Thermosensitive Magnetic Particles as Solid Phase Support in an Immunoassay

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SUMMARY : The adsorption and the chemical grafting of an antibody (anti-Alpha-fœto protein) onto thermosensitive magnetic latex particles has been investigated as a function of pH, ionic strength and temperature. The hydrophilic magnetic latexes bearing surface carboxylated groups have been prepared on the basis of an heterocoagulation concept. The adsorption of the antibody (anti-Alpha-fœto protein) was found to drastically decreased upon increasing the pH, whereas, the effect of ionic strength was found to be negligible in the studied range. The covalent coupling of the antibody was performed using two cardodiimide and succinimide derivatives. The prepared conjugates (latex-antibody) were evaluated by performing the classical Enzyme Linked Immuno Sorbent Assay (ELISA) for detecting the Alpha-fœto protein (AFP).

Introduction

Magnetic microspheres received considerable increasing attention in many fields especially in the biomedical field[1)]. As polymer particles, these materials exhibit a high specific surface area[2)], a narrow size distribution and versatility in surface functionality[3)]. Many routes have been described to obtain magnetic polymer particles : (i) batch emulsion polymerization in the presence of ferrofluid nanoparticles, (ii) preparation of ultrafine magnetic on preformed seed or porous latex particles and (iii) impregnation of seed latex or porous polymer microspheres with ferrofluid or magnetic powder followed by an encapsulation step using appropriate monomers.

The aim of this work is to evaluate the performances of hydrophilic monodisperse magnetic microspheres[4-5)] as solid phase of an immunoassay using the Alpha-fœto-protein (as inflammatory marker). Adsorption of the corresponding antibody-anti-Alpha-fœto (Ab-anti-AFP), was first performed as a function of pH, ionic strength and temperature, then its covalent binding was investigated. Finally, the immunological activity of the grafted antibody onto magnetic microspheres was studied using an ELISA (Enzyme Linked Immuno Sorbent Assay) test and the results were compared to that using automated systems.

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Experimental part

Preparation of magnetic microspheres. Hydrophilic magnetic particles were prepared according to a three-step procedure. Two cationic latexes bearing amine and amidine groups under cationic form were used as seed particles for the adsorption of iron oxide nanoparticles : poly(styrene) and core-shell poly(styrene/N-isopropylacrylamide)[6]. These two latexes were synthesized by emulsion-free emulsion polymerization using 2,2'-azobis(2-amidino-propane) dihydrochloride as initiator and aminoethylmethacrylate hydrochloride(AEM) as cationic monomer. Iron oxide nanoparticles (diameter~10 nm) prepared from ferric and ferrous chloride precipitation by sodium hydroxide were then adsorbed onto seed polymer particles via electrostatic interactions. The adsorbed nanoparticles onto seed latex were encapsulated through polymerization of N-isopropylacrylamide (NIPAM), N,N-methylenebisacrylamide (MBA), as cross-linker and itaconic acid (IA), as functional monomer and potassium persulfate (KPS) as initiator. For more information concerning the preparation of such magnetic latexes, see references 4 and 5 . The colloidal properties of the different microspheres used in this study are given in Table 1. It is worth noting that the used hydrophilic magnetic particles are noted ML1 (22.5 wt.% iron oxide content). All latexes are first cleaned by removing free iron oxide and electrolytes before performing the immobilization of antibody-anti-AFP.

Table 1. Number average diameter (D_n), diameter size distribution (PDI), and magnetic content of the latex particles (wt. % iron oxide).

Latex	D_n (nm)	PDI	wt. % iron oxide
CS1[a]	385	1.01	00
ML1[a]	330		22.5
PS[b]	320	1.01	00
CS2[b]	473		00

(a) Carboxylic and (b) Amine and amine poly(styrene/NIPAM) core-shell.

Adsorption of Antibody-anti-AFP. From 0 to 300 µL of the antibody solution, at a concentration of 1 mg/mL in phosphate buffer, were added to carbonate buffer (5 mM, pH9.3) solution containing 2 mg of latex particles. The final volume was then adjusted to 1 mL with the phosphate buffer. Adsorption of proteins was measured using the Bradford titration method[7,8]. The Ab-anti-AFP adsorbed amount corresponded to the difference of concentrations between the initial and the final Ab-anti-AFP amounts.

Covalent grafting of Ab-anti-AFP. A given amount of magnetic microspheres were dispersed

in the carbonate buffer as above described. The covalent coupling of the Ab-anti-AFP was performed at room temperature. Based on a previous work[8], the amount of activating agents were the following : 40 µL of N-hydroxysulfosuccinimide (NHSS, 10 mg/mL) and 1-ethyl-3-(N, Ndimethylaminopropyl) carbodiimide (CDI, 4 mg/mL) and a given amount of Ab-anti-AFP were added to particles dispersion. Then, the final volume was adjusted to 1 mL with a phosphate buffer (pH4.7, ionic strength 10 mM).

