Magnetic Poly-(Acrylonitrile-Co-Acrylamide) Microparticles for Immobilization of Trypsin

T. Ivanov¹, V. Ivanova², M. Kamburov¹

Abstract – Well-shaped spherical magnetic microspheres were prepared using a synthetic polymer of poly-(acrylonitrile-co-acrylamide). Their size varied from 100 up to 1000 µm proportionally to the flow rate of the polymer solution and their density was from 1030 to 1045 kg.m⁻³, depending on the magnetite content. Trypsin (EC 3.4.21.4) was chosen as model enzyme and immobilized on the acyl azide derivatives of microparticles with diameter approx. 500 µm. The impact of various parameters such as pH, initial protein/support ratio, magnetite content on the immobilization efficiency was investigated. The maximum amount of fixed protein was about 14.4 mg.g⁻¹ dry support. The relative activity of immobilized trypsin represented about 90% of the corresponding activity of free enzyme. Optimal pH for enzyme reaction and Km’ for substrate BAPNA remained unchanged after the immobilization. Copyright © 2009 Praise Worthy Prize S.r.l. - All rights reserved.

Keywords: Immobilized Trypsin, Magnetic Beads, Synthetic Polymer

I. Introduction

Magnetic particles exhibit unique physical and chemical properties, and hence have received much attention from scientists and researchers in different areas of biological sciences [1]. Although often referred to as magnetic, many of the particles currently used, are superparamagnetic, meaning that these particles can be easily magnetized with an external magnetic field and redispersed immediately once the magnet is removed. The magnetic carriers have found many applications in various areas of bioscience and biotechnology: in affinity chromatography, in human, animal and plant cell separation/concentration from water samples [2]; in protein separations and in assay procedures [3]; for biosorption and solid-phase extraction of water-soluble dyes and heavy metal removal [4]-[5]; for microbial and plant cell immobilization [6]-[7]; for detection of pathogenic microbial strains by magnetic-beads based magnetic biosensors [8]. They are also used in molecular biology for separation of specific and unspecific nucleic acid sequences and purification of plasmid DNA [9]. Magnetic microspheres have been used for entrapment and target delivery of drugs, chemotherapeutic agents (radionuclides) [10]. Biomagnetic techniques are mostly used for the immunomagnetic separations of target molecules and cells [10], in magnetic resonance imaging contrast agents, in magnetic fluid hyperthermia [11] and cancer treatment [12].

Enzymes, antibodies, receptors, avidin and streptavidin, lectins, enzyme inhibitors, RNA, DNA have been immobilized on magnetic carriers and are available commercially [3]-[13], [14].

Magnetite particles have been used as support material for binding of enzymes including yeast alcohol dehydrogenase and lipase [15] directly via carbodiimide activation. Recently, γ-Fe2O3 magnetic nanoparticles were used for binding lipase [16] after acetylation of thiophene functionalized nanoparticles, or through nitroso-derivative formed on the surface of the particles by reacting nitroso tetrafluoroborate in methylene chloride.

Glucose oxidase has been used to test various types of immobilization [17]. More recent studies examined the activity of cholesterol oxidase immobilized on magnetic particles using carbodiimide activation [18] and the stabilization of α-chymotrypsin by covalent immobilization on amino-functionalized super-paramagnetic nanogel [19].

Magnetic porous or non-porous beads can be manufactured combining inorganic materials and a number of natural or synthetic polymers [20] with some further surface derivatization to affect specific surface reactivity.

High mechanical strength, thermal stability, and solvent resistance are commonly desired features of magnetic beads and particles. Composite beads with magnetite as active component in a polymer matrix have been proposed [21].

Chitosan and synthetic polymers such as polystyrene, polymethyl methacrylate (PMMA), poly(vinyl butyral), poly(ethylene glycol), polyvinyl alcohol [20]-[22]-[23] have attracted much attention since they have a variety of functional groups and can be produced easily in wide variety of compositions.
The surface of magnetic particles could be modified with appropriate functional groups (e.g., hydroxy-, carboxy-, amino- or sulfhydryl-) and activated (e.g., carbodiimide, cyanogen bromide or tosyl activation) for the subsequent immobilization.

