Enhancing Biomarker Detection in Human Serum for Lung Cancer Diagnosis: Aqueous Biphasic Systems for Simultaneous Depletion of High-Abundance Proteins and Efficient Extraction of CYFRA 21-1

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Highlights

- PEG properties are investigated in the pretreatment of human serum using ABS.
- PEGs of several molecular weights are combined with phosphate buffer to form ABS.
- Serum pretreatment occurs by depleting IgG and HSA at the ABS interphase.
- Up to 99% of IgG and 70% of HSA can be depleted from serum.
- Simultaneous IgG/HSA depletion and CYFRA 21-1 extraction is enhanced with ABS formed by PEG 1500.

Enhancing Biomarker Detection in Human Serum for Lung Cancer Diagnosis: Aqueous Biphasic Systems for Simultaneous Depletion of High-Abundance Proteins and Efficient Extraction of CYFRA 21-1

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Abstract

Analysing biomarkers in human serum could be used as an effective and less invasive approach for the diagnosis of lung cancer; however, biomarker detection reliability is highly limited due to matrix effects. Herein, aqueous biphasic systems (ABS) are studied as platforms for human serum pretreatment, allowing the simultaneous depletion of high abundant proteins and biomarker extraction. By using ABS varying the polyethylene glycol (PEG) molecular weight between 400 and 6000 $g \cdot mol^{-1}$ and adopting phosphate buffer as the other phase-forming component, the depletion of the high abundance serum proteins immunoglobulin G (IgG) and human serum albumin (HSA) is induced at the ABS interphase, through precipitation, forming a three-phase partitioning system (ABS-TPP). Maximum depletion efficiencies of 99% for IgG and 70% for HSA were achieved in one step using PEG 1500-based ABS-TPP. On the other hand, lung cancer biomarkers, such as CYFRA 21-1, are extracted to the PEG-rich phase of the same ABS-TPP with recovery yields of 91%. This work shows that a proper selection of the PEG molecular weight in the ABS composition leads to the efficient depletion of high-abundance proteins and extraction of cancer biomarkers from human serum, in a single step, confirming the potential of ABS for sample pretreatment to improve biomarker analysis.

Keywords: CYFRA 21-1 • human serum pretreatment • aqueous biphasic systems • three-phase partitioning system • polyethylene glycol

1. Introduction

Currently, the deadliest (18% of the global burden) and the second most diagnosed (11% of all diagnosed) type of cancer worldwide is lung cancer.[1] Such cancer is commonly classified into two types – small cell lung cancer (SCLC) and non-small lung cancer (NSCLC), with the latter representing around 85% of all lung cancer cases.[2] Despite its lower frequency, SCLC is associated with faster growing rates, registering lower 5-year survival rates. According to the American Cancer Society (ACS), the 5-year survival rates of SCLC are 29% for localized cancer, 18% for regional cancer and 3% for distant cancer. NSCLC is instead characterized by 5-year survival rates of 64%

for localized cancer, 37% for regional cancer and 8% for distant cancer.[2] These survival rates, combined with the lack of effective screening strategies and the high metastasis rate, makes lung cancer hard to detect at early stages.[2] Thus, there is a critical need for an effective and less invasive early-stage diagnosis.

Lung cancer biomarkers are important players in early-stage diagnosis. In the search for appropriate lung cancer biomarkers, efforts to quantify in situ protein signals have been done.[3] In this field, the cytokeratin 19 soluble fragment (CYFRA 21-1) has been reported as a reliable serum marker.[4] CYFRA 21-1 is composed of 400 amino acids, with a molecular weight of 40 kDa and an isoelectric point of 5.2.[4] It is a polypeptide's epitope released into circulation after caspase-3 cleavage associated to cell death (apoptosis) within the tumour microenvironment.[4] CYFRA 21-1 is associated with the increase of the proteolytic activity in tumours such as NSCLC, especially for squamous-cell carcinoma, being considered a useful auxiliary test for diagnosis diagnosis and follow-up.[4] CYFRA 21-1 can be found in serum, [5] saliva[6] or even urine.[7] However, regarding early diagnosis and screening, human serum is the most widely adopted body fluid. The cut-off values of CYFRA 21-1 in serum are 3.3 $ng \cdot mL^{-1}$, above which it is considered abnormal and requires further testing.[5]

There are different strategies currently used to quantify CYFRA 21-1 in human fluids. The most used strategies are enzyme-Linked immunosorbent assay (ELISA)[8] and electrochemiluminescence immunoassay (ECLIA).[9] Despite their well-established and standardized application, the overall cost, procedure time and need for proprietary reagents remain limiting steps for a widespread implementation.[10] So far, the alternatives proposed in the literature to substitute conventional immunoassays comprise surface plasmon resonance (SPR),[11] immunoradiometric assay (IRMA) and electrochemical immunoassays.[12] However, these still face technical challenges, and the limit of detection (LOD), the sample matrix influence, and incompatibilities between the equipment and the sample/analyte need to be carefully addressed.[13] When concerning SPR, this technique requires antifouling polymers, salts and/or surfactants for the reduction of non-specific interactions.[11] As for IRMA, it presents radiation hazards, short half-life of iodinated labels, high costs and the need for large apparatus.[10] Lastly, electrochemical immunoassays use electrolytes that reduce the overall kit lifetime.[6]

