İSTANBUL TECHNICAL UNIVERSITY ★ INSTITUTE OF SCIENCE AND TECHNOLOGY

FUNCTIONAL POLY(VINYL ALCOHOL) AS PRECURSOR FOR BIOAPPLICATIONS

M. Sc. Thesis by
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Department : Chemistry

Programme : Chemistry

JANUARY 2010

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JANUARY 2010

İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

BİYO UYGULAMALAR İÇİN ÖNCÜL FONKSİYONEL POLİ(VİNİL ALKOL)

MASTER TEZİ
Bahadır GACAL
(509081202)

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January 2010 Bahadır Gacal

Chemist

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ABBREVIATIONS

PVA	**:** Poly(vinyl alcohol)
ABTS	**:** 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
GOx	**:** Glucose Oxidase
FAD	**:** Flavin Adenine Dinucleotide
EDC	**:** 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride
PVAc	**:** Polyvinyl Acetate
M_n	**:** Number Average Molecular Weight
M_w	**:** Molecular Weight
^{1}H-NMR	**:**.Nuclear Magnetic Resonance Spectroscopy
IR	**:** Infrared Spectrophotometer

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LIST OF SYMBOLS

R	**:** Radical
I	**:** Initiator
M	**:** Monomer
M_n	**:** The number average molecular weight
M_w	**:** The weight average molecular weight
M_w/M_n	**:** The molecular weight distribution

FUNCTIONAL POLY(VINYL ALCOHOL) AS PRECURSOR FOR BIOAPPLICATIONS

SUMMARY

Poly(vinyl alcohol)-pyrene-glucose oxidase (PVA-Py-GOx), a water soluble polymer possessing both fluorescent and oxidant sites in the structure is synthesized by "click" chemistry and modification processes and characterized. The morphology of PVA-Py-GOx was characterized with atomic force microscopy (AFM), and a heterogeneous morphology due to the incorporation of GOx was observed. The capability of PVA-Py-GOx to act as a bio-probe for fluorescence sensing of glucose is examined. The postulated fluorescence mechanism for glucose analysis is based on the consumption of glucose by dissolved oxygen and GOx present in the structure. Thus, the fluorescence intensity of pyrene groups of the probe increases by the elimination of fluorescence quenching by oxygen. Glucose concentration was analyzed quantitatively from 0.25 to 3.0 mM by the fluorescence measurement. The effect of pH and amount of PVA-Py-GOx was also studied. The proposed system was applied to analyze glucose in real samples and compared with those obtained from commercial kits.

Keywords: Poly(vinyl alcohol), Pyrene, "Click" Chemistry, Glucose, Glucose Oxidase.

BİYO UYGULAMALAR İÇİN ÖNCÜL FONKSİYONEL POLİ(VİNİL ALKOL)

ÖZET

Poli(vinil alkol)-piren-glikoz oksidaz (PVA-Py-GOx), yapısında hem floresan hem de oksidan bölgelere sahip olan, "klik" kimyası ve modifikasyon prosesleriyle sentezlenmiş ve karakterize edilmiş suda çözünebilen bir polimerdir. PVA-Py-GOx un morfolojisi atomik güç mikroskopisi (AFM) ile karakterize edildi ve GOx un katılımından dolayı heterojen bir morfoloji gözlemlendi. PVA-Py-GOx un glikoz floresans duyarlılığı için biyo araştırma kapasitesi incelendi. Glikoz analizi için kabul edilen floresans mekanizması yapıda çözünen oksijen ve GOx mevcudiyeti ile glikoz tüketimine bağlıdır. Bu nedenle piren gruplarının floresans yoğunluğu oksijence floresans kırılmasının elimine olmasıyla artar. Glikoz konsantrasyonu floresans ölçümlerde kantitatif olarak 0,25ten 3 mM e kadar analiz edildi. pH ın ve PVA-Py-Gox un miktar etkisi de araştırıldı. Önerilen sistem gerçek örneklerdeki glikoz analizini içermektedir ve ticari techizatlardan elde edilenlerle karşılaştırıldı.

Anahtar Kelimeler: Poli(vinil alkol), Piren, "Klik" Kimyası, Glikoz, Glikoz Oksidaz.

1. INTRODUCTION

Fluorescence sensing of chemical and biochemical analytes is the dominant analytical approach in medical testing, biotechnology and drug discovery [1-3]. This method has become one of the most sensitive and is often used for different bioanalytical purposes applying fluorescence dyes and quantum-dots as labels [4]. Some bioanalytical systems like real-time polymerase chain reaction [5] and some fluorescence sensors [6] are successfully exploiting combinations of fluorescence agents and quenching materials. The combination of a quencher with fluorescence agents may allow decrease of analysis time as well as an increase in selectivity and sensitivity of the methods. As well as polyaromatic hydrocarbons, ruthenium(II) complexes with ligands such as bipyridyl (Rubipy), 1,10-phenanthroline (Ru-phen), and 4,7-diphenyl-1,10-phenanthroline (Ru-dpp) were also successfully applied as oxygen probes due to their relatively long decay times and good photostability [3,7-9]. On the other hand, Platinum(II) and palladium(II) porphyrins represent another group of viable luminescent oxygen indicators because of their high chemical and photochemical stability, large Stokes' shifts, good brightness, as well as long luminescence lifetimes [3].

The development of specific sensors for biochemically relevant analytes via chemical synthesis is more challenging. However, chemical probe design for the complex analytes in biological samples cannot be approached via chemical synthesis alone due to their complex structures. Use of proteins and enzymes as components of sensors for biochemical analytes could be a good alternative to provide specific binding to the target molecules. The advantages of using proteins in probe design include relatively low costs in design and synthesis, water solubility, and the possibility of improving some of the properties by genetic engineering as a result of recent progress. Moreover, many of the important ligands in clinical medicine and in the food industry are relatively small so that the enzymes appear to be the proper class of proteins endowed with the highest specificity and affinity [1].

Glucose sensing takes an important place in controlling various food and

biotechnological processes as well as in diagnosing many metabolic disorders, mainly in diagnosis and therapy of diabetes [10]. In addition to the need for glucose monitoring in the case of patients with chronic diabetes, there is a critical need for monitoring glucose levels in non-diabetic acute care patients because large variations in blood glucose level have been observed also in non-diabetic patients as a common consequence of surgery or acute illness, mostly for those suffering from stress-induced hyperglycemia [11,12]. The crucial demand for continuous, accurate, and relatively noninvasive glucose sensing methods has motivated the design of various sensing materials as well as methodologies [11]. Various analytical methods such as amperometry, potentiometry, spectrophotometry or fluorometry are used for the enzymatic measurements of glucose concentration [13-16]. Among the these methods, those utilizing the glucose oxidase (GOx) enzyme are the most widespread especially in the fabrication of new glucose sensors [11]. The sensing mechanism and the enzyme immobilization method are crucial aspects of the biosensor design. Different methods have been used for immobilization of glucose specific enzymes such as thermal deposition, mechanical compacting, covalent attachment, surface adsorption, dip coating, spin coating, Langmuir-Blodgett (LB) and ionic binding [17]. In recent years, glucose sensors based on fluorescence intensity quenching has generated a great interest since it provides high sensitivity, and little or no damage to the host system. Various approaches including the fluorescence lifetime measurements and some fluorescence techniques, which provide information about the structure and micro-environment of molecules, could be also utilized for optical glucose sensors [18].

A sensor based on the variations in the emission fluorescence properties of GOx enzyme in the visible region due to the enzyme reaction was developed [19]. It is known that enzymes exhibit intrinsic fluorescence signals in the UV zone because of the tyrosine and thryptophan residues while a weak fluorescence due to flavine residues occurs in the visible region. For this reason, GOx has been used as glucose sensing mostly in the UV spectral region because of its intrinsic fluorescence features, while the effectiveness of the fluorescence of the GOx–FAD complex was the subject of a serious discussion. Some authors claimed that the FAD in the protein structure does not reveal any detectable emission. On the other side, it was reported [20-22] that oxidized free flavines exhibit a relatively strong fluorescence with an

emission maximum around 530 nm and the energy intensity of this emission depends on solvent polarity and temperature as well as on the formation of complexes with a variety of molecules [23,24].

As a fluorescence probe, pyrene (Py) has been attractive due to its high fluorescence yield, stability and characteristic excimer formation [25,26]. Particular attentions have been focused on the incorporation of Py units into polymers. For instance, living anionic [27-29], conventional radical polymerization [30], atom transfer radical polymerization [31,32] and ring opening metathesis polymerization [33] processes were successfully applied to prepare polymers with Py groups. We have recently reported a versatile method to synthesize Py functional polyvinyl alcohol (PVA) directly from the bare PVA using ‘‘Click” chemistry strategy [34]. It is conventionally considered that this polymer could be potentially useful for biomedical applications due to its high solubility in a range of solvents with different polarity including water.

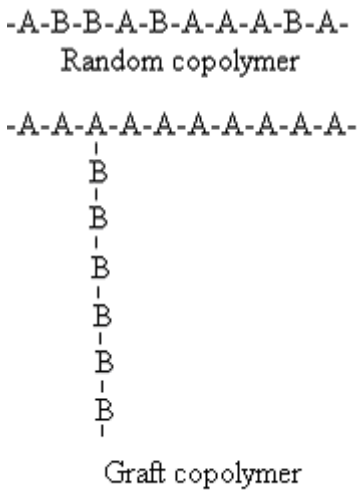
In this thesis, GOx was immobilized on the PVA-Py matrix prepared by “Click” chemistry and used as a water soluble probe molecule for the fluorescence sensing of glucose. Glucose detection was performed by following of the increase in fluorescence intensity due to the diminished quenching of the photo-excited Py molecules based on oxygen consumption through the enzymatic reaction.

2. THEORETICAL PART

2.1 Introduction

It would better to describe the main components of polymers. Polymer chemistry is based on monomers. Monomer is a small molecule that can construct molecules with high molecular weight. Monomers are bounded each other by covalent chemical bonds to form polymers. Polymers are also known as macromolecules due to their large molecular mass.

Polymers can be classified into two main groups: Homopolymers and Copolymers. Homopolymers are synthesized from suitably functionalized monomers. In addition polymers, they are formed only from one kind of monomer. In the case of condensation polymers, the two different monomers possessing antagonist groups contribute to the formation of the polymers. Thus, the repeating unit represents the structures of the both monomers. On the other hand, if the chemical structures of the repeating units are different, the polymer is called copolymer [35]. The mechanism and application of polymerization on two kinds of monomers can cause different arrangements [35].



(2.1)

Also, in graft polymerization, both of main and side chains can be homopolymer or copolymer chains.

Mostly, linear chains are a big fraction of polymers but branched structures such as dendrimers have important uses. The chain itself can build various forms. Dendrimeric structures are repeatedly branched, monodisperse and highly symmetric compounds. This molecule enables the synthesis of multifunctional polymers via changing the branch structures found on it. Dendrons are similar to dendrimers but they are not polymers (see Figure 2.1), they are only molecules with high molecular weights and chemical addressable groups.

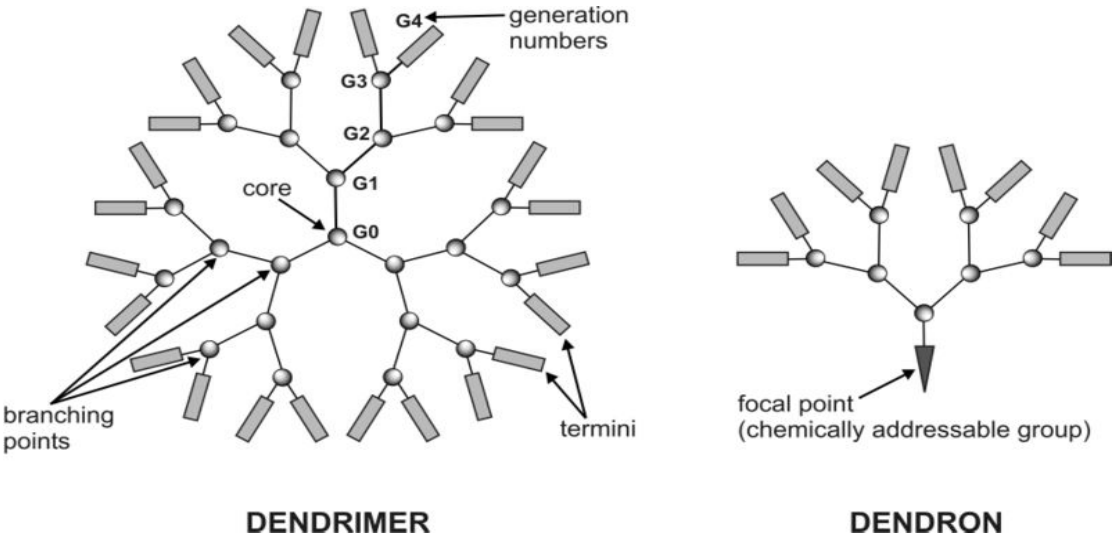
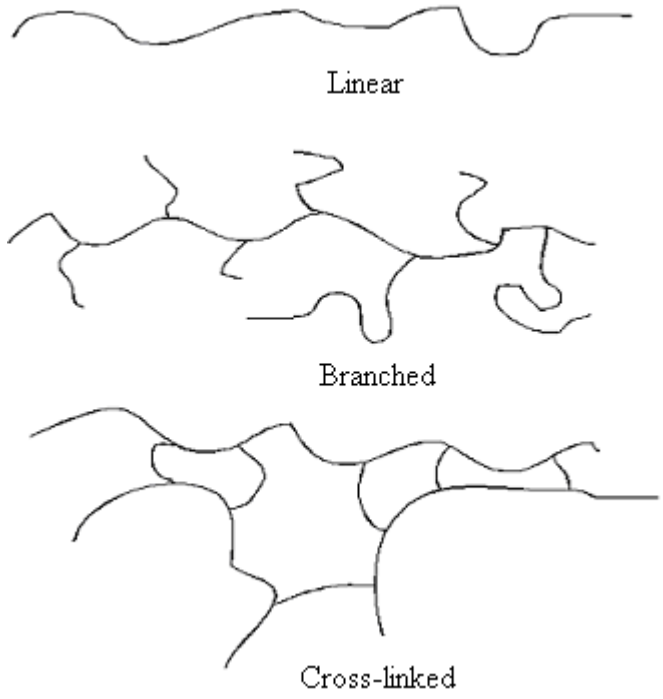


Figure 2.1 : Schematic presentation of dendrimer and dendron.



The polymer chain itself can have different structures

2.2 POLYMER CHEMISTRY

Polymer chemistry is concerned with the synthesis and analysis of polymers. There are 3 main methods which are used for polymer synthesis: Biological synthesis within living cells, modification of natural polymers and organic synthesis of polymers in laboratories.

Organic synthesis of macromolecules is consisted of 2 main methods: Addition (Chain Growth) Polymerization and Condensation (Step-Growth) Polymerization. A newer method, Plasma Polymerization is a good method for covering a surface by thin polymer film, this reaction is initiated by ionizing plasma source's attack on a monomer fragment which contains a vinyl group usually and found in gaseous phase.

2.2.1 Addition (Chain Growth) Polymerization

Addition Polymerization is a widely used method for obtaining commercially important polymers like polystyrene, polyethylene, polypropylene and poly (vinyl chloride) (PVC) [35]. Polymerization of vinyl chloride monomer will be explained in subsequent titles. In a general manner, addition polymerization can be studied in three levels: Initiation, chain growth, termination and chain transfer.

In initiation step; active centers are formed by initiators. Initiators are the molecules which activate the monomer particles. In cationic addition polymerization, carbocations are formed as active centers. Similarly, carbanions, organometallic complexes and free radicals are formed as active centers of anionic, coordination and free radical polymerization respectively.

In the second step, active centers increase the size of polymer chain by joining other monomers.

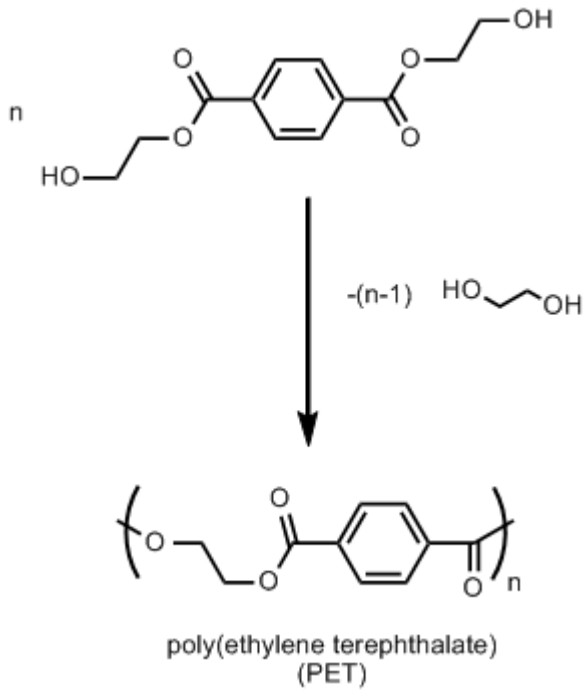
Termination is consisted of chain transfer or disproportionation. Mainly, in this step, active centers are quenched or transposed to another molecule like monomer, initiator, solvent molecule or within/without the polymer chain [35].

2.2.2 Condensation (Step-Growth) Polymerization

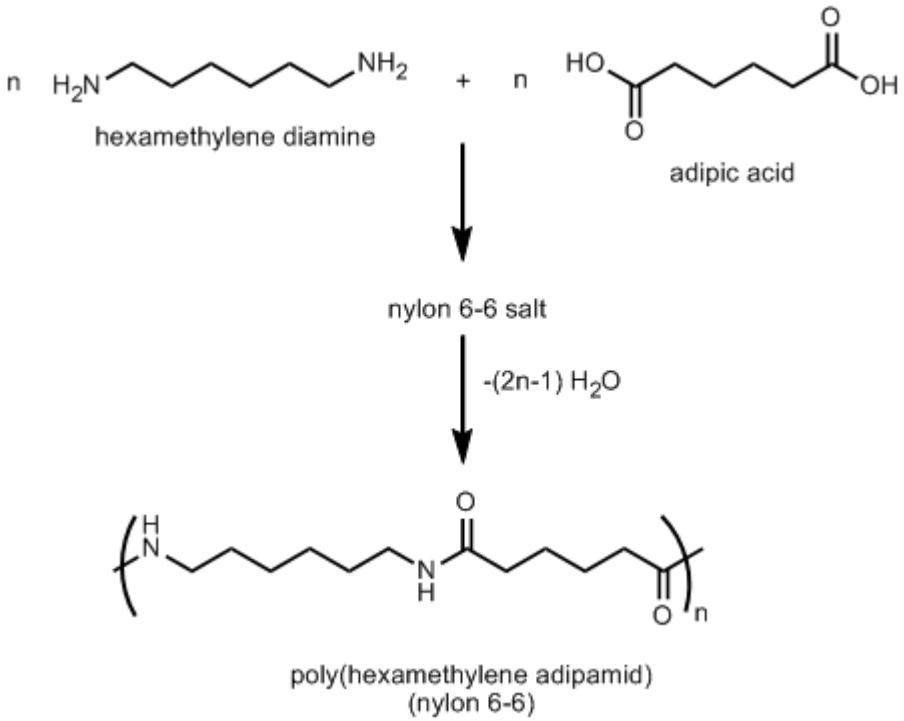
This type of polymerization is convenient for multifunctional polymer synthesis. It is called condensation reactions because during the polymerization, a small molecule leaves.