In bioprocesses magnetic bio-carriers are commonly organized in fixed or fluidized beds [13]-[14]-[24]. The former technique guarantees particle immobility, low stresses imposed on the beads, but a relatively low upper velocity limit imposed by the minimum fluidization where the particle mixing begins.

The fluidized bed ensures good mixing within a velocity range beyond the minimum fluidization velocity of the beads, but the immobilized cells or enzymes are subjected to intensive stresses caused by particle collisions and attrition.

An alternative approach combining the best features of both techniques is the magnetic stabilization [6]-[25]. The basic requirement of this fluidization technique is that particles should be magnetic.

An external magnetic field controls the particle mobility and beyond the minimum fluidization point in absence of a field, the bed expands without particle mixing motions. When the field lines are parallel to the fluid flow, the particle bed expands but the pressure drop across it remains constant [14]. Magnetically stabilized fluidized beds (MSFB) using magnetic carriers combine some of the best characteristics of fluidized beds (low pressure drop and high feed stream solid tolerances) and fixed beds (absence of particle mixing, high mass transfer rates, and good fluid-solid contact) [25].

This investigation was undertaken to study the formation of magnetic microbeads containing magnetite and a copolymer of acrylonitrile with acrylamide (PAN), and the possibility for enzyme immobilization by covalent coupling. We report on polymer synthesis, on immobilization conditions and on some properties of the fixed enzyme.

Trypsin was used as a model enzyme to measure the immobilizing character of the magnetic material. Immobilized proteolytic enzymes are of considerable interest because of their potential applications for studies in proteomics and on-line capillary electrophoresis peptide mapping of proteins [26]-[27]. Identification of proteins via peptide mapping is generally accomplished through proteolytic digestion with enzymes such as trypsin.

The use of immobilized trypsin for cleavage of proteins is advantageous in comparison with application of its soluble form. Enzymes can be immobilized on different supports and used in flow systems such as immobilized enzyme reactors (IMERs). Tryptic peptide mapping of human hemoglobin was performed by immobilized trypsin [28]. Immobilized trypsin can be used in bioaffinity chromatography for specific separation, isolation, purification and analytic characterization of trypsin inhibitors, i.e. processes that could be tested with magnetically stabilized beds.

II. Experimental

II.1. Synthesis of Poly-(Acrylonitrile-Co-Acrylamide) and Preparation of Bead

The polymerization ingredients (0.015 dm$^3$ of acrylonitrile, Mw 53.06; 0.006 dm$^3$ 50% acrylamide solution, Mw 71.08) were added to 0.174 dm$^3$ of water. The copolymerization via free radical mechanism was initiated by a redox system (0.010 dm$^3$ 1% (NH$_4$)$_2$SO$_4$, 0.010 dm$^3$ FeSO$_4$ solution (10 mg FeSO$_4$.3H$_2$O + 0.002 dm$^3$ conc. H$_2$SO$_4$ + 0.098 dm$^3$ H$_2$O); 0.010 dm$^3$ 1% Na$_2$S$_2$O$_5$) and was carried out at room temperature and mixing for 2 hours. Then the suspension was filtered, washed with large volumes of water and methanol, and dried in a vacuum oven at 50°C to a constant weight. Ten grams of dry polymer were after that dissolved in 0.100 dm$^3$ of dimethylformamide (DMF) and then the magnetic microbeads containing magnetite (as powder) were added. The ingredients were well-mixed, heated to 50-55°C and beads were formed using the device of Lochmuller [29]. The nozzle diameter was 0.5 mm. The regeneration bath, with a volume of 0.500 dm$^3$, contained water to ethanol (4:1) solution and 0.0005 dm$^3$ of surfactant Brij. The particles formed were filtered, rinsed with water and stabilized by heating to 70-75°C in water upon a vigorous mixing (500 rpm.min$^{-1}$) for 30 min. Finally, the particles were filtered and sieved by standard sieves. The wet particles were stored at 4°C for further use. Samples were dried in a vacuum oven at 40°C to constant weight for determination of dry weight.