The high abundance of immunoglobulin G (IgG) and human serum albumin (HSA) in human serum proteome may interfere within the analysis of biomarkers,[14] such as CYFRA 21-1, which is present at considerably lower concentrations. For instance, in immunoassays, immunoglobulins such as IgG have been documented to induce interference, resulting in erroneous positive or negative outcomes in both sandwich and competitive ELISA formats.[15,16] Furthermore, the elevated levels of albumin, characterized by its ability to avidly bind and release numerous ligands, have been demonstrated to significantly elevate background noise levels and diminish the sensitivity of ELISA assays.[17] A sample pretreatment step is thereby often required to remove high-abundance proteins extract target biomarkers, enabling more reliable results.[18] This step can be carried out using protein precipitating agents including polymers, salts, and organic solvents.[19] Protein precipitation is an easy-to-perform technique and has a low cost; however, it provides insufficient selectivity to comply with the requirements of working with clinically relevant proteins.[20] This issue can be solved using adsorption processes involving affinity ligands of high specificity and selectivity, but at expense of cost-efficiency.[21]

Liquid-liquid extraction (LLE) techniques can be considered alternative sample pretreatment strategies due to their technological simplicity and low cost.[22] Aqueous biphasic systems (ABS) are part of the LLE approaches, conventionally based on

ternary combinations of polymer–polymer–water and polymer–salt–water.[23] Due to the high-water content of ABS, they gained popularity over conventional LLE comprising volatile organic solvents as biocompatible separation techniques.[24] Polymer-salt ABS present some advantages over ABS formed by two polymers, including lower viscosities, and faster phase separation.[25] Based on the properties of the ABS phase-forming components, pH and temperature, molecules partition according to the most favourable interactions with the surrounding environment.[26] Among the polymers available for ABS formation, polyethylene glycol (PEG) is the most common, due to their high-water solubility, biodegradability, cost-efficiency, and low toxicity.[27] As salting-out agents, buffered salts represent the most suitable options due to controlled pH and stabilizing effects over biomolecules.[28,29] Recent research within the field of biomarker analysis has focused on PEG-salt ABS for the extraction of proteins[30,31] and nucleic acids[32,33] from complex biological samples such as blood[30,31,33] and cell cultures.[32] Overall, the application of ABS to improve biomarkers analysis using laboratory reliant equipment holds promise in biomarker discovery studies as well as in the detection of several pathologies including liver,[30] nutritional,[31] infectious[32] and oncologic diseases.[33]

The formation of three phase partitioning (TPP) systems based on ABS constituents (ABS-TPP) instead of t-butanol and ammonium sulphate is a promising alternative to separate proteins from complex samples.[34] ABS-TPP are characterized by the formation of two liquid aqueous phases and a solid interphase, usually rich in proteins.[34] Considering the advantages of ABS and TPP combined, a water-rich environment and a higher structural diversity is accomplished by playing with the pairs of components available.[34]

Due to the possible partition of target molecules between three coexistent phases, ABS-TPP are here shown as alternative strategies to achieve high selectivity and mild conditions for serum pretreatment, by depleting high abundance proteins such as IgG and HSA, with the simultaneous CYFRA 21-1 extraction, allowing to improve biomarker analysis. PEGs of different molecular weights were combined with a phosphate buffer salt (KH_2PO_4/K_2HPO_4 buffer at pH of ca. 7) and investigated as ABS-TPP components. The depletion efficiency of IgG and HSA at the system interphase and the extraction of CYFRA 21-1 was evaluated as a function of the polymer molecular weight.

2. Materials and Methods

2.1. Materials

Polyethylene glycol with a molecular weight of 400 $g.mol^{-1}$ (PEG 400) supplied from Sigma Aldrich, 1000 $g.mol^{-1}$ (PEG 1000) from Alfa Aesar, 1500 $g.mol^{-1}$ (PEG 1500) acquired from Acros Organics, 2000 $g.mol^{-1}$ (PEG 2000) from Alfa Aesar, 4000 $g.mol^{-1}$ (PEG 4000) and 6000 $g.mol^{-1}$ (PEG 6000) from Sigma Aldrich, were used as ABS-TPP phase-forming components. These PEGs were combined with a phosphate buffer (KH_2PO_4/K_2HPO_4 at ca. 40 wt% and pH of ca. 7) formed by potassium phosphate monobasic (KH_2PO_4, purity 99.5 wt%) and potassium phosphate dibasic (K_2HPO_4, purity > 98 wt%), both acquired from Sigma-Aldrich. Commercial human serum was acquired from Sigma-Aldrich (H4522- Lot # SLCD4040) and kept at - 20 °C until use.

The mobile phase for the Size Exclusion High Performance Liquid Chromatography (SE-HPLC) analyses was prepared with sodium di-hydrogen phosphate 1-hydrate, $NaH_2PO_4 \cdot H_2O$ (purity ≥ 99 wt%) and di-sodium hydrogen phosphate 7-hydrate, $Na_2HPO_4 \cdot 7H_2O$ (purity ≥ 99 wt%), both from Panreac Applichem. The water used was double distilled, passed by a reverse osmosis system and further treated with a Milli-Q plus 185 water purification apparatus. Purified human IgG (in solution at 29.4 mg/mL from Innovative Research, Inc, stored at -80 °C until use) and lyophilized powder of albumin from human (purity ≥ 96% from Alfa Aesar) were used to prepare the standards of the calibration curves. The CYFRA 21-1 enzyme-linked immunosorbent assay (ELISA) kit from Abnova® (KA4024 Lot #103 and #109A) was purchased from Sigma Aldrich and kept in storage at 4 ºC. CYFRA 21-1 antigen protein abx060980 (0.075 $mg \cdot mL^{-1}$) was supplied from abbexa® (Lot # L201609C899) and stored at - 20° C up to use.

