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Functional Magnetic Microdroplets for Antibody Extraction

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Antibodies play an essential role in modern medicine for diagnostic and therapeutic applications. Even though the production of antibodies is the fastest growing pharmaceutical industry area, the cost of antibodies remains high, which limits access to antibody-based medicine both in developing and developed countries. The bottleneck and major cost factor in the production is purification of the antibody. Here, a proof-of-concept is presented for antibody extraction using ferrofluid microdroplets. An external magnetic field splits oilbased ferrofluid droplets into an array of daughter microdroplets, which serve as a magnetically tunable, hydrophobic, liquid substrate with a relatively large surface area. A fusion protein (HFBI-Protein A), added to the solution surrounding the magnetic droplets, adsorbs strongly at the liquid-liquid interface by the hydrophobin HFBI moiety, creating a bifunctional monolayer that can catch antibody molecules. After adsorption at the liquid-liquid interface, these antibody molecules can be released by decreasing the pH of the solution. The antibody extraction process is investigated using confocal microscopy and gel electrophoresis. In addition, the effect of HFBI on the field-induced ferrofluid droplet splitting is examined. This study provides a proof of concept for utilizing liquid-liquid instead of a solid-liquid system in antibody handling.

1. Introduction

Due to their shape flexibility and ability to trap and orient surface-active molecules and nanoparticles, liquid-fluid inter-

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faces present an ideal platform for functional nanomaterial self-assembly and synthesis of functional bio-membranes.^[1] Therefore, controlling the physical and chemical properties of interfaces is of fundamental importance in different materials such as foams, emulsions, and proteins and nanoparticle self-assembly.^[2,3,4]

Proteins in general and especially surface-active proteins, such as hydrophobins, tend to self-assemble at liquidfluid interfaces (Figure S1 and S2, Supporting Information). Consequently, their behavior at interfaces has been extensively investigated, and their use as functional layers for bio-applications, such as protein purification, drug solubility, and tissue engineering, has received considerable attention.^[5,6,7]

Hydrophobins are a family of surfaceactive proteins produced by filamentous fungi, for example Schizophyllum commune, Magnaporthe grisea and Trichoderma reesei.^[8,9,10] Hydrophobins play a key role

in keeping the fungi alive in different environments: they lower the surface tension to help hyphae to penetrate air-water interfaces and allow the fungi to adhere onto both hydrophobic and hydrophilic surfaces due to their amphiphilic nature (Figure S3, Supporting Information).^[11] Due to their rigidity and small size, hydrophobins can be considered as biological nanoparticles. Hydrophobins are small (7-10 kDa) and are internally crosslinked through four disulfide bonds, tying the molecule into a compact, rigid, globular structure. Importantly, the disulfide bonds prevent the denaturation of hydrophobins at interfaces and keep them in an amphiphilic structure, which makes them highly surface-active proteins.[11] To add more functionality to the hydrophobin molecules, they can be covalently linked with different functional biomolecules, such as other proteins like Protein A, enzymes or suitable polypeptides, to form fusion proteins.^[12,6,5]

Antibodies are relatively large Y-shaped biomolecules (150 kDa) responsible for the recognition and the memory of the mammalian immune system. Antibodies form an essential part of the defense mechanism against invading pathogens like bacteria and viruses.^[13] Accordingly, they can be used as targeted drugs for a number of diseases, for instance, for cancer therapy and in diagnostics.^[14,15]

HFBI-Protein A is a fusion protein that consists of Protein A from Staphylococcus aureus and HFBI hydrophobin protein from Trichoderma reesei.^[12] Protein A has a high affinity to bind antibody molecules.^[16] Although it consists of five domains

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each able to bind a single antibody molecule the reported binding capacity of a single Protein A is approximately two antibody molecules, likely due to sterical hindrance.^[17]

The antibody purification process is often based on noncovalent interaction between the antibody Fc portion and Protein A.^[18] Protein A is a *Staphylococcus aureus*-derived protein that is typically covalently coupled to a solid agarose bead matrix. Antibody culture supernatant is forced through a column containing the Protein A-agarose beads. The bound antibodies are then released from the column by changing the conditions in the elution buffer.