II.2. Support Derivatization

Beads with size 500 µm were selected for enzyme immobilization. The first step was modification of amide groups from the matrix to hydrazide derivatives using the azide method [30]. Three grams dry beads (15 g, w.w$^{-1}$) were added gradually with stirring to 0.020 dm$^3$ aqueous solution of hydrazine (6M). The suspension was stirred slowly at room temperature. The reaction was stopped after 12 h by washing with large volumes of 0.2 M NaCl. The hydrazide derivatives were stored at 4°C. The second step was preparation of acyl azides. The beads were treated with 0.050 dm$^3$ chilled 0.5 N HCl and 0.050 dm$^3$ 0.5 N NaNO$_2$ by stirring (at about 0°C) for 20 min, followed by filtration and washing step with an abundant volume of cold 0.1 N HCl (4.0 dm$^3$). Dry weight of the derivatized support was determined. The obtained acyl azide is not stable and was used immediately for enzyme immobilization.

II.3. Enzyme Immobilization

Three grams of dry derivatized support were added to 0.020 dm$^3$ buffered trypsin solution and were stirred at 4°C for 18h. The enzyme solution was prepared in phosphate buffer, 0.1M, pH 6.3 and pH 7.5, and 0.1M borate buffer, pH 9.0. The trypsin quantity in the solution...
was from 10 to 40 g.dm⁻³ buffer. After the coupling step, the supports were washed with an abundant quantity of water until no protein was detectable in the filtrates, determined by the method of Bradford [31] using bovine serum albumin (BSA) as a standard. In order to remove the excess enzyme or the one which might be adsorbed, the beads were stirred at room temperature for 30 min in 0.500 dm³ of 0.2M NaCl solution and were washed again with distilled water. Bound protein quantity was determined after the end of washing steps. The enzyme-carrier complex was stored at 4°C in 0.1 M phosphate buffer, pH 7.8.

II.4. Assay Procedures

The analysis of the copolymer obtained was performed by FTIR spectroscopy (Perkin Elmer 1600) in KBr discs, in the range 3500-500 cm⁻¹, with sensibility 4 cm⁻¹. Bead density was measured picnometrically. The influence of the polymer solution flow on the particles size was investigated in a device similar to that, proposed by Lochmuller [29].

Bound protein was determined by a modified method of Lowry [32] using BSA as a standard. The beads (0.5 g, w.w⁻¹) were stirred with 0.005 dm³ cuprum-alkali reactive and 0.005 dm³ water at room temperature. After 2.5 h of treatment, 0.005 dm³ Folin-Ciocalteu reagent was added. The sample was stirred again for 30 min, until the absorbance at 750 nm was measured. Trypsin activity was determined spectrophotometrically using BAPNA (α-bensoyl-DL-arginine-4-nitroanilide, hydrochloride, Mw 434.89, casein, BRIJ, Fluka, Switzerland) as a substrate at 25°C. DL-BAPNA is a chromogenic substrate for proteolytic enzymes such as trypsin, amidase and balterobin. Hydrolysis of DL-BAPNA at the bond between the chromophore p-nitroaniline, which can be detected by colorimetric analysis of reaction products from native enzymes such as trypsin, amidase and balterobin.

Hydrolysis rates and varying the concentrations of BAPNA and trypsin activity were determined after the end of washing steps. The enzyme-carrier complex was stored at 4°C in 0.1 M phosphate buffer, pH 7.8.

