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Synthesis of bilirubin imprinted polymer thin film for the continuous detection of bilirubin in an MIP/QCM/FIA system

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Abstract

A bilirubin imprinted polymer (BIP) was coated on a thiol pretreated Au electrode on a quartz crystal microbalance (QCM) chip. The BIP thin film was synthesized using 4-vinylpyridine (4-Vpy) as the monomer, divinylbenzene (DVB) as the cross-linker, and benzophenone as the initiator. By using a photo-graft surface polymerization technique with irradiation by ultra-violet (UV) light, a thin BIP film was prepared, from which a biomimetic sensor for the detection of bilirubin was developed. The sensor was able to discriminate bilirubin in solution owing to the specific binding of the imprinted sites. The BIP/QCM chip has been repeatedly used for more than 7 months in many continuous experiments. The detection signal of bilirubin from the BIP thin film/QCM was compared with the non-BIP thin film/QCM. Biliverdin, an analogue of bilirubin, was used for comparison. The analogue comparison confirmed the binding specificity of the BIP film toward bilirubin. The selectivity can be as high as 31.2. The effect of pH on the detection of bilirubin is also discussed. With proper solvent for elution and recovery, flow injection analysis (FIA) could be applied to the system. The performance of the BIP/QCM chip was evaluated. A linear calibration of the bilirubin concentration with respect to the frequency shift was successfully obtained. The reproducibility of measurements from the same BIP/QCM chip was confirmed. In addition, repeatability of detection was also confirmed from different BIP/QCM chips. In conclusion, a combined BIP thin film/QCM/FIA method was successfully established for the detection of bilirubin concentration using a molecularly imprinted film.

Keywords: Bilirubin; 4-Vinylpyridine; Divinylbenzene; BIP/QCM chip; Biliverdin; FIA

1. Introduction

Bilirubin, a lipophilic and cytotoxic yellow-orange pigment, is produced from hemoglobin metabolism of aged red blood cells. It is transported to the liver as a complex with albumin and excreted into the bile as bilirubin glucuronides (Kamisako et al., 2000). Bilirubin can be regarded as an important index for judging liver functions and is used in this context to identify a variety of liver diseases. Disorders in the metabolism of bilirubin may cause a yellow discoloration of the skin and other tissues, called hyperbilirubinemia. High bilirubin concentration may even cause hepatic or biliary duct dysfunction, and also permanent brain damage or death in the most severe cases (Cotler et al., 2000).

Various methods have been developed for bilirubin analysis from clinical samples. The most commonly used method is diazo reaction. Bilirubin maybe detected using diazotized sulfanilic acid or directly using spectrophotometry, by measuring the absorbance at 440 nm. The measurement of bilirubin concentrations by the diazo reaction is highly selective, and accuracy in the determination of the bilirubin concentration is excellent. Direct measurement of bilirubin may involve the interaction with other heme containing proteins and pigments. Other analytical methods such as voltammetry (Doumas et al., 1987) and fluorometry (Wu et al., 1992; Andreu et al., 2002) have also been used for bilirubin analysis. Although the above methods may provide superior sensitivity, they are less selective than the diazo reaction. Therefore, bilirubin oxidase was used in an enzymatic assay to measure the conjugated bilirubin concentration in serum with high selectivity (Kurosaka et al., 1998). In addition, based on different types of transducers, there were sensors developed for bilirubin detection such as electrochemical amperometric sensors (Wang and Ozsoz, 1990) and fiber optic sensors (Li and Rosenzweig, 1997). Nevertheless, immobilization of bilirubin oxidase for the enzymatic oxidation reaction is the approach used most often for different types of bilirubin sensors to detect biliru-

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bin concentrations in aqueous solutions or in blood (Andreu et al., 2002; Wang and Ozsoz, 1990; Li and Rosenzweig, 1997).

Fischer et al. (1941) reported that bilirubin was an asymmetrically-substituted tetrapyrrole dicarboxylic acid consisting of two dipyrrole halves conjoined by a saturated -CH_2- group. Although covalently linked, the two halves are almost identical mirror images. However, Fischer's structure represents only the constitutional structure, not the three-dimensional structure or conformation. The three-dimensional structure of bilirubin is reported as a folded ridge-tile conformation, which is believed to be at the global energy minimum.