In condensation polymerization, chains of all sizes can combine without having the necessity of containing an active center [35].

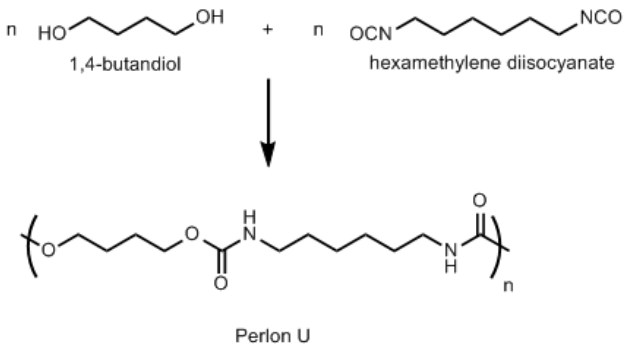
Multifunctional monomers form the basis of these reactions. These monomers are active on both of their sides where they give reactions and a multifunctional polymer is formed as a result of this. Polyesters, polyamides and polyurethanes are widely known types of polymers. Examples of all these kinds are shown respectively:



(2.2) Polyester



(2.3) Polyamide



(2.4) Polyurethane

Table 2.1: The differences between addition and condensation polymerizations [35]

Addition Polymerization	**Condensation Polymerization**
Active centers are required for chain growth	Any two molecules can provide chain growth
Monomers are joined on only active sites sequentially	Chains of every length can react
Monomer concentration decreases but never becomes zero	After a while, nearly all monomers are consumed
High molecular weight polymer is formed at the beginning and the weight does not change a lot during the polymerization	Weight of the polymer increases continuously
Conversion is directly proportional with the reaction time, but mass of polymer is independent of time	For obtaining high molecular weight polymer, long duration is needed
Only active centers react	Every molecule in the media can react with each other
Monomer, polymer and growing active chains are found in the media during the polymerization	Chains of every length are found in the media during the polymerization

Addition polymerization products are consisted of recurring monomer units. On the other hand, condensation polymerization yields polymers whose repeating units lack certain atoms present in the original monomer. During the polymer formation, small molecule elimination such as water, methanol or hydrogen chloride is carried out. During or after condensation reaction, the long chains of the formed polymers can react with each other and can form cross-link structures. These materials are tougher than the straight-chain polymer and can be designed for special purposes by regulation of the amount of cross-linking.

Another variation of a polymer molecule which is consisted of two or more monomer types is maintained bye carefully regulating the relative amounts of monomers, reaction conditions and initiators [36]. By this way, three types of copolymers can be formed:

Random copolymer: ABABBABAABBBA

Alternating copolymer: ABABABABABA

Block copolymer: AAAAABBBBB

A and B are different types of monomers.

2.3 Poly(vinylalcohol) (PVA)

Poly(vinyl alcohol) (PVA), a polyhydroxy polymer, is the largest volume, synthetic water-soluble resin produced in the world. It is commercially manufactured by the hydrolysis of poly(vinyl acetate), because monomeric vinyl alcohol cannot be obtained in quantities and purity that makes polymerization to PVA feasible [37].

Poly(vinyl alcohol) was discovered by Haehnel and Herrmann who, through the addition of alkali to a clear alcoholic solution of poly(vinyl acetate), were able to obtain the ivory-colored PVA [38]. The first scientific reports on PVA were published in 1927 [39,40].

The excellent chemical resistance and physical properties of PVA resins have resulted in broad industrial use. The polymer is an excellent adhesive and possesses solvent, oil, and grease resistance, properties matched by few other polymers.

PVA films exhibit high tensile strength, abrasion resistance, and oxygen barrier properties which under dry conditions are superior to those of most polymers. The

polymer's low surface tension provides for excellent emulsification and protective colloid properties.

The main uses of PVA are in textile sizing, adhesives, protective colloids for emulsion polymerization, fibers, production of poly(vinyl butyral), and paper sizing. Significant volumes are also used in the production of concrete additives and joint cements for building construction and water-soluble films for containment bags for hospital laundry, pesticides, herbicides, and fertilizers. Smaller volumes are consumed as emulsifiers for cosmetics, temporary protective film coatings, soil binding to control erosion, and photoprinting plates.

2.3.1 Solubility

Poly(vinylalcohol) is only soluble in highly polar solvents, such as water, dimethyl sulfoxide, acetamide, glycols, and dimethylformamide. The solubility in water is a function of the degree of polymerization (DP) and hydrolysis (Figure 2.2). Fully hydrolyzed PVA is only completely soluble in hot to boiling water. Partially hydrolyzed grades are soluble at room temperature, although grades with a hydrolysis of 70–80% are only soluble at water temperatures of 10–40◦C. Above 40 ^{o}C the solution first becomes cloudy (cloud point), followed by precipitation of PVA. The hydroxyl groups in PVA contribute to strong hydrogen bonding both intra- and intermolecularly, which reduces solubility in water. The presence of residual acetate groups in partially hydrolyzed PVA weakens these hydrogen bonds and allows solubility at lower temperatures. The hydrophobic nature of the acetate groups results in a negative heat of solution [41–43], which increases as the number of acetate groups is increased. This means that the critical θ temperature is lower, ie, the solubility decreases as the temperature is increased. Heat treatment or drying of a few minutes increases crystallinity and greatly reduces the solubility and water sensitivity (Figure 2.2). Prolonged heat treatment does not further increase crystallinity. The heat treatment melts the smaller crystals, allowing for diffusion and reformation of crystals with a melting point higher than that of the treatment temperature. The presence of acetate groups reduces the extent of crystallinity; thus, heat treatment has little or no influence on low hydrolysis grades. The influence of heat treatment is desirable in some applications such as adhesives and paper coatings where a greater degree of water resistance is needed, but is highly undesirable in textile warp sizing where the polymer must be removed after a drying cycle.

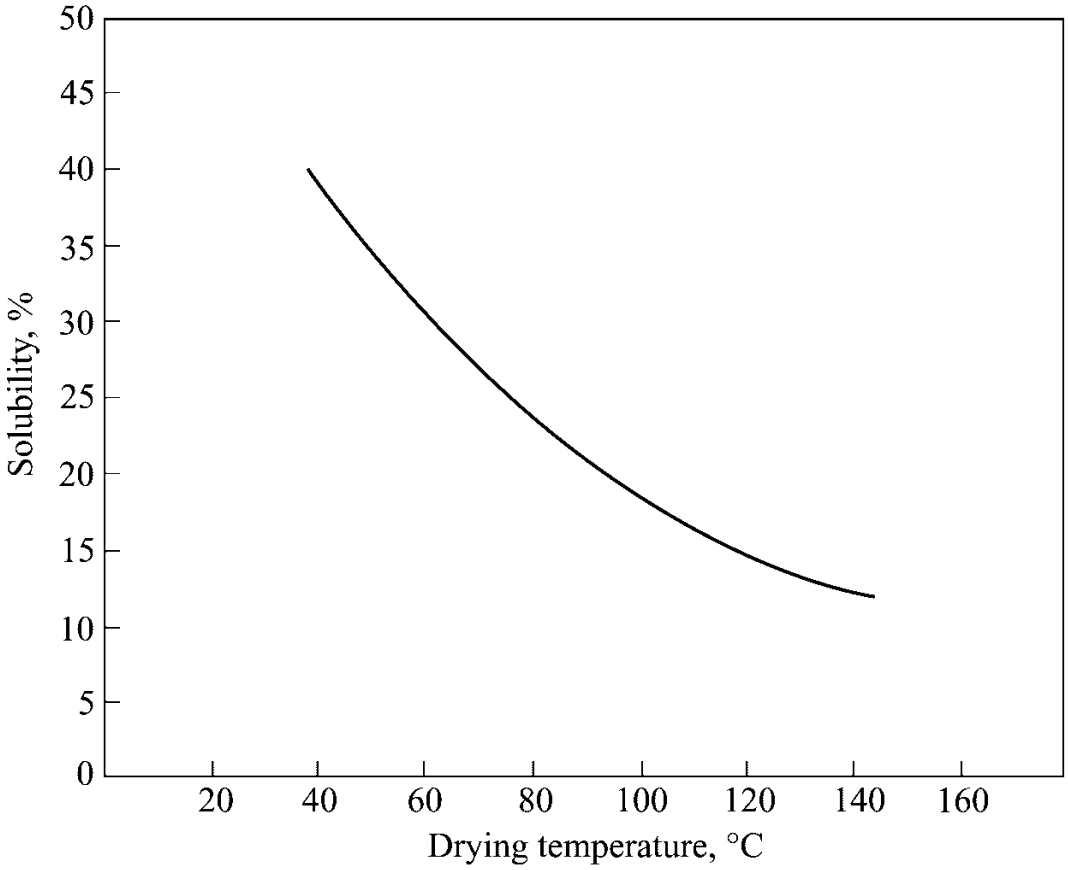


Figure 2.2 : Influence of heat treatment on solubility at 40◦C; DP = 1700, 98–99 mol% hydrolyzed.

2.3.2 Physical Properties

The physical properties of PVA are highly correlated with the method of preparation. The final properties are affected by the polymerization conditions of the parent poly(vinyl acetate), the hydrolysis conditions, drying, and grinding. Further, the term PVA refers to an array of products that can be considered to be copolymers of vinyl acetate and vinyl alcohol. The effect of hydrolysis and molecular weight is illustrated in Figure 2.3.

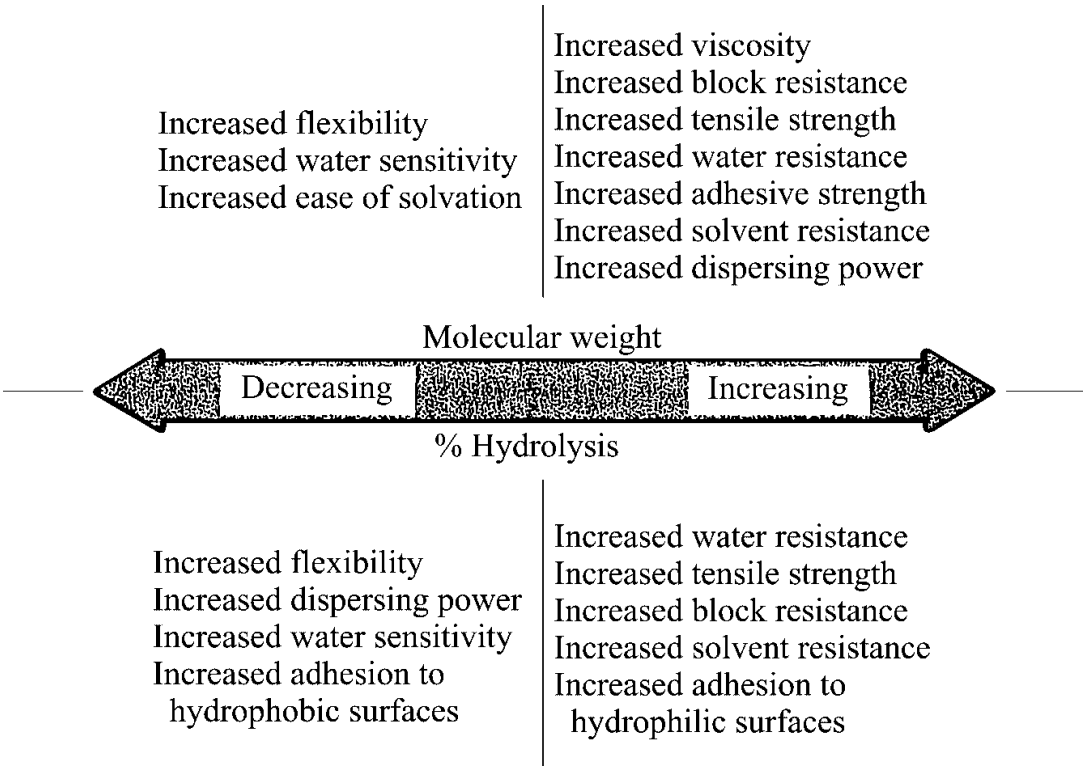


Figure 2.3 : Effect of molecular weight and hydrolysis on the properties of PVA [44].

2.3.2.1. Crystallization and Melting Point

The ability of PVA to crystallize is the single most important physical property of PVA as it controls water solubility, water sensitivity, tensile strength, oxygen barrier properties, and thermoplastic properties. Thus, this feature has been and continues to be the focal point of academic and industrial research. The degree of crystallinity as measured by x-ray diffraction can be directly correlated to the products density or the swelling characteristic of the insoluble part (Figure 2.4). The size of the crystals determines the melting point. Reported values for the melting point of PVA range between 220 and 267◦C for fully hydrolyzed PVA. Exact determination of the crystalline melting point using normal data techniques is difficult as decomposition takes place above 140◦C. The divergence in melting point may be ascribed to decomposition or prior treatment history. The melting point of PVA containing an appropriate amount of diluent or comonomer is less influenced by decomposition. Thus, the melting point of fully hydrolyzed PVA can be determined by the extrapolation of the measured values to 0% diluent.

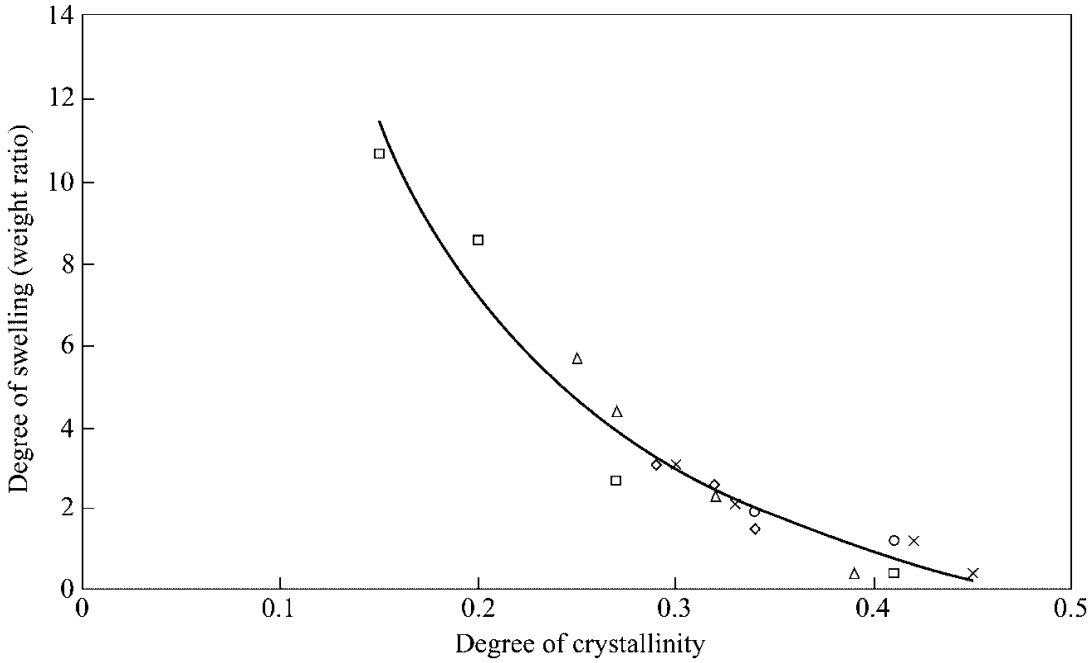


Figure 2.4 : Relationship between swelling and crystallinity.

A more reliable melting point is obtained in this manner. The melting points determined by the diluent method are 255–267◦C for commercial superhydrolyzed PVA (greater than 99% hydrolysis). The melting point determined by melting point depression caused by noncrystallizing comonomer units assumes as a first approximation that the vinyl acetate units are randomly distributed. This assumption usually does not apply to commercial PVA. The extrapolated values of heat of fusion and melting point obtained with this method are therefore highly dependent on the manufacturing method and the resulting blockiness (Figure 2.5). The heat of fusion, determined by either of the above methods, has been calculated as 6.82 □ 2.1 kJ/mol [45–47].

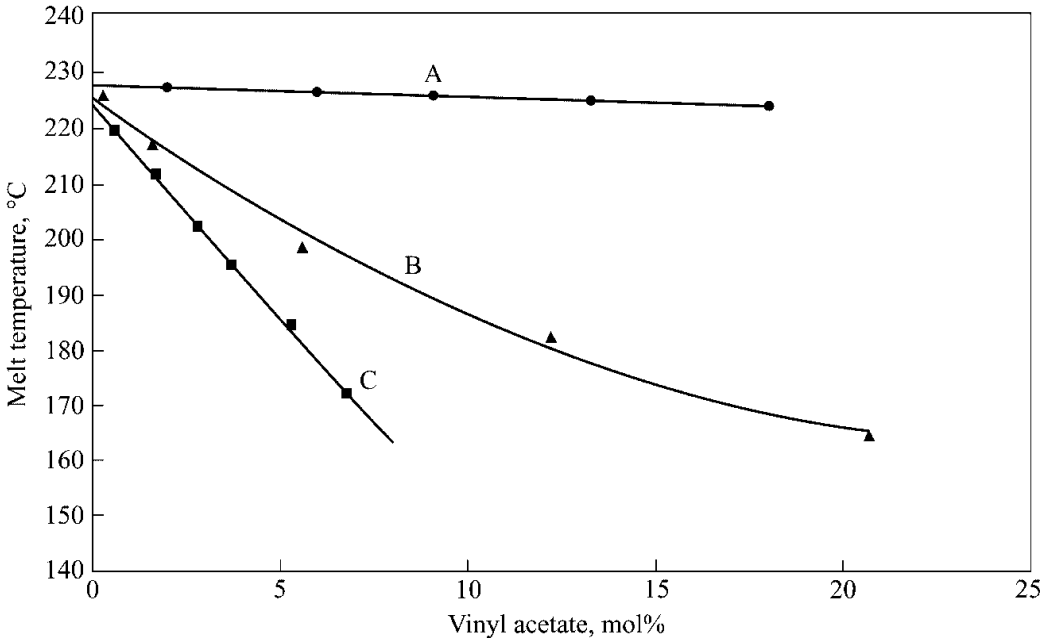


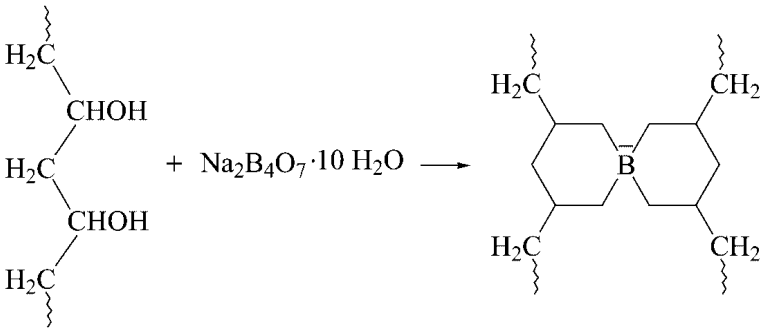
Figure 2.5 : Influence of vinyl alcohol–vinyl acetate copolymer composition on melting temperature [46], where A represents block copolymers; B, block copolymers; and C, random copolymers.