The binding (BY) yield and the relative activity (RA) of the preparations, Acalculated is the activity which the bound protein would have in solution. Kinetic parameters were estimated by direct linear method using the Lineweaver–Burk plot [36] of the initial BAPNA hydrolysis rates and varying the concentrations of BAPNA (0.202-1.0 mM) in 0.2M triethanolamine buffer, pH 7.8. The reaction temperature was 25°C. The quantity of native protein was 50 mg.dm⁻³ and the amount of biocatalyst was 10 mg (w.w⁻¹) per assay. The results were obtained automatically by fitting experimental points of linear plot using Origin 6.0 at 0.95 confidential levels. The pH-optima of fixed and native (free) trypsin were measured as described earlier [37] using 50 mg.dm⁻³ protein or 10 mg (w.w⁻¹) of biocatalysts per assay and the following buffers: 0.2 M phosphate buffer with pH 6.0 and 7.0; 0.2 M triethanolamine buffer pH 7.8 and 0.2 M borate buffer with pH 9.0 and 10.0.

The used chemicals were from Sigma, USA (trypsin, EC 3.4.21.4 from bovine pancreas; bovine haemoglobin with Mw 64.5 kDa); Merck, Germany (acyrilonitrile for synthesis and acrylamide, dimethylformamide); Fluka, Switzerland (BAPNA with Mw 434.89, casein, BRIJ, hydrazine, BSA).

III. Results and Discussion

III.1 Polymer Yield and Particle Size and Properties

Twelve grams of copolymer were obtained and the polymerization yield was 80%. The IR-spectrum showed sharp peaks at 1681.8 cm⁻¹, specific for the amide group, and at 2244.2 cm⁻¹, specific for the acrylic group. The beads were spherical and without a tendency to aggregate, revealed by optical microscopy. Particles lose about 80% of their weight after drying. Their density was from 1030 to 1045 kg.m⁻³ depending on the magnetite content (Table I, the weight ratio of magnetite in beads is calculated relatively to the dry polymer).

TABLE 1

<table>
<thead>
<tr>
<th>Magnetite content (w.w⁻¹)</th>
<th>Density (kg.m⁻³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>without magnetite</td>
<td>1015</td>
</tr>
<tr>
<td>10% magnetite</td>
<td>1030</td>
</tr>
<tr>
<td>20% magnetite</td>
<td>1045</td>
</tr>
</tbody>
</table>

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They exhibited a negligible remanent magnetism and do not form aggregates when the magnetic field was switched-off, tested by experiments in a system (fluidization column and a magnetic system) described earlier [6]-[14]. Their mean particle size varied within the range 100-1000 µm and the size distribution was reproducible in all the experiments performed.

The particle size depended on the conditions of production and was proportional to the flow rate of the polymer solution, as described earlier [38]. When the flow rate of polymer solution decreased, a spray of droplets was formed resulting in finer particles and vice versa, as it can be seen on Fig. 1.

Due to the high viscosity of the polymer solution, the flow rate cannot vary in a wide range. At polymer flow rate 0.001 dm³.min⁻¹, 48% (w.w⁻¹) of the particles were with diameter 200-400 µm and at flow rate 0.0015 dm³.min⁻¹ about 49% (w.w⁻¹) were with diameter 500-700 µm. The air flow rate in all experiments was 1.0 dm³.min⁻¹.

![Graph: Particle size distribution at two different polymer solution flow rates and at air flow rate of 1 L min⁻¹](image)

Particles with mean diameter of 500 µm were used in the immobilization experiments because this fraction exhibited the best fluidization performance - no aggregation in absence of field, satisfactory bed expansion with low hysteresis, when fluidized by water in an axial magnetic field.

Besides, it is well known, that particles within the range of 300-600 µm exhibit the best features of the magnetic stabilization technique [14]-[25].

### III.2 Immobilization of Trypsin and Characteristics of the Particles

The immobilization efficiency as a function of pH is shown in Table II. The higher binding yield (BY) was reached at pH 6.3.

At this pH value, the specific activity of immobilized protein was 0.67 U.mg⁻¹ bound protein, representing 90% of the corresponding enzyme activity in native state (RA, relative activity=90%).

