Immobilization of *Escherichia coli* outer membrane protein N (OmpN) on magnetic nanoparticles for application in the separation of water-in-oil emulsions

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Abstract

Biosurfactants are biomolecules capable of altering interfacial tension of emulsions. Since they are produced by different microorganisms and are biodegradable their use in water-in-oil separation is being studied. In this work, *Escherichia coli* outer membrane protein, OmpN, was immobilized on magnetic nanoparticles (MNPs) to explore their application as biosurfactant in the separation of water in oil emulsions. MNPs were synthesized via coprecipitation with a hydrodynamic radius of 122 nm in water, which increased to 196 nm upon immobilization of OmpN. SEM images showed a marked tendency of the MNPs to agglomerate after lyophilization processes. This can be addressed by sonication in a dispersion medium prior to their use. FTIR spectra demonstrated immobilization of protein
on the surface of silanized MNPs. TGA analysis showed a functionalization efficiency of 8%. The biosurfactant activity of free and immobilized protein was evaluated with the aid of pendant drop shape assays and direct separation of model emulsified media. Pendant drop shape assays showed that OmpN, interfacial tensions for the same media remained above 20 mN/m most likely due to the tendency of the protein to form clusters in both the free and immobilized state. Separation of model emulsions was carried out by adding 30,000 ppm of functionalized nanoparticles to diesel emulsions. Treated water was analyzed by UV-visible spectroscopy using pure water as blank. Nanoparticles were then washed using alkaline water (pH 10) and reused. Emulsion separation capabilities of the nanoparticles did not decrease after 3-4 reuses.

**Keywords:** Magnetic nanoparticles, membrane proteins, water-in-oil, biosurfactants, surface tension

1. **Introduction**

Fossil fuels such as oil and its derivatives are widely present in our daily lives for transportation, household purposes, power generation, and an ample variety of industry applications. Currently, it is estimated that around 90% of all vehicles work using these types of fuels, which corresponds to roughly 70% of all the oil used. Even though several renewable energy technologies such as biofuels, solar and wind energy, and nuclear power have been proposed to reduce pollution and replace or decrease oil use, most of these are still in early stages of development. Additionally, they face practicality/economic applications issues or cannot compete in terms of cost with fossil fuels, thereby making our society largely dependent on their use$^{1,2}$. 
The production of contaminant byproducts is considered one of the main problems associated with the exploitation and use of oil and its derivatives. Such contaminants are produced not only as a result of extensive use during daily life activities but also along exploration and refinement stages. Perhaps the resulting wastewaters are the principal pollutants derived from the exploration and extraction of oil towards the surface. These wastewaters account for about 96 to 98% of the total contaminants in the production of oil and gas. In the United States more than 5 barrels of contaminated wastewaters are produced per barrel of oil\(^3\). Even though most of these wastewaters are reinjected into the subsurface, about 2% of them are disposed in surface ponds to attempt evaporation into the atmosphere\(^3\). This approach is unattractive mainly due to the need of long evaporation times, contamination of the soil and the cost of building and maintaining such ponds.

Produced wastewaters are complex water-in-oil emulsions, which are rich in hydrocarbons, as well as organic and inorganic compounds such as barium, iron, manganese, mercury and zinc\(^4\). Both the volume and the values of physicochemical and chemical properties in the produced wastewaters might vary considerably depending on the location of the oil field, the geologic formation, the hydrocarbon extracted and the lifetime of the reservoir\(^4\). In a new well, the ratio of oil to water produced is high and decays as time progresses \(^4\)–\(^9\). Because of this time-dependent difference in composition, no absolute compositional definition exists for the produced wastewaters\(^10\).