2.3.3 Chemical Properties

Poly(vinyl alcohol) participates in chemical reactions in a manner similar to other secondary polyhydric alcohols. Of greatest commercial importance are reactions with aldehydes to form acetals, such as poly(vinyl butyral) and poly(vinyl formal).

2.3.3.1 Esterification

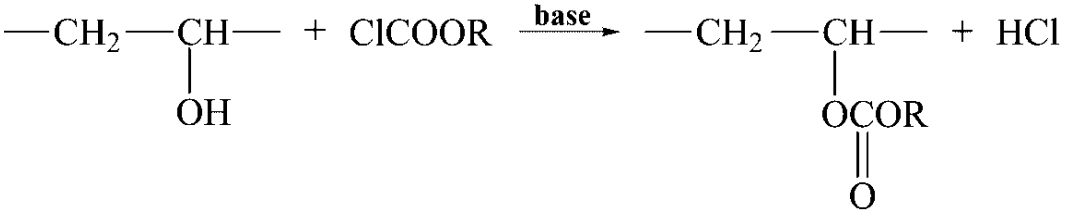
Boric acid and borax form cyclic esters with PVA [48]. The reaction is markedly sensitive to pH, boric acid concentration, and the cation-to-boron ratio **(2.5)**. An insoluble gel is formed at a pH above 4.5–5.0.



(2.5)

Similar complexes are formed between PVA and titanium lactate, titanyl sulfate, or vanadyl compounds. Poly(vinyl nitrate) has been prepared and studied for use in explosives and rocket fuel. Poly(vinyl alcohol) and sulfur trioxide react to produce poly(vinyl sulfate). Poly(vinyl alkane sulfonate)s have been prepared from PVA and alkanesulfonyl chlorides. In the presence of urea, PVA and phosphorus pentoxide or phosphoric acid yield poly(vinyl phosphate)s.

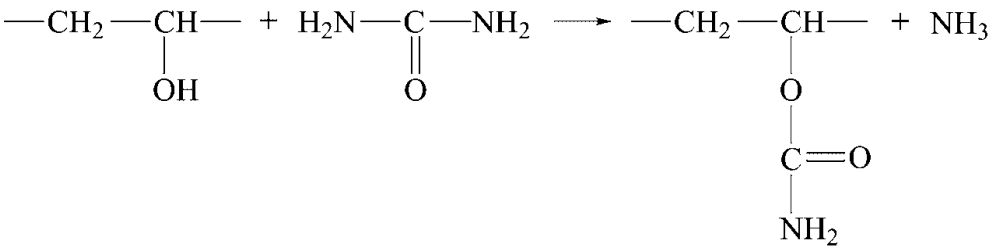
Organic Ester: An unlimited number of organic esters can be prepared by reactions of PVA employing standard synthesis [49,50]. Chloroformate esters react with PVA to yield poly(vinyl carbonates) **(2.6)**.



(2.6)

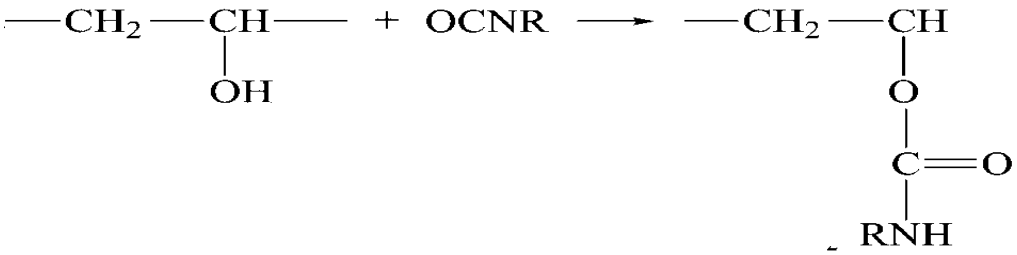
Poly(acrylic acid), poly(methacrylic acid), and maleic anhydride containing polymers react with PVA to form insoluble gels useful as absorbents for water, blood, urine, etc.

Urea and PVA form a polymeric carbamate ester **(2.7)**:



(2.7)

Reaction between PVA and isocyanates yields substituted carbamate esters (**2.8**):



(2.8)

2.3.3.2 Etherification

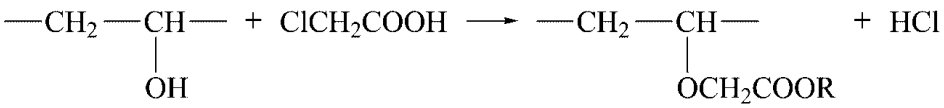
Ethers of PVA are easily formed. Insoluble internal ethers are formed by the elimination of water, a reaction catalyzed by mineral acids and alkali.

Ethylene oxide reacts with PVA under normal ethoxylation conditions. The resulting products have properties which make them useful as cold water-soluble films.

Cationic PVA has been prepared by the reaction of *N*-(3-chloro-2 hydroxypropyl)-*N*,*N*,*N*-trimethylammonium chloride and PVA and sodium hydroxide. Reactions between alkylidene epoxide and PVA in particulate, free-flowing form in an alkaline environment have been reported [51].

Poly(vinyl alcohol) undergoes Michaels addition with compounds containing activated double bonds, including acrylonitrile, acrylamide, *N*-methylolacrylamide, methyl vinyl ketone, acrolein, and sodium 2-acrylamido-2-methylpropanesulfonate. The reactions have been carried out under conditions spanning homogeneous reactions in solvent to heterogeneous reactions in the swollen powder or fiber.

Poly(vinyl alcohol) also reacts with monochloroacetates to yield glycolic acid ethers (**2.9**).

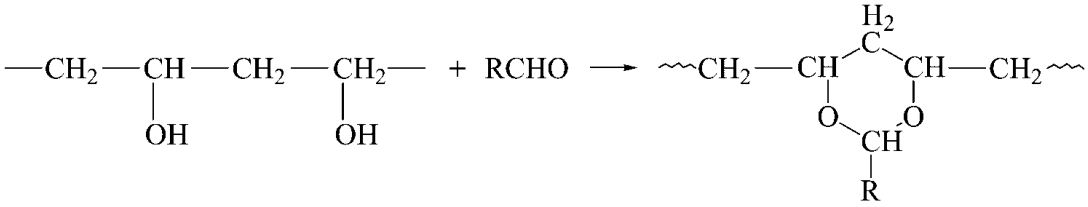


(2.9)

2.3.3.3 Acetalization

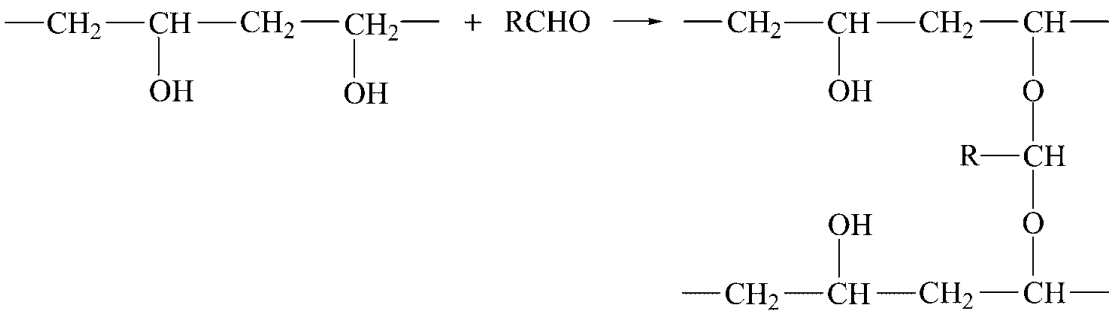
Poly(vinyl alcohol) and aldehydes form compounds of industrial importance.

Intramolecular acetalization **(2.10)**:



(2.10)

Intermolecular acetalization **(2.11)** :



(2.11)

Poly(vinyl butyral) prepared by reacting PVA with *n*-butyraldehyde finds wide application as the interlayer in safety glass and as an adhesive for hydrophilic surfaces . Another example is the reaction of PVA with formaldehyde to form poly(vinyl formal), used in the production of synthetic fibers and sponges. Poly(vinyl alcohol) is readily cross-linked with low molecular weight dialdehydes such as glutaraldehyde or glyoxal. Alkanol sulfonic acid and PVA yield a sulfonic acid modified product.

2.3.3.4 Biodegradation

Poly(vinyl alcohol) is one of the few truly biodegradable synthetic polymers with the degradation products being water and carbon dioxide. At least 55 species or varieties of microorganisms have been shown to degrade or take an active role in the degradation of PVA [52]. Poly(vinyl alcohol) degrading organisms consist not only of 20 different genera of bacteria, but molds, yeast, and fungi as well. The microorganisms which degrade PVA exist in most environments including activated sludge, facultative ponds, anaerobic digesters, septic systems, compost, aquatic systems, soil, and landfills. The time period for PVA to degrade is dependent upon the physical properties of the polymer, the form in which the product exists and the environment in which it is degraded. PVA is rapidly degraded by activated sludge especially if the sludge has already been adapted to

the PVA molecule. Many of the microorganisms which degrade PVA can be isolated from soil.

The mechanism of PVA degradation consists of a random oxidation of a hydroxyl group to a ketone through the influence of a secondary alcohol oxidize. The random oxidation is continued until a β-diketone is formed. This group is cleaved by a extracellular hydrolase, leading to a reduction in molecular weight and theformation of a carboxylic end group and a methyl ketone end group. Continued degradation eventually leads to the formation of acetic acid, which in turn is converted into carbon dioxide and water. The degradation is normally accomplished by means of symbiotic organisms. Symbiotic bacterial pairs known to degrade PVA include, for Type I, *Pseudomonas putida* VM 15A, *P. vesicularas* Va., *P. porolyticus* PH, and *P. alkaligenes*; and for Type II, *P. Sp.* VM15C, *P. vesicularis* XL, *P. vesicularis* XL, and *P. vesicularis* PD. More recent studies have shown that isotactic blocks in PVA molecule are more readily biodegraded than those having an atactic structure [53,54]. The biological oxygen demand for degradation of a 0.1% PVA solution is approximately 5 ppm.

2.3.4 Production of PVA

Poly(vinyl acetate) can be converted to PVA by transesterification, hydrolysis, or aminolysis. Industrially, the most important reaction is that of transesterification,

where a small amount of acid or base is added in catalytic amounts to promote the ester exchange.

The catalysts most often described in the literature [55-57] are sodium or potassium hydroxide, methoxide, or ethoxide. The reported ratio of alkali metal hydroxides or metal alcoholates to that of poly(vinyl acetate) needed for conversion ranges from 0.2 to 4.0 wt%. Acid catalysts are normally strong mineral acids such as sulfuric or hydrochloric acid. Acid-catalyzed hydrolysis is much slower than that of the alkaline-catalyzed reaction, a fact that has limited the commercial use of these catalysts.

The solution of poly(vinyl acetate) generated during the stripping operation is normally passed directly through to the alcoholysis system. This has limited the available solvents to methanol and ethanol [54, 56]. Substituting the alcohol used as solvent with the generated ester limits the hydrolysis and greatly affects how the remaining acetyl groups are distributed on the PVA chain. The distribution is often referred to as the blockiness of the PVA. The higher the methyl acetate concentration, the higher the degree of blockiness. The presence of catalyst residues, such as alkali hydroxide or alkali acetate, a by-product of the hydrolysis reaction, is known to increase the thermal instability of PVA. Transforming these compounds into more inert compounds or removal through washing are both methods that have been pursued. The use of mineral acids such as sulfuric acid, phosphoric acid, and *ortho*-phosphoric acid has been reported as means for achieving increased thermal stability of the resulting PVA.

Commercial Hydrolysis Process: The process of converting poly(vinyl acetate) to PVA on a commercial scale is complicated on account of the significant physical changes that accompany the conversion. The viscosity of the poly(vinyl acetate) solution increases rapidly and greatly as the conversion proceeds because the resulting PVA is insoluble in the most common solvents used for the polymerization of vinyl acetate. The outcome is the formation of a gel swollen with the resulting acetic acid ester and the alcohol used to effect the transesterification.

Continuous Saponification: There are several types of continuous systems, each with their own benefits and drawbacks. The basic premise is that continuous mixing is not required after the poly(vinyl acetate) and the caustic is mixed [58]. Several designs

for the high intensity mixing unit have been suggested in order to obtain efficient mixing and little fouling. In the belt process, the mixture is cast onto the belt or conveyer (high temperature polyethylene) where the gelling occurs. The gel is removed from the surface before syneresis, cut into smaller particles, and passed to a holding or washing tank (Figure 2.6). The liquid is removed from the precipitate using common chemical deliquefying methods, and the resulting particles are dried in a continuous dryer.

In the slurry process, the hydrolysis is accomplished using two stirred tank reactors in series. Solutions of poly(vinyl acetate) and catalyst are continuously added to the first reactor, where 90% of the conversion occurs, and then transferred to the second reactor to reach full conversion. Alkyl acetate and alcohols are continuously distilled off in order to drive the equilibrium of the reaction. The resulting PVA particles tend to be very fine, resulting in a dusty product. The process has been modified to yield a less dusty product through process changes and the use of additives. Partially hydrolyzed products having a narrow hydrolysis distribution cannot be prepared by this method.

In the screw conveyer process, solutions of poly(vinyl acetate) and catalyst are mixed in a high intensity mixer and continuously introduced to a screw-type saponification and conveyer system . Downstream details are similar to those found in the belt process.

Other methods also exist for handling the reaction mixture. For instance, an intermeshing, self-wiping, twin-screw extruder process has been proposed.

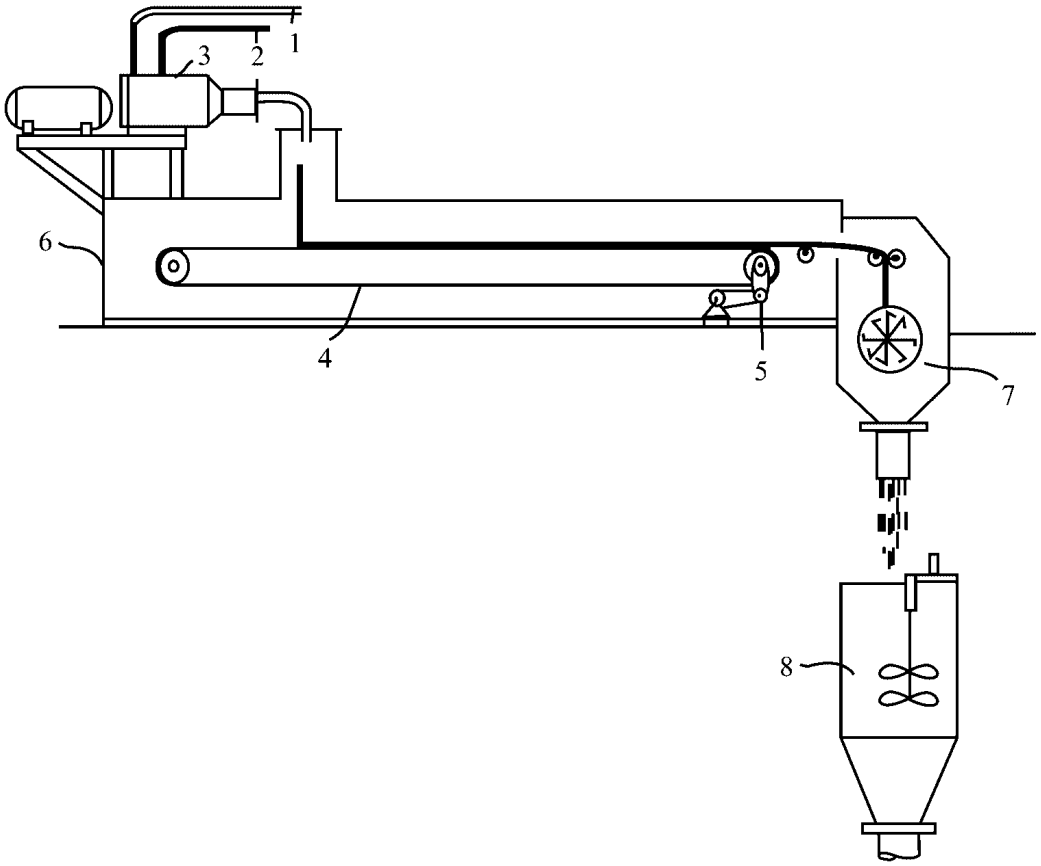


Figure 2.6 : The belt saponification process as used in the production of PVA. 1, Polyvinyl acetate/methanol mixture; 2, catalyst in methanol; 3, mixer; 4, belt; 5, drive; 6, housing; 7, cutter; 8, slurry tank (wash tank).

The advantages described include higher saponification temperature (less catalyst), increased water solubility, and fewer impurities.

Batch Saponificatio: Batch saponification, the oldest PVA manufacturing method, is mainly used for the production of specialty products. The process uses a kneader in which the hydrolysis, washing, and drying operations are performed. This is the simplest method of saponification, but the production rates are low, and producing the product quality needed by many end uses is difficult.