Enzyme Linked Immuno Sorbent Assay test (ELISA) evaluation. The microspheres bearing grafted Ab-anti-AFP were first washed using carbonate buffer (pH~11). This solution was then fractionated into 100 µL aliquots adjusted to 1 mL with PBS-Tween buffer. The immunological reaction between the grafted antibody and free antigen was performed using an ELISA test. From 0 to 20 µL of the AFP solution (0.31 mg/mL) were added onto 0.2 mg of the magnetic microspheres-Ab-anti-AFP conjugates at room temperature. After two hours incubation time, magnetic particles were washed 3 times using PBS-Tween buffer. Captured antigens amount was then determined by introducing the labeled antibody. After washing the excess of the labeled antibody through magnetic separation, the introduction of the para-nitrophenyl phosphatase at 37°C provided a rapid coloration measured by UV. at 650 nm wavelength.

Results and discussion

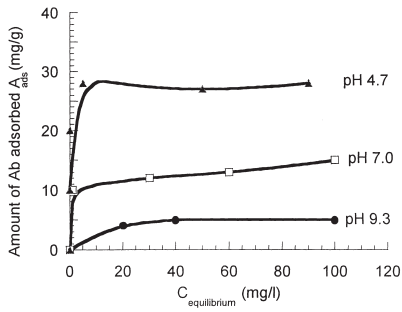

Fig. 1. Adsorption isotherms of Ab-anti-AFP onto magnetic latex particles (ML1) versus pH, at 20°C ionic strength 10 mM.

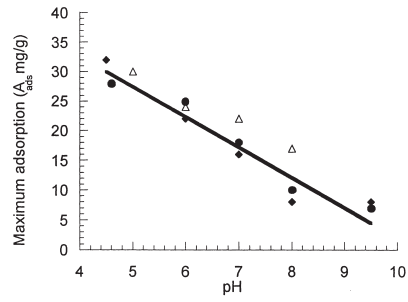

Fig. 2. Maximum Ab-anti-AFP adsorption onto magnetic latex particles (ML1) versus pH at various ionic strengths at 20°C. (Δ) 1mM, (●) 10 mM, (◆)100 mM.

Adsorption of Ab-anti-AFP. The adsorption isotherm of Ab-anti-AFP onto prepared carboxylated magnetic particles (ML1) was first carried out in a phosphate buffer as a

function of pH and at a constant ionic strength of 10 mM and the obtained results are reported in Fig. 1.

The initial slope reflects a decrease in affinity between Ab-anti-AFP and polymer particles as pH increases and the highest amounts are obtained at pH 4.7 which correspond to ~2.7 mg/m^2 a value lower than those reported for polystyrene particles (order of 6 mg/m^2 for IgG) (9). In this case, the overall charge of the antibody is positive whereas a large proportion of carboxylic groups are under dissociated form. Electrostatic interactions between the antibody and latex particles can explain the important adsorption amount at low pH. Since pH 7 is near to the isoelectric point (IEP) of the Ab-anti-AFP, electrostatic interactions are reduced and adsorbed amounts of antibody decrease. Further increase in the pH towards basic values cause the two reactants to exhibit a net charge of same nature, which result in a decrease of the adsorbed amount Ab-anti-AFP down to 0.5 mg/m^2. Two other possibilities have been reported to explain the adsorption of antibody (and proteins) onto thermosensitive[10] and polystyrene particles[11] : i), the presence of hydrogen bounding between COOH and amino groups of the antibodies enhance the adsorption, ii) the reconformation of adsorbed antibodies (or proteins) affects the surface coverage.

The influence of the ionic strength and pH on the adsorption was investigated in a limited concentration range (1 to 100 mM) in order to avoid particles aggregation. The adsorbed amount of antibody was measured at 60 mg/L concentration and as reported in Fig. 2, there is no influence of the ionic strength. This is indicative that electrostatic interactions are not only the driving forces in the adsorption of Ab-anti-AFP molecules. The adsorption behavior was also examined onto various non magnetic polymer particles exhibiting hydrophilic (CS2, cationic), (CS1, anionic) or hydrophobic (PS, cationic) surface and hydrophilic magnetic microspheres (ML1, anionic). The obtained adsorbed amounts of antibody are summarized in Table 2, leading to the following remarks. First, with the cationic hydrophilic latex core-shell (CS2), no adsorption is observed regardless of the pH because neither electrostatic interactions, nor hydrogen bonding can occur. Secondly, by replacing the hydrophilic surface by an hydrophobic one, the adsorption amount reaches a constant value which is no pH dependent, since hydrophobic interactions are predominant. The same trend is observed in the case of carboxylated latex particles. With the non-magnetic microspheres, the adsorbed amount is more important than in the case of magnetic microspheres at pH 4.6 due to either higher charge density or better charge accessibility.

