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Optical Absorption and Fluorescence of NADH Encapsulated Sol-Gel Silicate Gels

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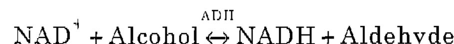
Reduced nicotinamide adenine dinucleotide (NADH) was encapsulated in transparent porous sol-gel silicate gels using by different organoalkoxysilane precursors. Characteristic optical absorption and fluorescence of NADH in the gels were examined with depending on NADH concentration and compared. Optical absorption in the amino-propyltrimethoxysilane (APTMS) gel is highest and remains constant during aging the gel. Thus, it is found that NADH in the APTMS gel is most stable and activated. On the other hand, methyltriethoxysilane (MTES) gel presents the lowest optical absorption diminishing with aging the gel. Measurable increase of fluorescence with raising the NADH concentration is observed except for the APTMS gel due to its solubility in the buffer during fluorescence measurement.

Key words: Sol-gel, Silicate gel, NADH encapsulation, Optical absorption, Fluorescence

I. Introduction

Sol-gel method has been used to encapsulate biomolecules such as enzymes and proteins with retention of activity in optically transparent porous silicate glasses for application of optical biosensors.[1-4] Biomolecules remain physically trapped in pores of the sol-gel glasses retaining their functional characteristics to a large content. Also, transparency of the matrix permits optical monitoring of the spectroscopic properties of the encapsulated biomolecules.

Oxidoreductases utilizing a soluble nicotinamide adenine dinucleotide (NAD^+) as a coenzyme can be used for optical sensors. NAD^+ acts as a hydrogen-transferring molecule in the respiratory chain forming the reduced form (NADH) by oxidation-reduction reaction with enzymes. While NAD^+ shows no optical absorption above 300nm, the reduced form NADH is a strong light absorber at 340nm and fluoresces in the blue spectral region with spectral maximum around 450nm.[5] Thus, the characteristic optical change of enzymatic reduction of the NAD^+ is feasible for measuring the course of enzymatic reactions. In actual, the exposure to its substrate of the encapsulated enzymes with NADH has shown increase in the NADH fluorescence.[3,4,6] NADH can be used as a coenzyme for the typical dehydrogenase enzyme reaction of the oxidation of alcohol to aldehyde by alcohol dehydrogenase (ADH) as shown in the following reaction.



$$NAD^+ + \text{Alcohol} \overset{ADH}{\leftrightarrow} NADH + \text{Aldehyde}$$

Recently, ADH/NAD^+ and ADH/NADH encapsulated sol-gel silicate glasses prepared using tetramethylorthosilicate (TMOS) were used in sensing of short-chained alcohols and aldehydes based on fluorescence of the NADH.[4] However, it was found that the enzymatic activity in the TMOS derived glasses is much lower than in the solution.

Sol-gel method allows organic components to incorporate into silicate glass network with high homogeneity using organoalkoxysilane precursors. These new prospective hybrid materials provide not only combining the contrary properties of inorganic and organic components but also showing the novel properties. Their molecular structures are the hybrids consisting of silicate network modified with organic component that has been called organically modified silicate (ORMOSIL).[7] Biomolecules can be also encapsulated in the ORMOSIL gels which will provide different environment around biomolecules from TMOS derived silicate gel. This may improve the stability and activity of the enzymatic reaction.

In the present study, NADH was encapsulated in transparent sol-gel silicate gels modified by different organic radicals using different organoalkoxysilanes. Optical absorption and fluorescence of the gels were examined and compared to find best sol-gel silicate matrix for better stability and activity of the encapsulated NADH.

II. Experimental

Tetramethylorthosilicate (TMOS, Aldrich), 3-aminopropyltrimethoxysilane (APTMS, Aldrich), and methyltriethoxysilane (MTES, Aldrich) were used as the precursors with different organic groups to fabricate the sol-gel silicate gels encapsulating NADH. Tri(hydroxymethyl)aminomethane (TRIS, Sigma) buffer solution was used as a catalyst to adjust solution pH.

