Conjugation of 5(6)-carboxyfluorescein and 5(6)-carboxynaphthofluorescein with bovine serum albumin and their immobilization for optical pH sensing

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\textbf{Abstract}

An approach for the immobilization of a pH indicator in optical pH sensors and biosensors was developed. Fluorescent dyes 5(6)-carboxyfluorescein and 5(6)-carboxynaphthofluorescein were conjugated with bovine serum albumin as a non-enzymatic scaffold protein and the conjugation procedure was optimized. Fluorescent properties, sensitivity to temperature and photostability of conjugates have been studied and characterized into details. The conjugates were immobilized on glass support by: (i) glutaraldehyde cross-linking or (ii) entrapment in sol-gel matrix ORMOCER with subsequent glutaraldehyde cross-linking. The response to pH and leaching potential were evaluated. The sensor layer, based on immobilized conjugates of 5(6)-carboxyfluorescein and bovine serum albumin, displayed rapid response over the pH range 4.0–9.0, making it compatible with a range of applications such as bioprocessing, clinical diagnostics or environmental monitoring. Immobilized conjugates of 5(6)-carboxynaphthofluorescein and bovine serum albumin showed an enhanced sensitivity in alkaline pH levels, response for glutaraldehyde cross-linking ranged from pH 9.1 to 11.0 and from 8.3 to 10.0 for the ORMOCER entrapment, however, control of the indicator leaching was essential.

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1. Introduction

pH is one of the most frequently measured properties of solutions because many biological, chemical and geochemical processes are dependent on this physico-chemical property. Determination of pH is routinely performed using the glass electrode \cite{1}. Optical pH sensors, particularly fluorescence-based, have proven to be an attractive alternative to electrochemical pH sensing \cite{2}. They are typically constructed by immobilization of a fluorescence pH indicator on the light guide from one or more optical fibers, which are used to couple light between the indicator and the measurement instrumentation \cite{3}. The resulting sensors offer high sensitivity, feasibility of miniaturization, possibility of remote sensing and in vivo measurement. pH can be measured in electrically noisy environments that would interfere with potentiometric-type electrodes \cite{2–4}. Application of optical fluorescence-based pH sensors has been reported in marine research \cite{5–7}, biomedical diagnostics \cite{8} and monitoring of biotechnological processes \cite{9,10}. Moreover, fluorescence-based pH sensors can be used as transducers in enzymatic biosensors for the determination of glucose \cite{11,12}, urea \cite{12–15}, creatinine \cite{12}, penicillin \cite{12,16–20}, acetylcholine \cite{21}, organophosphorous pesticides \cite{22} and halogenated hydrocarbons \cite{23}.

A wide range of indicator dyes are currently available for construction of fluorescence-based pH sensors and biosensors. Fluorescein is the most commonly used dye (Table S1) with a large variety of technical applications due to high quantum yield and its large absorption in the visible field \cite{24–26}. In most cases, fluorescein and its derivatives, e.g., 5(6)-carboxyfluorescein (CF, Fig. 1), are sensitive in an acidic or near neutral pH region. Good sensitivity in an alkaline pH range is known for another derivative 5(6)-carboxynaphthofluorescein (CNF, Fig. 1). Both dyes were exploited in the construction of fiber-optic pH sensors (Table S2) and published in the articles \cite{13,12,17,18}.

Selection of an immobilization technique for fluorescent dyes is a critical step in the development of a pH sensor. The sensors based on entrapment often suffer from indicator leaching due to small size of a fluorophore \cite{29}. Functional groups of the dye are useful
for covalent binding to the matrix or other molecules, e.g., proteins, which can be cross-linked to another one. Conjugation of the dye to the proteins, e.g., bovine serum albumin (BSA), offers further benefits compared to direct immobilization of the dye. The advantage comes from the two-step immobilization procedure: (i) covalent binding of the dye to BSA and (ii) formation of the sensor layer. BSA is inexpensive and possesses chemically well-defined structure. Incorporation of such a component improves reproducibility of the immobilization protocol. Both immobilization steps can be optimized separately without influencing each other. Therefore, variations in immobilization of dye conjugates, e.g., conjugate content or development of the enzyme layer, can be performed in a simple way. Although application of conjugated pH indicator (CFN) with proteins, BSA and organophosphorus hydrolase, was reported in optical biosensors [30,31], systematic investigation of conjugates’ properties has not been described.

The objective of this study was to compare and evaluate the differences in physicochemical properties of CF and CNF and their conjugates with bovine serum albumin (CF-BSA and CNF-BSA). Two methods for immobilization of conjugated fluorescent dyes were tested: (i) direct cross-linking with glutaraldehyde (GA) and (ii) the combination of entrapment into ORGanically MODified CERamics (ORMOCER) and cross-linking. These methods are generally applicable for the development of fluorescence-based pH sensors and enzyme-based biosensors. Detailed characterization of immobilized conjugates enables a better understanding of advantages and limitations of both immobilization techniques and facilitates their selection for optical sensing.

2. Experimental

2.1. Materials

All chemicals were of an analytical grade and used without further purification. Fluorescein, hydroxylamine hydrochloride, 2-mercaptoethanol and potassium dihydrogen phosphate were purchased from Fluka (Switzerland). Cresyl violet was obtained from AnaSpec (USA), hydrogen peroxide and sulfuric acid from Penta (Czech Republic). 2-(N-morpholino)ethanesulfonic acid was purchased from Carl Roth (Germany). The solution of ORMOCER AL657 and photoinitiator Irgacure were from Fraunhofer Institute for Silicate Research (Germany). Ethanol for the UV spectroscopy, acetone, methanol and toluene of HPLC grade were purchased from Chromservis (Czech Republic). All other chemicals were purchased from Sigma–Aldrich (USA). Aqueous solutions were prepared with MilliQ water obtained using a Water Purificator Simplicity 185 of Millipore (USA).

2.2. Preparation of conjugated pH indicators

Fluorescence pH indicators CF and CNF were covalently coupled to BSA (Fig. 2) using a procedure described previously [32,33] with following modifications. CF (5 mg) was dissolved in 1 ml of 0.1 M 2-(morpholino)ethanesulfonic acid with 0.5 M sodium chloride (pH 6.0). CNF (2 mg) was dissolved in 0.4 ml of methanol. Cross-linker 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (25.5 mg for CF and 8 mg for CNF) and N-hydroxysuccinimide (3.8 mg for CF and 1.2 mg for CNF) were weighed in a glass vial and the dye solution was slowly added. The reaction components were vortexed and reacted for 1 h (CF) and for 0.25 h (CNF) at room temperature. Cross-linker was inactivated by addition of 1.4 µl (CF) or 0.56 µl (CNF) of 2-mercaptoethanol. BSA (15 mg) was dissolved in 1.5 ml of 0.1 M sodium bicarbonate buffer (pH 8.3). The reactive dye solution (0.9 ml of activated CF and 0.3 ml of activated CNF, unless otherwise noted) was slowly added while the protein solution was continuously stirred. The reaction mixture was incubated for 1 h at room temperature with continuous mixing. The reaction was terminated by the addition of 0.15 ml of 1.5 M hydroxylamine (pH 8.5) to the mixture and incubated for 1 h at 25 °C. The mixture was filtered through the Millipore Millex syringe filter with the pore size of 0.45 µm (Millipore, USA). The conjugates were separated from non-reacted dye by size-exclusion chromatography using Sephadex G-25 Superfine (GE Healthcare, Sweden) as the stationary phase and 50 mM phosphate buffer (pH 7.5) as the eluent. Prepared CF-BSA and CNF-BSA were stored in the solution with addition of sodium azide (final concentration of 2 mM) or in a lyophilized form at 4 °C.

CF-BSA and CNF-BSA with different dye-to-protein ratio were synthesised using above mentioned procedure. Reactive dye solution (150, 300, 450, 600 and 900 µl) was mixed with BSA solution (15 mg of BSA dissolved in 1.5 ml of 0.1 M sodium bicarbonate buffer, pH 8.3). Dye-to-protein ratio was determined for CF-BSA and CNF-BSA eluted in 100 mM glycine buffer (pH 8.3).

Matrix-assisted laser desorption/ionization–time of flight mass spectrometry (MALDI–TOF MS) measurements were carried out using an Ultraflex III instrument (Bruker Daltonik, Germany). Samples (0.5 µl) were pre-mixed with 2.4 µl of the matrix solution composed of 20 mg/ml 2,5-dihydroxybenzoic acid in water, trifluoroacetic acid and acetonitrile in a ratio of 79:1:20 (v/v/v). This mixture (0.6 µl) was deposited on a stainless steel MALDI target. Analyses were acquired in a linear positive ion detection arrangement covering the mass range from 2 to 200 kDa.

2.3. Immobilization of conjugated pH indicators

CF-BSA and CNF-BSA were immobilized on glass slides (26 mm × 10 mm × 1 mm, Merck, Czech Republic) as a solid support.

Fig. 1. Chemical structure of 5(6)-carboxyfluorescein (CF) and 5(6)-carboxynaphthofluorescein (CNF).

Fig. 2. Conjugation of fluorescence pH indicator (F: CF or CNF) with BSA intermediated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS).
using two methods: (i) cross-linking with GA or (ii) entrapment in ORMOCER and subsequent cross-linking with GA.

(i) Cross-linking with GA

Glass slides were treated with 5% (v/v) hydrochloric acid overnight and then with a piranha solution composed of 95.0–97.5% (v/v) sulfuric acid and 30% (v/v) hydrogen peroxide in a ratio of 3:1 (v/v) for 30 min. Subsequently, they were washed thoroughly with distilled water and dried using paper cloth. The surface of the glass slides was modified using a procedure previously described [33] and based on a 2% (v/v) silane coupling agent 3-glycidoxypropyltrimethoxysilane (GOPTS) in 95% (v/v) ethanol. Lyophilized BSA (5 mg) and lyophilized CF-BSA or CNF-BSA (5 mg) were dissolved in 50 μl of 50 mM phosphate buffer (pH 9.5). This mixture (2 μl) was applied on the modified glass slide with a pipette. The glass slide was kept in the air for 5 min and then exposed to GA vapours for 30 min at 20 °C. The immobilized conjugates were stored overnight in 10 mM phosphate buffer (pH 9.0).

(ii) Entrapment in ORMOCER and subsequent cross-linking with GA

Glass slides were washed thoroughly with toluene, acetone, ethanol and 1 M sodium hydroxide. Then they were washed with distilled water and dried using paper cloth. The ORMOCER solution was prepared by mixing 1.25 g of ORMOCER AL657 and 0.0125 g of photoinitiator Irgacure equilibrated to room temperature. A well-vortexed mixture (2 μl) was applied on the glass slide using a pipette. The glass slides were placed below a high-pressure mercury lamp (100 W, Safibra, Czech Republic) for 60 min for hardening of the immobilized layer. Lyophilized BSA (5 mg) and lyophilized CF-BSA or CNF-BSA (5 mg) were dissolved in 50 μl of 50 mM phosphate buffer (pH 7.5). This mixture (2 μl) was applied on the glass slide with preformed sol–gel layer. The glass slide was kept in the air for 5 min and then exposed to GA vapours for 30 min at 20 °C. The immobilized conjugates were stored overnight in 10 mM phosphate buffer (pH 9.0).

2.4. Characterization of conjugated pH indicators

Fluorescence spectra were measured using spectrophotometer FluoroMax-4P (HORIBA Jobin Yvon, USA) with a 150-W xenon arc lamp as a light source. A square quartz cuvette with a 1-cm pathlength was used for dye solutions, and a triangular quartz cuvette for immobilized dyes on glass slides. Sample solutions were introduced into the cuvette by injection with a syringe. All measurements were carried out in 10 mM phosphate buffer at 20 °C (unless otherwise noted) minimally in triplicate. The pH meter ORION Star 2 (Thermo Scientific, USA) with pH-electrode Double Pore (Hamilton, USA) calibrated using three pH reference buffers (Hamilton, USA) was used for pH measurements. The pHs of solutions were adjusted by addition of 3 M hydrochloric acid or 3 M sodium hydroxide. The excitation and emission spectra were recorded at maximum emission and excitation wavelength (Table 1). Fluorescence quantum yield of free and conjugated pH indicators was calculated employing the comparative Williams’ method [34]. Dilute solutions of fluorescent dyes with an absorbance below 0.1 at the maximum excitation wavelength were prepared. Corrected emission spectra were recorded on a spectrophotometer set according to Table 1. Fluorescein in 0.1 M sodium hydroxide (quantum yield of 0.95) was used as a fluorescence standard for CF and CF-BSA, cresyl violet in methanol (quantum yield of 0.53) as a standard for CNF and CNF-BSA [35]. Response to temperature was measured with dye solutions prepared according to Table 1. Both dye solutions and cuvette were equilibrated for 20 min at a temperature of 1, 5, 10, 15, 20, 25, 30, 35 and 40 °C.

Two experimental procedures were used to evaluate photostability of free and conjugated pH indicators. The short-time photostability was determined by continuous recording of the emission spectra for 3 h at 20 °C, then the dye solutions were stored at 4 °C in dark for 24 h. Dye solutions were equilibrated at 20 °C for 30 min and spectra were acquired for each dye for 3 h, as described previously. Long-term photostability was evaluated for sterile dye solutions stored at 4 °C for 100 days shielded from ambient light.

Leaching was determined for immobilized conjugates stored in 10 ml of 10 mM sterile phosphate buffer (pH 9.0) at 4 °C. Leaching of conjugates was measured after 1, 3, 7, 14, 21 and 28 days. The amount of leached conjugates was assessed as the fluorescence of conjugates in storage buffer after removal of the glass slides.

3. Results and discussion

3.1. Free and conjugated pH indicators

3.1.1. Fluorescence properties

Spectrochemical properties of pH indicators, e.g., spectral maxima, pH sensitivity and quantum yield, can be substantially affected by conjugation. The effects of conjugation on pH-dependent excitation and emission spectra of free and conjugated indicators CF and CNF were evaluated (Figs. S1 and S2). The spectra exhibited the same shape, in all cases the fluorescence intensity increased with increasing pH. The excitation maximum was almost identical for free and conjugated CF, the emission maximum of conjugated CF was slightly shifted (Table 2). The excitation and emission maximum of conjugated CNF exhibited a slight shift to higher wavelengths in comparison to free CNF (Table 2).

Effect of dye-to-protein ratio on fluorescence intensity was investigated and an optimal labelling level was determined for each indicator (Fig. 3). The dye-to-protein ratio significantly influenced the fluorescence of conjugates. Moreover, CF-BSA and CNF-BSA revealed a different profile and optima of the dye-to-protein ratio. Fluorescence of CF-BSA continuously increased with increasing dye-to-protein ratio to the highest point at the value 5.4. This ratio was interpreted as the optimum when CF-BSA exhibited sufficient

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Table 1

Experimental conditions for fluorescence measurements with fluorescent dyes and their conjugates.

<table>
<thead>
<tr>
<th>Fluorescent dye</th>
<th>Preparation of sample</th>
<th>Amount of dye (μl) in 15 ml of phosphate buffer</th>
<th>Spectrofluorometer configuration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>λex (nm)</td>
</tr>
<tr>
<td>CF</td>
<td>Methanol solution (2 mg/ml)</td>
<td>1</td>
<td>491</td>
</tr>
<tr>
<td>CF</td>
<td>10</td>
<td></td>
<td>590</td>
</tr>
<tr>
<td>CF-BSA</td>
<td>As purified on Sephadex G-25 column</td>
<td>50</td>
<td>492</td>
</tr>
<tr>
<td>CNF-BSA</td>
<td>50</td>
<td></td>
<td>594</td>
</tr>
<tr>
<td>CF-BSA</td>
<td>Immobilized with GA</td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>CNF-BSA</td>
<td>Immobilized with ORMOCER + GA</td>
<td>NA</td>
<td>621</td>
</tr>
<tr>
<td>CF-BSA</td>
<td>Immobilized with ORMOCER + GA</td>
<td>NA</td>
<td>492</td>
</tr>
<tr>
<td>CNF-BSA</td>
<td>Immobilized with ORMOCER + GA</td>
<td>NA</td>
<td>615</td>
</tr>
</tbody>
</table>

NA – not applicable.
fluorescence and the consumption of the material is still reasonable. Synthesis of CF-BSA with a higher dye-to-protein ratio produced an excess of unbound indicator. An optimum dye-to-protein ratio for CNF-BSA (when the conjugate showed the highest fluorescence) was 1.6. The fluorescence intensity decreased with a further increase in the amount of indicator molecules. The decrease in fluorescence of CNF-BSA with increasing dye-to-protein ratio could be explained by self-quenching, as previous observations for fluorescent dyes bound to human serum albumin [36]. Conjugates with selected optimal dye-to-protein ratio were used in further experiments.

The fluorescence pH indicators CF and CNF show a significant difference in their fluorescence quantum yield (Table 2). CF exhibited a quantum yield identical to fluorescein (0.93), suggesting that the extra carboxy group in CF had no discernible effect on its quantum yield. Conjugation with protein reduced fluorescence quantum yield to 0.48. In comparison to CF, the quantum yield of CNF is low (0.05) and conjugation did not affect it. Low fluorescence quantum yield of CNF and CNF-BSA can lead to application problems with insufficient intensity of the produced signal. The fluorescence quantum yield of CF-BSA is sufficiently high for application in the biosensors even after the decrease caused by the conjugation.

The effect of pH on fluorescence response of indicators was tested with 10 mM phosphate buffer in pH ranging from 2.0 to 11.0 (Fig. 4, Table 2). The pH sensitivity of free dyes and conjugates was similar with slight broadening to higher pH, particularly in the case of CNF-BSA. CF and CNF-BSA are sensitive to pH changes in a slightly acidic and neutral pH region, i.e., pH range from 5.0 to 7.8. The most sensitive dynamic ranges of CNF and CNF-BSA occurs in a neutral and alkaline pH region from 6.7 (Table 2).

Measurement of MALDI–TOF mass spectra (data not shown) and the evaluation of spectral properties and pH sensitivity confirmed that CF and CNF were bound successfully to BSA. Moreover, the conjugation procedure did not destroy the optical properties of the pH indicator molecules.

### 3.1.2. Temperature response and photostability

The spectroscopic properties of fluorophores are temperature-sensitive; therefore, the temperature effect ranging from 1 to 40°C on free indicators and their conjugates was investigated (Fig. 5). A negligible temperature effect was observed for CF, CNF and

---

**Table 2**

<table>
<thead>
<tr>
<th>Fluorescent dye</th>
<th>Form</th>
<th>λ&lt;sub&gt;ex&lt;/sub&gt; (nm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>λ&lt;sub&gt;em&lt;/sub&gt; (nm)&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Quantum yield</th>
<th>pH sensitivity</th>
<th>pK&lt;sub&gt;a&lt;/sub&gt; value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CF</td>
<td>Free</td>
<td>491</td>
<td>513</td>
<td>0.93</td>
<td>5.0–7.8</td>
<td>6.36</td>
</tr>
<tr>
<td>CF-</td>
<td>Free</td>
<td>492</td>
<td>517</td>
<td>0.48</td>
<td>5.0–7.9</td>
<td>6.43</td>
</tr>
<tr>
<td>BSA</td>
<td>GA</td>
<td>502</td>
<td>523</td>
<td>NA</td>
<td>4.0–6.5: 6.5–9.0</td>
<td>7.69</td>
</tr>
<tr>
<td>CNF</td>
<td>Free</td>
<td>492</td>
<td>525</td>
<td>NA</td>
<td>4.0–6.0: 6.0–8.7</td>
<td>7.42</td>
</tr>
<tr>
<td>CNF-</td>
<td>Free</td>
<td>590</td>
<td>662</td>
<td>0.05</td>
<td>6.7–9.1</td>
<td>7.97</td>
</tr>
<tr>
<td>BSA</td>
<td>GA</td>
<td>594</td>
<td>668</td>
<td>0.05</td>
<td>6.7–9.5; &gt;9.5</td>
<td>8.09</td>
</tr>
<tr>
<td>CNF-</td>
<td>GA</td>
<td>621</td>
<td>677</td>
<td>NA</td>
<td>9.1–11.0</td>
<td>10.16</td>
</tr>
<tr>
<td></td>
<td>ORMOCER + GA</td>
<td>615</td>
<td>675</td>
<td>NA</td>
<td>8.3–10.0</td>
<td>9.30</td>
</tr>
</tbody>
</table>

NA – not applicable.

* Spectral maxima were determined in pH 9.0; the only exception was pH 11.0 for immobilized CNF-BSA since at this pH the dye exists in the deprotonated form, yielding maximal fluorescence signal.

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![Fig. 3](image) Fluorescence of CF-BSA and CNF-BSA at the emission maximum depending on the dye-to-protein ratio.

![Fig. 4](image) Fluorescence response of CF and CF-BSA, CNF and CNF-BSA to pH changes.

![Fig. 5](image) Fluorescence response of CF, CF-BSA, CNF and CNF-BSA to temperature changes.
CF-BSA. However, CNF-BSA showed a significant increase in fluorescence up to 190% upon a temperature elevation from 1 to 40 °C. For this reason, the proper control of temperature is strongly recommended when employing CNF-BSA in a sensor.

The short-term photostability of fluorescence pH indicators towards a 150-W xenon lamp was determined (Fig. 6). Fluorescent properties of CF and CF-BSA were similar during illumination. The fluorescence of CF was found unchanged after 3 h of illumination. The fluorescence intensity of CF-BSA remained at 98% of the initial intensity. The indicator exhibited similar behaviour during the second illumination after a 24 h recovery. CNF underwent considerable photobleaching under conditions of continuous illumination. Its fluorescence was reduced to 93% after illumination for 3 h, a further fluorescence decrease to 85% was observed during the second illumination. Interestingly, fluorescence of CNF-BSA increased significantly up to 152% during the first 20 min of illumination. The fluorescence intensity was even higher (211%) after incubation in dark in comparison to the level recorded at the end of the first illumination. Further illumination led to slow decrease of the fluorescence, detecting 202% of the initial intensity after 3 h.

The indicators and their conjugates were stored for 100 days at 4 °C to determine their long-term photostability (Fig. 7). While the fluorescence of free indicators remained nearly unchanged during the entire tested period, the behaviour of conjugates was different. During storage, fluorescence increased for both CF-BSA and CNF-BSA. Fluorescence of CF-BSA increased rapidly (to 145%) during the first 10 days of storage, followed by a gradual fluorescence rise to 166% during the following 90 days. A gradual fluorescence increase (to 143%) was observed for CNF-BSA during the course of the entire tested period.

3.2. Immobilized conjugated pH indicators

3.2.1. Immobilization

Conjugated pH indicators were immobilized using two methods: (i) covalent cross-linking with GA and (ii) physical entrapment in a sol–gel matrix ORMOCER with subsequent cross-linking with GA.

(i) Molecules of conjugates were cross-linked together via formation of Schiff bases using GA as the most common cross-linking agent [37]. When the cross-linked layer was formed on the glass slides cleared by toluene, acetone, ethanol and sodium hydroxide, the layer started to delaminate from the surface upon contact with buffer. The GOPTS-modified glass slides were used to solve this problem. GOPTS provided available epoxy groups for coupling amine-containing ligands, in this case CF-BSA and CNF-BSA. The immobilization of conjugates on GOPTS-modified surfaces led to a homogeneous layer without any cracks and delamination.

(ii) ORMOCER sol–gel matrix was used for immobilization of conjugates due to many advantages, such as superior chemical and mechanical stability, high optical transparency and material versatility thanks to organic components [38]. Fluorescent dyes can be immobilized in a sol–gel matrix by chemical doping, impregnation or covalent binding. Chemical doping refers to incorporation of the dye during formation of the sol–gel glass [39]. However, mixing conjugates with an ORMOCER solution led to loss of fluorescence indicator colour and as a result, the conjugates did not exhibit any fluorescent properties. The immobilization procedure based on impregnation was tested. The ORMOCER layer was formed first and hardened below UV lamp, after which the immobilization mixture containing the conjugates was applied on the preformed glass. Conjugates were entrapped in the porous polymeric structure into which protons can diffuse. The conjugates were subsequently cross-linked using GA to minimize the leaching problem.

3.2.2. Fluorescence properties

Spectral properties and pH sensitivity of immobilized conjugated pH indicators were studied in detail and compared with properties of conjugates in solution (Table 2). The pH-dependent excitation and emission spectra of immobilized CF-BSA were similar to the respective spectra of CF-BSA in solution (Figs. S1). The emission maximum is slightly shifted to higher wavelengths (Table 2). Significant change of shape of excitation spectra was observed for CNF-BSA immobilized both by cross-linking with GA and entrapment in ORMOCER with subsequent cross-linking (Figs. S2). The excitation maxima were significantly shifted (Table 2) in comparison to the excitation maximum of 594 nm for CNF-BSA in solution. The emission spectra of immobilized CNF-BSA retained its shape, the position of maximum was significantly pH-dependent, the difference between pH 4.0 and 11.0 was about 40 nm. The emission maximum determined at pH 11.0 exhibited only a slight shift in comparison to that of CNF-BSA in solution.

The effect of immobilization on the pH response of indicators was tested in pH ranging from 2.0 to 11.0 (Fig. 4). The pH sensitivity of immobilized conjugates was different when compared to conjugates in a solution, especially the pH response of immobilized CNF-BSA which shifted to very alkaline pH. Immobilized CF-BSA responds to pH over the range 4.0–9.0, with the most sensitive dynamic pH range from 6.0 to 9.0. The apparent pK_a of immobilized CF-BSA was one pH unit higher than the pK_a value of CF-BSA in solution (Table 2). The enhanced sensitivity of immobilized CNF-BSA at higher pH levels was observed. CNF-BSA entrapped in ORMOCER exhibited pH sensitivity from 8.3 to 10.0, CNF-BSA cross-linked
with GA from 9.1 to 11.0. The apparent $pK_a$ of immobilized CNF-BSA was significantly higher than that of CNF-BSA in solution. Spectral shift and change of pH sensitivity can be most likely attributed to the effect of immobilization on an indicator microenvironment.

3.2.3. Leaching

Leaching is an important factor for consideration since the immobilized conjugates are stored in hydrated form. Leaching of conjugates into the storage solution was monitored for 28 days (Fig. 8). Unexpectedly, the detected amount of leached conjugates was dependent on the type of conjugate more than on the immobilization method. Immobilized CF-BSA was stable with slight leaching in comparison to immobilized CNF-BSA. Leaching of CF-BSA was maximal in the first day of storage regardless of the immobilization technique (approximately 10%). The leached conjugate is assumed to result from the fraction of conjugate which was not covalently cross-linked. The amount of leached CF-BSA increased only slightly during the following days. The effect of leaching can be easily eliminated by reconditioning of the immobilized layer in buffer solution before using it in sensor measurement. Significant leaching of CNF-BSA from immobilized layer was observed for conjugates immobilized by cross-linking with GA. The amount of leached CNF-BSA was 22% after the first day of storage, increasing to 65% after 28 days of storage. The leaching of CNF-BSA from ORMOCER exhibited a different trend, maximal leaching was observed after the first day (approximately 50%), and the immobilized conjugate was then stable without any further leaching.