With the increase of pH above pH 6.3, the immobilized protein quantity per gram of support, the specific immobilized enzyme activity and the relative activity (RA) decreased. Lower pH values were not tested because of the specific reaction conditions of the method used.

All next immobilization experiments were carried out at pH 6.3.

#### TABLE II

<table>
<thead>
<tr>
<th>pH</th>
<th>Bound trypsin (mg protein.g⁻¹ dry support)</th>
<th>Specific activity (U.mg⁻¹)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6.3</td>
<td>9.20</td>
<td>0.67</td>
<td>90</td>
</tr>
<tr>
<td>7.5</td>
<td>7.65</td>
<td>0.54</td>
<td>72</td>
</tr>
<tr>
<td>9.0</td>
<td>8.80</td>
<td>0.47</td>
<td>63</td>
</tr>
</tbody>
</table>

Initial protein concentrations from 10 to 40 g.dm⁻³ buffer in the enzyme solution for immobilization were tested in order to determine the optimum immobilizations conditions (Fig. 2).

![Graph: Effect of enzyme/support ratio and magnetite content on the amount of immobilized protein](image)
dry support) increases up to about 13.0-14.0 mg.g\(^{-1}\) dry beads. Higher binding yield (BY) was reached at protein concentration 20.0 g.dm\(^{-3}\). The results for the maximal immobilized trypsin quantity and the binding yield are higher or comparable to the reported in other similar investigations [24]-[39]-[40]. Higher amounts of protein in the immobilization solutions (respectively higher enzyme/particle ratio) do not increase significantly the immobilized protein amount. Maximally up to 21\% of the added protein was immobilized depending on the magnetite content and the initial enzyme/particle ratio.

The magnetite content affected positively both the amount of immobilized trypsin and the enzyme activity when compared to the corresponding results for immobilization on nonmagnetic beads (Table III).

### Table III

<table>
<thead>
<tr>
<th>Magnetite content (w.w(^{-1}))</th>
<th>Specific activity (U.mg(^{-1}))</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>without magnetite</td>
<td>0.49</td>
<td>65</td>
</tr>
<tr>
<td>10% magnetite</td>
<td>0.65</td>
<td>87</td>
</tr>
<tr>
<td>20% magnetite</td>
<td>0.67</td>
<td>90</td>
</tr>
</tbody>
</table>

Specific enzyme activity (as U.mg\(^{-1}\) bound protein) increased from 0.49 (nonmagnetic beads) to 0.67 (magnetic beads, 20\% magnetite) and the corresponding relative activity (RA) increased from 65\% to 90\%. The effect of magnetite could be attributed to some influence on the formed chemical bonds during protein immobilization. The relative activity of bound trypsin (from 87 to 90\% of its activity in solution) was significantly higher than determined earlier RA 55.6\% for immobilized trypsin on activated glycidyl methacrylate-modified cellulose [34]. These results are similar to those for trypsin covalently bound to oxirane-acrylic beads [41] or covalently coupled to controlled pore glass [26].

The obtained curve for external mass transfer limitations for substrate BAPNA (Fig. 3) showed that at stirred speed (n) equal to 250 rpm.min\(^{-1}\) the relative enzyme activity reached 80\% (stirrer speed was varied from n=0÷350 rpm.min\(^{-1}\)). In this case the stirrer blade Reynolds number was Re=(nρfd\(^2\)/µf)=1664. Significant increase of the Re at stirrer speed beyond this value was not observed. This fact indicated that the diffusion of the synthetic substrate BAPNA with Mw 434.88 was not a rate limiting factor for the mass transfer processes. The hydrodynamic measures are very important when studying enzyme reactions with high molecular weight substrates. Such reactions are limited by the substrate diffusion in the particles and the external diffusion resistance to the mass transfer should be reduced. The experiments proved that the relative activity of immobilized trypsin for casein and hemoglobin decreased to 50\% and remained at this value at higher stirrer speeds as well.