TMOS(0.05 mole) was mixed with methanol(0.05 mole) and 0.05 M TRIS buffer(0.15 mole) for hydrolysis. As the amine group exists in APTMS as the basic species in aqueous solution, only distilled water(0.15 mole) was added into APTMS(0.05 mole). MTES(0.05 mole) was hydrolyzed with addition of 0.01 N HCl aqueous solution(0.15 mole) for 24 hrs. Then 1M TRIS buffer (0.025 mole) was added to the MTES sol for pH adjustment and faster gelation. NADH (5, 10, 15, 20×10^{-5} mole) was added to the buffered sols and stirred gently. Thus, the NADH concentration was settled on 1, 2, 3, and 4×10^{-3} moles per 1 mole of alkoxysilanes.(The concentration will be expressed 1, 2, 3, and 4 mM in this study) The NADH doped solutions were cast into polystyrene petri-dishes of 50 mm diameter and 10 mm thickness. The dishes were covered with Parafilm and stored within a desiccator at ambient temperature. All the samples were solidified in two days and transparent to perform the optical characterization.

Optical absorbance of the NADH encapsulated gel was examined using a UV/VIS/NIR spectrophotometer (Shimadzu UV-3101PC). The thickness of the bulk gel was measured to represent in a molar absorption coefficient. For fluorescence measurements, the powder samples crushed in a mortar were filled with addition of TRIS buffer in a quartz cuvette just before the examination. The fluorescence measurements were carried out with a spectrofluorometer (SLM Aminco 8100 series 2).

III. Results and Discussion

1. Optical Absorptions

It has been already known that the absorption spectrum of NADH in a medium presents two maxima at 260 and 340 nm.[5] The 260 nm absorption band is due to the adenine group which is a common spectral feature of NADH and NAD^+. On the other hand, the 340 nm absorption band corresponds to the electron transition of the reduced state of the coenzyme. It was reported that the extinction coefficients (ε) at the absorption maxima for NADH were $6.23\times10^{-3}cm^2mol^{-1}$ at 340 nm and $14.4\times10^{-3}cm^2mol^{-1}$ at 260 nm, respectively, with an intensity ratio of 1:2.31.[8]

Fig. 1 shows the optical absorption spectra of the encapsulated NADH in TMOS, APTMS, and MTES gels after 48 hours gelation with various NADH concentrations. It is clearly shown that two remarkable absorption peaks exist about 260 nm and 340 nm in the UV region. Both the peaks increase with increasing in NADH concentration for all the samples. However, the intensity ratios (I_{260nm}/I_{340nm}) of both bands are almost constant with 2.72, 2.69, and 3.23 in average for the TMOS, APTMS, and MTES gels, respectively as NADH concentration grows. The intensity ratio values of the TMOS and APTMS gels are very similar to the reference value in the solution. This can represent that NADH is settled in the gel matrixes. On the other hand, the value of the MTES gel is higher which means that NADH is likely to oxidize to NAD^+ since NAD^+ also exhibit optical absorption at 260nm. The absorption coefficient at maximum of the 340nm band which is the optical absorption characteristic of NADH is plotted as a function of NADH concentration as shown in Fig. 2. The absorption coefficients increase linearly for all the gels. The slopes of the plots which represent molar absorption coefficients (based on NADH molar concentration per 1mole of alkoxysilane) are 3688 $cm^{-1}M^{-1}$, 8544 $cm^{-1}M^{-1}$, and 2994 $cm^{-1}M^{-1}$ for the TMOS, APTMS, and MTES gels, respectively. The molar absorption coefficient as well as the absorption coefficient is highest for the APTMS gel. Thus, it is noted that NADH is best encapsulated and most activated in the APTMS gel. On the other hand, the MTES gel shows the lowest absorption and molar absorption coefficient. This presents that NADH is unstably encapsulated and less activated in the MTES gel. This

