Video Article

Hydrophobic Salt-modified Nafion for Enzyme Immobilization and Stabilization

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Abstract

Over the last decade, there has been a wealth of application for immobilized and stabilized enzymes including biocatalysis, biosensors, and biofuel cells.[1-3] In most bioelectrochemical applications, enzymes or organelles are immobilized onto an electrode surface with the use of some type of polymer matrix. This polymer scaffold should keep the enzymes stable and allow for the facile diffusion of molecules and ions in and out of the matrix. Most polymers used for this type of immobilization are based on polyamines or polyalcohols - polymers that mimic the natural environment of the enzymes that they encapsulate and stabilize the enzyme through hydrogen or ionic bonding. Another method for stabilizing enzymes involves the use of micelles, which contain hydrophobic regions that can encapsulate and stabilize enzymes.[4,5] In particular, the Minteer group has developed a micellar polymer based on commercially available Nafion.[6,7] Nafion itself is a micellar polymer that allows for the channel-assisted diffusion of protons and other small cations, but the micelles and channels are extremely small and the polymer is very acidic due to sulfonic acid side chains, which is unfavorable for enzyme immobilization. However, when Nafion is mixed with an excess of hydrophobic alkyl ammonium salts such as tetrabutylammonium bromide (TBAB), the quaternary ammonium cations replace the protons and become the counter ions to the sulfonate groups on the polymer side chains (**Figure 1**). This results in larger micelles and channels within the polymer that allow for the diffusion of large substrates and ions that are necessary for enzymatic function such as nicotinamide adenine dinucleotide (NAD). This modified Nafion polymer has been used to immobilize many different types of enzymes as well as mitochondria for use in biosensors and biofuel cells.[8-12] This paper describes a novel procedure for making this micellar polymer enzyme immobilization membrane that can stabilize enzymes. The synthesis of the micellar enzyme immobilization membrane, the procedure for immobilizing enzymes within the membrane, and the assays for studying enzymatic specific activity of the immobilized enzyme are detailed below.

Video Link

The video component of this article can be found at http://www.jove.com/video/3949/

Protocol

1. Modification of Nafion with Quaternary Ammonium Salts

1. Shake a bottle of 5% w/v Nafion suspension vigorously for approx. 30 seconds to ensure that Nafion is suspended uniformly in solution.
2. Pipette out 2 mL of the now re-suspended Nafion into a glass vial (vial volume could contain from 2.5 mL to 10 mL).
3. Measure a 3-fold molar excess (relative to the sulfonic acid groups on the Nafion polymer) of the alkyl ammonium bromide salt (appropriate masses are shown in **Table 1**) and add this to the vial containing the 2 mL of Nafion.
4. Vortex the vial at 1500 rpm for 10-15 minutes.
5. Pour the viscous solution into a plastic weighing tray that measures approx. 3 x 3 in., and use a pipette to transfer any residual solution from the vial to the weighing tray.
6. Allow the solvents to evaporate out of the weigh boat, leaving a yellow/brown, transparent film on the bottom of the weighing tray (**Figure 2**). The rate of solvent evaporation should be such that it takes more than 6 hours for all solvent to evaporate. If the solvent evaporates too fast, a white, crusty material will form instead of a transparent film indicating that the micellar structure of the polymer has been destroyed, and you must re-start the procedure. If solvent evaporation is too slow, a de-humidifier may be necessary, because too slow of evaporation typically leads to a tacky, orange gel and you must re-start the procedure. Typical temperature ranges for solvent evaporation are 20 - 37 °C. The actual conditions for drying are a function of the relative humidity and temperature in the room, but it is important that drying being slow to maintain micelle structure, but not too slow to allow for gel formation.
7. Fill the weigh boat with 18M Ωcm de-ionized water (10-20 mL of water), cover, and allow to soak for 12-24 hours to remove excess alkyl ammonium bromide salts and HBr.
8. Pour (or pipette) out the water and rinse 3 times with enough DI water to fill the tray each time. Be careful not to lose any of the polymer film during this step.
9. Allow the weighing tray to sit uncovered until the polymer is completely dry At this point, the polymer should be a clear and somewhat brittle plastic film. Again, if the air is very humid, a de-humidifier may be necessary to complete the evaporation in a timely manner.
10. Using a spatula, carefully remove the dried film from the weighing tray and transfer it into a clean glass vial.

11. Add 2 mL of ethanol and 3 ceramic mixing beads, and vortex for 4 hours or until the polymer film is completely re-suspended.

