator. Biotechnol J. 2018, *13*, DOI: 10.1002/biot.201700448.

[46] Roque, A.C.A., Pina, A. S., Azevedo, A. M., Aires-Barros, R., Jungbauer, A., Di Profio, G., Heng, J.Y.Y., haigh, J., Ottens, M. Anything but Conventional Chromatography Approaches in Bioseparation. Biotechnol J. 2020, *1900274,* DOI: 10.1002/biot.201900274.

Table 1. Summary of paramount parameters for affinity magnetic mAbs precipitation – Yield, purity and activity at the elution fraction having as precipitant 20% (w/v) PEG3350 in the presence of MP-22/8 and MP-dextran. **Legend: +**: Lower activity than the no MPs control sample; **++**: Same activity as the no MPs control sample; **+++**: Higher activity than the no MPs control sample

	Yield		Purity		Activity	
	MP-22/8	MP-dextran	MP-22/8	MP-dextran	MP-22/8	MP-dextran
Anti-TNFα mAb *	97%	31%	98%	52%	+	++
Anti-HER2 mAb *	100%	89%	63%	55%	+++	++
*Precipitant: 20% (w/v) PEG3350						

Figures Legends

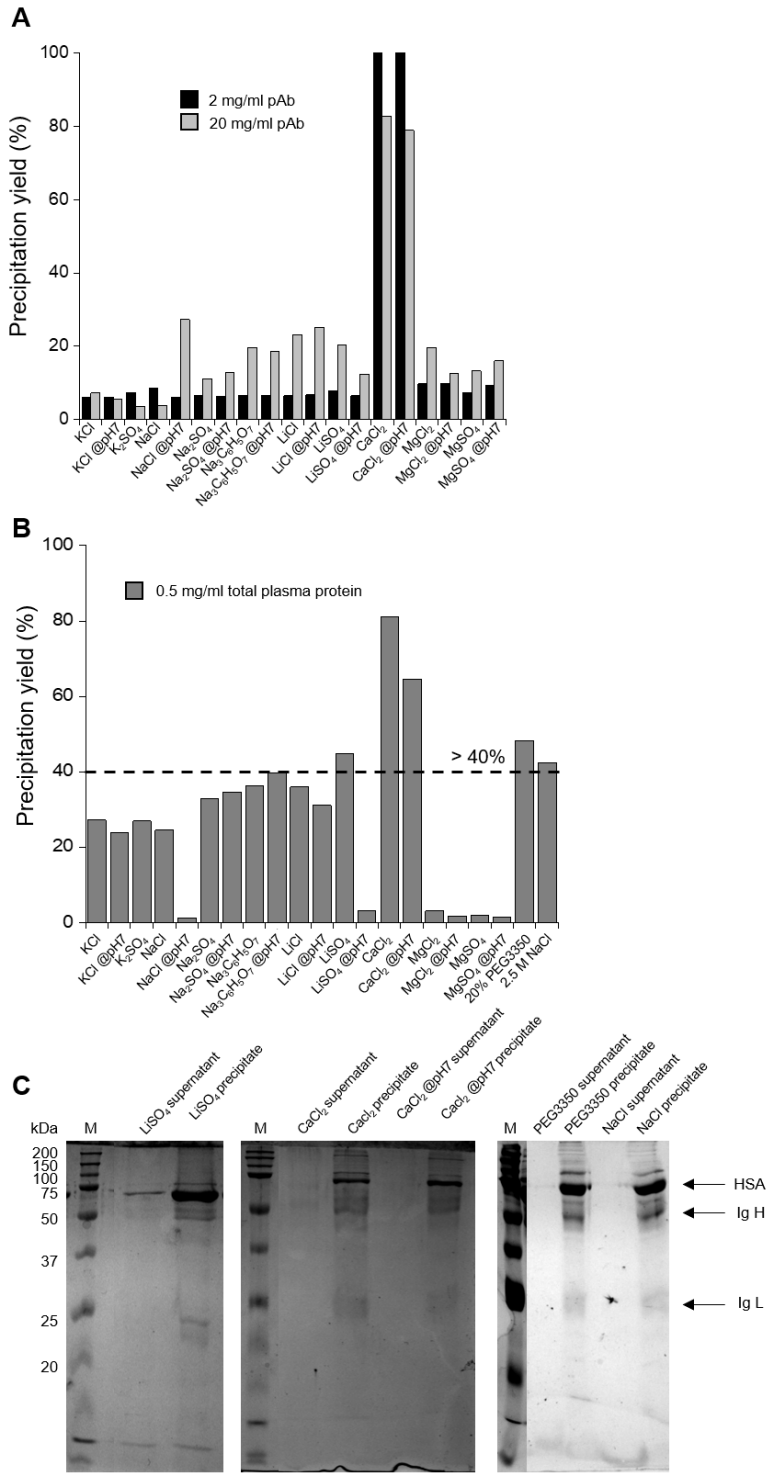


Figure 1. Precipitation screen for antibody precipitation using 300 mM precipitant, except if otherwise specified. Precipitation yield for (**A**) pure polyclonal antibody at 2 mg/ml (**black**) and 20 mg/ml (**grey**) and (**B**) human serum plasma at 0.5 mg/ml total protein with a dashed line at 40% precipitation yield threshold. (**C**) SDS-PAGE for the precipitant condition with a precipitation yield higher than 40% with the supernatant and precipitation samples for each condition. **Lane ID**: **M**: Precious Plus Protein™ Dual Color Standards; arrows indicate HSA (66 kDa), Ig heavy (Ig H; 50 kDa) and light (Ig L; 25 kDa) chains.