Aside from evaporation, one of the most common strategies to separate emulsions is the use of chemical surfactants that alter interfacial tension of emulsions. These synthetic surfactants tend to be toxic to the environment, not easily biodegradable, and their manufacturing processes and byproducts can be environmentally hazardous. Other techniques include the use of modified sponges\(^11\)–\(^12\), macroporous materials\(^13\), meshes and membranes\(^14\)–\(^16\) that are usually expensive and difficult to manufacture.
Recently, the use of biosurfactants, which are molecules of biological origin capable of altering the interfacial tension of such emulsions has attracted a lot of attention\textsuperscript{17}. Under certain operating conditions, biosurfactants might induce an interfacial tension reduction that ultimately leads to separation of the constituent phases. Biosurfactants are generally produced intracellularly or might be sometimes secreted to the extracellular space\textsuperscript{18}. Recent reports describe the production of several bacterial biosurfactants including rhamnolipids from a strain of \textit{Pseudomonas aeruginosa}\textsuperscript{19}. Li et al produced a lipopeptide biosurfactant by using a bacterial strain of \textit{Bacillus pseudomycoides} grown with soybean oil waste as its sole source of carbon and energy\textsuperscript{20}. Silva and coauthors optimized the production of two rhamnolipid biosurfactants, one that originated from \textit{P. cepacia} CCT6659 and the other from \textit{P. aeruginosa} UCP0992\textsuperscript{21,22}. Despite the inherent biodegradability of biosurfactants, their recovery upon phase separation is challenging because they generally remain fully soluble in one of the phases.

We have focused on biosurfactants from the family of \textit{E. coli} outer membrane proteins that can be produced by the overexpression of the specific gene. For example, OmpA behavior as a biosurfactant was analyzed by Molecular Dynamics simulations as well as experimentally when used as a stabilizer for decane/water emulsions. Both experimental and simulation results showed that OmpA remained stable in emulsion and had an inverse effect related to protein concentration on its stability. DNA shuffling of OmpA was carried out in order to improve its interface activity and candidates were selected to evaluate surface tension changes when exposing protein mutants to water/dodecane emulsions. Additional efforts included using Molecular Dynamics simulations to assess the interactions of peptide sequences that were obtained from the original sequence of the OmpA gene in \textit{E. coli}. It also has high homology with the commercial biosurfactant Alasan\textsuperscript{23–25}. 


Additionally, protein immobilization onto nanomaterials has proven to be an effective and useful technique since it improves the surface area and confers desired properties or specificity to nanoparticles so they can perform different tasks. For example, gold nanoparticles and their complexes with proteins are used for many analytical and therapeutic tasks\textsuperscript{26–28}. In a similar fashion, magnetic iron oxide nanoparticles are used as delivery vehicles due to their ability to interact with magnetic fields that can direct the nanoconjugate system to a desired location either in a body or a system like an water-in-oil emulsion\textsuperscript{29–32}.

Due to our group’s experience with outer membrane proteins as biosurfactants and the possibility of protein immobilization onto magnetic nanoparticles, an avenue to tackle the biosurfactant recovery issue is by immobilizing the biosurfactants on magnetic nanoparticles, which enables removal and constant reuse of these valuable molecules with the aid of magnetic fields. This opens the possibility for demulsification processes where biosurfactants can be recycled multiple times thereby leading to a more economically attractive operation.

Here we explored the immobilization of outer membrane protein (outer membrane protein N, OmpN) on magnetic nanoparticles and evaluated the ability of the prepared immobiizates in the separation of water-in-oil emulsions. Also, immobilizates were separated with the aid of a permanent magnetic field and subsequently reused to estimate their recyclability.

2. Materials and methods

2.1 Materials

All chemicals were of reagent grade and purchased from Sigma-Aldrich (USA). For production and purification of the proteins, sodium chloride (98%), tryptone, yeast extract,
agar-agar, lactose, chloramphenicol, NaH$_2$PO$_4$, Tween 20 and Tryton were used as received. The nanoparticle synthesis required FeCl$_2$ (99%), FeCl$_3$ (99%), sodium hydroxide (96%), tetramethyl ammonium hydroxide (TMAH), glutaraldehyde, and γ-aminopropyltriethoxy-silane (APTES).

2.2 Bacterial culture and protein production

Membrane proteins were recombinantly expressed in Escherichia coli (E. coli W3110/pCA24N OmpN) grown in Luria-Bertani (LB) medium supplemented with chloramphenicol. For this, isolated colonies of the modified E. coli strains previously grown in LB agar were transferred to 50 mL of LB nutrient broth, and maintained in an orbital shaker incubator overnight (MRC, UK) at 37 °C and 250 rpm. The broth was then transferred to a flask containing 500 mL of LB nutrient broth and grown under the same conditions as the 50 mL broth. Finally, this inoculum was transferred to a 4L Bioflo/Celligen 115 (New Brunswick, Canada) bioreactor and maintained under 250 rpm agitation at 37° C to achieve an Optical Density (O.D.) in the range of 0.6-0.8. Once the needed O.D. was achieved, 30 g of lactose were added to the bioreactor to induce the expression of the membrane protein. Bacteria were grown for five additional hours to maximize protein production yield. Finally, the medium was stored at 4 °C until further use.

