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Synthesis and characterization of microparticles based on poly-methacrylic acid with glucose oxidase for biosensor applications

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ABSTRACT

In the line of the applicability of biocompatible monomers pH and temperature dependent, we assayed poly-methacrylic acid (p-MAA) microparticles as immobilization system in the design of enzymatic biosensors. Glucose oxidase was used as enzyme model for the study of microparticles as immobilization matrices and as biological material in the performance of glucose biosensors. The enzyme immobilization method was optimized by investigating the influence of monomer concentration and cross-linker content (N′,N′-methylenebisacrylamide), used in the preparation of the microparticles in the response of the biosensors. The kinetics of the polymerization and the effects of the temperature were studied, also the conversion of the polymerization was determinates by a weight method. The structure of the obtained p-MAA microparticles were studied through scanning electron microscopy (SEM) and differential scanning microscopy (DSC). The particle size measurements were performed with a Galai-Cis 1 particle analyzer system. Furthermore, the influence of the swelling behavior of hydrogel matrix as a function of pH and temperature were studied. Analytical properties such as sensitivity, linear range, response time and detection limit were studied for the glucose biosensors. The sensitivity for glucose detection obtained with poly-methacrylic acid (p-MAA) microparticles was 11.98 mA M^{-1} cm^{-2} and 10 μM of detection limit. A Nafion® layer was used to eliminate common interferents of the human serum such as uric and ascorbic acids. The biosensors were used to determine glucose in human serum samples with satisfactory results. When stored in a frozen phosphate buffer solution (pH 6.0) at −4 °C, the useful lifetime of all biosensors was at least 550 days.

1. Introduction

Among numerous reports about glucose biosensors, the enzyme immobilization on electrodes is the first important step in fabrication, which also plays a vital role in the biosensor performance. Different immobilization methods have been assayed such as covalent attachment [1,2], entrapment in a suitable matrix [3], adsorption onto insoluble materials [4], conjugation [5], ionic-covalent hybridization [6,7], etc. Each method has its own advantages and limitations. Among these, polymeric entrapment presents several advantages that make it highly interesting. The enzymatic reporter molecule is less affected than when other methods are employed (i.e covalent attachment), because the reporter is entrapped in the matrix.

Emulsion polymerization is a well-known method for preparation of the latex polymers with defined structures. The synthesis of the microparticles usually does not lead to ideal core–shell morphology. Depending on the compatibility of the components in the interface, these can be mixed on the molecular level with continuous concentration gradient. Organic matrixes made of cross-linked hydrogels based on methacrylates have been proven non cytotoxic, non-immunoreactive and their porosity could be controlled by the amount of cross-linker used in their synthesis [8,9].

In the search for a suitable matrix for enzyme immobilization, other biocompatible polymers, pharmacologically inert were sought since they could have different responses to external stimuli such as temperature and pH [10–12]. After studying the pH-dependent polymer and temperature-dependent polymer (poly (ethylene glycol) methyl ether methacrylate (p-PEGMEM) and poly (dimethylaminoethyl) methacrylate (p-DMAEM), respectively [13,14], this production focuses on the study of the behavior of a polymer p-(methacrylic acid) (p-MAA) that has both features.

p-MAA is an ionizable hydrophilic polymer. Cross-linked

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p-MAA is able to swell in water. Its swelling behavior is greatly pH-dependent due to the ionization/deionization of the carboxylic acid groups [15,16]. At low pH, usually less than 5.5, the –COOH groups are not ionized and keep the p-MAA network at its collapsed state [17]. At high pH values, the –COOH groups are ionized, and the charged COO– groups repel each other, leading to p-MAA swelling. p-MAA exhibits thermosensitive properties as well, however, at high polymer concentrations (> 5 wt% in water); its LCST is equal to 50 °C [18]. For pH above the pK_a of MAA, the LCST transition is suppressed [19,20]. The reported values of T_g for p-MAA are 205 °C [21].

Amperometric enzyme electrodes and especially glucose oxidase-based biosensors had been described extensively during the almost 50 past years, and there are far more than 5000 publications related to these types of biosensors. Since the first report on glucose enzyme biosensors by Clark and Lyons [22], the analysis of glucose has attracted intense research interest due to the increasing incidence of diabetes in the population of developed countries [23,24]. In this scenario, amperometric biospecific enzyme glucose biosensors have been generally considered in terms of the simple operation, sensitive determination, fast response, and low cost [25–27]. The fabrication of biosensors with biocompatible materials will allow the monitoring in vivo of glucose and other compounds. Future progress in biosensors design will certainly focus upon the technology of new materials that solve the biocompatibility problem. Another decisive factor is the immobilization of enzymes, with complete retention of their biological activity, in matrices with good diffusion properties for substrates.