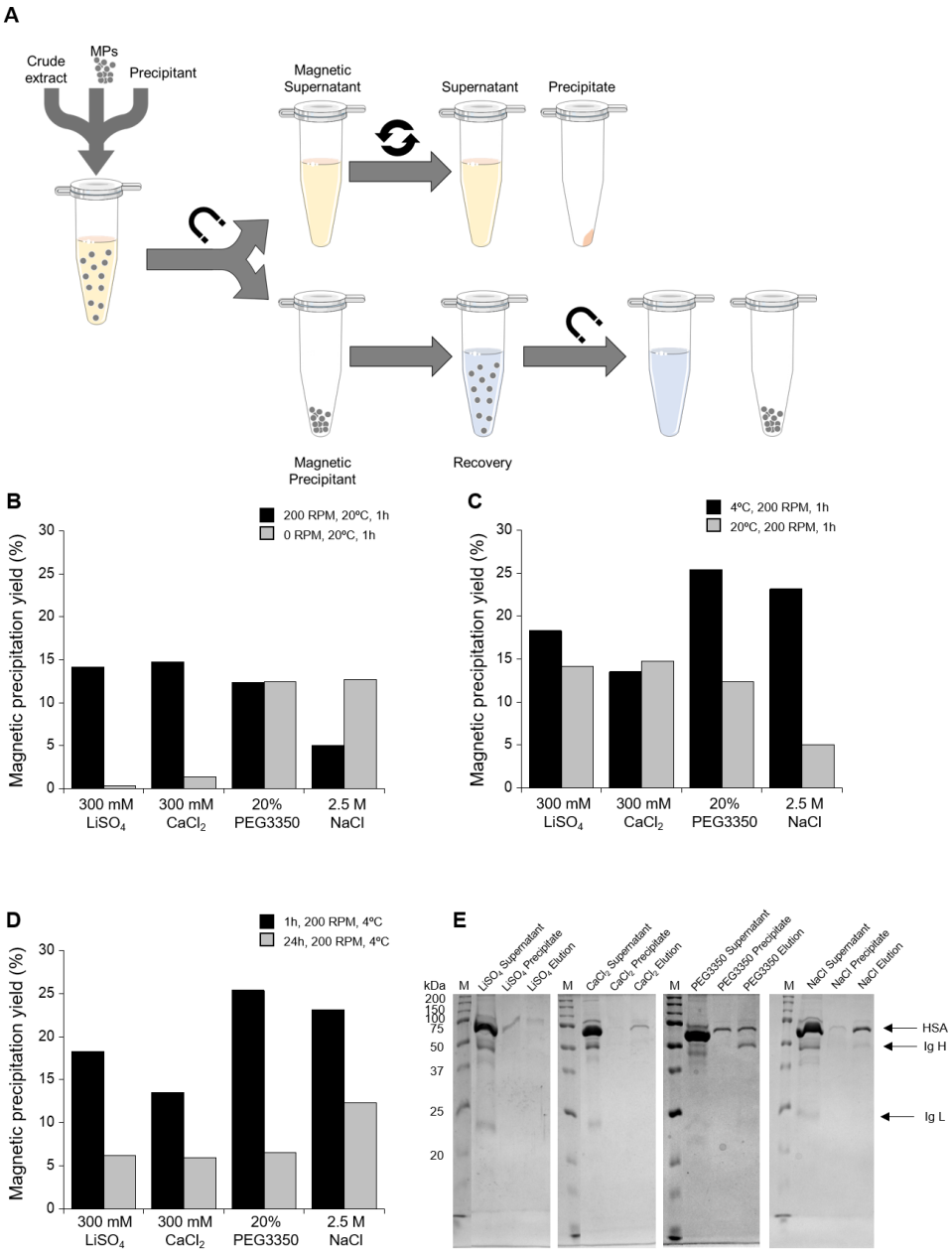


Figure 2. Magnetic precipitation optimization with MP-22/8. (**A**) Schematic representation of the Magnetic precipitation assay. (**B**) Optimization of the precipitation orbital shaking for 1 hour at 20ºC with 200 RPM (**black**) and 0 RPM (**grey**). (**C**) Optimization of the precipitation temperature for 1 hour at 200 RPM at 4ºC (**black**) and 20ºC (**grey**). (**D**) Optimization of the precipitation time at 4ºC with 200 RPM for 1 hour (**black**) and 24 hours (**grey**). (**E**) SDS-PAGE for the Magnetic precipitant at 4ºC for 1 hour at 200 RPM. **Lane ID: M:** Precious Plus Protein™ Dual Color Standards; arrows indicate HSA (66 kDa), Ig heavy (Ig H; 50 kDa) and light (Ig L; 25 kDa) chains.

