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Fluorinated NAD as an affinity surfactant

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Nicatinamide adenine dinucleotide (NAD) with an attached perfluoropolyether tail acts as an affinity surfactant in the extraction of the enzyme horse liver alcohol dehydrogenase (HLADH) from an aqueous medium into a fluorous solvent.

NAD is the coenzyme that dehydrogenase enzymes require to catalyze oxidation–reduction reactions. We synthesized a fluorinated NAD (FNAD) molecule composed of a hydrophilic NAD head group (MW 663.4 g mol^{-1}) with an attached fluorophilic perfluoropolyether tail (MW 2500 g mol^{-1}) (Fig. 1). Using previously defined procedures, NAD was first brominated, the bromine displaced using an excess of a diamine, then the amino-functional NAD coupled to a carboxy-terminal polyperfluoroether.[1] FNAD is soluble in fluorous solvents such as methoxynonafluoroubutane (HFE). Another interesting feature of FNAD is its amphiphilic character, and hence we investigated whether FNAD acts as a surfactant in HFE. In addition, the likelihood of using FNAD as an affinity surfactant to extract an enzyme with NAD-binding sites (HLADH) into a fluorous solvent was investigated.

Extraction of a hydrophilic dye is a good indication of reverse micelle formation.[2] Bromophenol blue, a water soluble dye, was prepared at 0.04 wt% in deionized H_2O. FNAD at different concentrations was dissolved in a total of 2 ml HFE in a 4 ml glass vial. Bromophenol blue, 100 µl, was added to each solution. The vials were shaken vigorously for 10 s, and then the phases were allowed to separate. The solutions were examined for dye extraction.

Fig. 2 shows a photograph of the dye extraction experiment. Five different concentrations of FNAD in HFE were used: 0, 0.015, 0.15, 1.5, and 15 mM. Concentrations of FNAD at 1.5 mM and above were able to extract significant amounts of the water-soluble dye. The critical micelle concentration of FNAD consequently appears to fall between 0.15 and 1.5 mM.

Extraction of an enzyme that requires NAD as its coenzyme, HLADH, from an aqueous buffer into HFE by FNAD was also investigated. A solution of HLADH (5 mg ml^{-1}) was prepared in 10 mM sodium phosphate buffer, pH 7.8. Different concentrations of FNAD in 2 ml of HFE were prepared in 4 ml glass vials. The enzyme solution (250 µl) was added to the vial; because it is not soluble in HFE it remained as a separate phase above the HFE. The vial was shaken vigorously for 10 s. The phases were separated by centrifugation for 5 min at 7000 rpm.

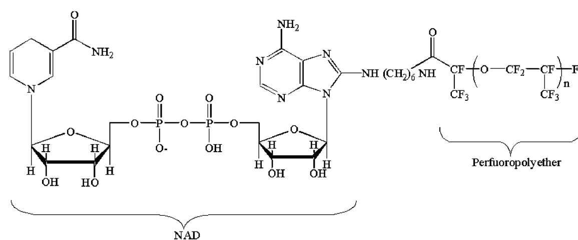

Fig. 1 Structure of FNAD.

Fig. 2 Extraction of bromophenol blue into HFE by FNAD. Concentrations of FNAD from left to right are 0, 0.015, 0.15, 1.5, and 15 mM.

The aqueous phase was assayed for its protein content using a Microprotein Determination Kit (Sigma).

Fig. 3 shows the results of protein extraction using HFE. Only the highest concentration of FNAD (15 mM) was able to extract measurable amounts of protein. FNAD at 1.5 mM was not able to extract protein, although dye could be extracted at that concentration.

Fig. 3 also shows the results of the extraction when 250 mM of both butyraldehyde and ethanol were included in the solvent. Butyraldehyde and ethanol are two viable substrates of HLADH when used in a regeneration (oxidation–reduction) reaction scheme. The addition of the substrates allowed a lower concentration of FNAD (1.5 mM) to extract HLADH. Butyraldehyde and ethanol thus appear to act as cosurfactants, assisting FNAD to extract HLADH. Likewise, alcohols have been used as cosurfactants in the formation of microemulsions containing HLADH in cyclohexane.[3]

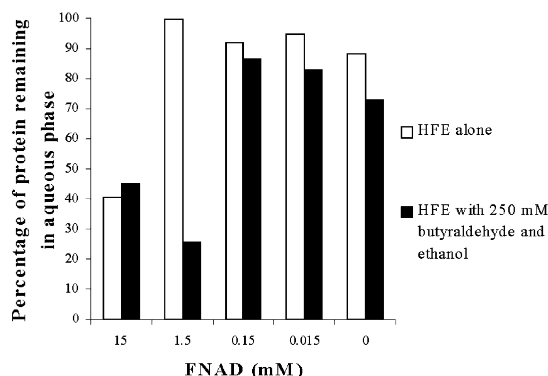

Fig. 3 Extraction of HLADH into HFE by FNAD.