During the last two decades, it has been reported that hydrophobins can adsorb at various interfaces, especially at hydrophobic surfaces, and Class II hydrophobins, such as HFBI and HFBII, can create a hexagonally structured monolayer as confirmed by atomic force microscopy (AFM).^[19,20,21,22] This raises the question whether hydrophobins could enable a large molecule like Protein A to become preferentially located at the interface and create a stable layer for added functionalities, like purification of antibody molecules such as immunoglobulin G (IgG). Kurppa et al.^[12] showed experimentally the ability of HFBI-Protein A fusion protein to catch antibody molecules and carry them from one aqueous phase to another using an aqueous two-phase system. Here, we demonstrate the ability of HFBI-Protein A to adsorb at oil-based ferrofluid microdroplets and functionalize them to serve as a shape and position controllable functional substrate for a novel antibody purification concept (Figure 1).

2. Results and Discussion

Ferrofluids are colloidal suspensions of superparamagnetic nanoparticles in a carrier liquid, which can be magnetically actuated and deformed due to their strong response to magnetic fields.^[23] In this work we used oil-based ferrofluid consisting of iron oxide nanoparticles stabilized with oleic acid dispersed in transformer oil.^[24] We created populations of small magnetic droplets with large surface area using a magnetic-fieldinduced instability.^[25,26] The oil-based ferrofluid was pipetted in a container with a glass bottom filled with phosphate-buffered saline (PBS) solution. The magnetic field was created by a cylindrical permanent magnet below the container. As the field is increased by slowly bringing the magnet closer to the container, the ferrofluid droplet elongates in the field direction and eventually splits into two and more droplets. This instability is governed by the critical wavelength λ_c , which depends on the magnetic field H and its gradient, the magnetization of the ferrofluid *M* and the interfacial tension σ between the ferrofluid and the PBS solution:^[25]

$$\lambda_c \approx 2\pi \sqrt{\frac{\sigma}{\frac{d}{dz} \left(\mu_0 H M\right)}} \tag{1}$$

where μ_0 is the vacuum permeability. Droplets with a diameter larger than λ_c are unstable and split into daughter droplets.



Figure 1. Schematic representation of the antibody extraction process. a) The process starts with magnetic-field-induced splitting of the ferrofluid droplets. b) The droplets are decorated with HFBI-Protein A fusion protein, after which excess HFBI-Protein A is washed out of the system (Washing 1). c) IgG antibody molecules with possible impurities are added to the buffer solution. d) IgG molecules are caught by Protein A. After that the excess IgG molecules and any impurities are washed out (Washing 2). e) IgG molecules are released from Protein A by decreasing pH of the solution. f) The purified antibody molecules are collected.

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The substrate bottom is a hydrophilic glass slide that is more easily wetted by the aqueous PBS solution than the oil-based ferrofluid, preventing any connecting ferrofluid film from remaining between the droplets. Instead, the droplets repel one another, as they are all magnetized in the same direction along the external field *H*. This repulsion combined with attraction to the field maximum at the magnet's axis leads to a self-assembled droplet pattern.^[25]

The droplets continue to split into smaller droplets as the field is increased further, creating a large population of tiny magnetic droplets (**Figure 2**a,b). After the magnet has stopped moving and the magnetic field remains constant, the rapid droplet splitting quickly stops. However, the droplets continue to split slowly, likely due to diffusion of iron oxide nanoparticles to the ferrofluid-PBS interface, which slightly lowers the interfacial tension according to Equation (1) (Figure 2c black dots). The experiments were repeated using PBS with 0.1 mg ml⁻¹ of HFBI-Protein A (Figure 2c, red dots). The ferrofluid droplets continued to split faster in a constant magnetic field compared to the experiments done in pure PBS, due to adsorption of HFBI-Protein A at the ferrofluid interface. HFBI-Protein A lowers the interfacial tension further, which

reduces the critical wavelength, making previously stable droplets unstable.