2.3.4.1 Drying/Solids Separation

Separation of the polymer gel from the methanol/methyl acetate liquid is an important step, accomplished by using standard pieces of equipment such as filters, screw presses, or centrifuges. Drying of the PVA is critical both to the color and solubility of the final product. Excessive drying temperatures result in high product color and an increase in the crystallinity, which in turn reduces the solubility of the

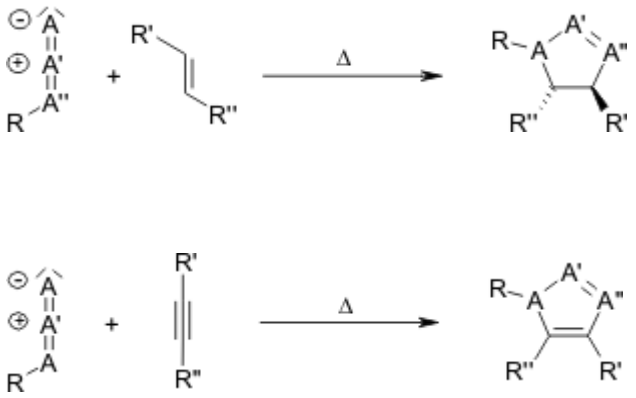
product. Drying is initially subjected to a flash regime, where the solvent not contained within the particles is flashed off. This first phase is followed by a period where the rate is controlled by the diffusion rate of solvent from the PVA particles. Because the diffusion rate falls as the material dries, complete drying is not practical. The polymer is, therefore, generally sold at a specification of 95% solids.

2.3.4.2 Solvent Recovery

A mixture of methanol and methyl acetate is obtained after saponification. The methyl acetate can be sold as a solvent or converted back into acetic acid and methanol using a cationic-exchange resin such as a cross-linked styrene–sulphonic acid gel. The methyl acetate and methanol mixture is separated by extractive distillation using water or ethylene glycol. Water is preferred if the methyl acetate is to be hydrolyzed to acetic acid. The resulting acetic acid solution is concentrated by extraction or azeotropic distillation.

2.4 CLICK CHEMISTRY

A new approach in organic synthesis that involves the successful achievement of a polymerization process represents an important task in macromolecular science. Cycloaddition reactions, among them the metal catalyzed azide/alkyne 'click' reaction which is a variation of the Huisgen 1,3-dipolar cycloaddition reaction between terminal acetylenes and azides were shown to be the most effective and versatile and thus became the prime example of click chemistry **(2.12)**.



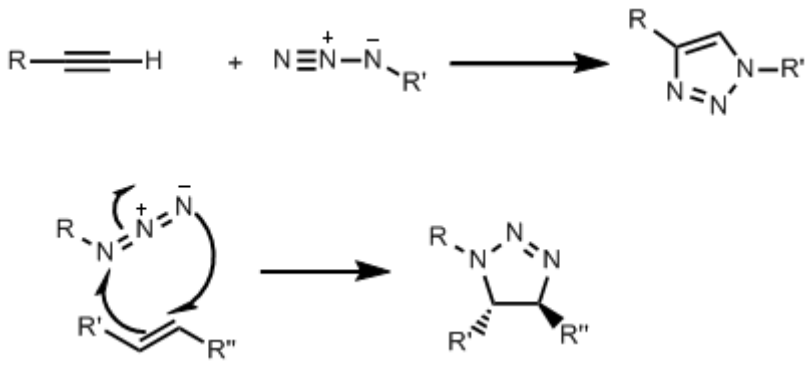
(2.12)

The Huisgen Cycloaddition is the reaction of a dipolarophile with a 1,3-dipolar compound that leads to 5-membered (hetero)cycles. Examples of dipolarophiles are alkenes and alkynes and molecules that possess related heteroatom functional groups (such as carbonyls and nitriles). 1,3-Dipolar compounds contain one or more heteroatoms and can be described as having at least one mesomeric structure that represents a charged dipole [60]. The reaction yield is above 95% with a high tolerance of functional groups and solvents, as well as moderate reaction temperatures between 25°C-70°C. Click reactions have gained popularity in materials science in recent years [61, 62].

The major scientific challenge of nanomaterial development is the requirement of high degree of structural order and defined properties. To meet this demand, the design of novel organic or inorganic molecules using sophisticated multistep experimental procedures is necessary. As a result, the discovery and selection of simpler and universal synthetic methods is essential.

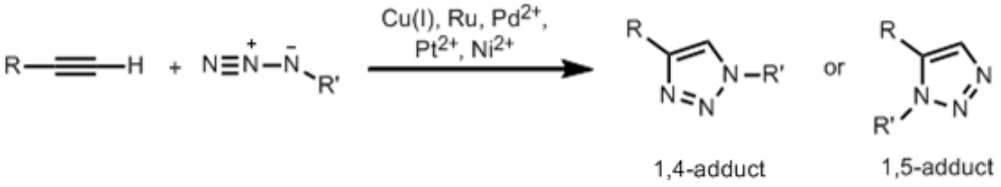
The azide/alkyne 'click' reaction which is an appealing concept proposed by Sharpless and co-workers is not a scientific discipline but rather a synthetic philosophy inspired by the simplicity and efficiency of the chemistry that takes place in nature. The objective of click chemistry is to establish an ideal set of straightforward and highly selective reactions [61-64]. Click reaction of azide/alkyne is a recent re-discovery of a reaction fulfilling many requirements for the affixation of ligands onto polymers by post-modification processes, which include often quantitative yields, a high tolerance of functional groups, an insensitivity of the reaction to solvents, irrespective of their protic/aprotic or polar/non-polar character, and reactions at various types of interfaces, such as solid/liquid, liquid/liquid, or even solid/solid interfaces [61].

The basic Sharpless-type click reaction is a variant of the Huisgen 1,3-dipolar cycloaddition reaction between C–C triple **(2.13),** C–N triple bonds, and alkyl /aryl-/ sulfonyl azides [65].



(2.13)

The central, purely thermal Huisgen process can be extremely accelerated by the addition of various metal species (Ru, Ni, Pt, Pd), but mostly by CuI species, within the reaction system. Click chemistry is widely recognized with copper-catalyzed Huisgen 1,3-dipolar cycloaddition of azides and terminal alkynes [66-70].This reaction is usually quite slow in the absence of an appropriate catalyst for alkynes are poor 1,3-dipole acceptors but in the presence of copper(I), which can bind to terminal alkynes, cycloaddition reactions are quite accelerated and regioselective **(2.14).**



(2.14)

Among many reactions tested, the 1,3-dipolar cycloaddition process has emerged as the method of choice to effect the requirements of ligating two molecules in a general, fast, and efficient process [61,62] . The gain of thermodynamic enthalpy of at least 20 kcal/mol [64], thus leading to reactions characterized by high yields (more than 95%), simple reaction conditions, fast reaction times, and high selectivity. Moreover, the copper-catalyzed azide–alkyne cycloaddition (CuAAC) can be performed in various solvents including water and in the presence of numerous other functional groups [68,69]. Sharpless and co-workers published a paper in 2002, where the formation of 1,2,3-triazoles by the CuI-catalyzed Huisgen reaction between non-activated alkynes and alkyl/aryl azides was described. A catalytic cycle

based on a concerted mechanism via a Cu acetylide intermediate was proposed, which has been recently revised to include a binuclear reaction mechanism on the basis of several observations [71]. Acetylide ion has a strong nucleophilic character and the critical 'invention' of this process is the transformation of a purely thermal 1,3-dipolar cycloaddition process to a 1,3- dipolar cycloaddition process catalyzed by metal salts which are mostly CuI salts, but recently also Ru, Ni, Pd, and Pt salts. As a result, the reaction runs at ambient temperature, is nearly solvent insensitive, and with an extremely high tolerance of functional groups [61].

Click chemistry is not a strategy only for organic synthesis, today it has an enormous potential in materials science, polymer chemistry, biological applications comprising drug discovery and biolabeling [61,62,72-74].

3. EXPERIMENTAL WORK

3.1 Materials and Chemicals

3.1.1 Polymers

Poly(vinyl) alcohol (PVA) (BDH) : PVA (Mn. 14000) was used as recieved.

3.1.2 Solvents

Methanol (Technical) : Methanol was used for the precipitation of polymers without further purification.

Chloroform (Sigma) : Chloroform was used without further purification.

Tetrahydrofuran (THF) (J.T.Baker) : Predried over magnesium sulfate followed by sodium wire and then distilled from sodium wire and benzophenone immediately before use.

N,N-dimethylformamid (DMF) (Merck) : Predried over magnesium sulfate followed by sodium wire.

Ethanol (Aldrich) : Ethanol was used as recieved.

Diethylether (Sigma-Aldrich) : Diethylether was used as recieved.

Dimethyl sulfoxide (DMSO) (J.T.Baker) : Dimethyl sulfoxide was used as recieved.

Anhydrous Pyridine (LAB-SCAN) : Anhydrous pyridine was used as recieved.

3.1.3 Other Chemicals and Reagents

Sodium Azide (Carlo-Erba) : It was used as received.

Copper(II) sulfate (CuSO4.5H2O) (Merck) : Copper(II) sulfate was used as received.

L-Ascorbic acid sodium salt (ACROS) : L-Ascorbic acid sodium salt was used as received.

Toluene-4-Sulfonic acid Monohydrate (PTSA) (Fluka) : Toluene-4-Sulfonic acid Monohydrate salt was used as received.

Sodium Hydride (Fluka) : Sodium Hydride was used as received.

Propargyl bromide (Fluka) : Propargyl bromide was used as received.

Pyrene methanol (Fluka) : Pyrene methanol was used as received.

Glucose oxidase (GOx; β-D-glucose: oxygen 1-oxidoreductase, E.C 1.1.3.4, Type II-S: from Aspergillus niger, 21200 U/g solid, 2.36 g solid) (Sigma-Aldrich) : Glucose oxidase was used as received.

1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC; $C_8H_{17}N_3 \cdot HCl$) (Sigma-Aldrich) : 1-Ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride was used as received.

2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) (Sigma-Aldrich) : 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) was used as received.

D-glucose (Sigma-Aldrich) : D-glucose was used as received.

3.2 Equipments

3.2.1 Nuclear Magnetic Resonance Spectroscopy (NMR)

^{1}H-NMR analyses were recorded on a Bruker 250 MHz NMR Spectrometer.

3.2.2 Infrared Spectrophotometer (IR)

IR spectra were recorded on a Perkin Elmer Spectrum One FT-IR Spectrometer.

3.2.3. Fluorescence Spectrophotometer

Fluorescence spectra were obtained by using a Perkin Elmer LS 50 Luminescence spectrophotometer.

3.3 Preparation Methods

1. 3.3.1 Partial Tosylation of PVA

Partial tosylation of PVA (PVA-Ts) with *p*-toluene sulphonyl chloride (*p*-TsCl; 1 : 1; in terms of hydroxyl moieties) in the presence of anhydrous pyridine at room temperature [75] yielded 10% PVA-Ts.

3.3.2 Synthesis of PVA-N_3 Coploymer

PVA-Ts was dissolved in *N,N*-dimethylformamid (DMF), NaN_3 (2 times excess to the mole of tosyl of poly(vinyl) alcohol) was added. The resulting solution was allowed to stir at 65°C for two days and precipitated into dietylether mixture (1/1 by volume).

3.3.3 Synthesis of popargylpyrene

To a solution of 9-anthracene methanol (1.0 equiv.) in dry 20 mL of THF was added to sodium hydride (60 w % dispersion in oil) (1.1 equiv.) and the reaction mixture was stirred at 0 °C under nitrogen for 30 min. A solution of propargyl bromide (1/2 equiv.) in toluene was added portion wise to the solution. The mixture was kept stirring at room temperature for 24 h. Then it was refluxed for 3 h in the dark. The resulting mixture cooled to room temperature and evaporated to half of its volume.. The solution was extracted with ethyl acetate, and the organic layer was dried over anhydrous $MgSO_4$. Evaporating ethyl acetate afforded light yellow product. The crude product was dissolved in toluene and was passed through a column of basic silica gel to remove unreacted 9-anthracene methanol. Toulene was removed by evaporating and the residue was dried in vacuum oven. (Yield: 55 %)

3.3.4 Synthesis of PVA containing pyrene side-group (PVA-Py)

In a flask, PVA-N3 (0.10 g), propargyl pyrene (0.1231 g, 0.45 mmol) dissolved in 5 ml of DMSO. Freshly prepared aqueous solution of sodium ascorbate (0.068g, 0.34mmol) was added followed by aqueous solution of copper(II)sulfate pentahydrate (0.017 g, 0.068mmol), so that the final concentrations of sodium ascorbate and copper(II)sulfate pentahydrate in the mixture 30 and 6 mM respectively. The ratio of azide and alkyne groups was 1. The mixture stirred for two days of ambient temperature. Functionlized polymer precipitated in dietyl eter (10 times excess),filtered and dried under vacuum.

3.3.5 Immobilization of GOx to PVA-Py

Before the immobilization of GOx on the polymeric matrix, the hydroxyl groups of PVA-Py (10 mg) were activated with EDC (0.5 %) coupling agent according to Chiou et al [76] After 2 hours activation, unreacted EDC was removed by using Microcon centrifugal filter units (3 000 NMWL, Millipore) at 10 000 x g for 10 mins. Afterwards, GOx (20 mg) in potassium phosphate buffer (50 mM, pH 6.0) was added to the activated PVA-Py solution (100 µl) and allowed to stand overnight at 4

^{o}C. Finally, immobilized enzyme solution was centrifugated for 15 min at 10 000 x g by using centrifugal filter units (100 000 NMWL) to remove unbound PVA-Py as well as other substances. The final volume of the immobilized enzyme was adjusted to 1 ml by potassium phosphate buffer (pH 6.0, 50 mM) which will be referred as working buffer solution in further steps. Before and after immobilization, the enzyme activity and protein content were also checked by ABTS [77] and Bradford assays [78] respectively. The response signals of water soluble immobilized enzyme (PVA-Py-GOx) through the enzymatic reaction of glucose substrate were monitored via fluorescence emission measurement.

3.3.5.1 GOx Assays

3.3.5.1.1 Detection of PVA-Py-GOx Response via Fluorescence Emission Measurements

GOx catalyzes the oxidation of glucose to gluconic acid and hydrogen peroxide. Since oxygen quenches photo-excited fluorescent molecules, the increase in fluorescence intensity of Py in the matrix is observed due to the oxygen consumption during the enzymatic reaction depending on the glucose concentration. The emission fluorescence spectra have been collected by means of a fluorescence spectrophometer purchased from Varian Cary Eclipse (USA). Sample excitation was performed at 340 nm, while the emission spectrum was recorded in the range 340–500 nm. The emission intensities were registered at 376 nm. All measurements were performed in 1x1 cm quartz cell at 25 ^{o}C. The spectra have been acquired with entrance and exit slit fixed at 5 nm and with a scan speed of 10 nm s^{-1}.

In the fluorescence emission assay, initially appropriate amount of immobilized enzyme and glucose from the stock solutions were transferred into the test tubes containing oxygen saturated working buffer solutions and allow to incubate in orbital shaker (175 rpm) at 25 ^{o}C for 15 min. Then, fluorometric response of PVA-Py-GOx as a result of enzymatic activity was measured as described before against to the blank containing the same reaction mixture except the glucose substrate. The response signal of PVA-Py-GOx was defined as the difference in fluorescence signals in the presence and absence of substrate and registered as arbitary units (Δ a.u). For the observation of effect of pH and enzyme amount, maximum signal value was assumed as 100% and other values calculated relative to this value.

3.3.5.1.2 ABTS Method

Before and after immobilization step, the activity was also checked according to ABTS method by enzymatically determining the concentration of the produced hydrogen peroxide by means of peroxidase (POD), according to the following procedure: 1 ml of ABTS (0.003 M), 0.3 ml of POD (6 U), 1 ml of glucose (0.3 M) and 0.6 ml of potassium phosphate buffer (50 mM; pH 6.0) were mixed and incubated for 5 min at 30 ^{o}C. Then 0.1 ml L of PVA-Py-GOx were added to reaction mixture and optical densities were followed for 1 min at 420 nm. The concentration of ABTS was in excess in respect to that of H_2O_2 so that the reaction was limited by the concentration of the latter product. H_2O_2 concentration was calculated by using the extinction coefficient of 43 200 $M^{-1}.cm^{-1}$ [77]. One unit (U) of enzyme activity was defined as the amount of enzyme which catalyzed the production of 1 μmol H_2O_2 per minute under the experimental conditions. Specific activity was calculated as the ratio of unit activity of free or immobilized enzyme to the protein content. Protein contents of GOx solution before and after immobilization procedure were found as 1.65 mg/ml and 1.0 mg/ml according to Bradford assay while specific activities were calculated as 41 and 36 U/mg protein, respectively. The spectrophotometric data have been collected by means of a Lambda 35 UV/VIS Spectrometer purchased from Perkin Elmer (USA).

3.3.5.1.3 Glucose Analysis

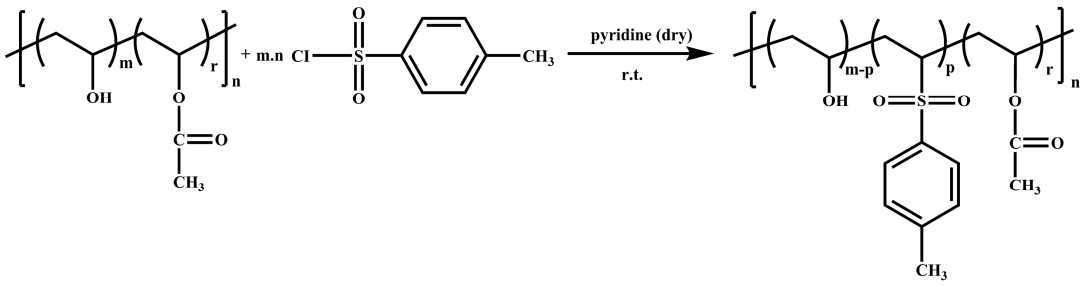
PVA-Py-GOx was used to analyze glucose content in real samples such as fizzy and orange juice that was purchased from the local markets. Additionally, a commercial enzyme assay kit based on spectrophotometric Trinder rection (Cromatest, Glucose MR, Cat. No. 1129010) was used as a reference method for independent glucose analysis. In this reaction, the glucose is oxidized to D-gluconate by the GOx with the formation of H_2O_2. In the presence of POD, a mixture of phenol and 4-aminoantipyrine (4-AAP) is oxidized by H_2O_2 to form a red quinine imine dye proportional to the glucose concentration in the sample [79]. No sample pretreatment was required for the analysis. Only appropriate amount of dilution was done prior to the analysis. Each trial was performed three times and data were presented as mean ± S.D (standard deviation).

4. RESULTS AND DISCUSSION

4.1 Synthesis and Characterization of Tosyl Functional PVA (PVA-Ts)

In the scope of this study, our main goal was to introduce "Click" chemistry approach for the modification of PVA. As stated in the introduction section, previous reports on the introduction of azide groups to PVA as the major click component involves an indirect route in which hydroxyl antagonist molecule possessing azide group was prepared separately. However, this approach has some limitations such as involvement of several independent steps and explosive nature of the azidation particularly on low molar mass compounds [63].

