NEW GLUCOSE OXIDASE - PAMAM CONJUGATE AS FLUORESCENT BIOSENSOR MATRIX IN ACETYLCELLULOSE MEMBRANE

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ABSTRACT

For the first time we report the covalent immobilization of glucose oxidase onto a fluorescent PAMAM dendrimer. A stable solid state membrane has been prepared using acetylcellulose and fluorescent enzyme-PAMAM membrane and its functional properties have been investigated. The new hybrid material obtained shows high enzyme activity. Thus it shows promising features as potential applications to the fabrication of high sensitive biosensors.

Keywords: dendrimer, 1,8-naphthalimide, acetylcellulose, Glucose oxidase, enzyme, biosensor, photophysis.

INTRODUCTION

The rapid detection of biological important compounds is of a particular interest for detecting different biologically active substances of metabolic or industrial origin. Fluorescence as a signal for identifying the presence and quality of organic compounds is widely used. For this purpose a number of appropriate sensors has been developed using fluorophores as signalling units [1-3]. Among them polymer sensors are considered as quite promising [4].

Enzymes as biological important substances have been widely used in field of medical, biochemical and food analysis. This is dictated greatest interest to enzymatic sensors in the recent years. In this case the enzyme stability is very important. Immobilization of the enzymes on biocompatible polymers is a good solution because they improve their stability, efficiency and sensitivity [5-7].

Dendrimers are a relatively new category of star shaped polymers [3]. A great deal attention has been paid to this class of macromolecules owing to their new form of structure organization, which combines the properties of low and high molecular weight compounds. Poly(amideamine) dendrimers (PAMAM) are mono disperse, well defined and developed three-dimensional structures comprising functional groups at high concentration in the dendrimer periphery [3]. In the core they possess amidic and tertiary amino functional groups. This makes them suitable materials with excellent biocompatibility and high capacity for carrying biomaterials, with potential in drug delivery and sensing applications. Our investigations on the synthesis and functional properties of some new PAMAM dendrimers decorated with 1,8-naphthalimide units in their periphery have been published recently [8-17]. Structurally modified PAMAM dendrimers with 1,8-naphthalimide derivatives are fluorescent dendrimers which can be applied as effective and selective sensors for different metal cations and protons in organic solvents [18]. This interesting property can be transferred to other polymer matrices for example textiles [19].

In this study we reports our first results on the possibility to incorporate of bio receptors on a fluorescent PAMAM dendrimer containing peripherally bonded 4-ethylamino-1,8-naphthalimide as signalling fragment. Glucose oxidase enzyme has been immobilised to the PAMAM dendrimer and acetyl cellulose has been used
for the preparation of solid state transparent matrix. The photophysical and biological properties have been investigated and discussed.

EXPERIMENTAL

Materials and methods

4-ethylamino-1,8-naphthalimide-labelled PAMAM dendrimer under study (Scheme 1) has been prepared recently [12]. N,N-dimethylformamide (DMF), ethanol and dichloromethane from Merck were of spectroscopic grade. Cu(NO₃)₂·3H₂O and Co(NO₃)₂·6H₂O, salts were the metal cation sources and used as obtained from Aldrich.

Acetyl cellulose has been used for preparation of membrane with 1% dendrimer by using a spin coating method.

Glucose oxidase [EC 1.1.3.4] from Penicillium chrysogenum was supplied by the plant of “Biovet”, Pestera, Bulgaria.

UV/vis spectrophotometric investigations were performed using “Thermo Spectronic Unicam UV 500” spectrophotometer. Fluorescence spectra were taken on a “Cary Eclipse” spectrophotometer. All spectra were recorded using 1 cm pathlength synthetic quartz glass cells at concentrations of 1.10⁻⁶ mol l⁻¹. For all fluorescent measurements, samples were excited at its absorption maxima.

The quantum fluorescence yield of dendrimer in all organic solvents has been calculated on the basis of the results obtained from the absorption and fluorescence spectra using equation 1:

$$\Phi_F = \Phi_u \frac{S_u}{S_s} \frac{A_u}{A_s} \frac{n^2}{n^2}$$  \hspace{1cm} (1)

where $F_u$ is the quantum yield of the reference, $A_u$ and $A_s$ represent the absorbance of the reference and the sample, respectively, $S_u$ and $S_s$ are the integrals of the emission of the reference and the sample respectively, and $n_u$ and $n_s$ are the refractive index of the reference and the sample, respectively. Fluorescein was used as reference ($F_0 = 0.85$).