Creating a bifunctional layer at a liquid-liquid interface was investigated using in-situ confocal laser scanning microscopy (CLSM) with the aid of Cy3 cyanine organic dye, which is a common fluorescent dye for labeling of biomolecules, including hydrophobin proteins.^[27]

In the first part of the experiment, 50 μ l of phosphate buffered saline (PBS) with labeled fusion protein (HFBI-Protein A-Cy3) at a concentration of 0.5 mg ml⁻¹ was added to the PBS solution near tiny ferrofluid droplets. Imaging was started immediately, as the molecules started diffusing in the buffer solution (**Figure 3**a) and continued for approximately 40 minutes. The results confirmed the adsorption of HFBI-Protein A at the ferrofluid-buffer interface (Figure 3b). As a control experiment, unlabeled wild type HFBI hydrophobin protein was used instead of HFBI-Protein A and the experiment was repeated as described above (Figure 3c).

In the second experiment, an unlabeled fusion protein (HFBI-Protein A) was used to decorate the interface of oil-based ferrofluid droplets. After placing the ferrofluid microdroplets on the bottom of a Petri dish, 5 μ l of HFBI-Protein A solution



Figure 2. Magnetic-field-induced ferrofluid droplet splitting (n = 4 experiments). a) Schematic of field-induced splitting. As the magnet is brought closer to the ferrofluid droplets, they become unstable and split into small daughter droplets. b) Top-view photographs of oil-based ferrofluid droplets immersed in PBS (top row) and PBS with 0.1 mg ml⁻¹ HFBI-Protein A (bottom row) at different magnetic field strengths *H* and vertical field gradients dH/dz. The images 1–4 are marked in Figure 2c. Scale bars: 2 mm. c) Normalized number of droplets N/N_0 as a function of time *t* and magnetic field strength *H*. First the magnet is brought closer to the droplets (t = 0-1.7 min), which induces droplet splitting. After the magnet has stopped moving (t > 1.7 min), *N* increases slowly for droplets immersed in pure PBS, likely due to diffusion of iron oxide nanoparticles to the ferrofluid-PBS interface. For droplets immersed in PBS with HFBI-Protein A the splitting continues faster due to diffusion of HFBI-Protein A. N_0 is the number of droplets in a constant magnetic field after the magnet has stopped moving. Black: droplets immersed in PBS, red: droplets immersed in PBS with 0.1 mg ml⁻¹ HFBI-Protein A.





with a concentration of 5 mg ml⁻¹ was added to 3 ml of PBS buffer solution. The solution was incubated for 40 min to make sure that HFBI-Protein A molecules had created a functional layer at the ferrofluid interface. Then 3 μ l of labeled antibody molecules (IgG-Cy3) with a concentration of 8.3 mg ml⁻¹ was added to the buffer and imaged for approximately 40 min (Figure 3d). The results directly show the adsorption of fusion protein in oil-based ferrofluid and its ability to catch antibody molecules.

HFBI-Protein A is a highly surface-active molecule, which can adsorb on any hydrophobic surface. To investigate antibody extraction using ferrofluid droplets, there was a need to ensure that all IgG molecules in the extraction step were extracted only from the ferrofluid interface and there were no molecules adsorbed on the walls of the container. This problem was solved by using a two-part system consisting of a substrate that can be covered totally by ferrofluid, and a disposable wall that can be changed after each step (Figure S4, Supporting Information). The two-part system with PBS buffer was placed on a cylindrical magnet. Subsequently, a layer of oil-based ferrofluid microdroplets was created on the bottom under the PBS buffer. To create the functional layer, an HFBI-Protein A fusion protein solution was added. The system was left to rest for approximately 60 min to ensure that the whole interface was covered with fusion protein molecules. then, one sample was taken from the solution before the washing step. After that, the disposable wall was changed and an IgG antibody molecule solution was added to the system. The system was left to rest for approximately 60 min to give the fusion protein enough time to catch antibody molecules, forming a HFBI-Protein A-IgG complex at the liquid-liquid interface. In this step, another sample was taken before the washing step and changing the disposable wall. To break the attraction between Protein A and IgG molecules, the pH of the solution was decreased to less than three with HCl-glycine buffer and HCl acid. As a result, IgG molecules were released from the fusion protein. The solution was collected and neutralized by adding Tris-HCl buffer (pH = 8.5), resulting in a highly purified IgG antibody solution.

