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IMMOBILIZATION OF HORSERADISH PEROXIDASE ON NANOPOLYANILINE SURFACE USING SELF-ASSEMBLY TECHNIQUES FOR BIOSENSOR APPLICATIONS

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***Abstract*— This review paper describes the immobilization of horseradish peroxidase (HRP) on nanopolyaniline (PANI) by using self-assembly technique. Some chemically synthesized conductive polymers such as PANI were used as support for HRP immobilization. Enzymes immobilized were depend on their properties of immobilize of HRP such as optimum pH, thermal stability and storage stability and all these were investigated. The structure of PANI was characterized by FTIR, FESEM and UV-VIS. The structure of PANI depends on the pH of the phosphate buffer and the type of organic solvent. Thus, the formation of monolayer especially nano-particles of PANI was prepared by using Langmuir-Blodgett technique. The contribution in the development of the biosensor application for hydrogen peroxidase (H_2O_2) based on the HRP modified electrode was described.**

Keywords*— *Horseradish Peroxidase; Polyaniline; Immobilization; Enzymatic; Langmuir-Blodgett

INTRODUCTION

Polyaniline (PANI) is a polymers that having its conductivity, reversible redox characteristics, electro chromic behavior and environmental stability in air [1]. PANI also was known as environmental stable polymer with good physical and chemical properties in last recent years because it can be used for conduct metric biosensors [2]. Based on these properties, chemically synthesized PANI can be a suitable polymeric supports for enzyme immobilization because of their chemical characteristics [2]. Their characteristics such as high synthesis yield, high stability in pH and temperature, ease of preparation, the simplicity of doping process, resistance to be attacked by microorganisms and its good redox recyclability enabling polymers with significant differences to be developed by simple acidic or basic treatments. [2 & 3]. The commercial use of PANI was limited because of harsh synthetic conditions and poor process ability [5]. In particular, PANI has been investigated for such applications as organic lightweight batteries, microelectronics, optical displays, antistatic coatings, and electromagnetic shielding materials [6].

Nowadays, the plant peroxidase families of enzymes have been used in the synthesis of a variety of polymers and have been extensively [7]. The classical plant peroxidases were found in horseradish, soybean, barley, tobacco as well as other plant systems [8]. Horseradish Peroxidase (HRP) is a type of an enzyme that mostly used as materials for the electrode modification [9]. An addition, HRP is also the most studied enzyme for use in decontamination processes [10]. HRP is a classic heme enzyme having widespread use in pollution control, biomedical research, and organic synthesis [11]. The enzyme HRP is used to polymerize aniline in the presence of a polyanionic template and sulfonated polystyrene [6]. HRP is an oxidoreductase which catalyzes the radical polymerization of aniline in the presence of an oxidizing reagent such as hydrogen peroxide [12]. Hydrogen peroxide (H_2O_2) plays a important role in various industrial applications [13] and it is an vital mediator in clinical, pharmaceutical, and environmental research [9]. The interaction of the H_2O_2 substrate with HRP immobilized on the surface of PANI produces a catalytic current, which flows from the electrode surface through the PANI [14]. Grennan et al [15] reported that the interaction of the H_2O_2 substrate with HRP produces a catalytic current which is indirectly proportional to the concentration of bound atrazine [14].

PROPERTIES OF THE IMMOBILIZED OF HRP

Immobilized enzymes are good in a soluble form and active in enzyme technology for industrial applications [19]. It is maybe using a support inexpensive synthesis and high binding capacity [5]. Recently, PANI was used for HRP immobilization because it showed some increment in the characteristics of HRP, such as pH, thermal and also storage [2]. Enzymes immobilized on nanoparticles showed a broader working pH and temperature range and also a higher thermal stability than the native enzymes [16]. The advantageous of immobilization of enzymes for commercial uses is due to easy in handling, ease of separation of enzymes from the reaction mixture and reuse, low product cost and a possible increase in thermal and pH stability [16].