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After the initial extractions of HLADH from an aqueous phase into HFE, the sample of HFE with 15 mM FNAD was shaken again with 1 ml of 10 mM phosphate buffer, pH 7.8. The phases were allowed to separate. The buffer phase (top phase) was then assayed for its protein content. HLADH was recovered in the buffer phase from the re-extraction (approximately 60% of that present in the HFE phase), further showing that protein had indeed been initially extracted into the HFE phase.

FNAD was able to strip HLADH from an aqueous solution into HFE; however, the HFE phase was not fully transparent even after centrifugation, suggesting that large micelles capable of scattering light were formed. Each HLADH enzyme molecule has two NAD binding sites, and it is highly probable that FNAD occupied those positions with the NAD head groups of other FNAD molecules aggregating around the hydration layer of the enzyme molecule. Such an NAD–HLADH aggregate would produce a large micellar structure responsible for the observed light scattering. Possibly, addition of a cosurfactant that has a smaller head group along with a lower concentration of FNAD would form smaller micelles that are optically transparent. Although the FNAD micellular solution was not optically transparent, the solution was thermodynamically stable. A solution containing micelles formed in HFE with 6 mM FNAD and 250 mM butyraldehyde and ethanol was stable for longer than one month.

Another method to demonstrate that FNAD was able to pull HLADH from an aqueous phase into HFE was to monitor the activity of HLADH in the HFE phase. HLADH activity in water-in-oil micelles in traditional organics has been studied. The nature of the surfactant has a major impact on the activity of HLADH in a microemulsion. HLADH demonstrated higher activity in microemulsions formed with nonionic surfactants.[4] Although little structural change of HLADH was reported in reverse micelles made with the common ionic surfactant AOT (sodium bis(2-ethylhexyl)sulfosuccinate),[5] surface adsorption or invasion of the active site by the ionic surfactant may account for decreased activity in AOT reverse micelles.[6] The major difference between previous studies of HLADH in microemulsions and this study is the surfactant. FNAD differs from conventional surfactants, either ionic or nonionic, in that it is an affinity surfactant. FNAD specifically binds to the active site of the enzyme, whereas ionic and nonionic surfactants act through ionic or non-specific interactions with the enzyme, respectively. Affinity surfactants increase the efficiency of protein extraction by reverse micelles.[7]

A solution of 15 mM FNAD in HFE containing 250 mM butyraldehyde and ethanol was used to extract HLADH. Activity was monitored by the reduction of butyraldehyde to butanol; ethanol was provided as the regeneration substrate. After extraction, the HFE phase was separated from the aqueous enzyme phase to prevent any biphasic catalysis. The solution was incubated at 30 °C with shaking at 250 rpm and the production of butanol was measured using GC. Since the aqueous enzyme phase was removed, any activity would be due to HLADH that was extracted into the solvent. Fig. 4 shows the results. The concentration of butanol increased with time indicating that HLADH was present in the HFE. After 5 h, the solution was filtered using a 0.2 μm syringe filter. The solution became transparent and the production of butanol was halted, signifying that the enzyme was removed from the solution. The other implication is that the micelles must be greater that 200 nm since they could be filtered from the solution using a 0.2 μm filter.

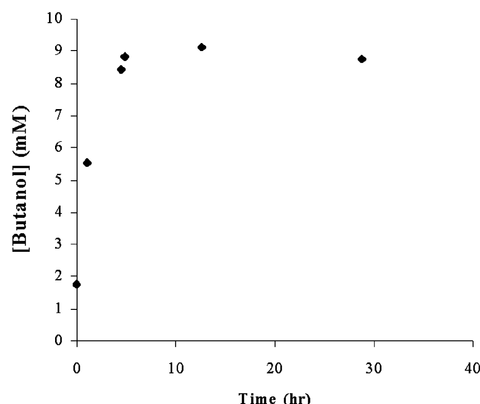

Fig. 4 Activity of HLADH extracted by FNAD in HFE.

In conclusion, FNAD was able to act as a surfactant in HFE due to its amphiphilic character. A solution of HFE with a concentration of at least 1.5 mM FNAD was able to extract the water-soluble dye bromophenol blue into the HFE phase. Furthermore, a solution of FNAD in HFE at a concentration of 15 mM was able to extract the enzyme HLADH into the fluorous phase. When 250 mM butyraldehyde and ethanol were added to the HFE, they acted as cosurfactants and a lower concentration of FNAD (1.5 mM) was able to extract HLADH into the fluorous phase. After extraction, the HFE phase was not optically transparent, but it was stable for at least one month. The HLADH contained in the microemulsion in HFE was found to be active towards some typical substrates.

Notes and references

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