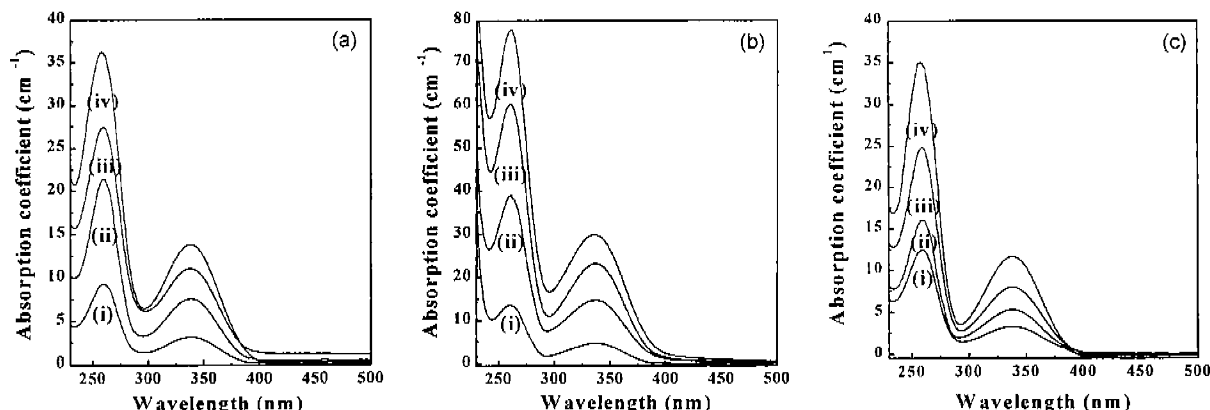


Fig. 1. Optical absorption spectra with various concentrations of NADH encapsulated in (a) TMOS (b) APTMS, and (c) MTES gels. The NADH concentrations are (i) 1 mM, (ii) 2 mM, (iii) 3 mM and (iv) 4 mM.

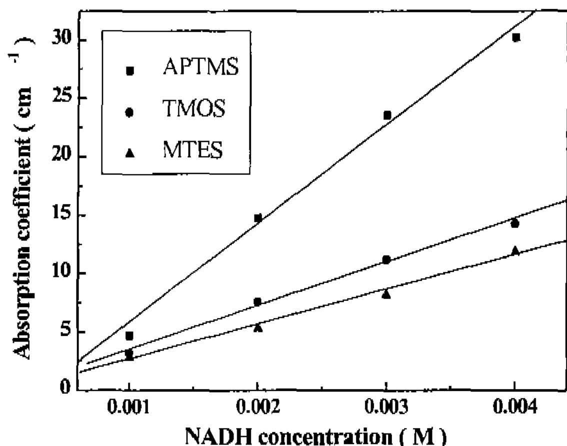


Fig. 2. Variation in absorption coefficient as a function of NADH concentration in the gels.

difference in optical absorption characteristic depending on the kind of alkoxysilanes can be described in many ways. For example, the variation in porous morphology due to different processing condition such as solution pH and mixture may cause different encapsulation state. Although the APTMS gel is strong basic in which enzymes can be denatured and large pores may be made, NADH in the APTMS gel shows the strongest optical absorption. It can be understood that hydrophilic amine ($-NH_2$) radical in the APTMS gel can encapsulate or fix NADH more effectively since NADH is aqueous soluble enzyme. Thus, the NADH encapsulated with hydrophilic environment in the APTMS gel makes more activity showing stronger optical absorption. On the other hand, NADH is unstably encapsulated around the hydrophobic methyl ($-CH_3$) radical in MTES gel. Thus, NADH in the MTES gel is not only unstable to be oxidized but also less active showing lower optical absorption.

2. Aging Effect on NADH Stability

Because NADH is prone to be oxidized to NAD^+ in air, the stable encapsulation in matrix without oxidation is important for biosensor application. Thus, we observed the absorption spectra of NADH in the gels stored in a desiccator at ambient temperature with depending on storing time.