A solar simulator (Suntest CPS+, HERAEUS), equipped with a 1.5 kW xenon arc lamp, protected with an adequate filter to simulate the solar spectrum between 290 nm and 800 nm, was used and the experiments were carried out in ordinary atmosphere.

RESULTS AND DISCUSSION

Photophysical properties

The modified PAMAM dendrimer has the structure presented in Scheme 1. It is seen that the 4-ethylamino-1,8-naphthalimide fluorophore fragments is covalently bonded to the dendrimer molecule. Table 1 presents the spectral characteristics of the 4-ethylamino-1,8-naphthalimide labelled PAMAM dendrimer in tree organic solvents with different polarity (N,N-dimethylformamide, ethanol and dichloromethane) solution: the absorption ($\lambda_a$) and fluorescence ($\lambda_f$) maxima, the extinction coefficient (e), Stokes shift ($n_a - n_f$) and quantum fluorescence yield ($F_f$).

In all organic solvents under study the dendrimer has yellow colour absorbing at $\lambda_a = 434-440$ nm and emitted green fluorescence with the fluorescence maximum at $\lambda_f = 512-528$ nm.

The molar extinction coefficient in the long wavelength band of the absorption spectra is $e = 19100-229000 \text{ l mol}^{-1} \text{ cm}^{-1}$. The dendrimer molar extinction coefficient is approximately 16 fold higher than that of the monomeric 1,8-naphthalimide derivative having the same substituent at C-4 position. This allows

\[ A = \text{NHCH}_2\text{CH}_3 \]

Scheme 1. Chemical structure of 4-ethylamino-1,8-naphthalimide-labelled PAMAM.
the suggestion that no ground state interaction occurs between the 1,8-naphthalimide chromophoric units.

The ability of the dendrimer to emit the absorbed light energy is characterized quantitatively by the quantum fluorescent yield $F$. It was determined on the basis of the absorption and fluorescence spectra of the dendrimer in organic solvents under study. From the tabulated data in Table 1, it is seen that the quantum fluorescent yields are $F = 0.26-0.32$. They are similar as other dendrimers having alkylamino substituents at C-4 position of the 1,8-naphthalimide fluorophores [8, 9, 13, 17].

The study also covered the spectral characteristics of dendrimer in thin acetyl cellulose membrane as well. In order to evaluate this membrane as substrate for biologically measurement, it is useful to study the functional properties of the material in solid state. In solid state the membrane has yellow-green colour with green fluorescence. Excitation and fluorescence spectra of the membrane are plotted in Fig. 1. The maximum of the excitation spectra is at 442 nm and the respective fluorescence maximum is at 510 nm. The fluorescence maxima of thin solid film differ significantly from those in polar organic solvents being hypsochromically shifted ($\Delta \lambda = 12-18$ nm) and is close in value to the non-polar solvent. From the Figure 1 it is also seen that the excitation spectrum is a mirror image of the fluorescence one with small overlap. This is indicative for the preserved planarity of the 1,8-naphthalimide chromophoric structure in the exited state.

Photodegradation of 4-ethylamino-1,8-naphthalimide-labelled PAMAM dendrimer in acetyl cellulose membrane

Photodegradation of the 4-ethylamino-1,8-naphthalimide-labelled PAMAM dendrimer has been measured comparing the fluorescence maxima of the acetyl cellulose membrane before and after irradiation. In Figure 2 are presented the kinetics of photodegradation of the dendrimer. It is seen then the fluorescence intensity decrease during the irradiation. No new emission maxima appear in the spectrum. Also there is not any change in the fluorescence maxima before and after the irradiation. This fact demonstrates that the products of photodestruction neither fluoresce in the spectral region

<table>
<thead>
<tr>
<th>Solvent</th>
<th>$\lambda_A$ (nm)</th>
<th>$\varepsilon$ (l mol$^{-1}$ cm$^{-1}$)</th>
<th>$\lambda_F$ (nm)</th>
<th>$\nu_f = \nu_A - \nu_F$ (cm$^{-1}$)</th>
<th>$\Phi_F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>N,N-dimethylformamide</td>
<td>440</td>
<td>194600</td>
<td>528</td>
<td>3787</td>
<td>0.26</td>
</tr>
<tr>
<td>Ethanol</td>
<td>438</td>
<td>191000</td>
<td>524</td>
<td>3747</td>
<td>0.24</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>434</td>
<td>198000</td>
<td>512</td>
<td>3510</td>
<td>0.32</td>
</tr>
</tbody>
</table>

Table 1. Photophysical properties of 4-ethylamino-1,8-naphthalimide-labelled PAMAM dendrimer in organic solvents (see text).
where the 4-ethylamino-1,8-naphthalimide-labelled PAMAM is photoactive, which is in agreement with the observations on the good photostability of some similar monomeric 1,8-naphthalimide derivatives and dendrimers [9, 12,19, 20].

