Synthesis and characterization of NAD\(^+\)-modified silica: a convenient immobilization of biomolecule via its phosphate group

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Abstract

The development of a practical tethering of \(\beta\)-nicotamide adenine dinucleotide (NAD\(^+\)) molecule on a silica surface is presented, leading to a functional biomaterial. The covalent attachment of the biomolecule was achieved through its phosphate group. The NAD\(^+\)-modified silica was mainly characterized by diffuse reflectance FTIR and \(^{13}\)C, \(^{31}\)P and \(^{29}\)Si solid state NMR spectroscopy. The electrochemical behaviour of the biomaterial was also studied using cyclic voltammetry (CV). The present method is convenient and easily applicable. Its importance is discussed and possible applications are suggested.

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Keywords: NAD\(^+\); Immobilization; Biocomposite material

1. Introduction

Bioimmobilisation provides a revolutionary way of use of biomolecules for electrochemical and optical sensing, catalysis and other applications [1,2]. Methods to immobilize biomolecules onto inorganic, organic or polymeric surfaces have typically been based on physical adsorption, covalent binding to surfaces, entrapment in semi-permeable membranes and microencapsulation into polymers and hydrogels [3].

An emerging route for bioimmobilisation involves either the entrapment of biological components into inorganic silicate matrices formed by the sol–gel-method [4], or the fixation of the biomolecules on chemically modified silica gels with organic functionalities [5]. The preparation of biomaterials generally requires the use of mild synthetic procedures to avoid the denaturation of the biomolecules and to maintain their biological function. The aim is to construct novel bioactive materials which bioactivity is comparable to other bioactive agents as, for example, drugs [6].

On the other hand, the development of ‘reagentless’ biosensors based on co-immobilisation of oxidoreductases and the coenzyme \(\beta\)-nicotamide adenine dinucleotide (NAD\(^+\)) still remains a challenge with great technological interest [7–9].

Very recently, we have developed a novel, mild and one-step procedure to immobilize vitamin B\(_1\) on a silica surface via its phosphate group leading to a very active biocatalyst [10]. The present work describes the extension of this synthetic procedure for the covalent tethering of NAD\(^+\) on a silica surface providing a new biocomposite material.

2. Materials and methods

NAD\(^+\) was purchased from Sigma and used without further purification. All other chemicals used were from Aldrich.

2.1. \(\beta\)-nicotamide adenine dinucleotide NAD\(^+\) spectroscopic characterization

IR (KBr, cm\(^{-1}\), selected peaks) 1695: amide I; 1640: amide II; 1586: ring stretching of pyrimidine + \(\delta\)NH; 1507: ring stretching of purine; 1434: amide IV; 1418: ring stretching of purine, \(C_4\text{–}N_9\text{–}C_8\text{–}H\); 1328: \(\nu\text{(PO}_2\text{)}\); 1129: \(\nu\text{(PO}_2\text{)}\); 1075: \(\nu\text{(PO}_2\text{)}\); 940: \(\nu\text{(P–O–P)}\); 721: P–O stretch.
2.2. Preparation of [NAD$^+$–OP$_2$O$_6$–SiO$_3$/$2$]$_n$–xSiO$_2$

In a methanol solution (10 ml) containing 0.5 g of NAD$^+$ and one drop of Et$_3$N, 1.25 g of silica gel (average pore diameter 150 Å) were added. The resulting slurred solution was refluxed for 2 h. The functionalized silica was isolated by filtration and extensively washed with MeOH. It was dried under reduced pressure at 60 °C.

2.3. [NAD$^+$–OP$_2$O$_6$–SiO$_3$/$2$]$_n$–xSiO$_2$ spectroscopic characterization

DRIFTS-IR (cm$^{-1}$, selected peaks): 1699: amide I; 1646: amide II; 1512: ring stretching of purine; 1480: ring stretching of purine. $^{13}$C CP MAS NMR (ppm) 164.4: CO–NH$_2$; 151.2(br): A 6, A 4, A 2; 149.1(br): N 4, N 6, A 8, N 2; 135.0: N 3; 134.1: N 5; 118.6: A 5; 100.7: N 1; 87.9: A 1; 77.2: N 2, A 2; 73.4: N 3, A 3; 67.6: N 5, N 6.

Infrared spectra were recorded on a Spectrum GX Perkin-Elmer FT-IR System. Thermogravimetric analyses were carried out using Shimadzu DTG-60 analyser. $^{13}$C, $^{31}$P and $^{29}$Si CP MAS NMR spectra were obtained on a Chemagnetics CMX 300 apparatus using the techniques of cross-polarization (CP), magic-angle spinning (MAS) and high-power proton decoupling at the resonance frequencies of 75.3425, 121.2790 and 59.5220 MHz, respectively. $^{13}$C and $^{31}$P chemical shifts were referred to those of tetramethylsilane (TMS) and 85% H$_3$PO$_4$, respectively.