Fig. 3 presents the variation in optical absorption spectra of the gels with gel aging time. In the TMOS and MTES gels, it is found that the 260 nm peak is raised but the 340 nm peak is reduced with storing time. After 7 days for the TMOS gel and 5 days for the MTES gel, the 340 nm absorption bands vanish. The decrease in the 340 nm absorption band may result from the discharge of NADH from the gels or the denaturalization of NADH itself during aging of the gels. However, in the cases of the discharge or the denaturalization of NADH, both the absorption bands at 260 nm and 340 nm should be reduced at the same time. Clearly, the reduced form of the pyridine ring in the NADH which represents characteristic optical absorption at 340 nm is oxidized to form NAD^+. This results in the increase of the 260 nm characteristic absorption band of the adenine group which exists in NAD^+ and NADH in common. On the other hand, both the bands at 260 nm and 340 nm of the APTMS gel remain almost constant with storing time. Fig. 4 represents the variations in intensity ratios of the peaks at 260 nm and 340 nm (I_{260nm}/I_{340nm}) with increasing gel aging time. While the intensity ratios in the MTES and TMOS gels increase rapidly, the ratio in the APTMS gel is maintained almost constantly. Because the lifetime of oxygen trapped

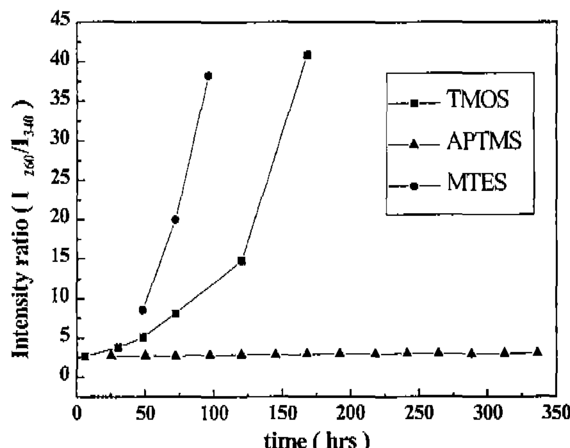


Fig. 4. Variation in intensity ratio (I_{260nm}/I_{340nm}) of absorption peaks with increasing gel aging time.

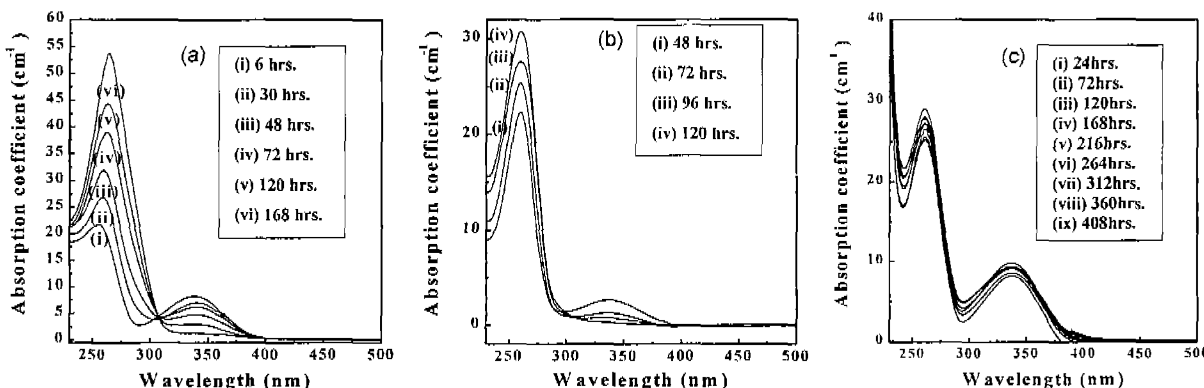


Fig. 3. Optical absorption spectra of NADH encapsulated in (a) TMOS, (b) APTMS and (c) MTES gels with gel aging time.

