ELSEVIER

Journal of Molecular Catalysis B: Enzymatic 18 (2002) 13–18

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

www.elsevier.com/locate/molcatb

Direct binding procedure of proteins and enzymes to fine magnetic particles

M. Koneracká [a,*], P. Kopčanský [a], M. Timko [a], C.N. Ramchand [b], A. de Sequeira [c], M. Trevan [d]

[a] *Institute of Experimental Physics, Slovak Academy of Sciences, Watsonova 47, 043 53 Košice, Slovak Republic*
[b] *University of Sheffield, Herries Road, Sheffield S5 7AU, UK*
[c] *School of Science and the Environment, Bath Spa University College, Newton Park, Newton St. Loe, Bath BA2 9BN, UK*
[d] *School of Biosciences, University of Westminster, 115 New Cavendish Street, London W1M 8JS, UK*

Received 25 April 2001; received in revised form 8 February 2002; accepted 8 February 2002

Abstract

Several clinically important proteins and enzymes (bovine serum albumin (BSA), glucose oxidase (GOD) (EC 1.1.3.4), streptokinase (EC 3.4.99.0), chymotrypsin (EC 3.4.21.1) and dispase (EC 3.4.24.3)), respectively, have been immobilised onto fine magnetic particles using carbodiimide as a coupling agent. The coupling reactions of these substances were carried out using various ratios of magnetic particles to protein, and different values of pH to determine the optimum conditions of immobilisation. The possible applications in biomedicine and biotechnology of this method of immobilisation are discussed.

Keywords: Immobilisation; Magnetic particles; Proteins; Enzymes; Biotechnology; Biomedicine

1. Introduction

One of the prerequisites for success of the application of drug targeting for the treatment of localised diseases is the development of an effective method to transport the drug to the target site in the organism. Biocompatible ferromagnetic particles have been used effectively as potential drug carriers since 1970 [1,2]. The targeting of drug-bearing magnetic particles to a specific part of the body has been studied using magnetic fluids (stable suspensions of highly dispersed particles of magnetic materials), unstable suspensions (well dispersed systems containing multi-domain magnetic particles) and magnetic microspheres (complex systems comprising special matrix materials like albumin, polysaccharide or containers like liposomes or erythrocytes) [3,4]. Most of these experiments used magnetic support materials in association with conventional support materials. Magnetic supports can be prepared by copolymerisation of magnetic particles, during the synthesis of the supporting polymer [5,6], allowing the entrapment of the magnetic particles within polymers, onto which the biomolecules can be immobilised. Magnetic particles can also be coated with layers of common support materials [7], e.g. agarose or dextran, and the biomolecules covalently linked to their surfaces.

The above systems are ideal for carrying small molecular weight pharmacologically active substances to a target area. However, these systems are

* Corresponding author. Tel.: +42-195-6336320; fax: +42-195-6336292.
E-mail address: konerack@saske.sk (M. Koneracká).

1381-1177/02/$ – see front matter
PII: S1381-1177(02)00016-4

not ideal for transporting enzymes to a target area for treatments of clot lysis in peripheral and coronary vascular occlusive diseases.

In the experiments reported here, the magnetic particles are synthesised by the co-precipitation method resulting in the formation of macroions. The specific adsorption of the amphoteric hydroxyl (–OH) group imparts superficial negative charges to the particles in an alkaline medium and positive charges in an acidic medium. Bacri et al. [8] have shown that the $–OH^-$ ligand will remain on the particles at a pH between 6 and 10. Thus, in the present case, the free hydroxyl group on the surface of the particles is responsible for the binding of the protein. The presence of 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride (CDI) modifies the carboxyl group of the protein at slightly acidic pH [9], which increases the percentage binding of the protein.

In an earlier study [10], it was found that it was possible to immobilise 97% bovine serum albumin (BSA) by covalently binding it to magnetic particles by coupling it to CDI, without loss of its properties. Several clinically important enzymes, like alkaline and acidic phosphatase and lactate dehydrogenase were also immobilised, and 50–80% activity was retained by the immobilised preparations.

In the present study, an attempt was made to link protein molecules (BSA, glucose oxidase (GOD), chymotrypsin, streptokinase and dispase) directly to magnetic particles using CDI as the coupling agent. Different values of pH and ratios of magnetic particles to protein were studied in order to establish the optimum conditions for immobilisation. The direct coupling of enzymes or bioactive molecules to the magnetic particles has a number of potential advantages.

