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Preparation and characterization of sensing membranes for the detection of glucose, lactate and tyramine in microtiter plates

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Abstract

In this work, sensing membranes for the detection of glucose, lactate and tyramine were successfully prepared by immobilizing enzymes and fluorophore on sol–gels. The membranes were fabricated on the bottom of the wells in a microtiter plate. Glucose oxidase (GOD), lactate oxidase (LOD) and tyramine oxidase (TOD) were immobilized on individual sol–gels or a mixture of different sol–gels (3-glycidoxypropyl-trimethoxysilane (GPTMS), methyl-triethoxysilane (MTES), aminopropyl-trimethoxysilane (APTMS)). The oxidation of the analytes specifically catalyzed by the enzymes resulted in the reduction of the oxygen concentration, which changed the fluorescence intensity (FI) of the oxygen sensitive ruthenium complex acting as the transducer. The linear calibration graphs were in the ranges of 0.0–5.0 g/l for glucose, 0.0–9.0 mg/l for lactate and 0.0–100 mg/l for tyramine. The values of the detection limit were found to be 0.10–0.52 g/l for glucose, 7.77 mg/l for lactate and 6.30–8.73 mg/l for tyramine. The covalent binding between the epoxy and amine groups of the sol–gels and enzymes, respectively, prevented the enzymes from being washed out and preserved the high stability of the sensing membranes. The different ratios of silanes in the sol–gels, which were used as the supporting matrix for the immobilization of the enzymes led to different responses of the sensing membranes to various concentrations of glucose, lactate and tyramine. The kinetic parameters of the enzymatic reactions, and the stability and other parameters for the sensing membranes were also investigated.
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1. Introduction

Microtiter plates have been extensively used for high-throughput screening applications, enzyme-immunometric assay or toxicity tests, etc. [1]. They offer the advantages of a small sample volume and simultaneous screening of high numbers of samples. For the simultaneous measurements of samples, microplate readers capable of quickly detecting the absorbance or fluorescence changes in the plate have also come into wide use in many works [1–4]. Furthermore, microplate readers have been applied to other functions such as the measurement of enzyme kinetics [3,5] and respiration of cells [4,7,10,12], as well as bio- and chemi-luminescence multiplexed quantitative assays [6]. In a microtiter plate, enzymes or analytes such as metabolites and proteins can be integrated with some indicators in a liquid medium [7–9] or in a thin layer at the bottom of the wells [10–12]. Their integration over the selected target areas allows for the quantitative evaluation of the emitted light, which is related to the identity and concentration of the analytes.

Many chemical and physical methods have been employed for the integration of enzymes or analytes into supporting materials. Among them, a few chemical immobilization methods, which involve the covalent coupling of the enzymes/analytes to functionalized carriers or the intermolecular cross-linking of the biomolecules are widely applied in the preparation of sensing membranes [13]. Among the various immobilization methods, the sol–gel technique, i.e. the encapsulation of the analyte/enzyme into a supporting material, has been intensively considered, since sol–gel solutions are chemically inert, physically stable and optically transparent materials [14]. Many sol–gel systems have been developed for the immobilization of enzymes or other biomolecules [15–21] to date. However, the relationship between the properties of the sol–gel systems and the resulting activity of the immobilized enzyme or biomolecule

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is not yet well understood. Moreover, there are few general sol–gel systems which can be used in similar applications. Therefore, it is often necessary to find optimal sol–gel systems for the effective immobilization of a certain enzyme or biomolecule onto the supporting materials.

Recently, the need to analyze glucose, lactate and tyramine in fermentation processes, food preparation and medical studies has increased. Glucose is by far the most important nutrient source for microorganisms in biotechnological processes. This carbon source is used in almost 95% of all fermentation processes. Therefore, the measurement of glucose concentrations is essential for the control of various biotechnological processes [22]. Moreover, glucose monitoring plays an important role in diagnosing many metabolic disorders, especially in the diagnosis and therapy of diabetes. For the measurement of lactate, the determination of D(−)- and L(+)-lactic acid is required whenever the quality of a food product has to be assessed. Levels of L(+)-lactic acid higher than 3 g/kg are usually considered as an index of microbial contamination [23,24]. This is the same principle as that used in the determination of tyramine in fermented foods. The presence of tyramine and other biogenic amines might pose a health risk to consumers who have a deficiency or lower activity of amino acid oxidase, due to the intake of inhibitory drugs, alcohol or gastrointestinal diseases [25].