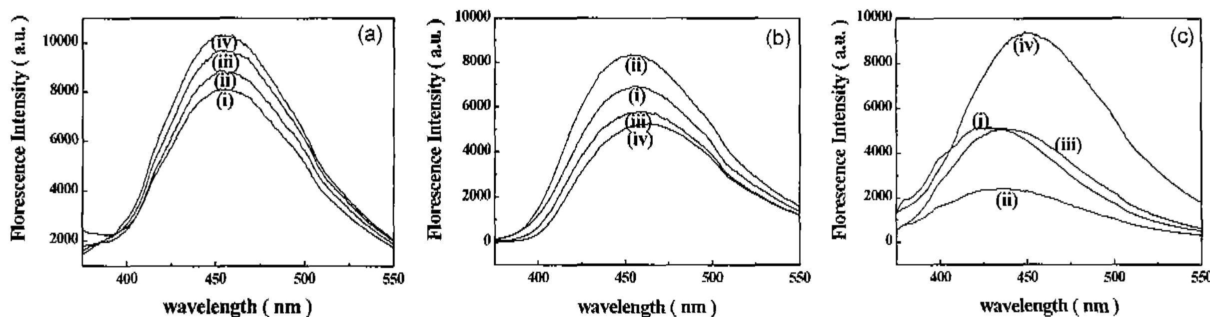


Fig. 5. Fluorescence spectra with various concentrations of NADH encapsulated in (a) TMOS, (b) APTMS and (c) MTES gels. The NADH concentrations are (i) 1 mM, (ii) 2 mM, (iii) 3 mM and (iv) 4 mM.

water,[9] NADH can be oxidized easily in the MTES (contains $-CH_3$ radical) and TMOS (uses methanol). However, since hydrophilic($-NH_2$) radical makes less oxygen trap as well as better encapsulation as discussed before, NADH in the APTMS gel is rarely oxidized to be stable in the gel.

3. Optical Fluorescence

Fig. 5 shows fluorescence spectra of the powdered gels with various NADH concentrations. As mentioned before, NADH absorbs strongly at 340 nm and fluoresces around 450 nm. All the samples show measurable fluorescence bands. The TMOS gels show symmetrical fluorescence bands and yield consistent growth in the fluorescence with increasing the NADH concentration. On the other hand, distorted fluorescence bands and inconsistent dependence of their intensities on the NADH concentration are observed in the MTES gel. This may be due to the unstable encapsulation in the gel as discussed previously. However, for the APTMS gel, nearly symmetrical fluorescence bands are shown but the peak intensity decreases with increasing the NADH concentration in the gel. Even though NADH is most stable and activated in the APTMS gel as found before, the fluorescence is reduced with increasing the NADH concentration. This indicates that the encapsulated NADH state changes during the fluorescence measurement. The fluorescence measurement was performed with the gel powder dissolved in TRIS buffer. Clearly, it was found that the APTMS gel was soluble in the TRIS buffer. It was also observed that the fluorescence disappeared as the dissolution time increased. Thus, it is noted that the dissolution of the gel encapsulating NADH in the TRIS buffer may deactivate the NADH in any reason. Specially, the alcohol in the gel produced during sol-gel reaction is dissolved in the TRIS buffer and can be involved in the reaction for the NADH to be deactivated. Because the encapsulating more NADH may deactivate NADH faster, the measured fluorescence spectrum for the higher NADH concentration can be lower. Therefore, it is not believed that the fluorescence spectra of the APTMS gel are inherent characteristic of the NADH in the gel. However, the solubility of the APTMS gel will be undesirable for sensor application although NADH encapsulated in the gel is most stable and active.

IV. Conclusion

NADH was encapsulated in transparent porous sol-gel silicate gels using TMOS, APTMS and MTES precursors. Characteristic optical absorption and fluorescence of NADH in the gels were examined depending on the NADH concentration and compared depending on organic radical in the gel. Optical absorption coefficient and molar absorption coefficient in the APTMS gel are highest and remain constant during aging the gel. Thus, it is found that NADH in the APTMS gel is most stable and activated. On the other hand, the MTES gel shows the lowest optical absorption coefficient and molar absorption coefficient vanishing with aging the gel. Thus, it is found that hydrophilic amine ($-NH_2$) radical encapsulates NADH stably and actively in the APTMS gel while hydrophobic methyl ($-CH_3$) radical in the MTES gel does not. All the gels encapsulating NADH show characteristic fluorescence. Measurable increases in the fluorescence with increasing the NADH concentration were observed for the TMOS and MTES gels. However, the NADH concentration dependence of the fluorescence for the APTMS cannot be found since the APTMS gel is dissolved in TRIS buffer during the fluorescence measurement. Thus, it is found that the APTMS gel has a problem to be soluble in aqueous solution. Therefore, it will be desirable to use APTMS and TMOS hybrid gels for the NADH-based sensor in order to obtain chemically stable gel as well as stable and active NADH encapsulation.

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