In this study we report a versatile method which allows converting hydroxyl groups into azide functionality by a simple two step reaction performed only on PVA. For this purpose, partial tosylation of PVA with *p*-toluene sulphonyl chloride (*p*-TsCl; 1:1; in terms of OH- moieties) were conducted as described in experimental section to obtain PVA with tosyl pendant groups (PVA-*Ts*) **(4.1)**.



(4.1)

Primarily, the extent of the modification was determined. In the ^{1}H-NMR spectrum of PVA-*Ts*, the new signals corresponding to C*H*$_3$ protons adjacent to phenyl ring at 2.34 ppm and the aromatic protons of *p*-toluene sulphonyl group between 7.38 and 7.85 ppm were detected (Figure 4.1). The signals at δ 3.69-4.00 and δ 1.19-1.78 ppm belong to resonances of the methine and metylene protons in the main chain. Protons corresponding to OCOC*H*$_3$ group from acetyl of pristine PVA appear at 1.8 ppm.

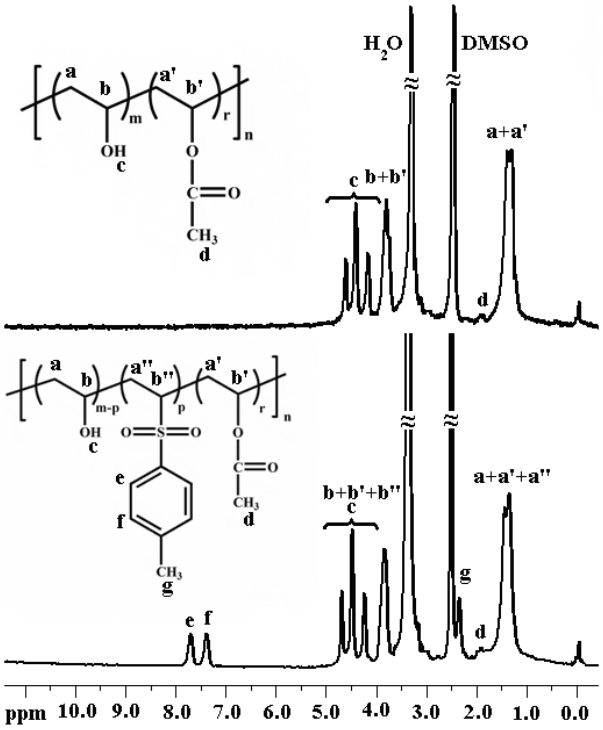


Figure 4.1 : ^{1}H-NMR (d_6-DMSO) spectra of PVA and PVA-*Ts*.

The composition of the polymers can be calculated by using the following equations:

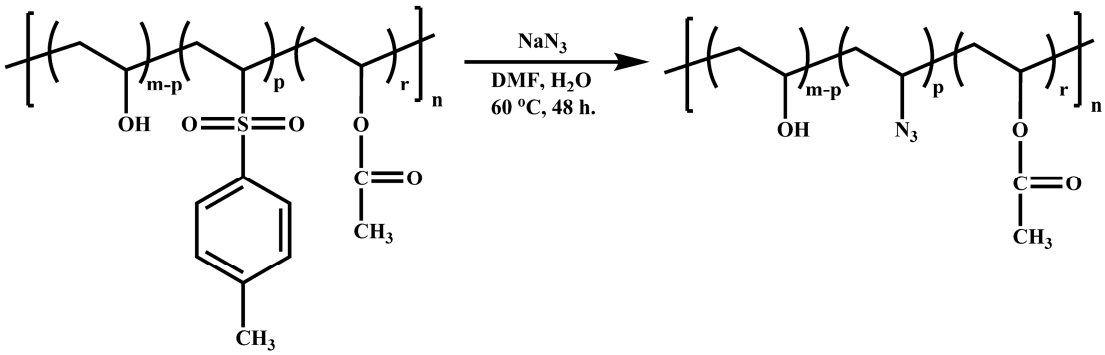
%Ts = *I*Ar100/4*I*CH and

%OCH_3 = *I*$OCH_3$100/3*I*CH

where %Ts and %$OCCH_3$ represent the amount of units with tosyl and acetyl groups, respectively, and *I*Ar, *I*OCH_3 and *I*CH represent the intensities of the integrals corresponding to the *p*-toluene sulphonyl, the $OCCH_3$ and CH protons of the main chain, respectively. The content of acetyl units of the starting PVA is about 2.82 %. After the tosylation, the content of tosyl groups is determined to be 10.03 %.

4.2 Synthesis and Characterization of Azide Functional PVA

The resulting PVA-*Ts* polymer was then quantitatively converted into PVA-N_3 in the presence of NaN_3/DMF at 60 ^{o}C **(4.2)**. Functionalization was kept deliberately at low level so as to preserve PVA properties.



(4.2)

In the ^{1}H-NMR spectrum, the disappearance of the signals at δ 7.38-7.85 and 2.34 ppm corresponding to aromatic and C*H*$_3$ protons of *p*-toluene sulphonyl side groups was indicative of quantitative conversion (Figure 4.2). Successful azidation was further supported by the observation of the azide streching band at 2094 cm $^{-1}$ in the FT-IR spectrum of PVA-*N*$_3$.

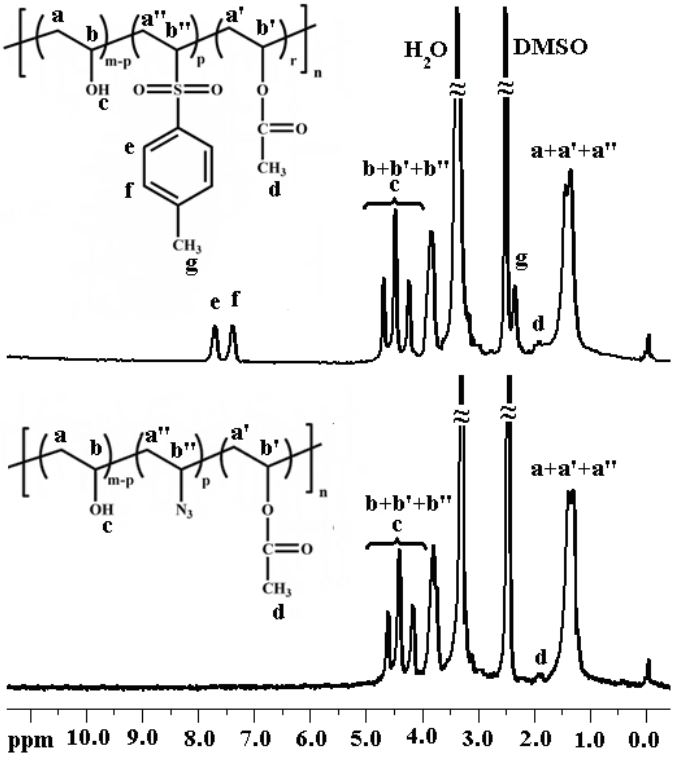
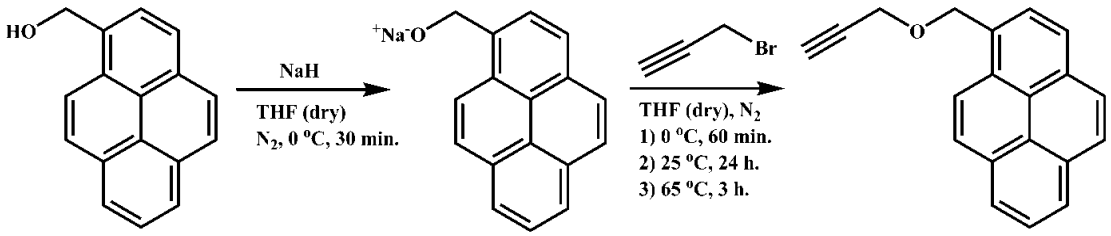


Figure 4.2 : ^{1}H-NMR (d_6-DMSO) spectra of PVA-*Ts* and PVA-*N*$_3$.

4.3 Synthesis and Characterization of Propargylpyrene

Propargyl*pyrene,* possessing both click functional group and chromophoric pyrene moiety, was prepared according to the following reaction **(4.3)**.



(4.3)

The chemical structure of propargyl*pyrene* was confirmed by both ^{1}H-NMR and FT-IR. The ^{1}H-NMR spectrum of propargyl*pyrene* showed two signals at 4.34 and 5.25 ppm which are assigned to CH_2 protons adjacent to pyrene ring and propargyl moiety, respectively. Notably, *H*C≡C proton of propargyl moiety and DMSO overlap and appear at 2.50 ppm. Also, aromatic protons of pyrene were detectable at 7.04-842 ppm (Figure 4.3). In the FT-IR spectrum, propargyl group was evidenced by characteristic bands of H-C≡C and -C≡C- appeared at 3277 and 2121 cm^{-1}, respectively.

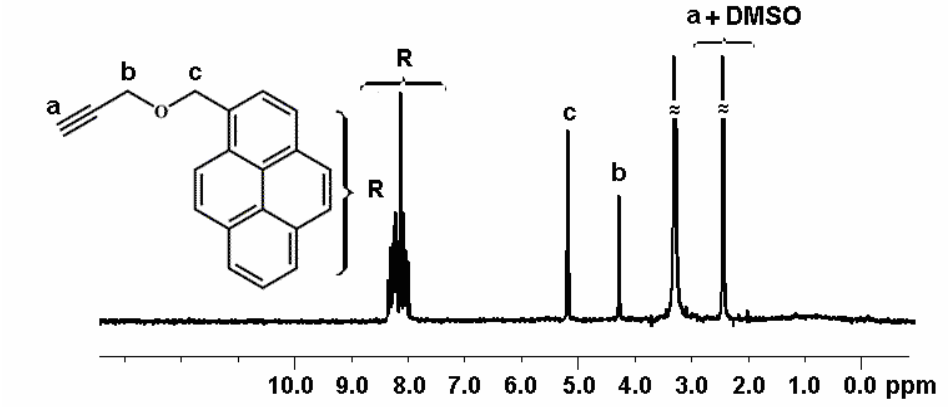
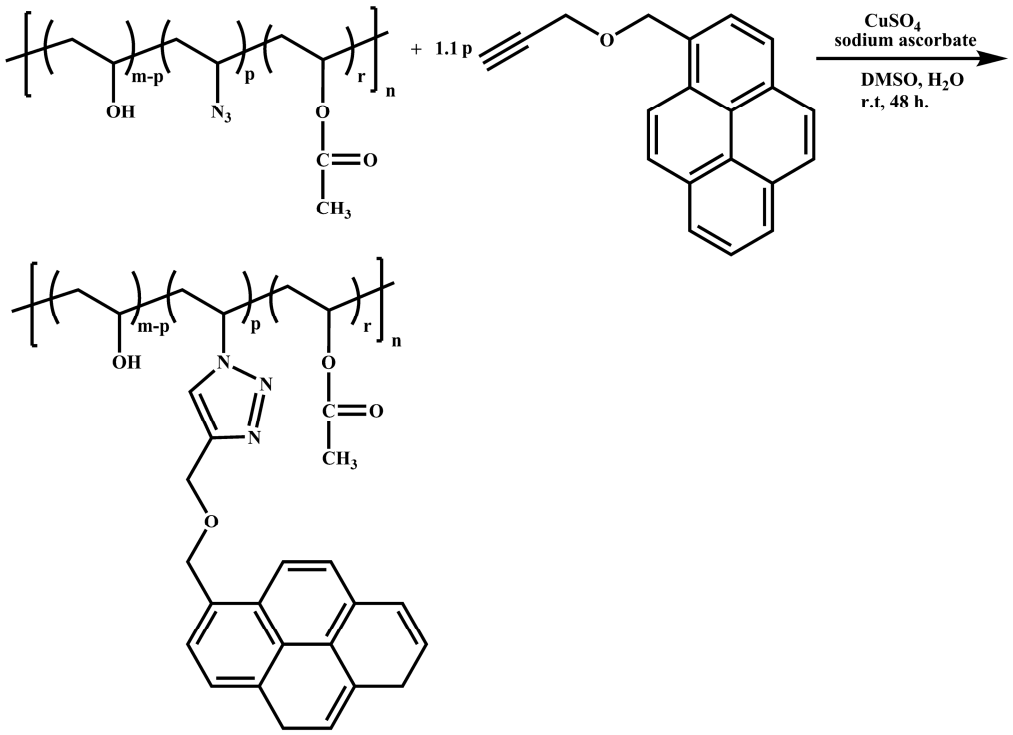


Figure 4.3 : ^{1}H-NMR (d_6-DMSO) spectra of propargyl*pyrene.*

4.4 Synthesis and Characterization of Pyrene Functional PVA (PVA-Py)

For the desired click process, the PVC-N_3 was dissolved in dimethyl sulfoxide (DMSO) and reacted with propargyl*pyrene* in the presence of aqueous solution of

sodium ascorbate and copper(II) sulfate pentahydrate at room temperature (**4.4**). The modified polymer was precipitated in diethyl ether and dried under vacuum.



(4.4)

Evidence for the occurrence of the "Click" reaction is obtained from ^{1}H-NMR and FT-IR spectroscopy. The extent of conversion of the side azido moieties to triazoles was monitored by observing the appearance of the new methylene protons adjacent to the triazole and pyrene ring at 4.10 and 5.26 ppm (triazole-CH_2-O-CH_2-Py) and the triazole proton (N-CH=C-) at 7.56 ppm. The peaks between 8.12-8.42 ppm, characteristic for aromatic protons of pyrene were also noted (Figure 4.4).

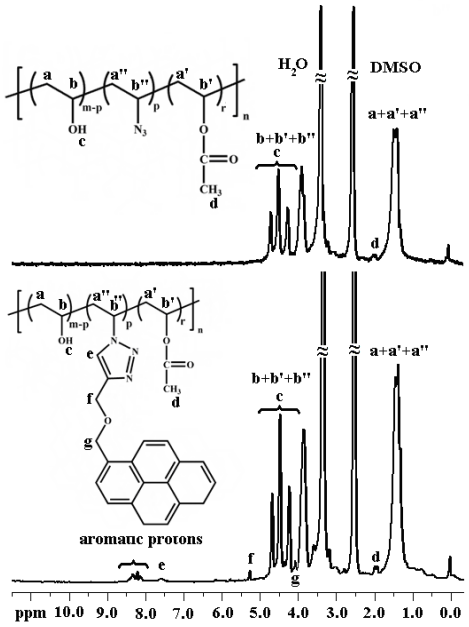


Figure 4.4 : ^{1}H-NMR (d_6-DMSO) spectra of PVA-*N_3* and PVA-*pyrene.*

Moreover, in the IR spectrum, the band corresponding to -N_3 group at 2105 cm^{-1} completely disappeared (Figure 5.5). These spectral characterizations clearly indicate that the side group click reaction was efficient, and near-quantitative functionalization was achieved.

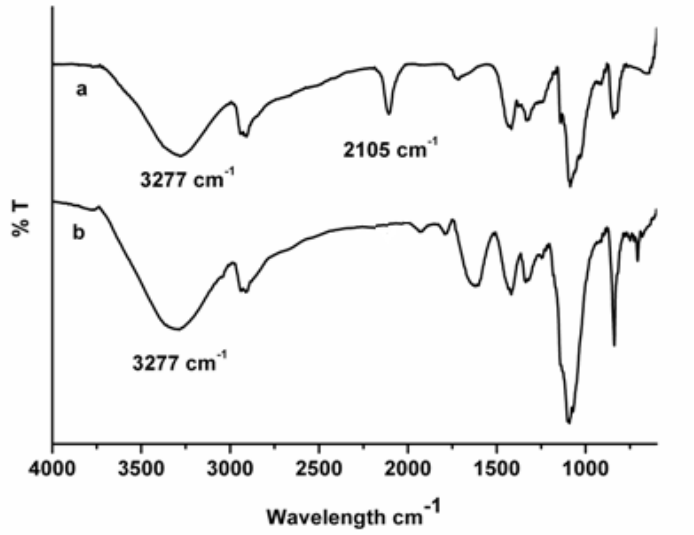


Figure 4.5 : FT-IR Spectra of (a) PVA-*N_3*, (b) PVA-*Py.*

4.5 Fluorescence Analysis

Playing the predominant role in labeling polymers, the fluorescence properties of the pyrene units incorporated to PVA side-chains are important and were also studied. The fluorescence spectrum of diluted solution of PVA-Py in DMSO excited at λ_{exc}=350 nm showed vibrational structures of pyrene chromophore (Figure 4.6). The observed emission property of PVA obtained this way is a striking advantage particularly for biomedical applications involving various polymer matrix-specific molecule interactions.

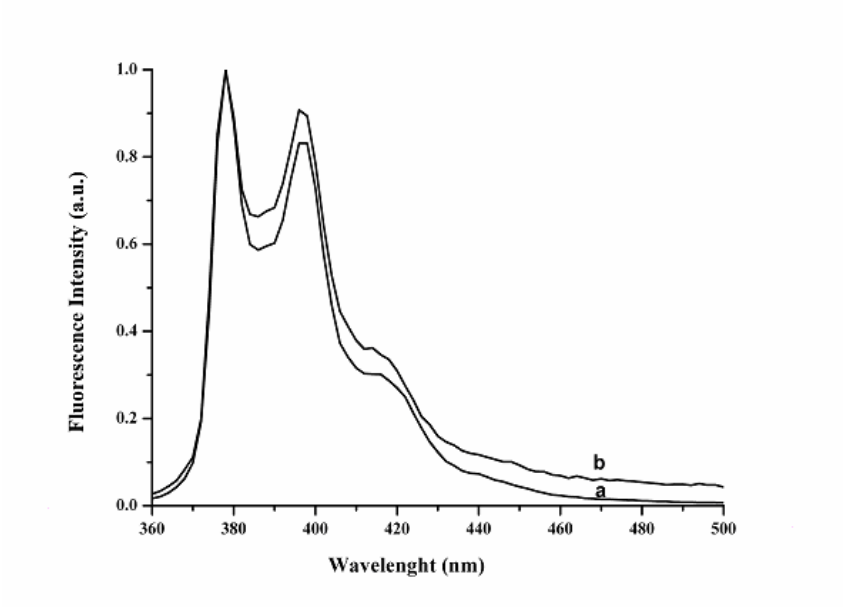


Figure 4.6 : Emission spectra of propargylpyrene and PVA-Py; λexc= 360 nm.

Fluorescence Lifetime Measurements

The typical temperature dependent fluorescence decay curves of PVA-*Py* in DMSO at several temperature is presented in Figure 4.7. It is seen that PVA-*Py* decays faster as the temperature is increased. Fluorescence decay curves were fitted to a single exponential.

$$I_p = A\exp(-t/\tau)$$

where τ is pyrene lifetime and A is the corresponding amplitude of decay curves. Here, it has to be noted that lifetime of pyrene, τ corresponds to the mobility of the PVA-*Py* chains in DMSO. Figure 4.8 shows a decay curve and weighted residuals.

