Preparation of polymer microspheres and their use in enzyme immobilization

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INTRODUCTION

Polymer microspheres show great potential in many applications of chemical and life sciences. These applications incule ion exchange, chromatography, controlled release, enzyme immobilization, biomedical diagnostics, coatings, paints etc (Arshady, 1999). Polymer microspheres are prepared either by in situ polymerization of respective monomer(s) or by physicochemical or mechanical processes from preformed polymers. Preparation of polymer microspheres by the former process includes techniques such as suspension, emulsion, dispersion or interfacial polymerization. The latter processes with performed polymers include spray drying, solvent evaporation and phase separation methods. Many applications as mentioned above, require polymer particles to be in a monodisperse spherical form in the nano to micron size range.

We have shown that polyurethane (PU) microspheres can be prepared by a novel non-aqueous method involving in-situ polymerization of diisocyanate with a diol (Shukla and Sivaram, 1998, 1999a). By employing suitable steric polymeric stabilizers PU spherical microspheres are prepared with uniform particle size in the range of 0.1 to 100 microns. The method developed was extended to prepare PU microcapsules containing soluble active agents (Shukla et al 1999b).

Enzymes which are biocatalysts have applications in various chemical reactions. Immobilized enzymes have importance in the industrial applications because of their advantages such as better process control, enhanced stability, enzyme-free products and can be reused several times and thus are cost effective. Polymer microspheres are found to be good substrate for enzyme immobilization. Surface property of polymer microspheres should be such that enzyme immobilization efficiency will be more.

In collaboration with scientists from material science and biochemical science groups from our laboratory, we demonstrated the direct assembly of colloidal gold particles on PU microspheres by interaction of polymer nitrogens with the gold particles (Ashwani et al. , 2003). This precludes the need for modifying the polymer microspheres to enable such nanoparticle binding. The gold nanoparticles shell - PU core (Au-PU) structures are found to be useful for enzyme immobilization. The polyurethane- nano-gold (PU-Au) core-shell material is then conjugated with the enzyme pepsin, leading to the formation of new class of biocatalyst. As compared to the free enzyme in solution , the new bioconjugate material exhibited significant enchanced pH and temperature stability. Similar results were obtained with enzyme endoglucanase (Phadtare Sumant et al. , 2004). The high surface area of the host gold nanoparticles renders the immobilized enzyme ‘quasi free’, while at the same time retains the advantages like easy separation from the reaction medium and reuse of the enzyme immobilized PU-Au core-shell particles over more than 5-6 reaction cycles.

In the present paper we describe variety of polymer microspheres prepared by different techniques and their use for immobilization of enzymes namely invertase and pepsin.

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MATERIALS AND METHODS

Enzymes: Invertase concentrate and pepsin were procured from BDH, UK and Sigma chemicals respectively. Enzyme immobilization efficiency, pH and temperature stability were carried out by standard enzyme assay procedures.

Polymer microspheres:
1) Poly (methyl methacyrlate) (PMMA) microspheres (PMMA microspheres) were prepared by solvent evaporation technique as follows. PMMA was dissolved in low boiling solvent (methylene chloride) to form an organic phase. This organic phase was then dispersed in aqueous phase containing suitable surfactant to form oil in water emulsion. Solvent was then evaporated by stirring the emulsion for 4 hours at 27^{o}C to form polymer microspheres which were then isolated by filtration or centrifugation, washed with water and dried in vacuum oven.
2) Polyurethane microspheres were prepared in non-aqueous medium by a novel in-situ polymerization method patented by us. Glycol (e.g. ethylene glycol) was dispersed in a parafiin oil containing suitable polymeric steric stabilizer and catalyst while stirring the mixture at 1000 rpm. Diisocyanate (toluene diisocyanate TDI) was then added drop-wise and then mixture was stirred at ~45 ^{o}C for 4-5 hours and then at room temperature for 20-22 hours. Obtained microspheres were isolated by filtration, washing with hexane and drying in vacuum oven.
3) Urea-formaldehyde (UF) microspheres were prepared by in-situ polymerization as follows. First UF prepolymer was prepared by refluxing urea and formalin with F/U molar ratio of 2.0. This prepolymer was then dispersed in aqueous phase containing suitable surfactant. Temperature of reaction mixture was then slowly raised to 70 ^{o}C and pH was lowered to ~3. After stirring at 1000 rpm for 3-4 hours, reaction mixture was further stirred for ~20 hours at 500 rpm at 25 ^{o}C. Reaction mixture was then neutralized and obtained microspheres were filtered, washed with water and dried in vacuum oven.
4) UF microspheres with methylol groups (UFM) on surface of microspheres were prepared with F/U molar ratio of 1.6. Here initial temperature of reaction mixture was kept at 40 ^{o}C for 2 hours , pH was then lowered to ~3 and reaction mixtures was further stirred for 2 hours at the same temperature. The speed was then reduced to 500 rpm and stirring was continued for 15-17 hours at room temperature. UFM microspheres were isolated as mentioned above.