Several advantages could be envisaged of such preparations where molecules are directly linked to a magnetic material, like ferrite, the lack of a polymer coat results in smaller particles, thus increasing the ratio of surface area to volume, allowing a greater response to any magnetic field; studies on the use of magnetic particles for cell-separation have revealed that the larger the particle size used for separation, the higher the extent of non-specific entrapment in the larger aggregates of magnetic particles, thus smaller magnetic particles hold the promise of greater specificity; small magnetic particles can exist as stable colloidal suspensions (ferrofluids) that will not aggregate, thus allowing for uniform distribution in a reaction mixture; the direct coupling method described here is both simple (one-step) and inexpensive.

2. Materials and methods

2.1. Preparation of magnetic particles

Magnetic particles (Fe_3O_4) were prepared by co-precipitating ferric and ferrous salts in an alkaline solution followed by washing in hot water. 27.8 g of $FeSO_4 \cdot 7H_2O$ and 54 g of $FeCl_3 \cdot 6H_2O$ were each dissolved in 100 ml double distilled water and thoroughly mixed and added to 75 ml, 8 M NH_4OH, with continuous stirring at room temperature. The particles obtained were black and exhibited a strong magnetic response. Impurity ions, such as chlorides and sulphates were removed by washing with copious amounts of hot distilled water. Finally, the magnetic particles were dispersed in slightly alkaline medium (pH = 8.9).

The amount of magnetic particles in a given volume of the ferrofluid was estimated by thermogravimetry and by magnetic measurements of magnetisation curves (VSM magnetometer).

The particle size distribution was determined by electron microscopy and magnetic measurements by means of Chantrell et al. [11] procedure. The particles were found to have a lognormal particle size distribution with a mean diameter of 10 nm and S.D., $\sigma = 0.23$.

2.2. Immobilisation

The reagents used in the immobilisation (BSA, dispase, GOD, chymotrypsin, streptokinase, and the coupling agent (CDI)) were obtained from Sigma, and the dye Coomassie Brilliant Blue from Fluka Chemical Company. The enzymes used for the immobilisations were freeze-dried preparations containing non-human enzyme stabilised in a buffered BSA base (Sigma).

BSA, GOD, chymotrypsin, streptokinase and dispase were immobilised onto magnetic particles using CDI. The coupling reactions were carried out under different conditions to determine the optimum conditions for immobilisation of proteins, i.e. change of the

Table 1
Effect of pH on the coupling of BSA to fine magnetic particles at ratio magnetic particles:BSA:carbodiimide—3:1:1, and on the immobilisation of GOD and its activity after immobilisation

Fe_3O_4:BSA:CDI[a]			Fe_3O_4:GOD:CDI[b]		
pH of buffer	Coating (%)	Proteins/Fe_3O_4 (mg)	pH of buffer	Coating (%)	Proteins/Fe_3O_4 (mg)
5.5	70	0.233	4.5	76	0.127
6	57	0.189	5	63	0.105
6.3	46	0.153	5.5	47	0.078
6.5	45.6	0.152	6.3	43	0.072

[a] Mass ratio (mg) = 3:1:1.
[b] Mass ratio (mg) = 6:1:2.

pH of the reaction mixture (Table 1) and proportion of magnetic particles to proteins (Fig. 3). The pH was varied using sodium and potassium phosphate buffers (0.003 M). The proteins to be coupled, and the CDI, were dissolved in buffer of the desired pH.

For a given value of pH (e.g. 6.3), 1 ml of protein dissolved in buffer pH 6.3 (20 mg/ml) was added to 1 ml of CDI (20 mg/ml) dissolved in the same buffer. A given volume of magnetite particles (3 ml) which had previously been washed copiously in buffer pH 6.3 were then added to the mixture. The reaction mixture was shaken at room temperature for 24 h, after which time the magnetic particles were separated out by placing a magnet under the vial containing the reaction mixture. The supernatant was decanted and the particles re-washed three more times in buffer. The supernatants were pooled and the total protein concentration measured using Bradford's dye binding assay [12]. The protein concentration in the original protein solution (before coupling) was measured by the same method and thus the quantity of protein bound to the magnetic particles could be calculated from the difference between the total protein added to the immobilisation mixture and the total protein recovered from the pooled washings. This procedure was repeated using different values of *X*:*Y*:*Z*.