Molecular imprinting (Shea and Sasaki, 1991; Wulff and Schauhoff, 1991; Kempe and Mosbach, 1995; Mosbach and Ramström, 1996; Ramström and Ansell, 1998; Haupt and Mosbach, 2000) is a technique for the fabrication of specific antibody-mimic binding sites. By means of a synthetic organic polymer matrix, the imprints of the template molecule are created in the polymer. The functional monomers, cross-linker, template, and the initiator are mixed in a solvent and then interact through the polymerization procedure. Thus, an imprinted polymer can be prepared. After the template is removed from the rigid polymer network, binding sites complementary in size, shape, and functional groups to the template molecule can be obtained. A molecularly imprinted polymer (MIP) offers a three-dimensional structure for the recognition of a template molecule from the shape complimentarity and the interaction between the template and the functional monomer. The role of the functional groups of the print molecule for complexation with the functional monomer was investigated (Sellergren et al., 1998). Studies have been performed which relate polymer structure to binding specificity (Piletsky et al., 2000). The hydrogen bond and hydrophobic effect on the recognition of the MIP was also investigated (Nicholls et al., 1995). The highly cross-linked polymeric nature of the MIP confers upon properties, such as physical robustness, high mechanical strength, and tolerance to heat and pressure. Additionally, it can be stored for a long period of time and reused for many times without loss of activity and stability. Owing to these favorable characteristics, MIPs are frequently applied to a variety of research areas such as solid-phase extraction (Muldoon and Stanker, 1997), by-products removal (Ye et al., 1998), pharmaceutical analysis (Zhou and He, 1999), immunoassays (Vlatakis et al., 1993), chiral separations (Shoji et al., 2003), and chemical sensing (Nicholls et al., 1995; Piletsky et al., 1994; Panasyuk-Delaney et al., 2001). Lately, the importance of polymerization time and initiation conditions on the structure of the imprinted polymer was reported (Piletsky et al., 2005). (−)-Ephedrine was used as the template. Different intensities of UV light and thermal effect were investigated. The binding mechanism was also studied using Van't Hoff analysis.

Searching for highly selective, low-cost, stable, and facile chemical sensors for a biological analytes has attracted a lot of attention. The QCM (Marx, 2003), a gravimetric sensor being portable, rapid, and sensitive, is well suited as a transducer element for chemical sensors. Initially, QCM was solely used in a vacuum chamber or air to measure the mass of a rigid film attached on the quartz crystal. The well-known Sauerbrey equation shows that the resonance frequency change of the quartz crystal, Δf, is directly proportional to the adsorbed mass on the QCM. For chemical sensing applications, a recognition element is added to the acoustic wave device capable of selectively binding the analyte to the device surface. The response from the device is then based on a decrease in the resonance frequency once a mass is attached to the device or the recognition element. QCM can be applied as a transducer in analytical chemistry (Windeln et al., 2001), biology (Mannelli et al., 2003), pharmaceutical detection (Zhou et al., 2002), environmental assays (Nakamoto and Sumitimo, 2003), and life science (Yamaguchi and Shimomura, 1993).

The fabrication of MIP films to detect certain compounds via a QCM transducer has been accomplished in recent years. A sialic acid imprinted polymer film was prepared and coated onto a 9 MHz AT-cut QCM chip. The selective response to sialic acid was obtained and a linear correlation of 20–250 nmol was achieved (Kugimiya et al., 2000). An MIP was packed in a cartridge attached to an SPE vacuum unit (MIP-SPE) so that extraction and pre-concentration could be done before detection. Microsystin-LR with a limit of 1 nM could then be detected by a piezoelectric sensor with an MIP receptor (Chianella et al., 2003). An MIP prepared from methacrylamidohistidine (MAH) monomer chelated with copper ion was proposed (Ersöz et al., 2005). It was used as a ligand to form a specific binding with glucose. QCM was further used to detected the adsorption of glucose by the ligand chelated MIP. The photo-grafted imprinted polymer using 2-(diethylamino) ethyl methacrylate as functional monomer, ethylene glycol dimethacrylate as cross-linker, and domoic acid (DA) as template was assembled as a monolayer onto the gold surface (Lotierzo et al., 2004). Surface plasmon resonance (SPR) was used to detect domoic acid. Contact angle measurements and atomic force microscope (AFM) imaging were used to optimize the grafting condition. Furthermore, the sensor was evaluated for its sensitivity, cross-reactivity and robustness.

In this study, combining the advantages of high selectivity from the MIP technique and high sensitivity from QCM detection, an MIP/QCM chip was assembled for the detection of bilirubin. In our previous work, the molecular imprinting technique for bilirubin and its associated binding kinetics were proposed (Syu et al., 2004; Syu and Nian, 2005). In this work, a bilirubin imprinted polymer (BIP) film was synthesized and characterized. The detection of bilirubin was performed by a continuous flow BIP/QCM system. The influence of pH and solvent on the frequency response was investigated and the performance of the BIP/QCM system for the detection of bilirubin was also evaluated.

2. Materials and methods

2.1. Chemicals

The functional monomer used in this work was 4-vinylpyridine (4-Vpy). The initiator for the polymerization was benzophenone. Both chemicals were purchased from Lancaster Synthesis Inc. (Morecambe, England). The cross-linking agent,