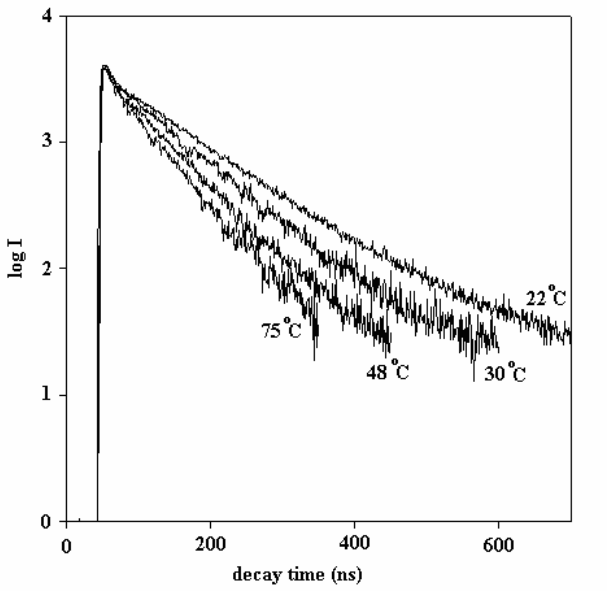


Figure 4.7 : Fluorescence decay profiles of PVA-Py in DMSO. Number on each decay curve presents solution temperature.

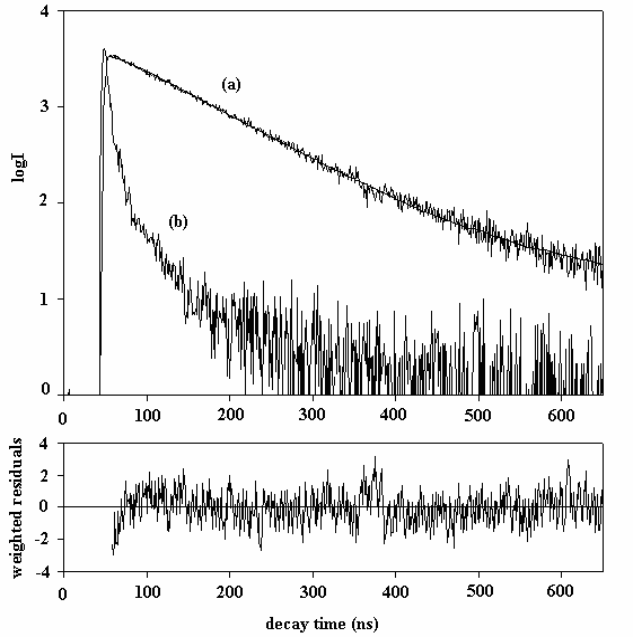


Figure 4.8 : Fluorescence decay curve of PVA-*Py* (a) in DMSO and the incident light pulse (b).

Measured lifetimes of propargyl*pyrene* and PVA-*Py* are presented in Figure 4.9, respectively. As seen in Figure 4.7, when the temperature increased the excited pyrenes decay faster and faster, indicating a probable collision between molecules which essentially results in fluorescence quenching. In another words, the DMSO acts as an energy sink for rapid vibrational relaxation which occurs after the rate-limiting transition from the initial state. An excellent linear relationship was observed between the lifetime values of pyrene and temperature in the range of 22-75 ^{o}C. The linear regression equation of the calibration graphs and a linear regression correlation coefficients were found to be $\tau = 338.96 - 0.823T$ and $\tau = 347.98 - 0.856T$, and 1 and 0.975 for propargyl*pyrene* and PVA-*Py,* respectively. These findings clearly indicate that free pyrene (propargyl*pyrene*) is more (even if slightly) mobile than attached one as expected.

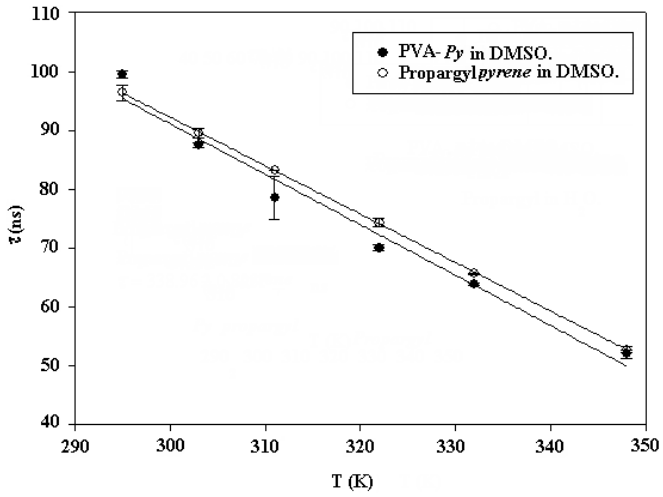


Figure 4.9 : The plots of the measured pyrene lifetimes (propargyl*pyrene* and PVA-*Py*), τ versus temperature.

Temperature Dependence of the Fluorescence Emission Intensity of PVA-Py

Figure 4.10 (a) and (b) show typical temperature dependent fluorescence emission spectra of *PVA-Py* (2 x 10^{-5} M) in water and propargyl*pyrene* (1 x 10^{-5} M) in DMSO at several temperatures, respectively.

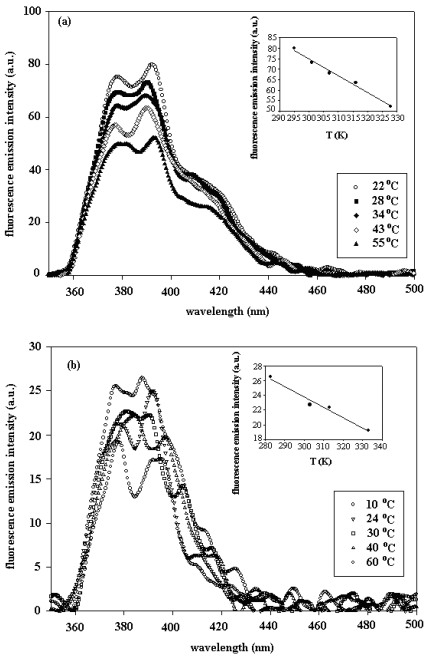


Figure 4.10 : Fluorescence emission spectra of PVA-*Py* in water (a) and propargyl*pyrene* in DMSO (b) for various temperature. Linear dependency of fluorescence intensity versus temperature is also presented in inset.

The fluorescence emission intensity of PVA-*Py* decreased as the temperature increased. This behavior is mainly controlled by a radiationless temperature-dependent process. As the temperature increases, probable collision among molecules results in fluorescence quenching and intersystem crossing reinforced with increasing temperature. Decreasing the fluorescence emission intensity should be a result of a convolution among photophysical process dependent on concentration, bimolecular quenching, unimolecular rate process, and photochemical processes decreasing the chromophore concentration and mobility of polymer chains [80]. An excellent linear relationship between the fluorescence emission intensity and temperature was observed and calibration graph was obtained by linear regression process. Obtained calibration equation are $Flu = 314.53 - 0.798T$ with a linear regression correlation coefficients of 0.9885 and $Flu = 66.93 - 0.142T$ with a linear regression correlation coefficients of 0.975 for Propargyl*pyrene* and PVA-*Py,* respectively (*Flu* stands for relative fluorescence intensity).

4.6 Thermal Analysis

Thermal stability of the PVA-*Py* was investigated by TGA and compared with pristine PVA. The TGA curves are presented in Figure 4.11 and weight loss

behaviors of the species are tabulated at Table 1. TGA data showed that the degradation for the bare PVA and pyrene functionalized PVA begins at temperatures close to 210 ^{o} C indicating that the general thermal degredation pattern of PVA was not influenced by the incorporation of pyrene units. On the other hand, the thermal data also reveals that the char yield of the PVA-*Py* is enhanced approximately 4 folds compared to unreacted PVA due to the presence of more rigid and bulky pyrene group. Another noticeable feature is that the weight loss difference at 800 ^{o} C between the polymer before and after modification. Interestingly, this value corresponds the pyrene content of the polymers.

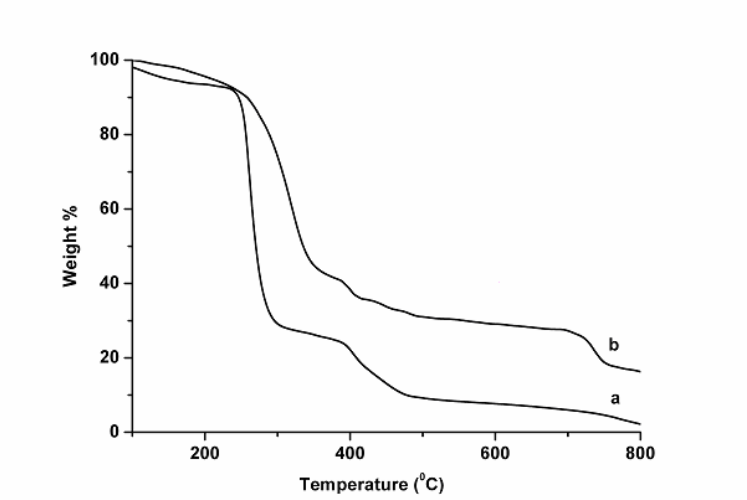


Figure 4.11 : TGA curves of PVA (a) and PVA-*Py* (b) recorded under nitrogen at heating rate of 10 ^{0}C/min.

Table 4.1: Thermal properties of PVA-*Py* and PVA.

Polymer	$T_{5\%}$ [a] (^{o}C)	$T_{10\%}$ [b] (^{o}C)	$T_{d\ max}$ [c] (^{o}C)	Y_c [d] at 800 ^{o}C (%)
PVA-*Py*	209.1	257.8	241.0	16.3
PVA	211.0	251.3	218.4	4.1

[a] $T_{5\%}$: The temperature for which the weight loss is 5%.
[b] $T_{10\%}$: The temperature for which the weight loss is 10%.
[c] $T_{d\ max}$: Maximum weight loss temperature.
[d] Y_c: Char yields.

4.7 Solubility of Modified PVA

The modification drastically changes the solubility behavior of PVA as the process results in a decrease in the number of hydroxyl groups contributing to strong intra- and inter-molecular hydrogen bonding. As can be seen from Table 2, the polymer with 10 % modification is soluble in highly polar solvents such as water, DMSO, and DMF as well as in the less polar solvents such as THF.

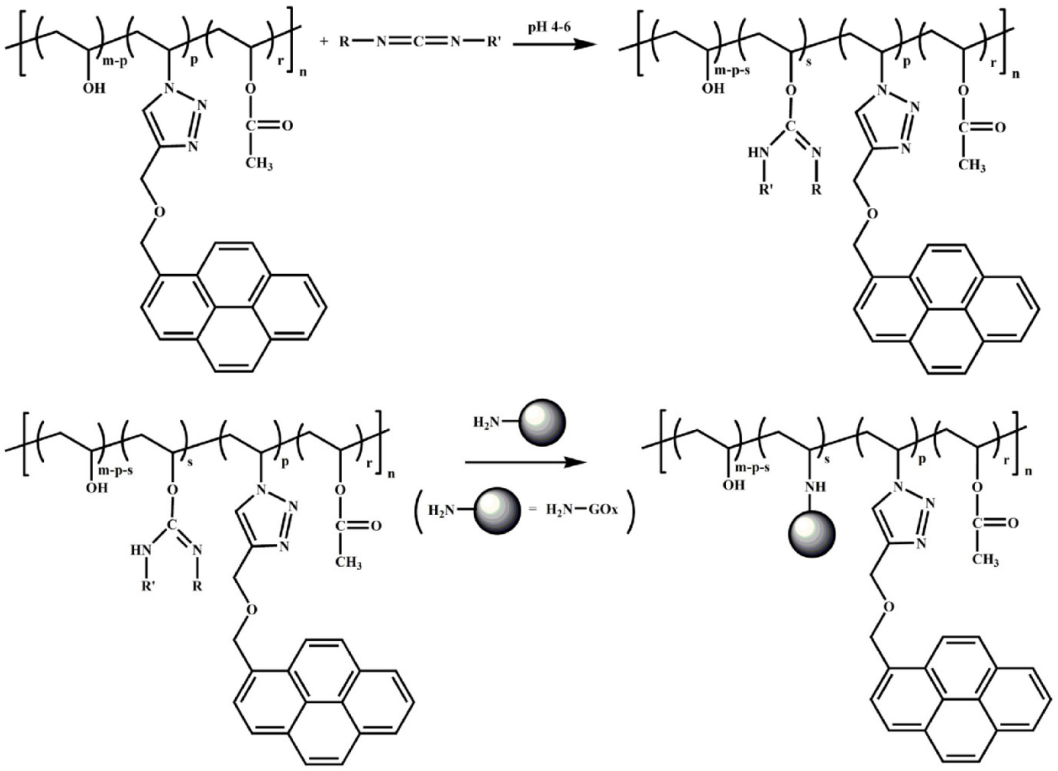
Table 4.2: Solubility[a] of PVA, PVA-N_3 and PVA-*Py* in solvents ranked according to dielectric constants. (S: soluble; SS: slightly soluble; NS: non-soluble)

Solvent Type	**Dielectric Constants[b]**	**PVA**	**PVA-N_3**	**PVA-*Py***
Water	80	SS	S	S
DMSO	46	S	S	S
DMF	36	SS	S	S
THF	7.6	NS	SS	SS

[a] 3 mg polymer in 4 ml solvent; solubility observed after 5 h.
[b] At 20 ^{o}C.

4.8 Synthesis and Characterization of GOx Functional PVA-*Pyrene* (PVA-*Py*-GOx)

Immobilization of GOx to PVA-Py was achieved according to **(4.5)**.



(4.5)

The portion of remaining hydroxyl groups after the click reaction was then reacted with GOx by using EDC as coupling agent. Initially, 1,1'-carbonyldiimidazole (CDI), a carbonylating reagent which has been shown to be suitable for the activation of free hydroxyl groups on the supports [81] was preferred to use as an activating agent prior to enzyme immobilization. However, this procedure caused an aggregation and water insoluble structure was obtained. This might be explained by the formation of the possible crosslinks with adjacent hydroxyl groups via diester bonds as previously reported [82]. Since carbodiimide (EDC) activation was practically and successfully adapted to our system, this method was preferred for the GOx immobilization.

The FT-IR and ^{1}H NMR spectra were used to confirm the structure. While the coupling reaction proceeds, the primary amines are converted to the secondary amines according as the characteristics of the reaction. The coupling process leads to one main change in the ^{1}H NMR spectra. As can be seen from Figure 4.12, in the case of PVA-Py-GOx, -N*H*$_2$ protons of bare GOx at 6.72 ppm disappeared and N-*H*

protons overlapped with the peaks appeared in the range of 4.1-3.1 ppm. The final product exhibits characteristic signals of the precursor PVA and Py.

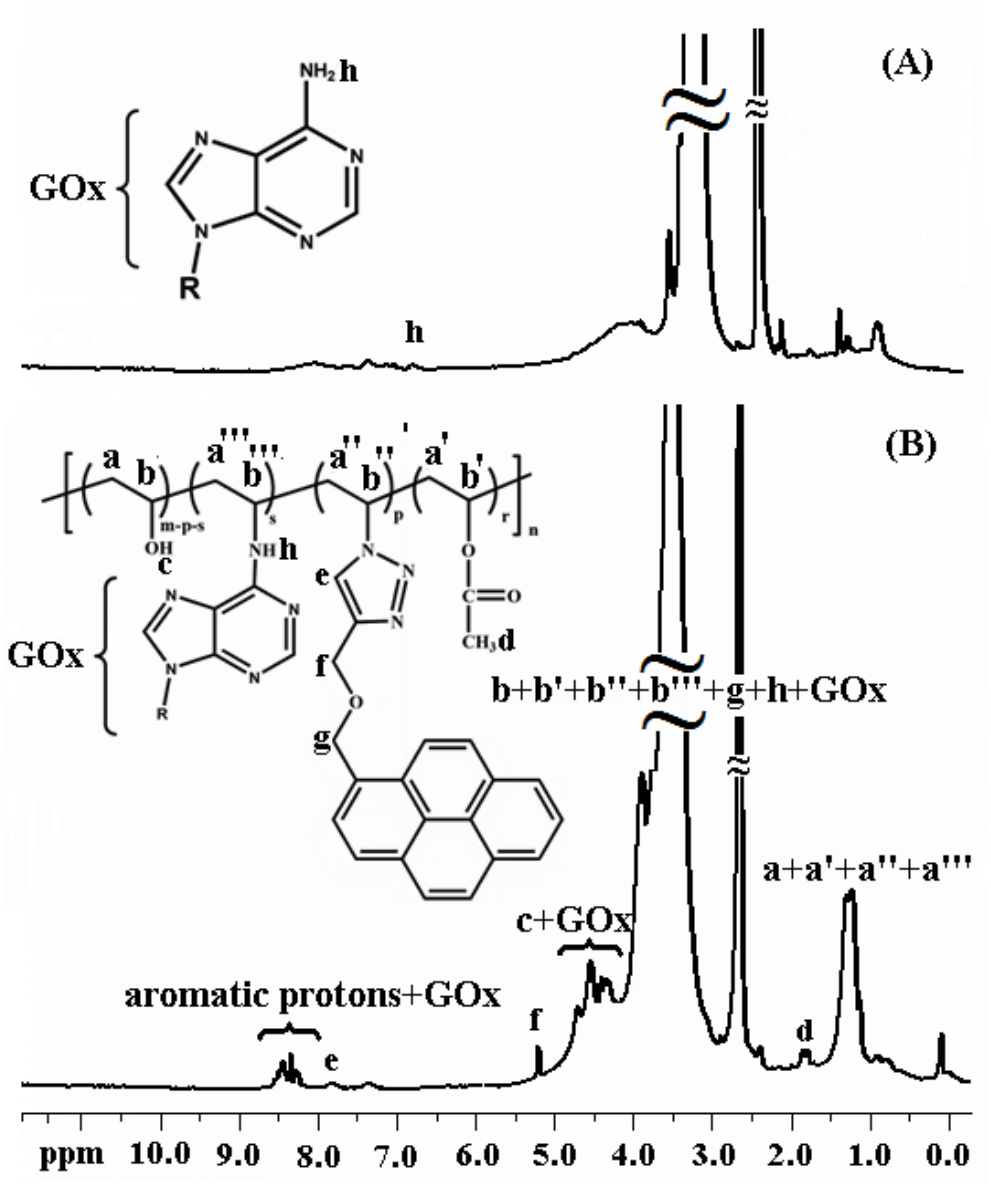


Figure 4.12 : ^{1}H-NMR (d_6-DMSO) spectra of GOx **(A)** and PVA-*Py*-GOx **(B)**.