The binding of the protein to the magnetic particles was examined by FTIR spectroscopy and electron microscopy.

The enzyme activity was determined in case of GOD by GOD–peroxidase (POD) coupled enzyme reaction [13].

Immobilised streptokinase was assayed by determining the smallest amount of streptokinase that will cause lysis of a standard fibrin clot within 10 min [14]. 100 μl of serial dilutions of streptokinase in gelatine buffer, 0.8 ml of standard human "fibrinogen" solution containing plasminogen and 0.1 ml of thrombin solution were mixed and placed in a water bath at 37 °C. The clotted fibrinogen starts to lyse due to the presence of streptokinase, which activates the plasminogen originally contained in the fibrinogen reagent. Using the above standard, the immobilised streptokinase was assayed to determine the percentage activity retained after immobilisation. The activity of other enzymes (chymotrypsin, dispase) were assayed according to the Sigma diagnostic procedures [15].

3. Results and discussion

3.1. Immobilisation of BSA onto magnetic particles

The extent of BSA binding to the magnetic particles was estimated by FTIR spectroscopy and electron micrography. It is clear from the electron micrograph (Fig. 1) that proteins are layered over the magnetic particles. Fig. 2 shows the FTIR spectral characteristics of BSA bound to magnetic particles. It is evident that the characteristic bands of protein at 1648 and 1540 cm^{-1} are present in pure BSA and in BSA bound to magnetic particles. The extent of coating of BSA onto the magnetic particles was determined by estimating the residual protein in the supernatant and washings. The estimation of protein content by Bradford's method [12] showed that the extent of coating onto the particles increased as the pH of the reaction mixture was decreased (Table 1). This is in agreement with the mechanism of the coupling reaction. Also, as expected, with a higher ratio between the magnetic particles and protein, the percent coating attained was higher (Fig. 3). The optimum conditions

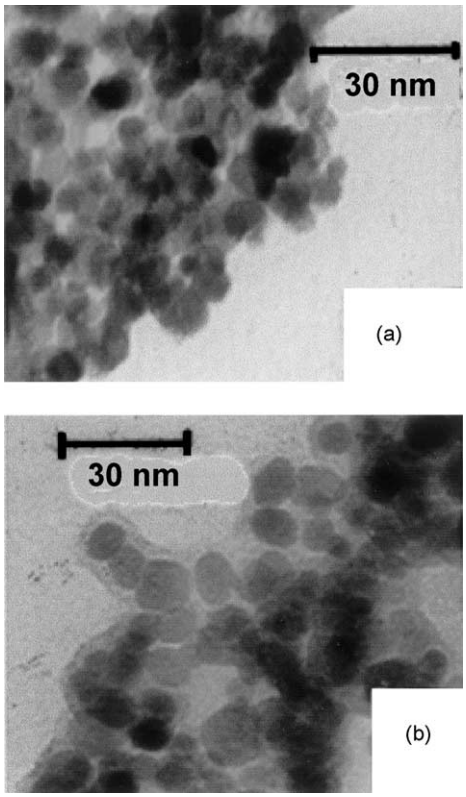

Fig. 1. Electron micrograph (a) pure magnetic particles and (b) protein bound to magnetic particles.