2.2. HSA and IgG depletion

The identification of the required amounts of PEG and salt yielding biphasic mixtures in water was based on the binodal data reported in the literature for the ABS composed of K_2HPO_4/KH_2PO_4 (pH ≈ 7) and the various molecular weight PEGs (at 25 ºC).[34–36] To investigate the depletion performance of all ABS-TPP a common mixture composition for all systems was selected, to which commercial human serum was added as a portion of the total ABS water content, yielding the following mixture composition: 30 wt% PEG + 12 wt% of K_2HPO_4/KH_2PO_4 (pH ≈ 7) + 10 wt% human serum + 48 wt% water.

After carefully weighing the required amount of each component (within ± 10^{-4} g), the resultant mixtures were mixed until complete dissolution and centrifuged (10 min at 3500 rpm). Following these steps, the formation of ABS-TPP was observed at room temperature, in which the top and bottom liquid phases are PEG- and salt-rich, respectively, whereas the solid interphase is rich on precipitated serum proteins. Both liquid phases were carefully separated using syringes to be further analysed by SE-HPLC. At least two replicates of each system were performed to infer on the average values and the corresponding standard deviations. The liquid chromatograph used was a Chromaster HPLC system (VWR Hitachi) with a diode array detector (DAD), equipped with an analytical column Protein KW- 802.5 (8 mm × 300 mm) coupled with a Protein KW-G 6B guard column, both from Shodex. 50 mM sodium phosphate buffer and 0.3 M NaCl solution was the mobile phase used. All analyses were performed using isocratic elution (at 0.5 $mL \cdot min^{-1}$ for 40 min), an injection volume of 25 µL and wavelength of 280 nm. The temperatures of column oven and autosampler were both controlled at 25 °C and 10 °C, respectively. Under the analytical conditions adopted, the retention times of IgG and HSA were approximately 15.21 min and 16.60 min, respectively. To quantify the amount of HSA and IgG in the original serum and each liquid phase, calibration curves (r^2 ó 0.99) were determined (0.1 - 1.0 $mg \cdot mL^{-1}$). Due to solubilization issues hindering the analysis of ABS-TPP interphases by SE-HPLC, mass balance was applied to the determination of HSA and IgG concentration at the interphase.

The recovery yield of IgG and HSA (RY_{IgG} and RY_{HSA}, %, respectively) in the top and bottom phases and depletion efficiencies of IgG and HSA (DE_{IgG}, % and DE_{HSA}, %, respectively) at the interphase were calculated by Equations 1 and 2, respectively.

$$RY_{IgG} = \frac{m_{IgG,top} + m_{IgG,inter} + m_{IgG,bottom}}{m_{IgG,initial}} \times 100 \quad \text{(Eq. 1)}$$

$$RY_{HSA} = \frac{m_{HSA,top} + m_{HSA,inter} + m_{HSA,bottom}}{m_{HSA,initial}} \times 100 \quad \text{(Eq. 2)}$$

where $m_{IgG,initial}$ and $m_{HSA,initial}$ represent the mass of IgG or HSA in the original commercial human serum and recovered or depleted in the top, inter and bottom phases, respectively.

2.3. CYFRA 21-1 extraction

For the evaluation of CYFRA 21-1 partition in ABS, the same experimental procedure adopted for the depletion studies was followed, with few adaptions. First, the addition of commercial human serum was replaced by the addition of 10 wt% of an aqueous solution of CYFRA 21-1 (1000 ng·mL^{-1}). This study allowed to first evaluate the partition of the biomarker in the system, with no interference caused by serum samples. Following the preparation of the systems and full separation of the two phases, these were diluted with Milli-Q water, i.e., 1:30 (top phase) and 1:20 (bottom phase), before the quantification of CYFRA 21-1 by ELISA. CYFRA 21-1 concentration was determined following the ELISA kit manufacturer's instructions, using an established calibration curve achieved with the supplied standards (0 - 50 ng·mL^{-1}, r^2 value ≥ 0.99). The interference of the ABS components in the CYFRA 21-1 quantification was assessed by blank controls.

CYFRA 21-1 extraction efficiency ($EE_{CYFRA\ 21\text{-}1}$%) to the top PEG-rich phase using ABS was determined through the following equation:

$$EE_{CYFRA\ 21\text{-}1}\ (\%) = \frac{m_{CYFRA\ 21\text{-}1,top}}{m_{CYFRA\ 21\text{-}1,top} + m_{CYFRA\ 21\text{-}1,bottom}} \times 100 \quad \text{(Eq. 3)}$$

where $m_{CYFRA\ 21\text{-}1,top}$ and $m_{CYFRA\ 21\text{-}1,bottom}$ are the total weight of CYFRA 21-1 in the PEG-rich top phase and in the salt-rich bottom phase, respectively.