2. Immobilization of Enzymes into TBAB-Modified Nafion for Activity Assays

1. For a dry enzyme, weigh out 1-10 mg of the enzyme into a 1.5 mL microcentrifuge tube, and add 1 mL of 100 mM pH 7 phosphate buffer to create a 1-10 mg/mL enzyme solution. For an enzyme that is in solution, use a bicinchoninic acid (BCA) assay[13] to determine the amount of protein, and add an appropriate amount of 100 mM phosphate buffer to bring the protein concentration to 1-10 mg/mL. 1-10 mg/mL typically corresponds to 1-50 nanomoles/mL.
2. To 120 µL of the 1 mg/mL enzyme solution, add 60 µL of the alkyl ammonium-modified Nafion solution, and vortex for 10 seconds. (This mixture can be scaled up for large numbers of replicates. Keep the enzyme-to-polymer solution ratio at 2:1.)
3. Pipette 60 µL of the enzyme/polymer solution into the bottom of 3 separate 1 cm^2 cuvettes, and allow to dry overnight.

3. Assay of Immobilized NAD-Dependent Dehydrogenase Enzyme

1. To the cuvette, add 1.3 mL of 50 mM sodium pyrophosphate (pH 8.8), 1.5 mL of 15 mM NAD (freshly prepared), and 0.1 mL water.
2. Place the cuvette in a UV/Vis spectrophotometer (i.e. ThermoScientific Evolution 260 Bio and Thermo Spectronic Genesys 20) set to a wavelength of 340 nm.
3. Zero the spectrometer, and then add 0.1 mL ethanol. Mix the reagents by gently pipetting the solution up and down 5 times. For a blank, use 0.1 mL of additional water instead of the 0.1 mL of ethanol.
4. Record the absorbance at 340 nm at 5 minutes after the reagents were added to the cuvette and 20 minutes after. Plot the two data points to get a slope that can be used for activity calculations.

4. Assay of Immobilized PQQ-Dependent Dehydrogenases

1. To the cuvette, add 1.5 mL of sodium phosphate (pH 7.3) and 200 µL of 600 µM PMS.
2. Place the cuvette in a UV/Vis spectrophotometer set to a wavelength of 600 nm and then zero the spectrometer.
3. Add 100 µL of 700 µL DCIP and 200 µL of the substrate of interest (ethanol, acetaldehyde, glycerol, glucose, or glyceraldehyde), and mix the reagents by gently pipetting the solution up and down 5 times. For a blank, use 200 µL of water instead of the substrate of interest.
4. Record the absorbance at 600 nm at 5 minutes after the reagents were added to the cuvette and 20 minutes after.

5. Assay of Immobilized Glucose Oxidase

1. To the cuvette, add 2.0 mL of a solution containing 0.2 M *p*-hydroxybenzoic acid, 0.02 % (w/v) sodium azide, 128 U peroxidase, 0.3 mM 4-aminoantipyrine, 1 M potassium phosphate, and 50 mM glucose. Mix the solution by pipetting up and down 5 times.
2. Place the cuvette in a UV/Vis spectrophotometer set to a wavelength of 510 nm.
3. Record the absorbance at 510 nm at 5 minutes after the reagents were added to the cuvette and again at 20 minutes after.

6. Representative Results

The micellar structure of the modified Nafion polymer can be disrupted by drying the original salt/polymer co-casted film too fast. **Figure 2** shows a salt/polymer mixture that has been dried correctly resulting in a transparent, light brown film. A film that dries too fast can result in opaque, white flakes of polymer due to the fact that the drying process can destroy the micellar structure.

Once the modified Nafion polymer and enzyme have been mixed and co-cast onto the bottom of a cuvette, enzymatic activity assays can be used to assess the stability of the enzyme within the polymer film. **Tables 2-4** show assay results of two dehydrogenase enzymes and glucose oxidase immobilized into various modified Nafion films, respectively. Note the higher activity of the enzymes that are immobilized vs. the enzymes in buffer solution, showing that modified Nafion polymers can actually enhance the activity of certain enzymes (called superactivity). Other enzymes have transport limitations that decrease their specific activity when immobilize them in the polymer (i.e. cellulases and amylases, whose substrates are quite large macromolecules).

Quaternary ammonium salt used	3 fold excess
T3A (tetrapropylammonium bromide)	32.37 mg/ml
TBAB (tetrabutylammonium bromide)	39.19 mg/ml
TPAB (tetrapentylammonium bromide)	46.01 mg/ml
TEHA (triethylhexylammonium bromide)	32.37 mg/ml
TMHA (trimethylhexylammonium bromide)	27.25 mg/ml
TMOA (trimethyloctylammonium bromide)	30.66 mg/ml
TMDA (trimethyldecylammonium bromide)	34.07 mg/ml
TMDDA (trimethyldodecylammonium bromide)	37.48 mg/ml
TMTDA (trimethyltetradecylammonium bromide)	40.89 mg/ml
TMHDA (trimethylhexadecylammonium bromide)	44.31 mg/ml
TMODA (trimethyloctadecylammonium bromide)	47.71 mg/ml

Table 1. Amounts of tetra-alkyl ammonium salts to use for Nafion polymer modification.