In this task, our first purpose was to encapsulate glucose oxidase (GOx) inside p-MAA microgels with different amount of cross-linker followed by the concentrated emulsion polymerization method [28], as well as the study of the influence of some polymerization parameters (monomer concentration, kinetics of polymerization). The chosen enzyme from *Aspergillus niger* (EC 1.1.3.4) was taken as a redox enzyme model because of its high stability, good catalytic ability, commercial availability, and moderate cost. Moreover, GOx is a widely studied enzyme that provides a suitable model system for the development of enzyme electrodes [29–35]. Secondly, we have investigated the properties of microparticles of GOx-immobilized in hydrogel microparticles, and the modifications induced by the enzyme in the glass transition temperature as well as in the microstructure of the gels. And finally, we have investigated the performance of the amperometric biosensors, based on glucose oxidase (GOx) immobilized in biocompatible microparticles, for the determination of glucose.

2. Experimental

2.1. Chemicals and buffers

Methacrylic acid (MAA), GOx (EC 1.1.3.4) (425 U mg^{-1}) from *Aspergillus niger,* D-(+)-glucose, ascorbic acid, uric acid and Nafion® 5 wt% (Nafion® purum 5% in a mixture of lower aliphatic alcohols and water), were purchased from Sigma (St. Louis, MO, USA). N,N´-methylenebisacrylamide (BIS) from Aldrich (St. Louis; MO, USA), ammonium persulfate (PSA), N,N,N′,N′ tetramethylethylendiamine (TEMED) and the surfactant Span 80 from Fluka (Buchs, Switzerland). Phosphate and Acetate/phosphate buffer solutions were prepared from stock solutions of sodium dihydrogen phosphate and sodium acetate (Panreac). The dialysis membrane (12,000–14,000 MWCO) was purchased from Spectrum Medical Industries. All reagents did not need any modification and the water was Milli Q quality (Millipore, Milford, MA, USA). Serum samples were kindly provided by Health Analysis Center belonging to the University Complutense of Madrid.

2.2. Apparatus and measurements

The microgel particles were examined using scanning electron micrographs (SEM) with a JEOL JSM-6400 microscope (JEOL, Japan) operating at an acceleration voltage of 20 kV and with 5000 magnification. The grid with the microparticles was dried, and replicas were produced by shadowing gold deposited with a Balzers Sputter Coater (SCD-004). Particle size measurements in the range 2–150 μm were performed with a Galai-Cis-1 particle analyzer system. The glass transition temperature and the degradation temperature of the microgels were investigated by DSC. The DSC measurements were carried out in a Mettler 820 differential scanning calorimeter equipped with a cooler operated by liquid nitrogen. The DSC cell was used for heat, treating the samples that weighted about 5.0 mg. The X-ray diffraction studies were performed with a Philips Xpert PW3050 diffractometer. The diffractograms were recorded covering an angular interval between $2\theta = 5$ and 50°, and using a step size of 0.01° with time per step of 1 s. The pH of the buffer solution was adjusted using a Metler Toledo MP-230 pH-meter. In addition, amperometric measurements at constant potential were carried out at a Metrohm Polarecord potentiostat, Model E-506. Electrochemical measurements were performed in 0.05M acetate/0.05M phosphate buffer and in 0.1 M phosphate buffer, using a three-electrode cell with a platinum working electrode, a SCE reference electrode and a platinum counter electrode. All experiences were carried out in thermostated cells with the help of a circulation thermostat Julabo (−50, 200 °C). Calibration plots were obtained by measuring the current response, after successive additions of substrate solution into a stirred electrolyte solution (10 mL), corresponding to the enzyme saturation concentration. Sensitivity was expressed as the slope of the calibration curve. Detection limit was calculated according with the criterion of ratio signal-to-noise equal 3. The response time was the time needed to reach 95% of the steady-state current after a substrate addition. In order to study the catalytic enzyme immobilization system efficiency, a comparative study of the measured absorbances of solutions generated once the enzymatic reaction with a UV–visible spectrophotometer CARY 300 Bio was performed at 353 nm.

2.3. Sample preparation

p-MAA microgels with varying amount of cross-linker N,N´-methylenebisacrylamide (BIS), have been prepared using the concentrated emulsion polymerization method [28]. The immobilization of GOx was carried out by adding the enzyme (425 U mg^{-1}) in the aqueous phase of the concentrated emulsion. The amount of cross-linker, ($\eta = n_{BIS}/(n_{BIS} + n_{monomer})$% where n_{BIS} and $n_{monomer}$ are the number of moles of BIS and monomer respectively) was varied between 0.13% and 1.48%. During the synthesis of the microgels (around 1.5 h) the temperature was controlled and kept below 25 °C to preserve the enzyme properties. The polymeric reaction of p-MAA and BIS is shown in the Scheme 1.

2.3.1. Control of the temperature of polymerization

A key factor in the immobilization of enzymes is the temperature to which the polymerization is carried out. The polymerization is exothermic and the temperature reached depends on the amount of initiator used in the synthesis, in this task it was PSA. Therefore, we have prepared different syntheses varying the amount of initiator from 9.86, 10.96 and 21.92 mM. Fig. 1 shows the range of polymerization temperature for the different concentration of initiator. In all cases the temperature of polymerization increases when increasing the concentration of initiator, keeping the temperature between 19.0 and 23.0 °C for