To investigate the influence of serum in the partition of CYFRA 21-1 as well as the ability of ABS-TPP to simultaneously perform HSA and IgG depletion and biomarker extraction, ABS were prepared using spiked human serum at the same concentration. In these systems, three phases are formed instead of only two, and the amount of CYFRA 21-1 that precipitated at the interphase was assessed by mass balance.

The recovery yield of CYFRA 21-1 in the top phase, interphase and the bottom phase ($RY_{CYFRA\ 21\text{-}1}$, %) was determined according to Equation 4:

$$RY_{CYFRA\ 21\text{-}1}\ (\%) = \frac{m_{CYFRA\ 21\text{-}1,top} + m_{CYFRA\ 21\text{-}1,inter} + m_{CYFRA\ 21\text{-}1,bottom}}{m_{CYFRA\ 21\text{-}1,initial}} \times 100 \quad \text{(Eq. 4)}$$

where $m_{CYFRA\ 21\text{-}1}^{top/inter/bottom}$ is the total weight of CYFRA 21-1 in the top, inter or bottom phases and $m_{CYFRA\ 21\text{-}1}^{spiked\ serum}$ is the total weight of CYFRA 21-1 in the spiked human serum.

3. Results

ABS composed of PEGs of several molecular weights and K_2HPO_4/KH_2PO_4 (pH ≈ 7) were studied regarding their aptitude to deplete high-abundance serum proteins – IgG and HSA - through their precipitation at the interphase. Regarding biomarker extraction, due to their high-water content, these systems can be foreseen as amenable routes enabling extraction of biomarkers at low concentration without compromising biomarker stability or leading to integrity losses. In addition, by the variation of the PEG molecular weight, it is possible to determine the most appropriate PEG properties to the successful design of ABS-TPP for serum pretreatment, comprising both the depletion of high abundance proteins and CYFRA 21-1 extraction.

To achieve the proposed goal, ABS respective binodal curves were first examined to define compositions within the biphasic region. Such examination led to the selection of mixtures bearing 30 wt% PEG + 12 wt% of K_2HPO_4/KH_2PO_4 (pH ≈ 7) + 10 wt% human serum + 48 wt% water to investigate the depletion of high-abundance serum proteins at the interphase of these systems.[34–36] Subsequently, the more suitable sample pretreatment systems were established based on their ability to concurrently deplete IgG and HSA, while efficiently extracting CYFRA 21-1 in one of the ABS aqueous phases. Figure 1 summarizes the process of developing a serum pretreatment strategy based on ABS-TPP, particularly envisaging its application towards CYFRA 21-1 extraction.

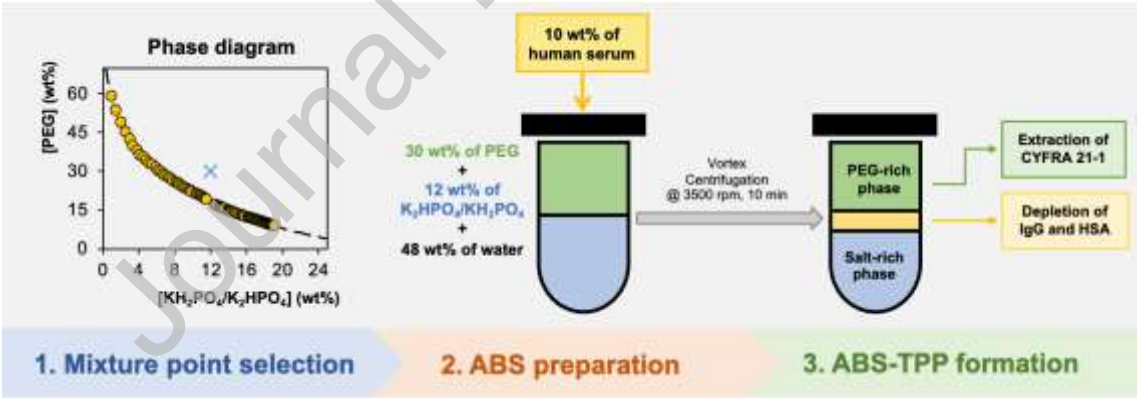

Figure 1. Development of the ABS-TPP-based approach proposed in the current work for application in the pretreatment of human serum samples and CYFRA 21-1 extraction.

3.1. Depletion of HSA and IgG using ABS-TPP

The depletion efficiencies of both high-abundance serum proteins, i.e., IgG and HSA (DE_{IgG} (%) and DE_{HSA} (%)) achieved with ABS-TPP are represented in Figure 2. The average depletion efficiencies in the interphase and recovery yields (RY_{IgG} and RY_{HSA}, %) in the top and bottom phases for both proteins and standard deviations are shown in full detail in Table S1 in the Supporting Information. The depletion efficiencies range between 23% and 99% for IgG, and between 18% and 70% for HSA. When

concerning IgG, the depletion efficiency can be ranked according to PEG 400 < PEG 4000 ≈ PEG 6000 < PEG 1000 ≈ PEG 2000 ≈ PEG 1500. For HSA, the depletion efficiencies increase as follows: PEG 400 < PEG 1000 < PEG 4000 < PEG 6000 < PEG 2000 < PEG 1500. Overall, these results show that the PEG molecular weight exerts a higher influence on the depletion of HSA over IgG.