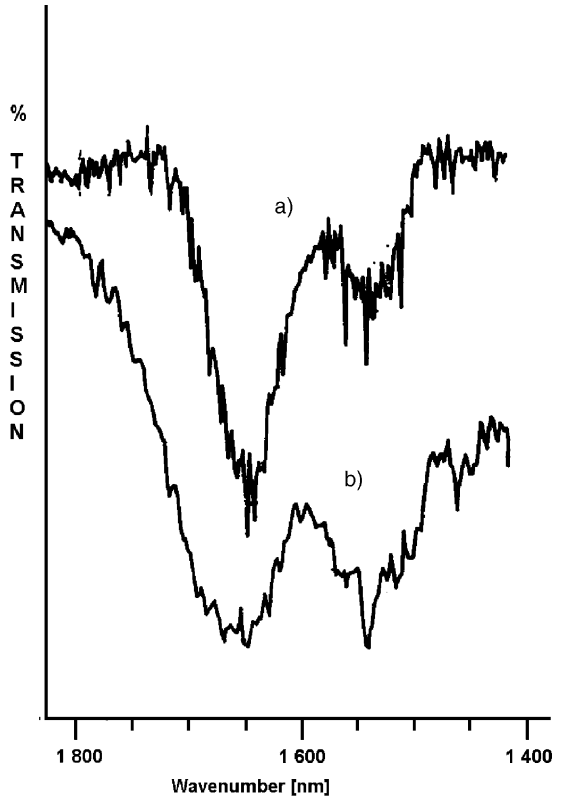

Fig. 2. FTIR spectrum of (a) pure protein and (b) protein bound to magnetic particles.

for the binding of BSA to the magnetic particles were observed at a ratio of magnetite:BSA:CDI of 2:1:2 at room temperature and pH = 6.3, respectively.

3.2. Immobilisation of glucose oxidase onto the magnetic particles

The coupling reaction was allowed to proceed at room temperature or at lower temperatures (0–4 °C). The extent of coating was determined by estimating the residual protein and enzyme activity in the supernatants and washings, and hence expecting that the protein/enzyme activity not accounted for in the supernatants and washings, had been coated onto the particles. The activity of the enzyme coated onto the magnetic particles was also estimated. In all cases, protein was estimated by Bradford's method [12] using GOD as the standard. The enzyme activity

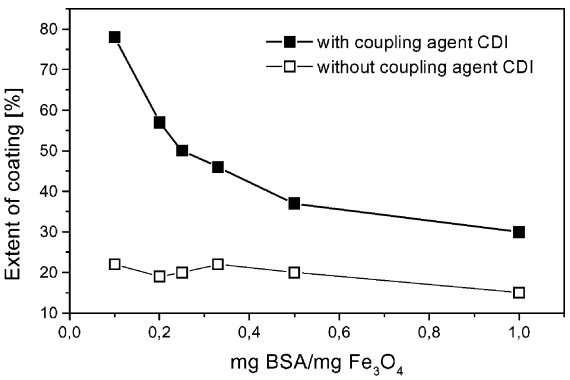

Fig. 3. Effect of ratio of magnetic particles to protein to carbodiimide on extent of coating of BSA (phosphate buffer 0.003 M, pH = 6.3).

was monitored by the GOD–POD coupled enzyme reaction. When coupling was attempted at room temperature all of the added protein was recovered in the washings and the particles did not show any enzyme activity. It is therefore reasonable to assume that no protein was coupled. However, the coupling could be achieved by using a lower temperature (0–4 °C); at pH 6.3, 43% protein was coated onto the particles, while almost 76% protein was coated onto the particles at pH of 4.5. The optimum ratio of magnetic particles:GOD:CDI was found to be 6:1:2. The higher magnetic particle to protein ratio used here helped to minimise losses of the enzyme.

Whilst GOD could be bound to the magnetic particles at pH 6.3, no enzyme activity was observed: maximum activity was observed where the coupling reaction was carried out at pH 4.5 (Table 1). Although maximum protein binding was obtained for a coupling reaction pH value of 4.5, over 70% of the enzyme's specific activity was lost (for the amount of enzyme bound 70 units of activity should have been observed, but the measured figure was 24). This is a common observation for enzymes immobilised by covalent bonding.

This apparent loss of specific activity of the enzyme can be attributed to the various alterations in the properties of enzymes, which often accompany their immobilisation. Random covalent bonding of an enzyme to the surface of a solid matrix could result in multiple orientations of the enzyme on that surface. Thus, coupling may result in a conformation of the enzyme where the active site is partly or totally obscured by the immobilisation matrix and thus not available to the substrate. Alternatively, even if the active site is exposed, the binding of the enzyme molecule to the support could restrict its ability to undergo any necessary change in conformation on binding the substrate. Yet another possibility is that covalent bond formation could take place through residues, which though not actually present at the active site may be essential for maintaining the active conformation of the active site. One or more of these factors could have contributed to the loss of activity.

Dispase, chymotrypsin and streptokinase were immobilised to fine magnetic particles, in a similar manner as described above. The extent of coating and enzyme activities were measured in these cases. The best achieved results are summarised in Table 2.