Type of Nafion	Enzyme activity (U/g)
Buffer (no polymer)	16.63 ± 8.11
Nafion (un-mod.)	9.25 ± 2.21
TMTDA	3.23 ± 2.92
TBAB	3.93 ± 3.33
TMDDA	4.19 ± 1.04
TMOA	3.51 ± 1.11
TMDA	8.00 ± 4.53
TMHA	1.68 ± 1.39
TMHDA	4.83 ± 0.99
TMODA	10.45 ± 3.20

Table 2. NAD-dependent glucose dehydrogenase activity immobilized in selected modified Nafion polymers (note: immobilized activity is a function of initial specific activity of the enzyme).

Type of Nafion	Enzyme activity (mU/g)
Buffer (no polymer)	7.18 ± 0.51
Nafion (un-mod.)	70.1± 0.5
TMTDA	133 ± 6
TBAB	244 ± 4
TMDDA	221 ± 6
TMOA	1.78 ± 0.63
TMDA	206 ± 5
TEHA	40.1 ± 50.6
TMHDA	0
TMODA	1.45 ± 0.06

Table 3. PQQ-dependent glucose dehydrogenase activity immobilized in selected modified Nafion polymers (note: immobilized activity is a function of initial specific activity of the enzyme).

Type of Nafion	Enzyme activity (U/g)
Buffer (no polymer)	103.61 ± 3.15
Nafion (un-mod.)	19.93 ± 10.10
TMTDA	247.25 ± 12.49
TBAB	152.27 ± 5.29
TMDDA	262.05 ± 6.26
TMOA	129.18 ± 2.31
TMDA	141.23 ± 1.97
TMHA	131.75 ± 2.89
TMHDA	132.50 ± 1.18
TMODA	136.50 ± 0.96

Table 4. Representative glucose oxidase specific activity immobilized in selected modified Nafion polymers (note: immobilized activity is a function of initial specific activity of the enzyme).

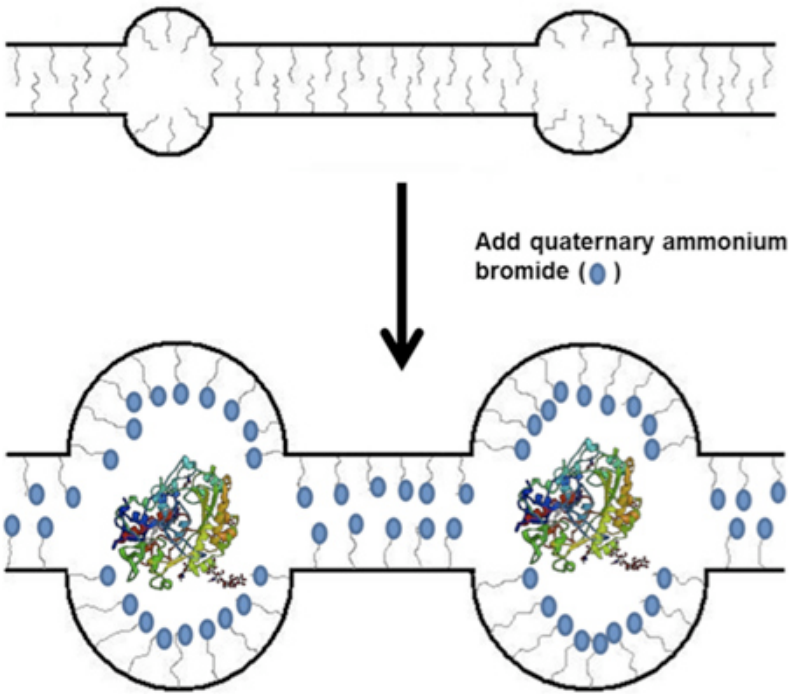

Figure 1. Schematic of TBAB incorporation into Nafion polymer and subsequent use in enzyme immobilization.