It is well-known that proteins can partition or precipitate in ABS due to a combined effect of multiple interactions with the phase-forming components.[37] In the ABS-TPP under appraisal, the molecular weight of the PEG and its hydrophobicity combined with the salting-out ability of K_2HPO_4/KH_2PO_4 (pH ≈ 7)[38] seems to be determining the extent of depletion. PEG-induced protein depletion can be explained by the excluded volume or the attractive depletion theories.[39,40] According to the former, PEG and proteins compete in solution for the solvent, reducing proteins solubility and inducing its precipitation.[40] According to the latter, protein precipitation occurs because PEG molecules are excluded from the protein surface, inducing an osmotic imbalance.[40] Regardless of the actual molecular mechanism behind such phenomena, protein-protein interactions are responsible for protein depletion in the presence of PEG due to reduced water availability and increased effective protein concentration until precipitation Moreover, this effect is intensified by increasing the molecular weight of PEG.[41] In ABS-TPP, however, in addition to the role of proteins, water and the PEG, the salting-out effect also needs to be considered.

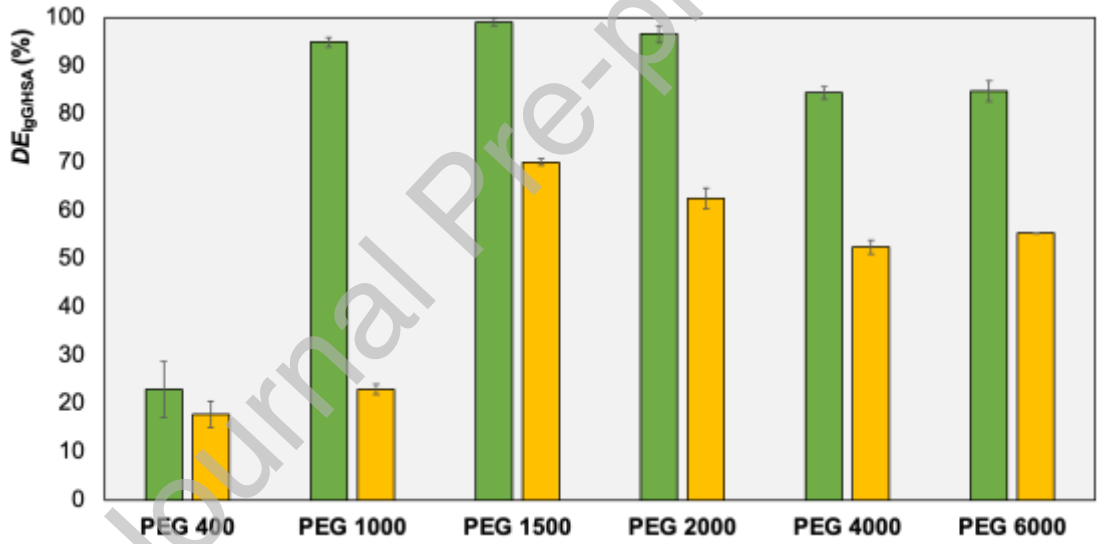

Figure 2. Depletion efficiencies obtained for IgG (DE_{IgG}, %) (green bars) and HSA (DE_{HSA}, %) (yellow bars) at the interphase obtained with ABS-TPP composed of 30 wt% of PEG + 12 wt% K_2HPO_4/KH_2PO_4 + 10 wt% human serum + 48 wt% water (mean ± SD, n=3). Data for the ABS-TPP composed of PEG 1000 is taken from the literature[38].

Based on the overall results, the lighter the PEG, particularly for molecular weights below 1500 $g.mol^{-1}$, the lower the depletion efficiencies. In fact, for short chain PEGs, such as PEG 400, the proteins partition preferentially to the top PEG-rich phase (RY_{HSA} and RY_{IgG} ≥ 77%). This agrees with previous observations by Samanta et al.[41], who have shown that, for PEG 400, interactions between the hydrophobic amino acids of HSA and the PEG chain affect the tertiary structure and stability, leading to a higher solubility in the PEG-rich phase.[41,42] On the other hand, for higher molecular weight PEGs, namely PEG 1000, 1500, 4000 and 6000, protein-protein interactions that promote protein precipitation are favoured.[42,43] Our results comply with those observations since both IgG and HSA precipitated mainly at the

interphase. Also, for PEGs with molecular weights above 1500 g·mol^{-1}, smaller proteins such as HSA require higher concentrations of PEG to precipitate, and this phenomenon is more pronounced at high concentrations of proteins.[44,45] Our results are in accordance with these studies, since it was observed that at 30 wt% of PEG, PEG 4000 and 6000 were outperformed by PEGs 1500 and 2000 for both proteins. Additionally, when using PEG 4000 and PEG 6000, DE_{HSA} values are higher than the values obtained when using PEG 1000. Hence, PEG 1000 better solubilized HSA when compared to PEG 4000 and PEG 6000 that induced protein-protein interactions and consequently their precipitation at the interphase. These results suggest that each protein responds differently to the polymers molecular weight. However, as ranked above, a similar tendency for both IgG and HSA proteins is registered among all PEGs, suggesting that there is a threshold of the PEG molecular weight/hydrophobicity that, once passed, limits the simultaneous depletion of the studied proteins. Overall, the ABS-TPP comprising PEG 1500 was the system with the highest depletion efficiencies for both proteins (DE_{IgG} = 99.2% and DE_{HSA} = 70.1%), once again showing the critical role of the PEG molecular weight.