To ensure that the container walls nor the bare surface of ferrofluid droplets did not contribute to antibody adsorption, control experiments were performed without the fusion protein. These showed no antibodies in the extraction step (**Figure 4**a). Furthermore, Figure 4b shows that no antibodies are obtained in the extraction step when the native type of hydrophobin is used. In contrast, Figure 4c shows that antibodies do originate from the surface of magnetic microdroplets decorated with HFBI-Protein A fusion protein. Sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) was used to



Figure 3. Confocal microscopy. a) Schematic of the experiment: an oil-based ferrofluid droplet on a glass petri dish immersed in PBS buffer containing HFBI-Protein A-Cy3, or HFBI/ IgG-Cy3, or HFBI-Protein A/ IgG-Cy3. b) Schematic and confocal images of the adsorption of HFBI-Protein A fusion protein molecules labeled with Cy3 fluorescent dye at the interface of the ferrofluid. The fusion protein is pipetted near the ferrofluid droplet, leading to adsorption to the ferrofluid interface and diffusion to PBS buffer. c) There are no IgG-Cy3 molecules adsorbed at the interface of the ferrofluid decorated with a native type of HFBI hydrophobin protein. d) The HFBI-protein A fusion protein adsorbs at the ferrofluid interface and catches the labeled IgG-Cy3 antibody molecules. Scale bars: 50 µm. (See the Supporting Movies S1–S3).



analyze the samples from all antibody purification steps. The SDS-PAGE analysis also shows that the functional magnetic microdroplet interface releases the IgG molecules when the pH is decreased to less than three in the extraction step (Figure 4c). SDS-PAGE confirmed that there are two bands at 50 and 25 kDa positions (Figure 4c), corresponding to the heavy chain (Hc) and the light chain (Lc) of the antibody molecule.

Liquid-liquid interfaces are flexible and can adsorb and orient surface-active molecules and nanoparticles. Furthermore, they are easily deformable in contrast to solid-liquid interfaces. For instance, Pickering emulsions and bijels are in principle liquidliquid systems, but particle jamming reduces their interfacial elasticity, which makes them more like solid-liquid interfaces. Therefore, they are not the best choice for applications that require control over the shape of the interface.^[28,29,30] On the other hand, oil-based ferrofluid in aqueous media offers a suitable alternative, which can act as a functional substrate featuring both shape controllability and fluidity.

The oil-based ferrofluid used here is a dispersion of hydrophilic nanoparticles in oil. This dispersion is not thermodynamically stable unless the nanoparticles are coated with a suitable surfactant, such as oleic acid or lauric acid.^[24] However, any excess surfactant can lead to the formation of an oleic acid double layer, which promotes nanoparticle adsorption on the oil-water interface and increases the rigidity of the interface due to nanoparticle jamming.^[31] Therefore, the ferrofluid used in this project is purified to minimize the amount of the excess surfactant (see Supporting Information for details about the synthesis of ferrofluid).

Protein A consists of five domains, each being able to bind a single molecule.^[32] The reported experiments have shown that the binding ratio between Protein A and IgG is 1:2, meaning that each protein A molecule can catch only two IgG molecules in solution.^[20] In the case of HFBI-Protein A fusion protein adsorbed on a solid substrate. Kurppa et al. reported that the binding ratio is $1:1.5 \pm 0.3$ HFBI-Protein A to antibody molecules.^[12] On the other hand, Soikkeli et al. reported that the binding ratio is roughly 1.9:1 HFBI-Protein A molecules to IgG antibody molecules, meaning that only every other HFBI-Protein A molecule can catch an IgG molecule.^[6] Here the ratio widely varied, even though both of them had utilized the same technique, quartz crystal microbalance (QCM). That means the binding ratio is highly sensitive and it could be affected by different experimental factors. IgG molecules are relatively large (150 kDa) compared to the size of the fusion protein (44.2 kDa) and steric hindrance could be one of the reasons for the low binding ratio.