From this point of view, we designed optical planar biosensors for the detection of glucose, lactate and tyramine using a microtiter plate. The principle of the measurement is based on the oxidation of the analytes (glucose, lactate, tyramine) specifically catalyzed by enzymes:

$$\text{Analytes} + O_2 \xrightarrow{\text{Enzyme}} \text{Oxidized products} + H_2O_2$$

This reaction leads to a reduction in the concentration of oxygen, which has an effect on the fluorescence intensity (FI) of the ruthenium complex acting as the transducer. The enzymes (glucose oxidase (GOD), lactate oxidase (LOD), tyramine oxidase (TOD)) and fluorophore were immobilized on an individual sol–gel or a mixture of sol–gels consisting of 3-glycidoxypropyl-trimethoxysilane (GPTMS), methyl-triethoxysilane (MTES) and aminopropyl-trimethoxysilane (APTMS). In this work, we prepared three different sol–gel systems for the immobilization of the ruthenium complex and enzymes and investigated the performances of these sol–gel systems to detect the concentrations of glucose, lactate and tyramine.

2. Experimental

2.1. Materials

Glucose oxidase (from *Aspergilus niger*, 47.2 U/mg solid), lactate oxidase (from *Pediococcus* sp., 20 U/mg solid), tyramine oxidase (from *Arthrobacter* sp., 3.9 U/mg solid), 3-amino-propyl-trimethoxysilane, 3-glycidoxypropyl-trimethoxysilane and methyltriethoxysilane were purchased from Sigma–Aldrich Chemical Co. (Seoul, Korea). Tris(4,7-diphenyl-1,10-phenanthroline) ruthenium complex was synthesized in our laboratory. All other chemicals were of analytical grade and used without further purification.

Table 1
Mixture ratio of silanes used for the preparation of the sol–gels

Sol–gel	GPTMS (v/v, %)	APTMS (v/v, %)	MTES (v/v, %)	EtOH (v/v, %)	35% HCl (μl/ml)
GT	37.5	–	–	62.5	20
GA1	12.5	6.25	–	81.25	40
GA2	25.0	6.25	–	68.75	40
GM1	12.5	–	12.5	75.0	40
GM2	12.5	–	25.0	62.5	40

2.2. Preparation of the sensing membrane

The sensing membrane consisted of a transducer (ruthenium complex + sol–gel) and a biological detection element (enzyme + sol–gel). The sol–gels used were prepared by the hydrolyzation and polymerization of mixtures of GPTMS and APTMS or GPTMS and MTES or GPTMS alone in 99% ethanol solvent. The mixture ratio of these silanes and the volume of 35% hydrochloric acid added to the mixture solution are shown in Table 1. After adding hydrochloric acid, the sol–gels were kept at room temperature for at least 2 h before being used in the next steps.

The preparation of the transducers was done by adding 50 μl of a 0.044 mM ruthenium complex solution to 200 μl of sol–gel GM1 or GM2. The mixtures of the ruthenium complex and the sol–gels were vortexed and stored at room temperature for 2 h. The 5 μl of these mixtures were deposited on the bottom of a well in a 96-well microtiter plate (NUNC Co., Denmark) and dried at 95 °C for 18 h. After the heat treatment, the transducers were covered by different sol–gels (GT, GA1, GA2, GM1 or GM2) on which 40 μl of enzyme solution (GOD: 100 U, or LOD: 1 U, or TOD: 0.005 U) were added to one well of a 96-well microtiter plate. The enzyme immobilization was performed at room temperature for 18 h.

2.3. Characterization of the sensing membrane

2.3.1. Immobilization efficiency

The efficiency of the enzyme immobilization in the well was calculated by dividing the amount of immobilized enzyme protein by the total amount of enzyme protein used for the immobilization. The amount of immobilized enzyme protein was determined by subtracting the amount of un-immobilized enzyme protein from the total amount of enzyme protein used. The un-immobilized enzyme was removed by washing several times with 300 μl of 0.1M phosphate buffer (pH 7), for which the protein values were determined by the Bradford method. The enzyme immobilization and protein measurements were performed in triplicate for each type of sol–gel.

2.3.2. Effect of pH and temperature

The effect of pH and temperature on the sensing membrane was investigated in the range of pH from 4.5 to pH 10, and also

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