In all tested ABS-TPP, higher depletion efficiencies were obtained for IgG rather than for HSA. These results follow the molecular weight of both proteins, in which the higher the molecular weight the higher the depletion efficiency (IgG ~ 150 KDa[46] vs. HSA ~ 66 KDa).[47] Accordingly, IgG has a higher surface, being more prone to be affected by the PEG hydrophobicity, pH, ionic strength of the ABS and precipitate. These results are in line with the relative specificity of PEG in the precipitation of immunoglobulins and their complexes.[40,48] Additionally, the pH of the ABS-TPP (pH ~ 7 due to the K_2HPO_4/KH_2PO_4) is closer to the isoelectric point of IgG (pI = 7-9.95)[49] than HSA (pI = 4.7).[50] Under these conditions, ABS-TPP provide a more favourable environment to the precipitation of IgG because of the reduced repulsive electrostatic forces.[51,52] As mentioned above, this effect is particularly observed for PEGs with molecular weights higher than 400 g·mol^{-1} (DE_{IgG} Ó 84%). Accordingly, a work by Thompson et al.[53] has shown that PEG-induced precipitation is less efficient at pH far from the target protein pI. However, HSA solubility seems to be less affected by the PEG molecular weight (DE_{HSA} values fluctuated between 18% to 70%) and its depletion is more likely to be governed by electrostatic interactions.

3.2. Extraction of CYFRA 21-1 using ABS and ABS-TPP

The partition of CYFRA 21-1 using the proposed ABS was initially investigated using an aqueous solution of CYFRA 21-1 at a known concentration. These studies allow to address the interactions and affinities controlling the CYFRA 21-1 partition in ABS, without the influence of human serum. The results obtained are shown in Figure 3 and provided in detail in Table S2 in the Supporting Information. Regardless of the polymer molecular weight (from 400 to 6000 g.mol^{-1}), a preferential partition of CYFRA 21-1 to the PEG-rich top phase ($EE_{CYFRA\ 21\text{-}1}$ Ó 93%) was obtained. These results suggest that the salting-out effect of the K_2HPO_4/KH_2PO_4 is the driving force for the partition of CYFRA 21-1 in PEG-salt ABS. Still, a slight decrease in CYFRA 21-1 partition for the top phase takes place when using PEGs with molecular weights above 2000 g·mol^{-1}. This trend suggests that the affinity for water of PEGs also plays a role in the partition of CYFRA 21-1 in PEG-salt systems and should not be disregarded.

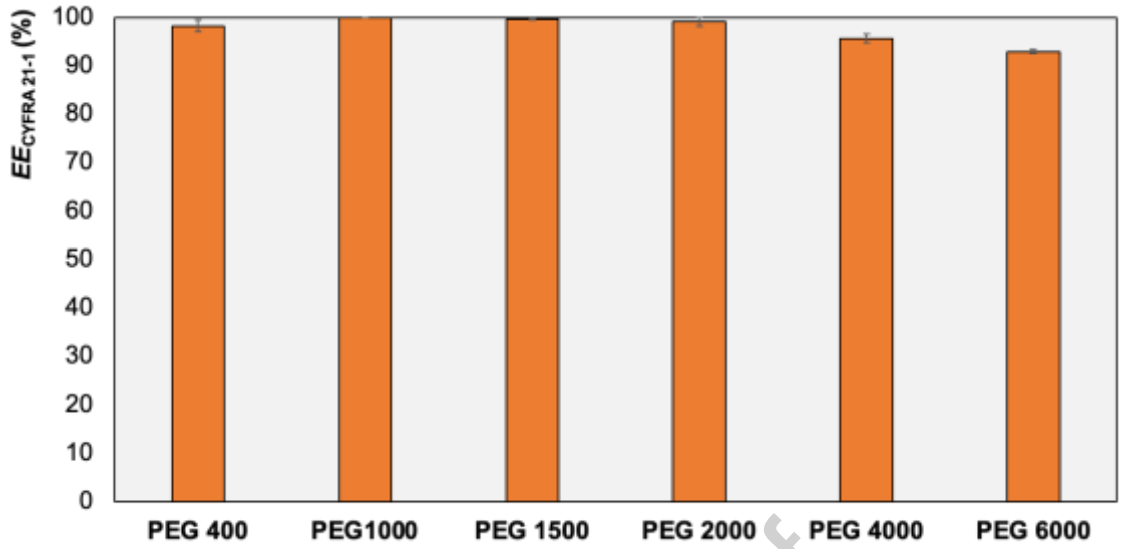

Figure 3. Extraction efficiencies of CYFRA 21-1 ($EE_{Cyfra21-1}$, %) obtained with ABS composed of 30 wt% of PEG + 12 wt% K_2HPO_4/KH_2PO_4 + 48 wt% water + 10 wt% CYFRA 21-1 aqueous solutions (1000 ng·mL^{-1}) (mean ± SD, n=2).

After investigating the partition of CYFRA 21-1 in ABS, extraction studies involving spiked human serum were conducted to infer on the simultaneous ability to deplete IgG and HSA and extract CYFRA 21-1. For this study, the ABS-TPP composed of PEG 1000, PEG 1500, PEG 2000, PEG 4000, and PEG 6000 were tested according to the highest depletion efficiencies obtained for both IgG and HSA. The recovery yields of CYFRA 21-1 ($RY_{CYFRA\ 21-1}$, %) in the top, inter, and bottom phases are depicted in Figure 4, being provided in detail in Table S3 in the Supporting Information.