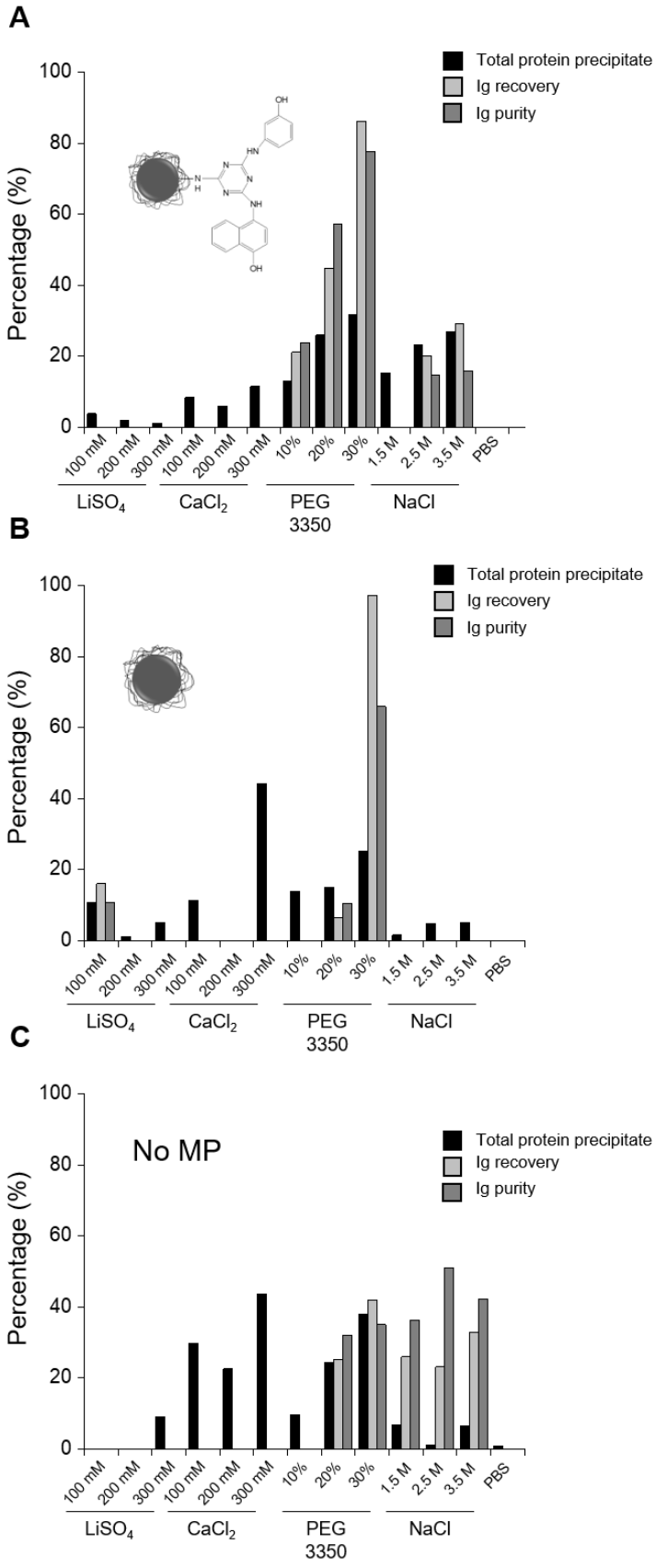


Figure 3. Magnetic precipitation of human serum plasma for pAb recovery. In the presence of (**A**) MP-22/8, (**B**) MP-Dextran and (**C**) No MP, using the four main precipitants - $LiSO_4$, $CaCl_2$, at 100, 200 and

300 mM; PEG 3350 at 10, 20 and 30% (w/v) and NaCl at 1.5, 2.5 and 3.5 M. **Legend**: Total protein precipitate (**black**), Ig recovery (**light grey**) and Ig purity (**grey**).

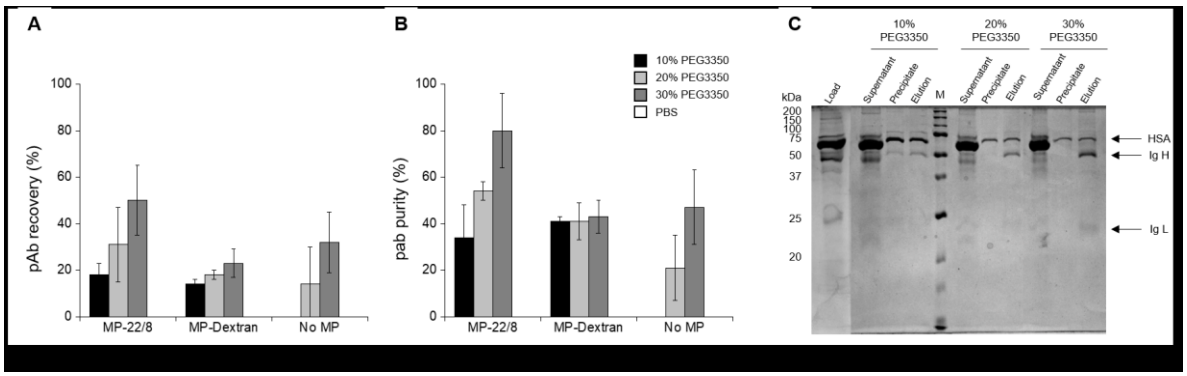


Figure 4. Magnetic Affinity Precipitation of human serum plasma crude extract for pAb purification. (**A**) pAb recovery and (**B**) pAb purity using MP-22/8, MP-Dextran and No MP with 10 (**black**), 20 (**light grey**) and 30% (w/v) (**grey**) PEG3350. (**C**) SDS-PAGE for the best affinity magnetic precipitation condition tested – MP-22/8 with 10, 20 and 30% (w/v). **Lane ID**: **M**: Precious Plus Protein™ Dual Color Standards; arrows indicate HSA (66 kDa), Ig heavy (Ig H; 50 kDa) and light (Ig L; 25 kDa) chains.