i. Optimum pH

Enzymes actually operate only in a narrow pH range. If the pH moves below or over the optimal range, enzymes can stop working and denature due to conformational changes [20]. The optimum pH values of the enzyme activity were found as 8 and 7 for the free HRP and the immobilized HRP respectively [21]. Based on Torabi et al [22] covalently immobilized HRP on perlite surface activated with 3-aminopropytriethoxysilane via glutaraldehyde can cause change optimum pH of enzymes to the lower pH exactly. On the other hand, Fernandes et al [23] also reported that pH range of HRP increased after immobilization. Immobilized enzyme activity was tested acidic and the alkaline pH [20]. Compared to C. H. Lim et al [12] reported that the structure of PANI was influenced by the pH of the buffer and the level of protonation at the aromatic amine groups was involved in polymerization mechanism and had an effect on the

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structure of the resulting polymer. When pH of the phosphate buffer increased, the ratio also increased [12].

Based on immobilization of HRP to polyvinyl-alcohol glutaraldehyde coated with polyaniline using glutaraldehyde (PVAG-PANIG), the good pH for immobilization was 5.5. Compared to Fernandes et al [2], he got pH 6 as optimum pH in immobilization HRP on PANIG. The other authors also reported that pH 6 is the optimum pH for immobilization of HRP on PANIG [2]. In order to maintain constant pH, the solutions are buffered using a wide range of organic or inorganic weak acid/base pairs [7].

ii. Thermal Stability

Many authors reported that thermal stability closely related with HRP immobilization. The thermal stability of the free HRP and immobilized HRP reported a same behaviour, although the immobilized HRP have higher relative activity in the range from 50 to 60˚C [21]. The higher thermal stability was showed in the PVAG-PANIG-HRP whereas the soluble enzyme activity decreased faster than the immobilized preparation when both were incubated at 50◦C and 70◦C [4]. It is because after 15 min at 70◦C, the soluble enzyme lost all its activity, whereas the immobilized one retained about 20% of its initial activity [4]. U. Bora et al. [24] reported that the thermal resistance increased after immobilization of HRP where the activity of the HRP was found to decrease beyond the incubation time and temperature. Weng et al. [25] reported that the heat stability of authentic HRP is well-known and can be used as a marker for balancing in food production. In fact, a statistical analysis of available showed that only 16% of the enzymes studied gave a decrease in stability after immobilization [26].

iii. Storage Stability

Based on previous researchers, they stated that some examples of immobilized of HRP with stability varying from 5 days until 6 months [23]. Immobilization improved stability of the PANIG-HRP stored in phosphate buffer, freely of the temperature [23]. Table 1 showed that the activities remaining after storage of the PANIG-HRP and also free enzyme. Fernandes et al [23] stated that when stored at 4˚C, the immobilized enzyme still maintained 100% of its activity until 70 days of storage and 50% of its initial activity at room temperature. An addition, the decrease in the electro catalytic response with increasing storage time, as well as the decrease in sensitivity, are attributable to the denaturation of HRP on the nano composite layer and some leaching of the enzyme from the electrode surface while stored at 4 °C [27]. Besides that, immobilized HRP on PANI only maintain 83 % of its original activity even after 8 weeks of storage at 4 °C, while the free enzyme lost its initial activity after 3 weeks of storage period [28]. Temocin and Yigitoglu [21] reported that the immobilized HRP showed higher storage stability than the free HRP in their studies.

TABLE 1: STORAGE STABILITY OF THE FREE AND IMMOBILIZED HRP

Enzymatic aspect	**Storage**			
	Buffer		**Dry**	
	Room temperature	4˚C	Room temperature	Lyophilised
Free HRP	Inactive (after 5days)	Active 44% (after 70days)	Inactive	Active
PANIG-HRP	Active 50% (after 70days)	Active 100% (after 70days)	inactive	Inactive

CHARACTERIZATION OF PANI

PANI was used for HRP in a mixture of phosphate buffer and organic solvent and its structure was analyzed by elemental analyses such as UV-VIS spectra, TEM, Fourier Transform Infrared (FTIR) Spectroscopy and etc. [12]. Based on some authors, the UV-VIS spectra reported the result of polyaniline emeraldine base (PANI-EB) with and without HRP that PANI-EB have higher rate absorption when add with HRP [19]. This is because PANI-EB has ability to absorb electromagnetic radiation because of the existence of its electron valence that can be improved to higher level. Manigandan et al. [29] reported that the high transmission electron microscopy (TEM) analysis exposed to the variation of the sub-phase pH crucially determines the type of nano-structures of PANI. In 1997, some authors have tried to transfer molecular film of PANI with camphor suphonic acid at pH 6 and have failed to obtain uniformly [29]. Based on the Manikandan et al [29] studies, it can expect that non-uniformly over surface may be caused by the formation of lump type nano-structure pH 6.