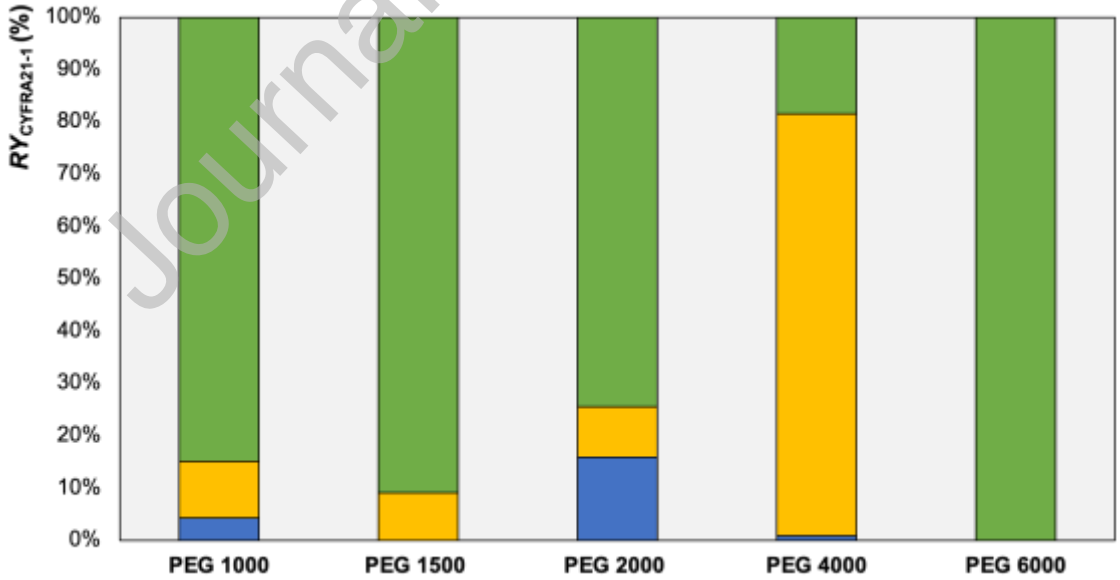

Figure 4. Recovery yields of CYFRA 21-1 ($RY_{CYFRA\ 21-1}$, %) for the top (green bars), inter (yellow bars), and bottom (blue bars) phases of the ABS-TPP composed of 30 wt% of PEG + 12 wt% of K_2HPO_4/KH_2PO_4 + 10 wt% of CYFRA 21-1 spiked human serum + 48 wt% of water (mean ± SD, n=3).

Due to the protein size difference between CYFRA 21-1 (~ 40 KDa) and the high-abundance serum proteins IgG (~ 150 KDa) and HSA (~ 66 KDa), as well as their different concentration range in the spiked serum (1000 $ng \cdot mL^{-1}$ for CYFRA 21-1 vs. 50 $mg.mL^{-1}$ for HSA[54], and ranging from 7 to 16 $mg.mL^{-1}$ for IgG[55]), different partition/precipitation patterns are anticipated. These proteins properties together with the tunability of the ABS-TPP afforded by the PEG molecular weight can lead to the extraction of the biomarker in a single phase. Using PEGs 1000, PEG 1500, PEG 2000 and PEG 6000 there is a clear preferential extraction of CYFRA 21-1 to the PEG-rich top phase ($RY_{\text{CYFRA 21-1}}$ Ó 74%), with PEG 6000 achieving a complete extraction (100%) of CYFRA 21-1. On the other hand, when resorting to PEG 4000, only 18% of CYFRA 21-1 partitioned to the top phase. In spite of the similar DE_{IgG} and DE_{HSA} results obtained for PEG 4000 and PEG 6000, these results do not imply a similar profile for CYFRA 21-1 extraction. Since different polymers' molecular weights induce different extraction/depletion profiles, optimization needs to be carried for each protein. These results agree with the evidence reported by Tubio et al.[56] for similar structured proteins.

Overall, both high abundance protein depletion and biomarker extraction steps are contingent on the molecular weight and affinity for water of the PEGs under appraisal, stressing the importance of the PEG properties in the development of one-step serum pretreatment and biomarker extraction strategies. Since the ability to simultaneously perform an efficient depletion of high-abundance serum proteins and extraction of the target biomarker is a desired feature in the field of biomarker analysis, the optimal PEG molecular weight is set here at 1500 $g \cdot mol^{-1}$ (DE_{IgG} = 99% and DE_{HSA} = 70%; $RY_{\text{CYFRA 21-1}}$ = 91%). Due to technical limitations inherent to the used ELISA, the method was developed using serum spiked with 1000 $ng \cdot mL^{-1}$ of CYFRA 21-1, whereas concentrations above the cut-off 3.3 $ng \cdot mL^{-1}$ are indicative of cancer.[5] . Nevertheless, it should be remarked that demonstrating that CYFRA 21-1 does not suffer depletion at higher concentrations can be seen as a benefit, proving that the phase in which it is enriched is not saturated. However, it is crucial to address matrix effects, which may not be relevant at higher biomarker concentrations, but could impact results at lower biomarker concentrations. This consideration emphasizes the importance of future studies for a robust validation across a range of clinically relevant concentrations to ensure the method's applicability in diagnostic settings.