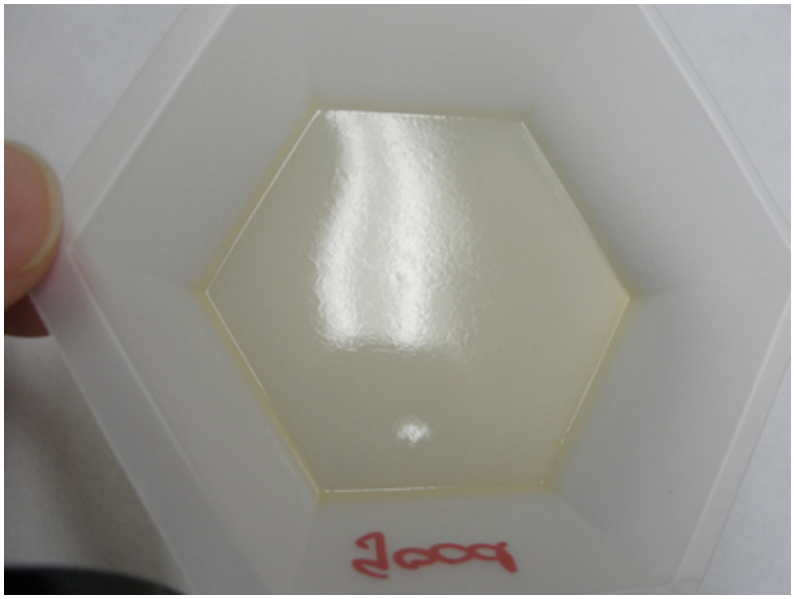

Figure 2. Optical photograph of initial co-cast films of Nafion and TBAB. Slow drying yields a transparent, light brown film covering the bottom of the weighing tray.

Discussion

In the described procedure, tetra-alkyl ammonium salts are used to modify commercial Nafion to create micellar polymers that can be used to immobilize and stabilize enzymes. The assays described in the procedure show that the polymer can be used to immobilize a wide variety of enzymes with a high retention of activity. If the enzyme of interest has very low activity or is impure, a higher concentration may be required and should not affect the immobilization process, unless immobilizing enzymes in concentrations greater than 10 mg/ml. The simplicity of the procedure separates it from other enzyme immobilization techniques in that no synthetic steps (such as polymer synthesis or cross-linking) are required. Also, these synthetic steps frequently denature proteins or dramatically decrease their activity. The protein concentration of 1mg/mL is merely a suggested concentration for high enzyme loading. Lower enzyme concentrations can always be employed, but will result in less volumetric catalytic activity. In theory, higher enzyme concentrations can be used as long as the enzyme dissolves.

Because the polymers are soluble in lower aliphatic alcohols such as methanol, ethanol, and propanol, a high percentage of alcohol must be present when mixing the polymer suspension with an enzyme. In many cases, this is not a problem as long as the ethanol is evaporated in a timely manner once the enzyme-encapsulated film is cast. However, one limitation of this immobilization can occur if an enzyme is not at all alcohol-tolerant and denatures or precipitates upon the addition of the modified Nafion suspension. In rare cases, enzymes will precipitate or denature when mixed with the modified Nafion suspension, usually indicating that the enzyme has become denatured and will not function. It is possible to decrease the alcohol content in the suspension by resuspending the polymers in alcohol/water mixtures, but lower aliphatic alcohols are required in significant concentrations (>25%) in the resuspension solution, so this immobilization technique does not work for enzymes/ enzyme solutions that cannot tolerate these concentrations of alcohol.

The enzymatic assays for each of the polymers with each of the three enzymes show that the trends in relative specific activity compared to enzyme in solution is a function of the enzyme system. This is expected, since each of the enzymes is a different size, different pI, different optimal pH, as well as the fact that PQQ-dependent glucose dehydrogenase is a membrane associated protein and therefore needs a very different chemical microenvironment that cytosolic proteins. Therefore, the hydrophobically modified micellar Nafion gives a more membrane-like environmental for stabilizing the active PQQ-dependent glucose dehydrogenase than buffer and shows superactivity, which is rare in immobilized enzymes. Another issue to consider is that polymer membranes decrease transport of large molecules and although glucose (the substrate for all three tests shown here) is small, the coenzyme NAD needs to diffuse in and out of the membrane for NAD-dependent dehydrogenases and this decreases the observed enzymatic activity. Overall, it is important to note that the exact polymer needed for each enzyme has to be optimized, because of the differences in size, charge, optimal pH, and transport of substrate/cofactors for each of the enzyme systems.

Other than immobilized enzyme assays, the primary application that has been explored with this enzyme immobilization method is the fabrication of enzymatic biosensors and biofuel cells. When modified Nafion polymers containing encapsulated redox enzymes are cast onto electrode surfaces, bioelectrocatalytic processes can occur in the presence of appropriate substrates and cofactors, producing an electric current response. Bioanodes fabricated with modified Nafion have been used in biofuel cells that utilize ethanol, methanol, pyruvate, and glycerol, as described in the introduction.

Disclosures

No conflicts of interest declared.

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