Hydrophilic Stimuli-Responsive Particles for Biomedical Applications

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SUMMARY: Hydrophilic and stimuli-responsive submicronic latex particles based on polyalkyl(meth)acrylamide can be prepared owing to simple radical-initiated polymerizations in heterogeneous media using a water-soluble initiator and a crosslinker (methylenebisacrylamide). The paper aims at reviewing the synthesis and properties of functionalized polystyrene-polyN-isoprpylacrylamide core-shell particles or polyN-isopropylmethacrylamide microgel particles. Particle size of analysis showed that a short nucleation period afforded the synthesis of highly monodispersed latexes. The dramatic change of the colloidal properties (particle size, electrophoretic mobility) was found to reflect the thermal sensitivity of such particles. The hydrophilic nature of the particles below the volume phase transition temperature was found to drastically reduce the physical adsorption of proteins. Some examples of biomedical applications of these stimuli-responsive particles are briefly reported.

Introduction

In the past decade, stimuli-responsive aqueous microgel particles attracted much increasing interest from both academic and practical points of view[1,2]. In this domain, such particles produced by radical-initiated precipitation polymerization of amphiphile-like monomers such as alkylacrylamide derivatives proved to exhibit unique properties, mainly because of the thermal sensitivity of the corresponding polymer. Moreover, it was shown that such latex particles in the submicron size exhibited faster response to stimuli than macrogels. As recently reviewed[3], they provide a large potentiality for application, especially in medicine and biological fields, mainly due to the suitable environment they offer for the immobilization of biomolecules.

As a part of a research program devoted to the development of hydrophilic latex particles suitable for biomedical applications, our work aims at focusing on the synthesis and characterization of various functionalized poly[alkylacrylamide]-based microgel particles. The incorporation of various reactive monomers in the polymerization recipe was found to confer several advantages of particular interest to the final latex particles in addition to their thermal sensitivity: control of the particle size; sensitivity of the microgels to pH and ionic

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strength; high electrosteric stability of the particles against electrolyte concentration (especially below the volume phase transition temperature VPTT). Moreover, the presence of surface functional groups makes these microgel particles appropriated supports for the immobilization of various biomolecules (nucleic acid probes, peptides, proteins) through a careful control of experimental conditions (pH, ionic strength, buffer, temperature, etc) allowing to reduce physical adsorption.

In this paper, it will be first emphasized the main features related to the precipitation polymerization kinetics of two alkylacrylamide derivatives in the presence of a crosslinking agent (methylene bisacrylamide), a functional comonomer and using potassium persulfate or 2,2'-azobis(2-amidinopropane) dihydrochloride as initiators. Second, some of the colloidal properties of these stimuli-responsive microgel latexes (particle size and size distribution, electrophoretic mobility) will be presented together with their variation upon changing temperature, pH and ionic strength. Finally, a brief review will be given illustrating some applications in which such particles can be involved in diagnostics .

Preparation of latex particles

In relation to the type of application, various models of stimuli-responsive particles were designed and developed through radical-initiated polymerization either under emulsifier–free emulsion conditions or by a precipitation process : i) polystyrene(PS)-polyN-isopropylacrylamide (poly[NIPAM]) core-shell particles; ii) polyN-isopropylmethacrylamide (poly[NIPMAM]) microgel particles, i.e, latex particles which are expected to show different (VPTT), 32 and 44°C, respectively. In addition, such particles were functionalized with a reactive monomer for subsequent covalent immobilization.

In the case of the core-shell particles, we adopted a recipe of Makino et al.[4] for making PS/poly[NIPAM] latex particles and using aminoethyl methacrylate hydrochloride (AEM)[5] to provide amino surface groups under protected form. It was indeed found that the introduction of this functional monomer offered outstanding advantages during the synthesis of such latexes as well as for conferring improved colloidal electrosteric stability. As illustrated in Figure 1, batch emulsion polymerization of styrene in the presence of NIPAM (with and without AEM) is characterized by a fast consumption of NIPAM early in the reaction (80%