In our case, the substrate is a liquid-liquid interface, so our results are not directly comparable with the results of Kurppa and Soikkeli.

Figure 4c shows that the ferrofluid-HFBI-Protein A system was able to catch and release the antibody molecules. By estimating the surface area present in the experimental system, we evaluated that theoretically, the test bed would be able to bind 10.4 μ g of IgG. Our densitometric quantification showed that we were able to recover 0.74 μ g of IgG (see Supporting Information for details). This leads to approximately 7% recovery of the theoretical maximum. The recovered antibody molecules concentration is about 148 ng ml⁻¹, which is clearly



low compared to the commercialized protein A resin purification method that has a capacity of 30 mg human IgG per ml.^[33] Even though the recovery is low, it confirms the ability of oilbased magnetic microdroplets to bind, and release antibody molecules. The reason for the low concentration reported here is most probably related to the nature of the layer at interface and washing steps before extracting step. According to Soikkeli the spacing between HFBI-Protein A molecules at the solid interface is approximately 2 nm, implying that the layer is not as close-packed as native type HFBI hydrophobin, showing hexagonal packing. Therefore, the complex molecules (HFBI-ProteinA-IgG) could leave the interface during the washing steps.

3. Conclusion

A new approach for extracting antibody molecules using a complex functional layer at the liquid-liquid interface instead of the commonly used solid-liquid interface was investigated. This approach combines the unique properties of two synthetic functional materials: the ability of ferrofluid to create a relatively large liquid surface area due to the external magnetic field, and the self-assembly and antibody catching of the fusion protein. To our best knowledge, this is the first time when a remotely controllable liquid material like ferrofluid and self-assembling proteins have been applied for antibody handling.

The feasibility of the extraction method was confirmed via direct and indirect evidence, including field-induced ferrofluid droplet splitting, confocal microscopy and SDS-PAGE. The decrease in ferrofluid interfacial tension, indicated by the increased number of split microdroplets, indirectly shows that the fusion protein adsorbs at the ferrofluid interface to create a functional layer. On the other hand, confocal microscopy provides a direct proof of the bifunctionality of the fusion protein (Figure 3). The SDS-PAGE analysis also shows that the bifunctional layer at magnetic microdroplet interface catches and releases the IgG molecules when the pH is decreased to less than three in the extraction step. The antibody yield could be improved by increasing the surface area by reducing the size of the microdroplets and modifying the adsorbed layer by adding some native type HFBI hydrophobin molecules, which can serve as fillers between HFBI-Protein A molecules to facilitate the fusion protein creating a denser layer. The results presented here open the door for applications that require complex functional layers in liquid-liquid systems with high final morphology controllability.

4. Experimental Section

HFBI-Protein A fusion protein and HFBI hydrophobin protein were produced and purified by VTT Technical Research Centre of Finland Ltd (Espoo, Finland) as described in Kurppa et al. 2018 and Paananen et al. 2003, respectively.^[12,19] The Cy3 labeling kit was purchased from Biovision (California, USA). Hexadecane (99%), dodecane (99%) and IgG antibodies were purchased from Sigma-Aldrich (Missouri, USA). The oil-based ferrofluid was obtained from the Laboratory of Magnetic Fluids (Timisoara, Romania) (see Supporting Information and Figure S5, Supporting Information for details).







Figure 4. SDS-PAGE experiments for extracting antibody molecules. a) IgG molecules do not adsorb at the bare ferrofluid interface. b) Ferrofluid droplets decorated with native type HFBI protein do not catch antibody molecules. c) Ferrofluid droplets decorated with HFBI-Protein A fusion protein catch and release IgG antibody molecules successfully. In the extraction step, there are two bands at 50 and 25 kDa, which refer to heavy and light chains of the antibody molecule. The numbers on the left side of the gels refer to molecular sizes in kilodalton, these numbers represent the same sizes in the marker used in the SDS-PAGE for these experiments (see Figure S6, Supporting Information).