The structure of PVA-Py-GOx was also confirmed by FT-IR analysis (Figure 4.13). The development of the band at 3168, 1323 and 1222 cm^{-1}, which were not present in the precursor polymer and enzyme, can be attributed to the secondary amine structure[41] formed as a result of the coupling reaction. Moreover the disappearance of primary amine bands [83] at 3434 and 1537 cm^{-1} also indicates successful immobilization of GOx.

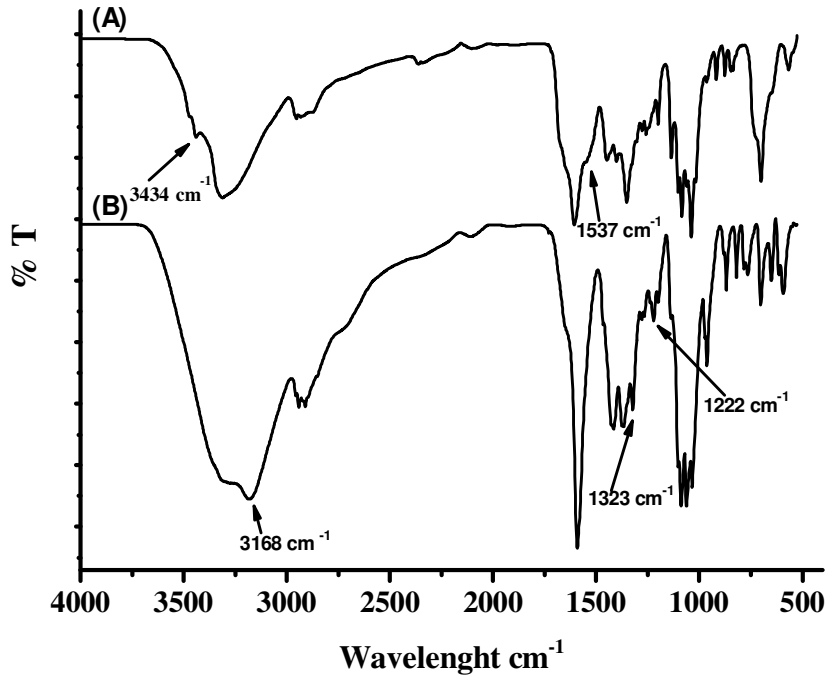


Figure 4.13 : FT-IR spectra of GOx **(A)**, PVA-Py-GOx **(B)**.

The incorporation of GOx into polymer matrix was further evidenced by surface morphology analysis. Atomic force microscopy (AFM) is a powerful means to observe microscopic polymer surface morphology. Typical AFM images of PVA-Py before and after modification with GOx in non-contact mode were shown in Figure 4.14. PVA-Py matrix exhibited a homogenous surface structure with an average size of 67 nm (Figure 4.14A). After biomolecule immobilization, the change in morphology of the polymeric composition was clearly seen in Figure 4.14B. In this case, the height of matrix was found to be 312 nm due to the presence of GOx in the structure.

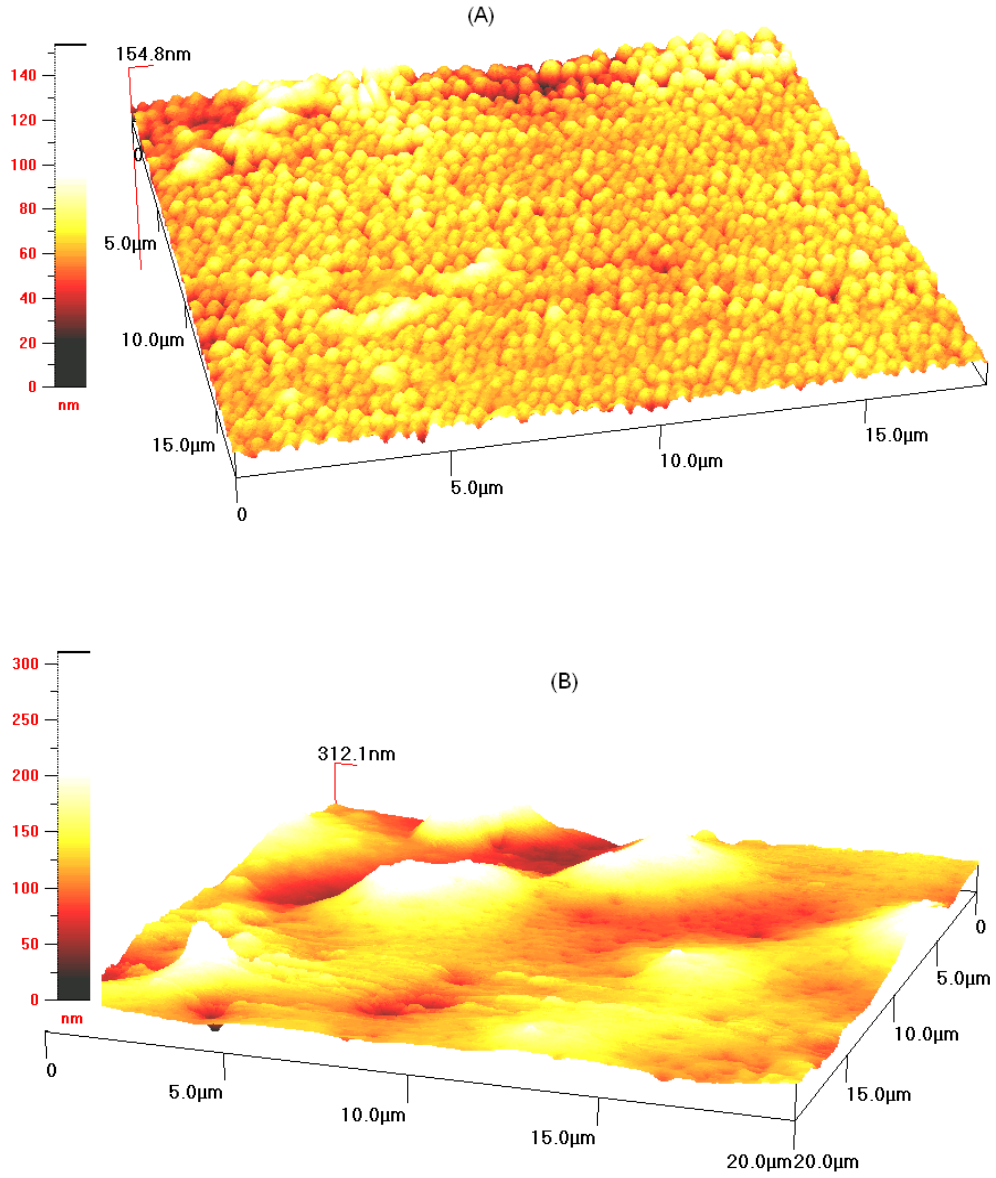
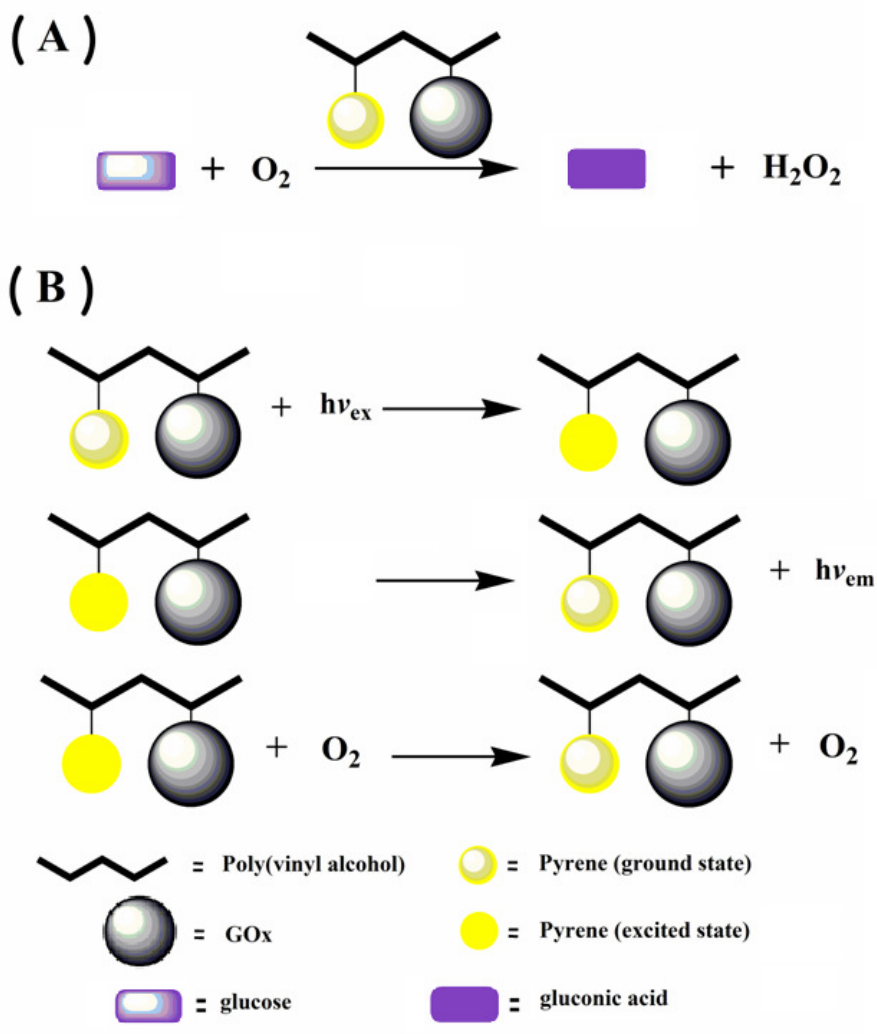


Figure 4.14 : Three dimensional AFM height images of the surfaces of PVA-Py **(A)** and PVA-Py-GOx **(B)**.

In this biosensing approach, glucose is oxidized by the enzyme bound to the Py labeled matrix, and oxygen is simultaneously consumed **(4.6 (A))**, and the change of dissolved oxygen in the medium causes a quenching fluorescent intensity **(4.6 (B))**.

Figure 4.15 shows the fluorescence emission spectra of immobilized GOx in the absence (A) or in the presence (B) of glucose (2.5 mM). An increase in fluorescence signal (about 40%) was observed when glucose was added into the reaction medium. The mechanism responsible for the enhancement of the fluorescence intensity is shown in **(4.6 (B))**.


(A)
+ O2
+ H2O2
(B)
+ hνex
+ hνem
+ O2
+ O2
= Poly(vinyl alcohol)
= Pyrene (ground state)
= GOx
= Pyrene (excited state)
= glucose
= gluconic acid

(4.6)

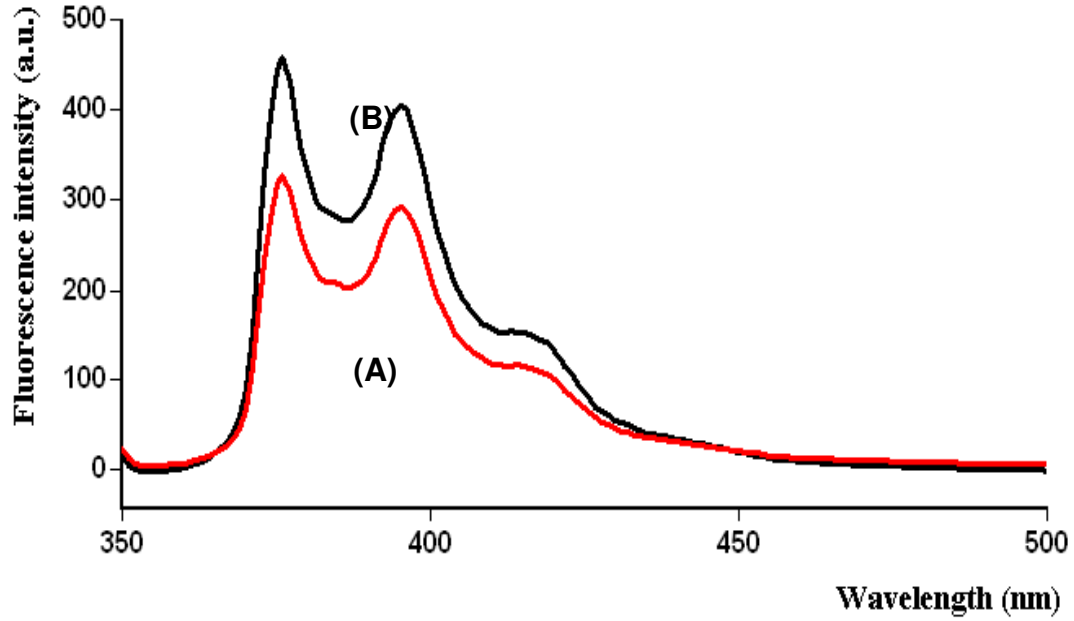


Figure 4.15 : Fluorescence spectra of PVA-Py-GOx in the absence **(A)** and presence **(B)** of glucose (1.5 mM) in potassium phosphate buffer (pH 6.0; 50 mM).

As mentioned before, protein content and GOx activity were checked according to Bradford and ABTS method after immobilization step and 89 % recovery in activity was obtained. The recovery was calculated in considering with the specific activity values which is expressed as U/mg-protein. Moreover, a blank experiment was carried out by the same immobilization procedure except activation step of the matrix to observe non-specific binding of the enzyme, and no fluorescence signal was observed in the experimental conditions. Further trials with the immobilized GOx were carried out via fluorescence intensity measurements.

The pH dependency of immobilized enzyme was investigated by monitoring the fluorescence emission signals at 390 nm by using sodium acetate (pH 4.5-5.5; 50 mM) and potassium phosphate buffers (pH 6.0-6.5; 50 mM) in the presence of glucose 3.0 mM. In order to eliminate the pH effect on the fluorescence of Py even though it was previously reported as non-sensitive to pH change and the ionic strength, the blank signals without glucose were registered initially for each pH values and then, the increments were followed after incubation with the glucose. The highest increase in the response signal was achieved at pH 6.0 (Figure 4.16) and all

experiments were conducted with this value which was similar to the free enzyme reported in previous studies [84].

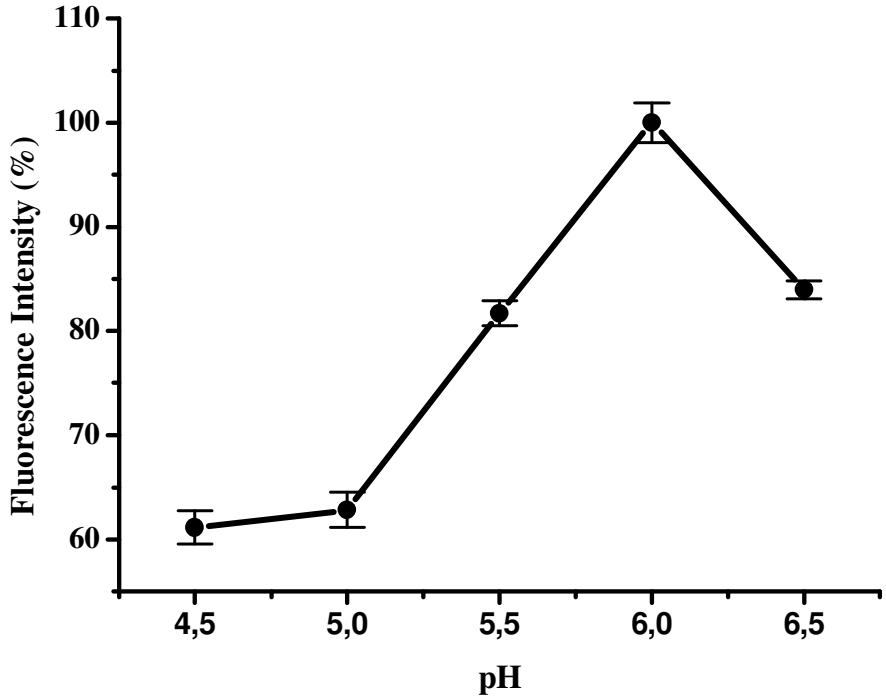


Figure 4.16 : Effect of pH in sodium acetate (pH 4.5-5.5; 50 mM) and potassium phosphate buffers (pH 6.0-6.5; 50 mM), in the presence of glucose (3.0 mM) at 25 ^{o}C. Error bars show the standard deviation.

To search the effect of the amount of PVA-Py-GOx on the fluorescence signal, varying amounts of immobilized enzyme between 2.5 and 100 µl (which equal 2.5-100 mg/ml protein contents) were used in the measurements of fluorometric responses in working buffer solution while the substrate concentration was kept constant at 3.0 mM. As can be seen in Figure 4.17, saturation was observed after 75 µl (75 mg/ml protein) and the signals remained constant.

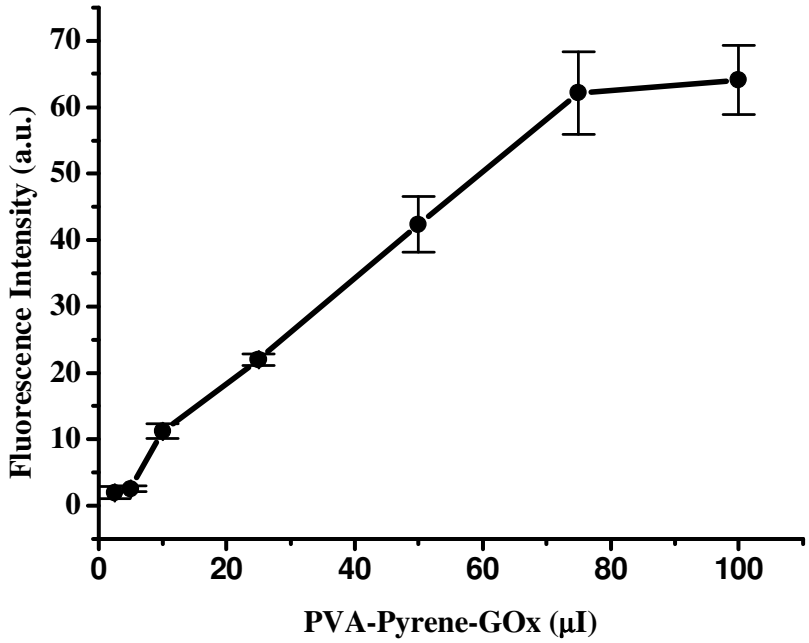


Figure 4.17 : Effect of amount of PVA-Py-GOx in potassium phosphate buffer (pH 6.0; 50 mM), in the presence of glucose (1.0 mM) at 25 ^{o}C. Error bars show the standard deviation.