Cyclic voltammetry (CV) experiments were performed with a computer-controlled potentiostat (AUTOLAB, Eco Chemie, The Netherlands), by using a voltammetry cell (VC2, BAS, IN) consists of a glassy carbon electrode (MF-2070, 3 mm diameter, BAS) as the working electrode, a Ag/AgCl/3 M KCl reference electrode (BAS) and a Pt wire as auxiliary electrode with a gold connecting pin (BAS). All experiments were carried out at 25 °C. The working solution was a deaerated 0.05 M phosphate buffer solution in 0.05 M KCl pH 7.5. Before use, the working electrode was polished with aluminum oxide according to a previously reported method [11]. Ten milligrams of bare silica or NAD$^+$-modified silica were placed over the electrode surface and covered with an aliquot of 10 μl of a 4% cellulose acetate membrane [11], which was then left to air-dry for 30 min.

3. Results and discussion

The modified silica was prepared by reaction of NAD$^+$ with silica gel (Fig. 1). The loading achieved is ca. 0.1 mmol

![Fig. 1. Preparation of the immobilized NAD biomolecule.](image-url)
determined by thermogravimetric analysis. The TGA profile showed one main weight loss in the range 260–320 °C due to loss of the organic groups from the surface.

Diffuse reflectance FTIR (‘DRIFTS’) data of the material showed absorption bands of the NAD$^+$ at ca. 1699, 1646, 1512 and 1480 cm$^{-1}$, which are attributed to the characteristic amide I and amide II vibrations and to stretching vibrations of the purine ring of adenine.

The $^{13}$C CP MAS NMR spectrum of the tethered NAD$^+$ (Fig. 2) contains the signals that characterize the immobilized biomolecule and found to be close to the $^{13}$C signals of NAD$^+$ in D$_2$O solution. It is pointed out that the $^{13}$C signals of carbons next to –NH$_2$ groups found to be also close to the $^{13}$C signals of NAD$^+$ in D$_2$O solution excluding the anchoring of NAD$^+$ via the –NH$_2$ groups. More particularly, the observed peak at 164.4 ppm is attributed to the $^{13}$C resonance of the carboxyl group, while the intense and broad signals at 149.1 and 144.9 ppm are assigned to the A$_6$, A$_4$, A$_2$ and N$_6$, A$_8$, N$_2$ carbon resonances of the NAD$^+$, respectively. The peak at 135.0 ppm is assigned to N$_3$ carbon atom, at 100.7 ppm to N$_{1'}$, at 87.9 ppm to A$_{1'}$ and at 67.6 ppm to A$_{2'}$ and N$_{5'}$ carbon atoms.

The $^{31}$P CP MAS NMR spectrum of untreated silica (Fig. 2) gave a typical spectrum for a silica sample showing two major resonance peaks at −101 and −111 ppm assigned to Q$^3$ (Si(OSi)$_3$OH) and Q$^4$ (Si(OSi)$_4$) groups and a third, quite weak signal, at −92 ppm attributed to Si with two OH groups (designated Q$^3$). In the CP MAS $^{29}$Si NMR spectrum of modified silica (Fig. 3) an enhance-
ment of the $Q^4$ peak and absence of the $Q^2$ signal were observed indicating a decrease in the number of Si–OH groups; this is consistent with the loss of protons on the OH groups of the silica upon phosphorylation. There was, however, no new signal arising from the Si–O–P moiety of the solid. Either this peak is too weak to be distinguished from the noise, or it is obscured by the strong $Q^3$ and $Q^4$ signals [15].

Besides the spectral studies, the electrochemical behavior of the modified silica was also investigated by performing CV experiments. Fig. 4 shows the CVs for an unmodified (scan a) and NAD$^+$-modified (scan b) silica. Modified silica gave rise to a relative small reductive current at $-1$ V, indicating clearly the presence of an irreversible oxidative agent. This should be the anchored NAD$^+$ biomolecule.

Evaluating the synthetic procedure presented to covalently anchor NAD$^+$ on a silica surface, we point out that it is a mild and one-step synthesis, which does not require silica pre-modification. The attachment of the biomolecule occurs via its phosphate moiety leaving unaffected its functional groups, providing in this way the basis for a wide range of covalently heterogenised biomolecules.

As far as NAD$^+$-immobilized loading is concerned, this method results in a rather low content. This fact does not limit potential applications but rather favors them, such as further silica post-modification. That is, NAD$^+$-modified silica could be further modified by a gentle silane coupling technique, e.g. $n$-aminopropyl-triethoxysilane; this will provide reactive amino-ends for cross-linking proteinic molecules via the glutaraldehyde method. In this context, the present method offers the basis to construct bifunctional packed-bed reactors in order to develop reagentless enzymic systems.
4. Conclusions

The overall conclusion is that NAD$^+$ immobilization on a silica surface through its phosphate moiety was achieved by a convenient and easily applicable method. This method results in NAD$^+$-modified silica with covalently attached biomolecules. The prepared material was mainly characterized by diffuse reflectance FTIR and $^{13}$C, $^{31}$P and $^{29}$Si solid state NMR spectroscopy. The electrochemical behaviour of the biomaterial was also studied using cyclic voltammetry. In general, the immobilized biomolecules on silica surface through its phosphate moiety could have considerable practical value in modification of silica surface.

References