After immobilization the bound trypsin retained the same pH-activity profile as the soluble enzyme (Fig. 4). The optimal pH for enzyme reaction remained unchanged at pH 7.8.

Trypsin immobilized on magnetic beads showed high relative activity and similar apparent values of the Michaelis constant (Km) 1.5.10\(^{-3}\) M and Vmax for substrate BAPNA when compared to free enzyme (Fig. 5). Km values can significantly vary from enzyme to enzyme and even for different substrates of the same enzyme. Hence, the immobilization of trypsin to poly-(acrylonitrile-co-acrylamide)-magnetite particles does not affect the enzyme affinity to its substrate BAPH.
Thus, the results proved that no structural changes of the enzyme molecule occurred during the immobilization procedure on this support. Immobilization does not cause low accessibility of the substrate to the enzyme active sites as it was reported for bacterial proteases and trypsin [42]-[43].

In this investigation, porous magnetic poly-(acrylonitrile-co-acrylamide) microparticles were produced by modified aerosol-jet technique. The obtained polymer was water-insoluble and the presence of amide groups allows application of different types of immobilization procedures. The main advantage of the technique employed for bead formation was the possibility to produce easily and rapidly large quantities of particles. Changing the flow rate one can change the size of magnetic particles easy from 100 to 1000 µm.

Immobilization of enzymes by covalent binding methods usually leads to strong binding of the protein due to the high number of functional groups on the surface of its molecule being able to interact with the highly functionalized polymer surfaces. Binding close to the active site, however, may cause a decrease of the enzyme activity. It reflects the effect of binding on protein molecule and respectively the activity loss due to the immobilization. Complete inactivation of some enzyme molecules, changes of catalytic parameters such as Km’ and kcat values or diffusion limitations of the reaction rate may be the mean reasons for these activity losses. Steric limitations could also be observed when the immobilized enzyme catalyses a reaction with a high molecular weight substrate. The physical structure and chemical composition of support can also influence the microenvironment of the immobilized enzymes and consequently their biological properties.

The high immobilized amount of trypsin (13.4-14.4 mg protein.g⁻¹ dry support) is comparable to the values reported for similar immobilization procedures [24]-[39]. Magnetite influenced the specific enzyme activity. Both the optimum pH of the trypsin and apparent Km’ for the low molecular weight substrate BAPNA were not modified by the employed covalent immobilization as observed in other investigations [34]-[44]-[45].

IV. Conclusion

In enzyme immobilization generally a small particles are used because of their high immobilization area, but packed-bed reactors with small particles show high pressure drops and insufficient flow rates for large scale applications. These disadvantages can be eliminated by using the magnetically stabilized bed (MSB) technique. The stabilized bed has a packed bed structure but a flexibility to modify it within a certain velocity range beyond the minimum fluidization velocity in absence of a field. Employing such MSB’s reactor the magnetic supports are easily separable from the reaction medium and stabilized in a magnetic bed by applying an external to the reactor magnetic field. Besides, higher particle-fluid relative velocity can be used to prevent product film formation around the enzyme-magnetic beads, or more precisely to decrease the product inhibition effect on the process. Also, the substrate transfer through the surface of the immobilized-enzyme-beads could be facilitated and the immobilized enzyme activity in the bioreactor could be controlled. All these features form specific requirements to the beads such as size and density relevant to both the stabilized bed hydrodynamics and mass transfer processes, and they predetermine the use of narrow fraction of particles.

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The relative activities (RA) of the immobilized trypsin for substrate BAPHA was 87-90%, but decreased to 50% for high molecular weight substrates such a hemoglobin and casein. These results could be attributed to effects of steric and micro-environmental substrate diffusion limitations. The present results form the first level of studies towards investigations in MSB with various field orientations and variations of macroscopic process parameters such as bed depth, fluid velocity and bed expansion controlled by the filed intensity. Obviously the next step is the beads performance in MSB reactor as an object of further studies.

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