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Ferrofluid microdroplet populations were created using fieldinduced droplet splitting.^[25] Approximately 20 μ l of oil-based ferrofluid was pipetted in a polystyrene box with a glass bottom filled with 8 ml of phosphate-buffered saline solution (PBS). Cylindrical permanent magnet (radius 10 mm, height 42 mm) underneath the box was slowly brought closer to the ferrofluid using a computer-controlled linear stage, increasing the magnetic field affecting the ferrofluid from 1.3 kA m⁻¹ to 390 kA m⁻¹, leading to sequential droplet splitting and self-assembled droplet population.

The confocal laser scanning microscopy CLSM (LSM 710 from Zeiss, Oberkochen, Germany) experiments were performed using an excitation wavelength of 561 nm and a 571–715 nm emission wavelength. A small magnet (d = 6 mm, h = 1.5 mm, and magnetic field strength = 90 mT) was placed under the glass Petri dish that contained 3 ml of PBS buffer in order to keep oil-based ferrofluid microdroplets under the PBS solution on the Petri dish. The HFBI-Protein A fusion protein and IgG antibody molecules were labeled by Cy3 cyanine organic dye. The labeling procedure was executed according to the protocol in the labeling kit (Thermo Fisher Scientific).

The antibody extraction experiments were performed as below: the two-part system (Figure S4, Supporting Information) was placed on a cylindrical magnet (3 cm diameter). Then, 3 ml of PBS was injected at the bottom. Subsequently, approximately 100 µl of oil-based ferrofluid was injected to the bottom (the glass slide) under the PBS buffer. Immediately, the ferrofluid splits to several hundreds of microdroplets that self-assemble on the substrate. Then an additional 5 ml of PBS was added as a prewashing step, and the first sample was taken (1 ml) to ensure there were no contaminants that could affect the results. After that, the rest of the 5 ml of PBS was also removed. To create the functional layer, 5 ml of HFBI-Protein A fusion protein solution was added (0.1 mg ml⁻¹). The system was left to rest for approximately 60 min to ensure that the whole interface was covered with fusion protein molecules. 1 ml was taken as a sample and 4 ml was removed. Next, 5 ml of PBS was added and subsequently removed to wash out the free fusion protein molecules. The washing step was repeated ten times. In each washing step, 1 ml was taken as a sample. After that, the disposable wall was changed and 5 ml of IgG antibody molecule solution (0.1 mg ml⁻¹) was added to the system. The system was left to rest for approximately 60 min to give the fusion protein enough time to catch antibody molecules, forming a HFBI-Protein A-IgG complex at the liquid-liquid interface. 1 ml was taken as a sample and 4 ml was removed. Then, the system was washed ten times with PBS, while taking samples each time, and the disposable wall was changed. To break the attraction between Protein A and IgG molecules, 1 ml of HCl-glycine buffer with a pH of approximately 3 and 180 μ l of HCl (0.25 M) were added to decrease the pH of the solution below Three. As a result, IgG molecules were released from the fusion protein. 3 ml of the solution was collected and neutralized by adding 75 μ l of Tris-HCl buffer (pH = 8.5), resulting in highly purified IgG antibody solution.

All samples were concentrated from 1 ml to 100 μ l using a vacuum centrifugal concentrator for approximately 4 h at room temperature.

The SDS-PAGE experiments were carried out using a TGX AnykDa polyacrylamide gel in a Criterion Cell from BIO-RAD (California, USA). First, 60 μ l of sample solution was mixed with 20 μ l of buffer that contained SDS and β -mercaptoethanol. Then the sample was placed in boiling water (100 °C) for approximately 10 min to denaturize the proteins. After the sample cooled down to the room temperature, 13.5 μ l of the sample was placed in the electrophoresis cell at 200 V voltage for approximately 30 min. Finally, the polyacrylamide gel was stained using a silver staining protocol (see Supporting Information for details).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Keywords

antibody extraction, ferrofluid, fusion protein, hydrophobin, magnetic microdroplets, protein A

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