Table 2. Antibody adsorbed amount, A_{ads} (mg/m^2) onto different latex particles at various pHs.

Latex	pH=4.6	pH=7	PH=9.3
CS1[a]	4.2	1.9	0.4
ML1[a]	2.7	1.4	0.5
PS[b]	3.8	3.4	3.8
CS2[b]	0	0	0

(a) Carboxylic and (b) Amine and amine

The adsorption was also investigated above and below the LCST of poly(NIPAM)~32°C and the measured adsorbed amounts as reported in Table 3, show that adsorption is higher at 40°C than at 20°C. This behavior can be attributed to an increase of hydrophobic interactions irrespective of pH[12]. When temperature is decreased from 40 to 20°C, a small desorption is observed, due to reduction of the hydrophobic character of the particles except at pH 4.7, where electrostatic interactions are stronger since the antibodies and particles are oppositely charged. Such a behavior has already been observed by Kawaguchi et al.[12,13].

Table 3. Adsorbed (A_{ads}) and desorbed amount (A_{des}) of Ab-anti-AFP expressed in mg/m^2 (using magnetic particles).

	pH 4.7	pH 7	pH 9.3
A_{ads} (20°C)	2.7	1.4	0.5
A_{ads}(40°C)	3.6	1.9	1.7
A_{des} (20°C)	00	0.6	0.5

Covalent Grafting of Ab-anti-AFP. Covalent coupling of Ab-anti-AFP was then performed under conditions where pH and ionic strength strongly favor adsorption, (i.e. pH 4.7 and ionic strength 10 mM). Preliminary experiments were carried out in order to examine the accessibility of the grafted Ab-anti-AFP molecules onto functionalized magnetic microspheres. Covalent coupling of Ab-anti-AFP was first compared to adsorption: the two activating agents were added only in the first sample. After one hour reaction, both samples were washed using a carbonate buffer. In the absence of activating agents, desorption is complete at pH 11 buffer. In contrast, after covalent coupling, the desorption of Ab-anti-AFP is partial, proving that 30% of the total immobilized Ab-anti-AFP are chemically fixed.

Enzyme Linked Immuno Sorbent Assay test (ELISA). ELISA tests were then carried out on both bare and magnetic microspheres bearing antibody BSA being commonly used as a non-specific "antigen". In all cases, the optical density was found to be around 130 a.u. corresponding to the reference value. Since these preliminary experiments showed specific

AFP recognition, ELISA tests were performed at various AFP concentrations and sensitized magnetic particles (ML1): (i) At a very diluted range of concentrations, the lowest AFP concentration which gives a optical density (OD) signal above the reference value is equal to 15 ng/mL (Table 4). Such a value is close to the detection limit reached using automated systems. (ii) Upon increasing the amount of AFP (target), optical density increases and the saturation detection is reached at an antigen concentration of 4.5 µg/mL (Fig. 3). This upper limit corresponds to a ratio of Ab-anti-AFP over AFP molecules of 0.05. This low efficiency compared to automated systems can be attributed to the non optimized AFP detection conditions and to the low antibody grafted amount.

Table 4. Minimum amount of AFP using magnetic particles bearing antibody with 21 mg/g Ab-anti-AFP.

ML1 amount	0.2 mg					0.5 mg		
C_{AFP} (ng/mL)	00	3.1	15	31	77	0	3.1	9.3
OD (a.u)	127	135	145	240	310	140	264	291

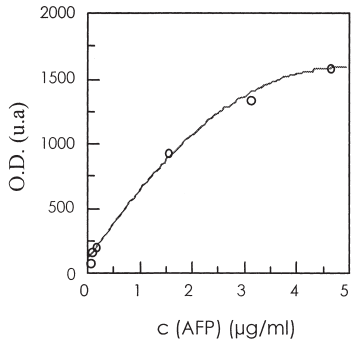

Fig. 3. Optical density versus AFP concentration. Immobilized antibody amount: 21 mg/g.

Conclusion

Submicron thermosensitive magnetic latex particles prepared via heterocoagulation process have been used as a solid phase in an ELISA test. It is worth noting that no free magnetite is released during the immobilization process of antibody onto the magnetic particles. The adsorption of antibody (Ab-anti-AFP) was strongly influenced by pH of the buffer. At acidic pH, electrostatic interactions favor adsorption. The covalent grafting of antibody (Ab-anti-

AFP) was carried out at pH 4.7, in which the adsorption was favored and the non chemically immobilized molecules were desorbed using basic buffer (pH~11). The magnetic particles bearing antibody were evaluated using ELISA test and an encouraging sensitivity was evidenced. Any way, a better sensitivity could be reached by optimizing the covalent binding study of the antibody and also by controlling both the grafted amount and the latex-antibody concentration in the ELISA test. Such thermosensitive magnetic particles can be used as solid phase support in immunoassay incorporated in automated apparatus based on magnetic separation.

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