It has been observed that when the PVA-Py-GOx amount was kept constant at 75 µL in the saturation level while the substrate concentration was gradually increased, the signals were found to be increased until it reached a maximum at 3.0 mM (Figure 4.18). After this point saturation was observed at 4.5 mM glucose and then, increases in substrate concentration to 6.0 mM caused a drop on the response (approximately 50 %). In order to show the advantage of using immobilized form of the enzyme on the PVA-Py in terms of glucose analysis, another calibration curve has been also plotted by using PVA-Py and free GOx (Figure 4.18). As can be seen from the Figure, lower fluorescence signals were obtained in case of free enzyme in the assay. As it is expected, higher efficiency for the elimination of fluorescence quenching by oxygen can be provided by combining all molecules into the same structure so that even small variations in the physical properties of microenvironment surrounding the enzyme due to the biocatalytic reaction could be easily monitored as a signal.

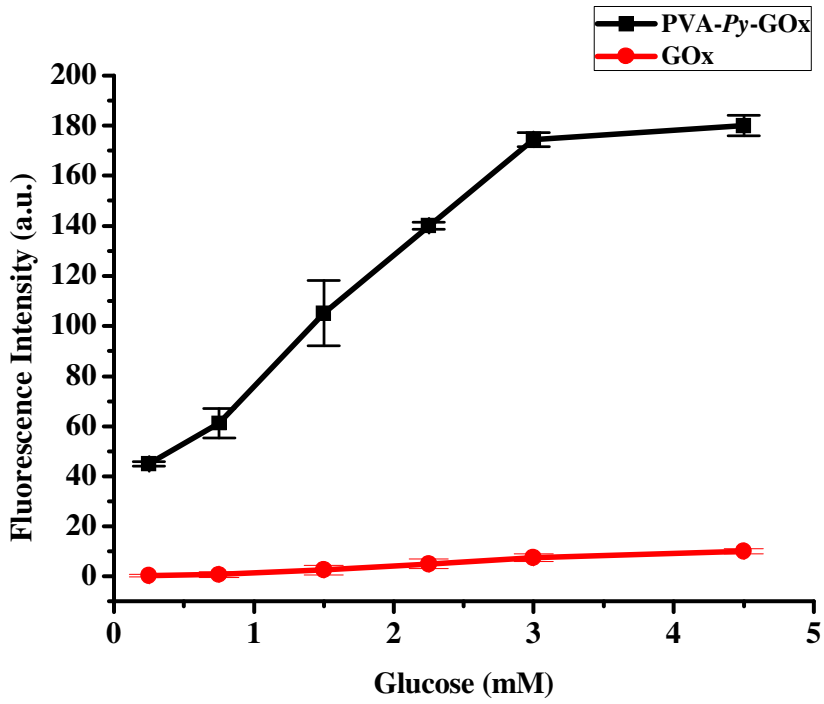


Figure 4.18 : The effect of glucose concentrations (in potassium phosphate buffer (pH 6.0; 50 mM at 25°C)). Error bars show the standard deviation.

It was reported that GOx normally does not allow for substrate inhibition except at very low oxygen levels. In that case, glucose could cause inhibition in the enzymatic catalysis [85] so that our data could be due to the oxygen limitation in the reaction medium that required for the activity of GOx as well as the possible inhibition effect of gluconic acid as described in the previous study [86]. Evaluation of these findings are important for the analytical characterization of PVA-Py-GOx preparation which would be a useful fluorescent probe model for glucose analysis in different matrices. The linear response of the immobilized GOx was observed between 0.25 and 3.0 mM glucose with equation of y = 48.38x + 30.173 (R^2 = 0.996) where x was glucose concentration in mM and y was in arbitary unit (a.u) and defined as the difference in fluorescence emission signals, in the presence and absence of substrate. Also, by applying the S/N=3 criterion, the limits of detection (LOD) was calculated as 0.19 mM. Tatsu and Yamamura designed the chemically modified GOx by using sulfonyl chloride derivatives of Py for the direct attachment to the amino groups of the enzyme and applied for the glucose detection in range of 1.1-17.4 mM. 7-hydroxycoumarin-4-acetic acid labeled GOx as well as genetically engineered proteins were previously investigated as fluorescent probes for glucose analysis [87]. It can be concluded that use of Py-labeled PVA provides a good platform for design

of a water-soluble fluorescent sensing system because of its possible applications in clinical chemistry and biotechnological processes. To evaluate the repeatability of the biosensor, five replicate measurements were carried out by using 2.5 mM of glucose solution. S.D and variation coefficient (c.v) were as follows, ±0.107 mM and 4.25 %, respectively.

In order to determine the shelf life, a freshly prepared immobilized enzyme solution was stored at 4 ^{o}C. And, the response signals were measured by using 3.0 mM glucose at different time intervals during 1 month. And no drop in the fluorescence signals was observed. It is obvious that, good stability of the sensing system could be mainly due to the excellent chemical resistance of PVA backbone [86].

The proposed system was applied to analyze glucose in real samples. For the glucose sensing by means of PVA-Py-GOx in fizzy and orange juice, samples with the appropriate dilutions were added into the reaction cell instead of substrate. Then, the signals were recorded as already described for fluorometric assay and data were calculated from the linear curve between glucose concentration and fluorescence intensities. According to the procedure glucose content in fizzy and orange juice samples were found as 7.98±0.56 g and 3.46±1.13 g per 100 ml. Additionally, the commercial kits based on spectrophotometric method was used as the reference method and the amounts were calculated as 8.18±0.61 g and 3.26±0.997 g per 100 ml. According to data, the recovery values for samples were calculated as 98 % and 106 %, respectively. It can be said that these values were very closer to 100% which means that the matrix of the sample didn't affect the measurements.

4. REFERENCES

[1] **Staiano, M., Bazzicalupo, P., Rossi, M. and D'Auria, S.,** 2005: *Molecular Biosystems,* **1**, 354–362.

[2] **McDonagh, C., Burke, C. S. and MacCraith, B. D.,** 2008: *Chemical Reviews,* **108**, 400-422.

[3] **Borisov, S. M. and Wolfbeis, O. S.,** 2008: *Chemical Reviews* **108**, 423-461.

[4] **Gac, S., Vermes, I. and Berg, A.,** 2006: *Nano Letters*, **6**, 1863–1869.

[5] **Higuchi, R., Fockler, C., Dollinger, F. and Watson, R.,** 1993: *Biotechnology,* **11**, 1026–1030.

[6] **Xu, M. H., Lin, J., Hu Q. S. and Pu, L.,** 2002: *Journal of the American Chemical Society,* **124**, 14239–14246.

[7] **Xu H., Aylott J. W. and Kopelman R.,** 2002: *The Analyst* **127**, 1471-1477.

[8] **Moreno-Bondi, M. C., Wolfbeis, O. S., Leiner, M. J. P. and Schaffa, B. P. H.,** 1990: *Analitical Chemistry*, ***62***, 2377–2380.

[9] **Anders, K. D., Wehnert, G., Thordsen, O., Scheper, T., Rehr B. and Sahm B.,** 1993: *Sensors and Actuators B-Chemical,* **11**, 395-403.

[10] **Tatsu, Y. and Yamamura, S.,** 2002: *Journal of Molecular Catalysis B-Enzymatic,* **17**, 203-206.

[11] **Muscatello, M. M. W., Stunja, L. E. and Asher, S. A.,** 2009: *Analitical Chemistry,* **81**, 4978–4986.

[12] **Zhang, T. and Anslyn, E. V.,** 2007: *Organic Letters*, **9**, 1627–1629.

[13] **Lepore, M., Portaccio, M., De Tommasi, E., De Luca, P., Bencivenga, U., Maiuri, P. and Mita, D. G.,** 2004: *Journal of Molecular Catalysis B-Enzymatic,* **31**, 151-158.

[14] **Sahmetlioglu, E., Yuruk, H., Toppare, L., Cianga, I. and Yagci, Y.,** 2006: *Reactive and Functional Polymers*, **66**, 365-371.

[15] **Yildiz, H. B., Kiralp, S., Toppare, L. and Yagci, Y.,** 2005: *International Journal of Biological Macromolecules,* **37**, 174-178.

[16] **Ozturk, G., Timur, S. and Alp, S.,** 2008: *Analitical Letters,* **41**, 608-618.

[17] **Corres J. M., Sanz A., Arregui F. J., Matías I. R. and Roca J.,** 2008: *Sensors and Actuators B-Chemical,* **131**, 633–639.

[18] **Wang X. D., Zhou T. Y., Chen X., Wong K. Y. and Wang X. R.,** 2008: *Sensors and Actuators B-Chemical,* **129**, 866–873.

[19] **Trettnak, W. and Wolfbeis, O.S.,** 1989: *Analytica Chimica Acta*, **221**, 195–203.

[20] Bastiaens, P. I. H. and Visser A. J. W. G., 1993: In *Fluoresence Spectroscopy: New Methods and Applications*, Wolfbeis, O. S. Ed., Springer-Verlag, Heidelberg.

[21] Ghisla, S., Massey, V., Lhoste, J. M. and Mayhew, S. G., 1974 *Biochemistry*, **13**, 589-597.

[22] Wolfbeis, O. S., Posch, H. E. and Kroneis, H., 1985: *Analitical Chemistry,* **57**, 2556-2561.

[23] Swoboda, B. E. P., 1969: *Biochimica et Biophysica Acta,* **239**, 365-379.

[24] Haouz, A., Twist, C., Zentz, C., Kersabiec, A. M., Pin, S. and Alpert, B., 1998: *Chemical Physics Letter,* **294**, 197-203.

[25] Prado, E. A., Yamaki, S. B., Atvars, T. D. Z., Zimerman, O. E. and Weiss, R. G., 2000: *Journal of Physical Chemistry B,* **104**, 5905–5914.

[26] Kim, S. K., Lee, S. H., Lee, J. Y., Lee, J. Y., Bartsch, R.A. and Kim, J. S., 2004: *Journal of American Chemical Society,* **126**, 1649–1650.

[27] Winnik M. A., Redpath A. E. C., Paton K. and Danhelka J., 1984: *Polymer,* **25**, 91-99.

[28] Slomkowski S., Winnik M. A., Furlong P. and Reynolds W. F., 1989: *Macromolecules,* **22**, 503-509.

[29] Strukelj M., Martinho J. M. G., Winnik M. A. and Quirk R. P., 1991: *Macromolecules*, **24**, 2488-2492.

[30] Hepuzer Y., Guvener U. and Yagci Y., 2004: *Polymer Bulletin,* **52**, 17-23.

[31] Kom M. R. and Gagne M. R., 2000: *Chemical Communication,* **18**, 1711-1712.

[32] Erdogan M., Hepuzer Y., Cianga I., Yagci Y. and Pekcan 0., 2003: *Journal of Physical Chemistry A,* **107**, 8363-8370.

[33] Scherman O. A., Kim H. M. and Grubbs R. H., 2002: *Macromolecules,* **35**, 5366-5371.

[34] Gacal B. N., Koz B., Gacal B., Kiskan, B., Erdogan M. and Yagci Y., 2009: *Journal of Polymer Science Polmer Chemistry Edition,* **47**, 1317-1326.

[35] Saçak, M., 2004: Polimer Kimyası, 2nd Edition, Gazi Kitabevi, Ankara.

[36] Austin, T.G., 1984: Shreve's Chemical Process Industries, 5th Edition, Graw-Hill Book Company, New York.

[37] Novak, B. N. and Cederstav, A. K., 1994: *Journal of American Chemical Society,* **116**, 4073.

[38] Ger. Pat. 450,286 (1924), W. Haehnel and W. O. Herrmann (to Consortium f ¨ ur Elecktrochemie, GmbH).

[39] Hermann, W. O. and Haehnel, W., 1927: *Berichte der Deutschen Chemischen Gesellschaft,* **60**, 1658.

[40] Staudinger, H., Frey, K. and Stark, W., 1927: *Berichte der Deutschen Chemischen Gesellschaft,* 60, 1782.

[41] **Sakurada, I., Sakaguchi, Y. and Ito, Y.,** 1957: *Kobunshi Kagaku,* **14**, 141.

[42] **Timasheff, S. N.,** 1951: *Journal of American Chemical Society,* **73**, 289.

[43] **Satake, K.,** 1955: *Kobunshi Kagaku,* **12**, 122.

[44] *Airvol Poly(vinyl alcohol) Manual*, Air Product and Chemicals, Inc., Allentown, Pa.,1990.

[45] **Tubbs, R. K., Inskip, H. K. and Subramanian, P. M.,** 1968: *Properties and Applications of Polyvinyl Alcohol* Monograph No. 30, Society of Chemical Industry, London, p. 88.

[46] **Tubbs, R. K.,** 1965: *Journal of Polymer Science*, Part A, **3**, 4181.

[47] **Kikukawa, K., Nozakura, S. and Murahashi, S.,** 1968: *Kobunshi Kagaku,* **25**, 19.

[48] **Lorand, J. P. and Edwards, J. O.,** 1959: *Journal of Organic Chemistry,* **24**, 769.

[49] **Noma, S.,** 1955: in *First Osaka Symposium, Kobunshi Gakkai*, Tokyo, pp. 81–103.

[50] **Finch, C. A.,** Ref. 39, pp. 183–202.

[51] **Stober, R., Kohn, E. and Bischoff D.,** 1989: U.S. Pat. 4,822,851 (Apr. 18), (to DegussaAktiengesellschaft).

[52] **Axelrod R. J. and Phillips, J. H.,** 1991: *Plastic Waste Management Workshop*, New Orleans,La., Dec. 9.

[53] **Fukae R. and co-workers,** 1994: *Polymer Journal,* **26**, 1381.

[54] **Matsumura, S., Shimura, Y. and Toshima, K.,** 1995: *Macromolecular Chemistry and Physics,* **196**, 3437.

[55] **Inskip, H. K. and Adelman, R. L.,** 1972: U.S. Pat. 3,689,469 (Sept. 5), (to E. I. du Pont de Nemours & Co., Inc.).

[56] **Marten, F. L.,** 1986: U.S. Pat. 4,618,648 (Oct. 22, 1986), (to Air Products & Chemicals, Inc.).

[57] **Sakurada, I. and Sakaguchi Y.,** 1956: *Kobunshi Kagaku,* 13, 441.

[58] **Germain, L. M.,** 1953: U.S. Pat. 2,643,994 (June 30), (to Shawinigan Chemicals Ltd.).

[59] Anon. 2007. http://www.organic-chemistry.org/namedreactions/huisgen-1,3-dipolar-cycloaddition.shtm

[60] **Binder, H. W. and Sachsenhofer, R.,** 2007: 'Click' Chemistry in Polymer and Materials Science, *Macromolecular Rapid Communication*, **28**, 15–54.

[61] **Lutz, J. F.,** 2007: 1,3-Dipolar Cycloadditions of Azides and Alkynes: A Universal Ligation Tool in Polymer and Materials Science, *Angewante Chemie International Edition,* **46**, 1018 – 1025.

[62] **Kolb, H. C., Finn, M. G. and Sharpless, K. B.,** 2001: *Angewante Chemie,* **113**, 2056 – 2075.

[63] Kolb, H. C., Finn, M. G. and Sharpless, K. B., 2001: *Angewante Chemie International Edition,* **40**, 2004-2021.

[64] Huisgen, R., Szeimies, G. and Möbius, L., 1967: *Chemische Berichte*, **100**, 2494; for a general review see Gothelf, K. V. and Jo¨rgensen, K. A., 1998: *Chemical Reviews,* **98**, 863.

[65] Huisgen, R., 1963: *Angewante Chemie*, **75**, 604 – 637; 1963: *Angewante Chemie International Edition English,* **2**, 565 – 598.

[66] Huisgen, R., 1963: *Angewante Chemie*, **75**, 742 – 754; 1963: *Angewante Chemie International Edition English*, **2**, 633 – 645.

[67] Rostovtsev, V. V., Green, L. G., Fokin, V. V. and Sharpless, K. B., 2002: *Angewante Chemie*, **114**, 2708 – 2711; 2002: *Angewante Chemie International Edition,* **41**, 2596 – 2599.

[68] Tornoe, C. W., Christensen, C. and Meldal, M., 2002: *Journal of Organic Chemistry,* **67**, 3057 – 3064.

[69] Bock, V. D., Hiemstra, H. and van Maarseveen, J. H., 2006: European Journal of Organic Chemistry, 51 – 68.

[70] Rostovtsev, V. V., Green, L. G., Fokin, V. and Sharpless, K. B., 2002: *Angewante Chemie International Edition,* **41**, 2596.

[71] Hawker, C. J. and Wooley, K. L., 2005: *Science*, **309**, 1200 – 1205.

[72] Binder, W. H. and Kluger, C., 2006: *Current Organic Chemistry,* **10**, 1791–1815.

[73] Wang, Q., Chittaboina, S. and Barnhill, H. N., 2005: *Letters in Organic Chemisry*, **2**, 293.

[74] Köhn, M. and Breinbauer, R., 2004: *Angewante Chemie*, **116**, 3168.

[75] Ankush, B., Nicholas, A. and Peppas, A., 1998: Journal of Applied Polymer Science, **70**, 817–829.

[76] Chiou, S. H. and Wu, W. T., 2004: *Biomaterials*, **25**, 197-204.

[77] Leitner, C., Volc, J. and Haltrich, D., 2001: *Applied and Environmental Microbiology,* **67**, 3636–3644.

[78] Bradford, M. M., 1976: *Analitical Biochemistry,* **72**, 248-254.

[79] Trinder, P., 1969: *Annals of Clinical Biochemistry,* **6**, 24-27.

[80] Wang, B., Guan, X., Hu, Y. and Su, Z., 2007: *Polymers for Advanced Technologies,* **18**, 529-534.

[81] Hearn, M. T. W., 1987: In *Methods in Enzymology*, *1,1'-Carbonyldiimidazole Mediated Immobilization of Enzymes and Affinity Ligands*; Moshbach, K., Ed., Academic Pres: New York, **135,** pp 102–117.

[82] Salamone, J. C. and Rochester, B. L., 1996: In *Polymeric Materials Encyclopedia,* Salamone, J. C. Ed., CRC Press: *New York*, **3**, pp 2126.

[83] Pretsch, E., Bühlmann, P. and Affolter, C., 2000: In *Structure Determination of Organic Compounds;* Springer: Heidelberg New York, pp 268-271.

[84] Liu, J.-Z., Yang, H. Y., Weng, L.-P. and Ji, L. N., 1999: *Letters in Applied Microbialogy,* **29**, 337-341.

[85] Nicol, M. J. and Duke, F. R., 1966: *Journal of Biological Chemistry,* **241**, 4292-4293.

[86] Mirón, J., González, M. P., Vázquez, J. A., Pastrana, L. and Morado, M. A., 2004: *Enzyme and Microbial Technology,* **34**, 513-522.

[87] Sierra, J. F., Galban, J., Marcos, S. and Castillo, J. R., 1998: *Analytica Chimica Acta,* **368**, 97-104.

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