4. Conclusions

This study clearly demonstrates the critical role of the PEG molecular weight in the development of serum pretreatment and lung cancer biomarker extraction resorting to ABS-TPP. Initially, ABS composed of various molecular weight PEGs and K_2HPO_4/KH_2PO_4 at pH ≈ 7 were applied to the depletion of two high-abundance serum proteins – IgG and HSA. Upon the addition of serum, ABS-TPP were formed, allowing the depletion of IgG and HSA at the interphase. As a combined result of the polymer molecular weight, salting-out effect and electrostatic interactions, the highest depletion efficiencies were obtained by the ABS-TPP composed of PEG 1500 (i.e., DE_{IgG} = 99.2% and DE_{HSA} = 70.1%). Regarding the extraction of CYFRA 21-1, results using aqueous solutions showed that the biomarker has higher affinity for the PEG-rich phase. However, different affinities were achieved when spiked serum was added to the systems, suggesting the matrix effect on CYFRA 21-1 extraction. Such variable partition patterns are dependent on the PEG molecular weight. While the ABS-TPP

composed of PEG 4000 led to a significant biomarker loss at the interphase (81%), the remaining PEGs were able to extract 74+% of CYFRA 21-1 to the PEG-rich phase.

Envisioning highly accurate biomarker analyses, the ABS-TPP composed of PEG 1500 holds the greatest potential among all systems by providing the best compromise between the depletion efficiency of high abundance serum proteins and recovery yield of CYFRA 21-1 (91%) simultaneously achieved. Our findings show that, if the PEG is properly designed, ABS-TPP are efficient serum pretreatment and biomarker extraction tools that may potentially improve the accuracy and sensitivity of currently available strategies for the detection of lung cancer biomarkers. Furthermore, these systems could extend their scope of application in proteomic studies, particularly in targeted biomarker analysis, by optimizing ABS-TPP composition and extraction/depletion profiles. However, it is important to acknowledge their limited applicability in proteomic studies due to the complexity and variability of protein abundance and characteristics.

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Conflict of interests

The authors declare no commercial or financial conflict of interest.

Author Contributions

M.E.R.: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing-original draft, Visualization; M.S.M.M.: Methodology, Validation, Formal analysis, Investigation, Data Curation, Writing – review & editing; D.C.V.B.: Methodology, Investigation; J.A.P.C.: Writing – review & editing, Supervision; F.A.eS.: Writing – review & editing, Supervision, Funding Acquisition, Project Administration; M.G.F.: Conceptualization, Writing – review & editing, Supervision, Funding Acquisition.

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Figure captions

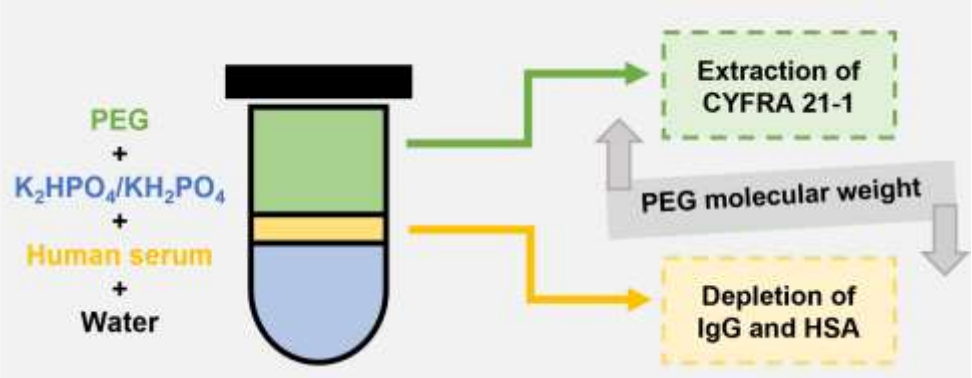
Figure 1. Development of the ABS-TPP-based approach proposed in the current work for application in the pretreatment of human serum samples and CYFRA 21-1 extraction.

Figure 2. Depletion efficiencies obtained for IgG (DE_{IgG}, %) (green bars) and HSA (DE_{HSA}, %) (yellow bars) at the interphase obtained with ABS-TPP composed of 30 wt% of PEG + 12 wt% K_2HPO_4/KH_2PO_4 + 10 wt% human serum + 48 wt% water (mean ± SD, n=3). Data for the ABS-TPP composed of PEG 1000 is taken from the literature[38].

Figure 3. Extraction efficiencies of CYFRA 21-1 ($EE_{Cyfra21-1}$, %) obtained with ABS composed of 30 wt% of PEG + 12 wt% K_2HPO_4/KH_2PO_4 + 48 wt% water + 10 wt% CYFRA 21-1 aqueous solutions (1000 $ng \cdot mL^{-1}$) (mean ± SD, n=3).

Figure 4. Recovery yields of CYFRA 21-1 ($RY_{CYFRA\ 21-1}$, %) for the top (green bars), inter (yellow bars), and bottom (blue bars) phases of the ABS-TPP composed of 30 wt% of PEG + 12 wt% of K_2HPO_4/KH_2PO_4 + 10 wt% of CYFRA 21-1 spiked human serum + 48 wt% of water (mean ± SD, n=3).

Graphical abstract



Declaration of interests

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: