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

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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.

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 [1]. Optical pH sensors, particularly fluorescence-based, have proven to be an attractive alternative to electrochemical pH sensing [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 [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 [2–4]. Application of optical fluorescence-based pH sensors has been reported in marine research [5–7], biomedical diagnostics [8] and monitoring of biotechnological processes [9,10]. Moreover, fluorescence-based pH sensors can be used as transducers in enzymatic biosensors for the determination of glucose [11,12], urea [12–15], creatinine [12], penicillin [12,16–20], acetylcholine [21], organophosphorous pesticides [22] and halogenated hydrocarbons [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 [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 [13,23,27,28].

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 [29]. Functional groups of the dye are useful

Abbreviations: BSA, bovine serum albumin; CF, 5(6)-carboxyfluorescein; CF-BSA, conjugates of 5(6)-carboxyfluorescein with bovine serum albumin; CNF, 5(6)-carboxynaphthofluorescein; CNF-BSA, conjugates of 5(6)-carboxynaphthofluorescein with bovine serum albumin; GA, glutaraldehyde; MALDI–TOF MS, matrix-assisted laser desorption/ionization–time of flight mass spectrometry; ORMOCER, ORganically MOdified CERamics.

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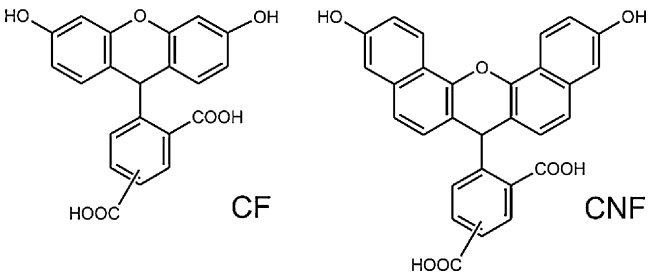

Fig. 1. Chemical structure of 5(6)-carboxyfluorescein (CF) and 5(6)-carboxynaphthofluorescein (CNF).

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 (CNF) 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 (0.6 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, Merci, Czech Republic) as a solid support

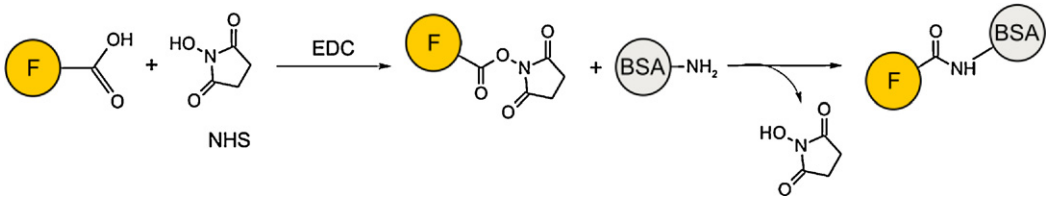

Fig. 2. Conjugation of fluorescence pH indicator (F: CF or CNF) with BSA intermediated by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) and N-hydroxysuccinimide (NHS).