4. Conclusions

Conjugates of CF and CNF with BSA are excellent candidates for use as fluorophores in optical pH sensors and biosensors. CF-BSA had similar fluorescence properties to free CF, the fluorescence quantum yield decreased after conjugation being the only difference between the free and conjugated form. On the contrary, CNF-BSA differs from free CNF substantially in photostability, pH and temperature sensitivity.

Two methods were investigated for immobilization of conjugates on an optical transducer which is a crucial factor for obtaining a high fluorescence signal, fast response and good stability. Both the cross-linking of CF-BSA and entrapment of CF-BSA, followed by cross-linking, is useful for biosensing applications due to minimal spectral changes, negligible leaching and good mechanical stability. The sensitivity in acidic and the near-neutral pH region is compatible with a range of applications such as bioprocessing, clinical measurements or environmental monitoring. CF-BSA cross-linked with GA has already been used for construction of enzymatic pH biosensor for the detection of halogenated compounds [23]. The performance of CNF-BSA was affected significantly by immobilization. The immobilized conjugate exhibited substantial differences in spectral properties and pH sensitivity. The enhanced sensitivity of immobilized CNF-BSA to a strong alkaline pH can be particularly useful for pH measurements, however, it will be necessary to eliminate leaching of the dye into the storage solution.

Immobilized conjugates are generally applicable for the development of optical sensors. They can be used for optical fiber tip coating as well as in evanescent wave type sensors. Conjugated pH indicators can be co-immobilized with enzymes in one layer via BSA molecules to produce an effective biosensor within a close proximity of the sensing elements and thus minimal diffusion limitations. Moreover, BSA can facilitate immobilization of enzymes with a low content of surface residues carrying the amine functional groups.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.snb.2011.09.046.

References

Biographies

Sarka Bidmanova is a Ph.D. student of Environmental Chemistry at Masaryk University in the Czech Republic. She received her M.Sc. degree in Microbiology in 2007 from Masaryk University and is currently working on the development, optimization and characterization of optical enzyme-based biosensors for military-defence and environmental applications. Her research interests also include immobilization and characterization of enzymes and fluorescence pH indicators.

Antonín Hlavacek is a Ph.D. student of Biochemistry at Masaryk University in the Czech Republic. He obtained his M.Sc. degree in Biochemistry in 2007 from Masaryk University. His research is mainly focused on the immobilization of biomolecules and the synthesis of quantum dot bioconjugates for bioanalytical applications.

Jiri Damborsky is the Josef Loschmidt Chair Professor at the Masaryk University in the Czech Republic. He obtained his M.Sc. degree in Microbiology in 1993 and Ph.D. degree in Microbiology in 1997 from Masaryk University. His research group develops new concepts and software tools, e.g., CASTER and HOTSPOT WIZARD, for protein engineering, and uses them for the rational design of enzymes for biocatalysis, biodegradation and biosensing. He has published more than hundred original articles in the field of protein engineering and enzymology. He is a co-founder of the national Centre for Biocatalysis and Biotransformation and biotechnology spin-off company ‘Enantis Ltd’.

Zbynek Prokop is a team leader in the Loschmidt Laboratories at the Masaryk University in the Czech Republic. He received his M.Sc. degree in Environmental Science and Ecotoxicology in 1998 and Ph.D. degree in Environmental Chemistry in 2001 from Masaryk University. Zbynek Prokop extended his expertise in biochemistry and biophysical chemistry during his research stays at the University of Gent in Belgium, at the University of Groningen in the Netherlands, at the European Molecular Biology Laboratory and at the University of Hannover in Germany. Zbynek Prokop is the co-author of twenty-nine publications and two international patents in the field of biocatalysis and protein engineering. He is a co-founder of the biotechnology spin-off company ‘Enantis Ltd’.