Table 2
The best results of extent of coating of dispase (EC 3.4.24.3), chymotrypsin (EC 3.4.21.1) and streptokinase (EC 3.4.99.0) and their activity after immobilisation at pH = 6.3

Proteins/enzymes	A:B:C (mass ratio)	Proteins/ Fe_3O_4 (mg)	Units proteins/ Fe_3O_4 (mg)
Dispase	2:1:2	0.41	86
Chymotrypsin	2:1:2	0.28	50
Streptokinase	2:1:2	0.26	50

A: magnetic particles, B: proteins and C: CDI.

4. Discussion

The present findings have several applications in medicine and biotechnology. One of the important applications worth exploring is the treatment of coronary thrombosis and peripheral arterial occlusions. By drawing magnetic streptokinase to the target site of a thrombosis by applying powerful magnetic fields to the patient, it should be possible to lyse clots using reduced quantities of the enzyme. This procedure can certainly avoid the unwanted side-effects of high doses of streptokinase. The present procedure could also be used to remove carcinogenic cells from the circulation to prevent metastases by using magnetic antibodies specific for tumour cell surface antigens. The direct binding of enzymes and proteins to magnetic particles has several other potential applications in the field of biotechnology. The procedure could be used to immobilise the whole cells, using the cell surface proteins, which can be used in chemical and drug transformation. Microbial cells with the capacity to degrade toxic aromatic hydrocarbons could be immobilised to magnetic particles and used for industrial waste treatment.

The method can be applied to immobilise antibodies used in radio immunoassays, thus avoiding the time-intensive centrifugation step. Affinity ligands can be directly bound to magnetic particles linked to protein for the use of enzyme and antibody purification avoiding time-consuming chromatographic procedure.

5. Conclusion

The present findings clearly show that it is possible to bind proteins onto magnetic particles in the presence of CDI without the aid of a primary coating. The binding is due to the presence of hydroxyl groups on

the surface of fine magnetic particles freshly prepared from Fe_3O_4. The optimal conditions for the immobilisation of the various proteins and enzymes to fine magnetic particles depend on the kind of immobilised proteins and enzymes, the pH of the reaction mixture and the ratio of each reagent in the reaction mixture, respectively.

Acknowledgements

Funds for this research were provided in part by the Slovak Academy of Sciences (within the framework of Project No. 7020) and NATO (within the framework of Project No. LST. CLG. 977500).

References

[1] K. Mosbach, U. Schröder, FEBS Lett. 102 (1979) 112.
[2] K.J. Widder, A.E. Senyei, D.G. Scarpelli, Proc. Exp. Biol. Med. 58 (1978) 141.
[3] E.K. Ruuge, A.N. Rusetski, J. Magn. Magn. Mater. 122 (1993) 335.
[4] U.O. Häfeli, G.J. Pauer, J. Magn. Magn. Mater. 194 (1999) 76.
[5] M. Horisberger, Biotechnol. Bioeng. 18 (1976) 1647.
[6] P.J. Robinson, P. Dunnill, M.D. Lilly, Biotechnol. Bioeng. 15 (1973) 603.
[7] A.N. Rusetski, E.K. Ruuge, J. Magn. Magn. Mater. 85 (1990) 299.
[8] J.C. Bacri, D. Salin, R. Massart, J. Magn. Magn. Mater. 85 (1990) 27.
[9] L. Packer, S. Tristram, J.M. Herz, C. Russel, L.C. Borders, FEBS Lett. 108 (1979) 243.
[10] R.V. Mehta, R.V. Upadhyay, S.W. Charles, C.N. Ramchand, Biotechnol. Tech. 11 (1997) 493.
[11] R.W. Chantrell, J. Popplewell, S.W. Charles, IEEE Trans. Magn. Mag. 14 (1978) 975.
[12] M.M. Bradford, Anal. Biochem. 72 (1976) 248.
[13] The Enzymatic Colorimetric Determination of Glucose, Sigma Technical Bulletin No. 510, Sigma Chemical Co., St. Louis, 1983.
[14] G. Reber, P. Kappus, in: J. Bergmeyer, M. Graßl (Eds.), Methods of Enzymatic Analysis, Enzymes 3: Peptidases, Proteinases and Their inhibitors, Vol. 5, 3rd Edition, Hans Rappold Offsetdruck, Germany, 1984, p. 433.
[15] K. Slavik, R. Smetana, Chem. Listy 46 (1952) 648.