2.3 Protein purification

Bacteria with the expressed protein were concentrated using a tangential filter of 0.22 μm to obtain a volume of retentate (biomass of the microorganism) of 400 mL. The collected biomass was then transferred to 50 mL Falcon vials and centrifuged at 4500 rpm for 1 hour to form pellets. A lysis buffer (NaH$_2$PO$_4$, NaCl, Tween 20, Tryton) was then added to the pellets in a 6:1 ratio (6mL of buffer per gram of pellet) and resuspended by vortexing vigorously. The samples were then subjected to sonication using a Vibra-cell VCX-750
(Sonics & materials inc, USA) ultra-sonic processor with a 37% amplitude for 40 cycles (20s on, 40s off) to ensure disruption of *E. coli* cells and the release of the bacterial proteins. The lysate was then centrifuged at 1400g for 1 hour and the collected supernatants subjected to purification via an IMAC Proximity Nickel resin (Bio-Rad, USA) by making use of the His-Tags present in the proteins. The presence of the protein was verified by SDS-page and the protein was quantified using a ThermoScientific™ Nanodrop in protein A280 mode. Finally, the protein was freeze-dried for 24 hours and stored at 4°C until further use.

2.4 Magnetic nanoparticle synthesis

The magnetic nanoparticles were produced via chemical co-precipitation as described by Reimers and Khelafalla\textsuperscript{33} and Massart\textsuperscript{34}. Briefly, NaOH is added to a mixture of ferric (FeCl\textsubscript{3}) and ferrous (FeCl\textsubscript{2}) chlorides in a 1:2 ratio to yield iron oxide according to the following reaction:

\[
\text{Fe}^{2+} + 2\text{Fe}^{3+} + 8\text{OH}^- \rightarrow \text{Fe}_3\text{O}_4 + 4\text{H}_2\text{O}
\]

Typically, 3.24 g of FeCl\textsubscript{3} were added to a solution of FeCl\textsubscript{2} (1.26 g in 100 ml H\textsubscript{2}O\textsubscript{mQ}) and then transferred to a vacuum flask containing 250 mL of H\textsubscript{2}O\textsubscript{mQ}. The mixture was placed in an orbital shaker at 150 rpm, then a solution of NaOH (3.2 g in 100 mL of H\textsubscript{2}O\textsubscript{mQ}) was injected at a rate of 2 mL per minute. The mixture was then kept under 150 rpm agitation, and room temperature for 3 hours. Finally, the MNPs were washed 5 times with H\textsubscript{2}O\textsubscript{mQ} via magnetic decantation and freeze-dried until further use.

2.5 Protein immobilization

To immobilize OmpN on the nanoparticles, 200 mg of freeze-dried MNPs were dissolved in 60 mL of H\textsubscript{2}O\textsubscript{mQ} (pH 8.0) and sonicated for 20 min. Then 4 mL of TMAH solution (25%) were added followed by sonication (35% amplitude, 40 s off, 20 s on) for 5 minutes. Next, 200 μL of glacial acetic acid were added to the mixture followed by 5 additional minutes of sonication
under the same conditions. This was followed by the addition of 400 μL of γ-amine-propyltriethoxy-silane (APTES) and sonication for 10 more minutes under the same conditions. APTES was added to render free amine groups on the surface of the MNPs. Next, 2 mL of glutaraldehyde (2% v/v) were added and after 30 minutes at room temperature under stagnant conditions, 2 mL of the protein solution (0.083 g/mL) was added and left overnight at room temperature and no agitation. Finally, functionalized nanoparticles (i.e., immobilizates) were washed 5 times with H$_2$O$_{mQ}$, freeze-dried and stored at 4°C until needed.