Enzyme immobilization on polymer microspheres
1) UF, PMMA and PU microspheres: Polymer microspheres (500 mg) were soaked in 1.5 mL of enzyme (invertase) solution made in 50 mM acetate buffer (pH 4.5) containing 35000 units of enzyme per mL. The whole setup was kept overnight at 10 ^{o}C with constant slow stirring. Polymer microspheres immobilized with enzyme were then thoroughly washed 2-3 times with distilled water to remove all unbound enzyme.
2) UFM microspheres: UFM-pespsin bioconjugate was prepared as described earlier. UFM microspheres (200 mg) were dispersed in 3 mL of KCl-HCl buffer (pH 2) containing 2.5 mg of pepsin. The setup was kept under slow stirring for 10-12 hours at room temperature. UFM-pepsin biocnjugate was isolated by centrifugation and by washing several times with KCl-HCl buffer.

RESULTS AND DISCUSSION

1) Polymer microspheres immobilized with enzyme- invertase: Different polymer microspheres, their method of preparation and size and enzyme immobilization efficiency are given in Table 1. Highest enzyme immobilization efficiency was obtained with PU microspheres followed by UF microspheres. PMMA showed lowest enzyme immobilization efficiency. PU and UF have secondary or tertiary amino groups in the polymer structure which are good binding sites for the

emzyme. PU showed maximum efficiency than UF. PU has mostly secondary amino groups (-NH-COO- , urethane linkage) while UF with F/U ratio of 2.0 has more number of tertiary amino groups than secondary amino groups. This was shown by ^{13}C NMR studies (Bhaskar et al.,1994). Secondary amino groups seem to have better binding capacity for the enzyme through C-terminal. UF microspheres showed lowering of activity from 100 to 41 % after 4 reuse cycles whereas PU microspheres could maintain 73% activity after 7 recycles. This again showed that enzyme has better binding capacity for PU microspheres (Table 2) Table 3 shows temperature stability of PU microspheres. Increase in percent efficiency of bioconjugate as compared to free enzyme increases with temperature. At lower temperature 30 -50 ^{o}C there is marginal increase of 5-12 % but at 90^{o}C there is 70% increase in percent efficiency of polymer enzyme bioconjugate as compared to free enzyme.

Polymer microspheres	Method of preparation	Size range (microns)	Immobilization efficiency (%)
PMMA	Solvent evaporation	10-20	71
UF	In-situ polymerization	2-5	75
PU	In-situ polymerization	2-10	84

Table 1: Preparation and properties of polymer microspheres and their immobilization efficiency for Invertase

Reuse cycle →	I	II	III	IV	V	VI	VII	VIII
UF microspheres	100	56	46	41	-	-	-	-
PU microspheres	100	80	75	74	73	74	73	-
UFM	93	92	84	78	63	59	53	48

Table 2: Reusability of Invertase immobilized on UF and PU microspheres and pepsin on UFM microspheres

Temp ^{o}C →	30	40	50	60	70	80	90
% increase in efficiency	5	10	12	45	50	60	70

Table 3: Increase in % efficiency of Invertase immobilized on PU microspheres as compared to free enzyme at different temperatures

2) UFM microspheres: As UF microspheres as described above showed less immobilization efficiency and reusability as compared to PU microspheres, we thought of preparing UF microspheres with methylol groups on the surface (UFM). It has been shown that N-hydroxymethylol groups can undergo condensation reaction with amino acid residues of enzyme (Fraenkel-Conrat and Olcott, 1948). We explored this property for better enzyme immobilization on UF microspheres with hydroxymethylol groups on the surface (Viond, 2006). Pepsin –UFM bioconjugate when evaluated for reusability (Table 2) a small, monotonic decrease in the biocatalytic activity of the enzyme with reuse was noted. After eight cycles of reuse, activity decreases to 48% of the starting activity. Temperature and pH stability studies are shown in Fig 1. As compared to free enzyme (solid circles), UFM-enzyme bioconjugate (solid triangles) exhibited enhanced stability towards high pH (6 and 8) and higher temperature (60^{o}C).

CONCLUSION

Polymer microspheres have great potential application in the area of enzyme immobilization. PMMA microspheres which do not have suitable functional groups for enzyme immobilization showed less immobilization efficiency as compared to UF and PU microspheres. PU microspheres showed higher immobilization efficiency than UF microspheres which can be attributed to presence of more number of secondary amino groups in PU than UF. UFM microspheres with methylol groups showed good binding capacity to enzyme. PU and UFM microspheres showed significant reuse utility and good pH and temperature stability.

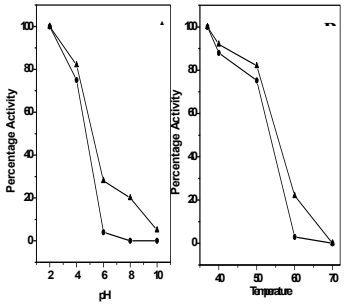

Fig 1: pH and temperature stability of UFM-pepsin conjugates

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