**Biological investigations**

Glucose oxidase enzyme is widely applied in biochemistry, analytical chemistry and clinical diagnostic as well as in the development of microreaction biosystems. In our study glucose oxidase was used as a model enzyme to test the feasibility of its use in the design of the membrane and construction of the biosensors respectively. This enzyme was covalently immobilized on the dendrimer membrane by preliminary oxidation of carbohydrate residues of the glucose oxidase with periodic acid [22]. It was supposed that the enzyme reacts with amidic functional groups from the dendrimer core. Thus the model enzyme and dendrimer form a stable conjugate with a high activity and were used to create a stable and flexible fluorescent transparent film. In this regard, acetyl cellulose has been used as solid polymer membrane whit thickness of 30 mm. High relative activity of the immobilized enzyme (84.69 %) at pH = 6.0 has been obtained. The amount of the bonded protein to the dendrimer in the acetyl cellulose membrane was 22.48 mg/g.

In our previous study we obtained that in DMF solution Co$^{2+}$ metal cations quench the fluorescence intensity of this dendrimer [12]. The quenching effect of the Co$^{2+}$ was 56% at 4x10$^{-5}$ mol l$^{-1}$ concentration of Co$^{2+}$. In this case the coordination occurring in the cores of the dendrimer molecules is followed by a photoinduced electron transfer processes or energy transfer to the periphery of the molecule which quenches the fluorescence without any change in the maxima of the fluorescence and absorption spectra.

The ability of the new membrane to detect biologically important metal cations as Cu$^{2+}$ and Co$^{2+}$ has been tested in aqueous solution at pH=6 at metal cations concentration c = 10$^{-5}$ mol l$^{-1}$. Fig. 3 plot the change in the fluorescence intensity of the membrane in the presence of Cu$^{2+}$ and Co$^{2+}$ metal cations ($l_{ex}$ = 442 nm and $l_{em}$ = 510 nm). It is seen than the fluorescence intensity decrease in the presence of these cations. On the other hand the quenching of the fluorescence emission has weak dependence from the nature of the metal cations. Slightly higher quenching effect is observed in the presence of Co$^{2+}$ cations. The sensing properties of this system are based on the complexing ability of the PAMAM. These results indicate that during the immobilization of the enzyme on PAMAM dendrimer not all groups in the dendrimer core are reacted with the enzyme. This means that the membrane remain some active centers in the dendrimer structure, which can detect metal ions. The dendrimer at the membrane and the metal cations form a non fluorescent complex containing more than one metal per ligand according to the following relationship:

\[
\text{Dendrimer} + n \text{M}^{2+} \rightleftharpoons [\text{Dendrimer M}_n]^{2+}\text{NON-FLUORESCENT}
\]

Evidence comparing these results with results obtained in solution, we can conclude that the dendrimer retains its sensor properties after immobilization of the enzyme.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Amount of bound protein (mg/g carrier)</th>
<th>Specific activity (U/mg)</th>
<th>Relative activity (%)</th>
<th>pH opt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free</td>
<td>-</td>
<td>64.72</td>
<td>-</td>
<td>6.0</td>
</tr>
<tr>
<td>Immobilized</td>
<td>22.48</td>
<td>54.63</td>
<td>84.69</td>
<td>6.0</td>
</tr>
</tbody>
</table>
CONCLUSIONS

For the first time we have developed a novel enzyme-PAMAM fluorescent solid state composite when glucose oxidase and dendrimer form a stable conjugate with a high activity. The photophysical and biological investigations shows that the dendrimer membrane possesses promising properties for applying in biosensor constructions with fluorescent detection. Also the results show that the immobilised dendrimer could act as fluorescent sensor for detection of biological important metal cations as Cu$^{2+}$ and Co$^{2+}$.

REFERENCES