2.6 Nanoparticle characterization

Microscopy analysis was carried out on dried samples with a scanning electron microscope SEM Phenom Pro XTM (ThermoFisher Scientific, USA) at 10 kV. For this, 50 mg of the lyophilized nanoparticles were deposited over a stub covered with carbon tape and observed at 250X. For the resuspended nanoparticle solution, 5 μL of the solution were deposited over the carbon tape and dried in a convection oven at 50°C. This was followed by imaging at 2150X. Immobilization of OmpN on MNPs was confirmed via Fourier transform infrared spectroscopy (FTIR) using a Bruker Alpha II FTIR Eco-ATR (Bruker Optik GmbH, Germany). Spectra were obtained in a range of 600-4,000 cm$^{-1}$ with a resolution of 1 cm$^{-1}$ and 64 scans per sample. Immobilized amounts were also estimated via thermogravimetric analysis (TGA) using 15 mg of each sample in a simultaneous TGA/DSC (TA instruments, USA). For this, a temperature ramp of 10°C/min starting at room temperature and up to 800°C was used under a nitrogen atmosphere with a gas flow rate of 100 mL/min. TGA data is shown as weight percentage loss as a function of temperature.

2.7 Interfacial tension measurements
Inverted pendant drop shape technique was used to determine the interfacial tension of a drop of decane in a protein solution, while in the case of immobilized protein, the regular pendant drop shape technique was used. Briefly, a drop of liquid is allowed to hang from the end of a capillary until it adopts an equilibrium profile that is a unique function of the tube radius, the interfacial tension, the liquid density and the gravitational field as reported by Chen et al\textsuperscript{35}.

Interfacial tension testing was conducted on solutions containing different concentrations of OmpN (4300 and 8600 ppm) and OmpN immobilizates (4300 and 8600 ppm). To form the drop for the measurements, the protein solution was added into a quartz cuvette (heavy phase) while decane (light phase) was added using a small tube. In the case of the immobilizates and due to their dark-brown color, the cuvette was filled with decane (light phase) and the drop for measurements was formed from the immobilize solutions (heavy phase). The change of the drop profile was monitored with an Attension theta optical tensiometer (Biolin Scientific, Sweden) and recorded as a function of time.

2.8 Emulsion separation assays

To evaluate the separation capability of the immobilizates at a concentration of 35,000 ppm, diesel emulsions were prepared at different concentrations (0.5, 1 and 3% v/v) by sonication of a mixture of diesel fuel and water for 5 minutes similar to the process described by Wang et al\textsuperscript{36}. Untreated emulsions were stable for 1 week without observable phase separation. The oil-water separation induced by the immobilizates was determined by measuring light absorbance of the water recovered after the treatment with the aid of a GENESYS 10S UV-Visible spectrophotometer (Thermo Fisher Scientific, USA) scanning a range of 190-1100 nm with a 1 nm interval using deionized water as a blank. After the addition of the corresponding amount of nanoparticles to 10 mL of the emulsion, immobilizates were shaken by hand for 30 s to assure full dispersion in the diesel-water interface similar to the
procedure described by Lü et al. After treatment, the immobilizates were removed by applying an external magnetic field. Finally, To evaluate whether the immobilizates were reusable, after a separation test. They were collected via magnetic decantation with the aid of a permanent neodymium magnet (0.37 T) and subsequently washed with alkaline water (pH 10) followed by resuspension in neutral water. The recovered immobilizates were tested again as described above. This procedure was repeated for three cycles for each sample.

3 Results and discussion

3.1 Magnetic nanoparticles synthesis

SEM micrographs showed that the powder obtained after lyophilization of the MNPs presented agglomeration and formed clusters of around 300 μm as shown in Figure 1a. Additionally, Figure 1b shows that after resuspension in milliQ water clusters of approximately 30 μm were still present as well as agglomerated MNPs with diameters of about 100 nm. Sonication reduced clustering for the MNPs, which exhibited a hydrodynamic diameter of 122 nm (data not shown). This indicates that sonication is required prior to using the immobilizates in the separation experiments. This was also the case for the sonicated immobilizates, which showed a hydrodynamic diameter of 196 nm (Data not shown).
3.2 Protein purification and immobilization

Protein purity was confirmed using SDS-page in which isolated bands at around 39 kDa were detected, indicating successful purification of OmpN. Protein concentration in the purified fractions approached 1.5 mg/ml.