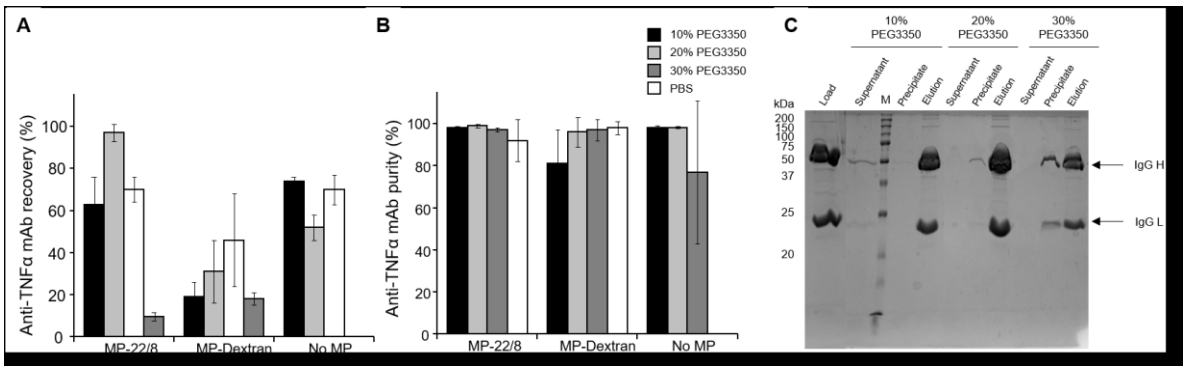


Figure 5. Magnetic Affinity Precipitation of anti-TNFα mAb crude extract. (**A**) mAb recovery and (**B**) mAb purity using MP-22/8, MP-Dextran and No MP with 10, 20 and 30% (w/v) PEG3350. (**C**) SDS-PAGE for the best affinity magnetic precipitation condition tested – MP-22/8 with 10, 20 and 30% (w/v). **Lane ID**: **M**: Precious Plus Protein™ Dual Color Standards; arrows indicate IgG heavy (Ig H; 50 kDa) and light (IgG L; 25 kDa) chains.

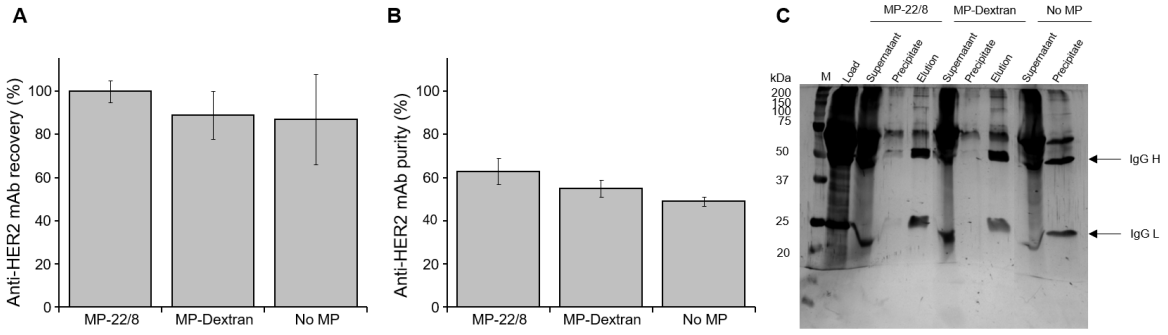


Figure 6. Magnetic Affinity Precipitation of anti-HER2 mAb crude extract with 20% (w/v) PEG3350. Anti-HER2 (**A**) recovery and (**B**) purity assessment using MP-22/8, MP-Dextran and No MP. (**C**) SDS-PAGE for the precipitant conditions tested. **Lane ID**: **M**: Precious Plus Protein™ Dual Color Standards; arrows indicate IgG heavy (Ig H; 50 kDa) and light (IgG L; 25 kDa) chains.