On the other hand, formation of the polymers, presence of a functional group on the polymer backbone or change in the protonation-deprotonation equilibrium of emeraldine can be concluded by the presence of corresponding bands in the FTIR spectrum [30,31]. Polymers showed differences in the FTIR spectra and it was focused in the relative intensities of the benzoid and quinoid related bands [2]. Based on others author, FT-IR analysis showed the characteristic bands of polyaniline and those

related to modifications introduced by treatment with glutaraldehyde and magnetization in Table 2 [18].

TABLE 2: COMPARATIVE FT-IR BANDS OF PANI, PANIG, AND PANIMG

Polymer	Composites	Band
	NH from benzoid ring	1500 cm−1
PANI∗	NH from quinoid ring	1600 cm−1
	doping grade	1100 cm−1
PANIG∗∗	aldehyde hydroxyl	1720 cm−1
	assimetric stretching of CH2	2740 cm−1
PANImG	angular deformation	830 cm−1
	superficial hydroxyl group	584 cm−1

*Data from Fernandes 2003 [18]
**Data from Azevedo et al [32]

SELF-ASSEMBLY OF TECHNIQUE

Conducting polymers in the form of nano-fibers, nano-rods and nano-tubes were stated as the best sensing materials due to their high surface volume ratio [29]. Recently, Langmuir-Blodgett (LB) technique has been reported the largely successful in preparing their monolayer [33]. LB technique has many potential applications in molecular electronics, non-linear optics and conducting thin films [34]. LB technique for the preparation of ultra-thin films of various organic, metallorganic and polymeric compounds plays a vital role as an action of producing molecular materials at the microscopic level [34]. The most important advantage of LB is that the characteristics of the films can be varied by changing LB parameters such as temperature, and molar composition [34].

S. Manigandan et al [29] also reported that the self-assembly of technique to produce several nano-structures of PANI without aid of any templates was using by LB technique. By previous authors, LB technique was stated as a potential technique for the production of ultra-thin films with controlled molecular orientation. The LB technique supported rigid nanomaterial to be aligned in particular structures through a flexible assembly process at liquid interfaces [35]. LB technique was used to see the formation of PANI-EB monolayer before deposited on Indium Tin Oxide (ITO) glass [19]. This technique demonstrated the nanostructures reorient themselves and align paralleling to the trough barrier, finally forming a closely packed monolayer [33,36].

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BIOSENSOR APPLICATIONS

Biosensors are chemical sensors that its recognition system utilizes a biochemical mechanism [37]. An addition, biosensor is a sensing device made up of a combination of a certain biological element and also a transducer [37]. As in many different technological sections, nanomaterials have showed their suitability for bio sensing applications [38]. The electrodeposited polymers nanoparticles can be tested by scanning electron microscopy (SEM), Fourier transform infrared (FT-IR) spectroscopy, and atomic force microscopy (AFM) [39 & 40]. Morrin et al. [40] reported that PANI was very useful in the development of biosensors. Thus, every biomolecules will adsorbed electrostatically onto a surface such as thin film for biosensor application [40]. The biosensor format was showed for H_2O_2 sensing [40]. The advantages of biosensor are simple to use, high specificity analytical tools, very portable in data-processing technologies [41].

CONCLUSION

This paper was to elaborate the immobilization of HRP on nanopolyanine by using a self-assembly technique for biosensor applications. The properties of immobilize of HRP such as pH, thermal stability and storage were important to support immobilization of HRP on PANI. Thus, PANI was characterized by several analysis machines such as FTIR, FESEM, UV-VIS and etc. Due to this, Langmuir-Blodgett was demonstrated to produce the monolayer. The interaction of HRP and PANI can generate the electricity through biosensor applications.

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