To validate the presence of OmpN in the immobilizates, FTIR spectra of the samples were taken as shown in Figure 2. Two peaks can be observed at 1500 cm\(^{-1}\) and 1600 cm\(^{-1}\) corresponding to amide-II and amide-I, respectively. Thermogravimetric analysis (TGA) was chosen as an additional tool to assess the amount of immobilized proteins.

TGA results indicated that all samples had an initial weight loss of around 3% due to dehydration. A second loss corresponding to physically absorbed organic compounds of 1% and, 7% was observed for bare MNPs and, OmpN MNPs, respectively. Finally, the
detachment of OmpN from the functionalized nanoparticles is represented by the final weight loss (8%), which also corresponds to the immobilized amount of protein (Figure 3).

![FTIR spectra of OmpN, MNPs and functionalized nanoparticles](image)

**Figure 2** FTIR spectra of OmpN, MNPs and functionalized nanoparticles
3.3 Interfacial tension measurements

Due to the inherent hydrophobicity of OmpN, interfacial tension measurements proved to be difficult since solubilization at high concentrations was quite difficult. Due to aggregation of both free and immobilizates of OmpN, measurements at concentrations above 8600 ppm were not possible. Figure 4 shows that for 4300 and 8600 ppm, the interfacial tension was reduced from approximately 25 mN/m to 20 mN/m. For OmpN immobilizates the experiment started at higher interfacial tension values but after 1200 seconds similar values to those of the pure protein were achieved (Figure 5).
Figure 4 Inverted pendant drop shape results for a drop of decane in an OmpN-in-water solution at 4300 and 8600 ppm
The performance of immobilizates was evaluated by measuring the absorbance of the water obtained after separating diesel-in-water emulsions. **Figure 6** shows the UV-visible spectra of the water obtained after treatment of diesel emulsions with OmpN immobilizates using deionized water as a blank. Samples treated with bare nanoparticles showed spectra similar to that of the initial emulsions. Samples treated with the immobilizates had the appearance of clear water and showed a significant reduction in intensity for a major peak observed at 350 nm. According to Shimamoto and Tubino, this peak is typically observed in diesel...
samples\textsuperscript{39}. The reduction corresponded to a removal of approximately 92% of diesel components, or in other words to a separation efficiency of 92%. After the first separation test, the immobilizates were then collected using a magnetic field and washed with alkaline water (pH 10) to carry two additional tests. The system did not show a change in its efficiency after three reuse cycles. This proves not only that OmpN immobilizates are capable of separating the constituent phases of diesel emulsions but also that immobilizing this protein onto MNPs allows the system to be reused without change for at least three cycles opening the possibility for its implementation at a larger scale.
Figure 6 Uv-visible Spectra of water recovered after treatment of Diesel-in-water with OmpN immobilizates a) 0.5% (v/v), b) 1% (v/v) and, c) 3% (v/v). Insets shows the samples collected after treatment with bare MNPs and immobilizates
4 Conclusions

The synthesized nanoparticles were successfully functionalized with OmpN and presented hydrodynamic radius of 122 nm, in the cases of MNPs and OmpN functionalized NPs, respectively. SEM micrographs of both the lyophilized and suspended nanoparticles showed the presence of clusters indicating the need of sonication of the samples prior to their evaluation in the emulsions. In the FTIR spectra the samples presented both amide I and amide II indicating the presence OmpN on nanoparticle surfaces. Additionally, TGA analysis showed an immobilization efficiency of 8% and confirmed protein presence on the nanoparticles surface. Both pendant drop and inverted pendant drop analysis indicated the ability of OmpN functionalized nanoparticles to reduce the interfacial tension of the emulsions, showing that the systems behaved as biosurfactants. Separation tests demonstrated the ability of the OmpN functionalized systems to break diesel-in-water emulsions with an efficiency of 92% and the possibility to obtain clear and minimally contaminated water after treatment. The difference between treated and pure water was minimal as observed by the UV-Vis spectra. These nanosystems showed no decrease in their performance for at least three recycles. These results indicate that OmpN functionalized nanoparticles are promising for their use in the separation of emulsions.

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