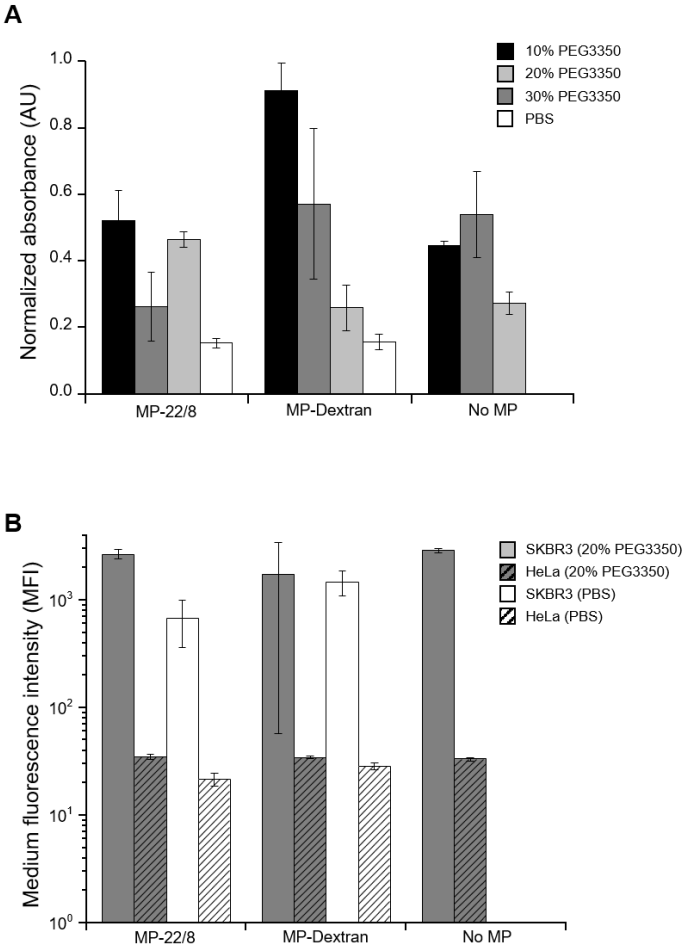
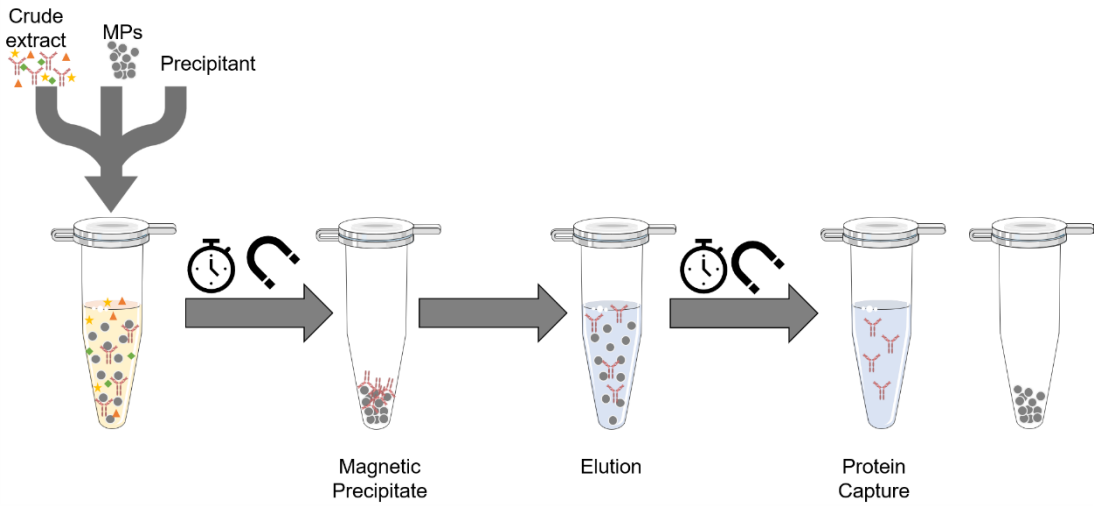


Figure 7. Activity of anti-TNFα and anti-HER2 mAbs purified by magnetic precipitation. (**A**) Indirect ELISA of the elution fraction of anti-TNFα mAb in the presence of MP-22/8, MP-dextran and No MP at different PEG3350 concentration (10%, 20% and 30% (w/v)) and PBS. (**B**) Indirect flow cytometry of the elution fraction of anti-HER2 mAb in the presence of 20% (w/v) PEG3350 and PBS for SKBR3 and HeLa cells.



Direct antibody precipitation from crude extract as a capture step in antibody downstream processing is still a great challenge. In this work, two non-chromatographic methods were merged to work in synergy: precipitation and magnetic separation enabled the selective capture of antibodies from crude extract without any previous conditioning.