NIPAM consumed before styrene starts to polymerize). The effect is even more pronounced when AEM was used. Such an effect was attributed both to the water solubility of NIPAM and AEM and the huge absolute propagation rate constant k_p for NIPAM. TEM examination of the particles along with the conversion showed that polymerization of such a system occurred through a two-step mechanism. At first, due to its favorable partitioning in water, NIPAM rapidly reacts leading to polymer chains which precipitate at very low conversion (ca, order of 2%) as tiny precursors, then producing by coagulation larger particles (50/ to 90nm) exhibiting an already narrow size distribution (<10% conversion). Above 15% conversion, more than 80% of the NIPAM has already polymerized and styrene begins to react through diffusion of the monomer in the hydrophobic (at the reaction temperature) poly[NIPAM]–rich particles. The formation of PS-rich chains inside the particles leads to a phase separation and formation of small PS–rich inclusions. Further polymerization of styrene results in the expelling of most of the previously formed NIPAM at the particle outerlayer. Such a mechanism has also been postulated in the case of emulsifier-free copolymerization of styrene with an hydrosoluble monomer[6].

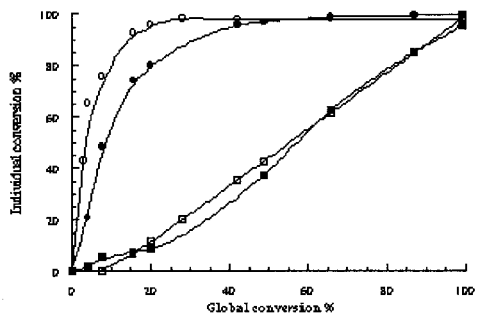
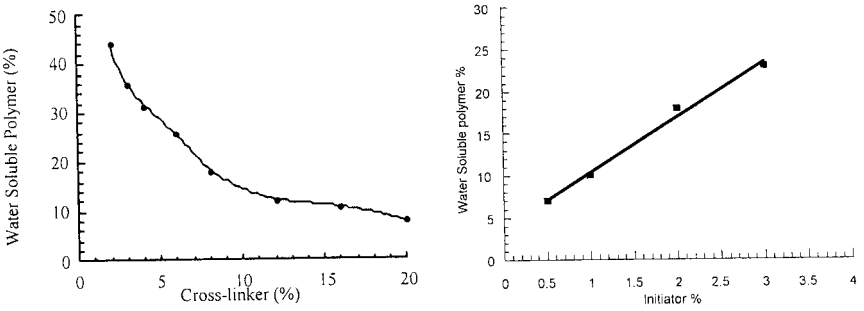


Figure 1: Individual conversion vs. Overall conversion for batch emulsion copolymerization of styrene and NIPAM with and without AEM[5]: (■) styrene without AEM; (●) NIPAM without AEM; (□) styrene with AEM; (○) NIPAM with AEM.

Using this latex as seed, particles having a higher surface density and thicker hydrophilic layer were produced through a shot-growth process consisting in polymerizing at high conversion a mixture of NIPAM, AEM (with variable amounts) and a crosslinker (methylene bisacrylamide) (MBA)[5].

In the case of poly[NIPMAM] microgels particles, a systematic study was conducted allowing to investigate the influence of crosslinker (MBA) and initiator (potassium persulfate) concentration as well as temperature on the kinetics of precipitation polymerization of this monomer[7].

The crosslinker is indeed necessary to favor the particles formation by crosslinking the precipitated poly[NIPMAM] chains and to avoid the solubilization of the polymer upon lowering the temperature below the VPTT. It is worth mentioning that on increasing the crosslinker concentration in the batch polymerization recipe led to reduce the final amount of water soluble polymer (Figure 2-a). In addition, the water soluble crosslinker did not significantly influence the polymerization rate as well as the final particle size[7] but on the contrary it affected the swelling ability of the particles. According to the higher reactivity of the crosslinker (MBA), the final internal structure of hydrogel particles was heterogeneous with a gradient composition of MBA (looser and looser from the core to the shell) as recently evidenced by Guillermo et al.[8] by analyzing the transverse relaxation of protons by NMR.



(a) (b)

Figure 2: Effect of the crosslinker (MBA) (a) and initiator (potassium persulfate) (b) on water soluble polymer formation for NIPMAM polymerization[7].

Concerning the initiator effect, the polymerization rate was found to increase with increasing the potassium persulfate (KPS) concentration similarly to what is observed in conventional emulsion polymerization, a behavior which has been attributed to the increase of the polymerization loci. It is also worth mentioning that the initiator concentration increase also made the water soluble polymer formation (as low M_W oligomers) (see Figure 2-b) to increase and the final particle size to decrease provided the initiator concentration was lower than the critical coagulation concentration of the primary formed particles (no coagulation step induced by too a high initiator concentration).

The polymerization temperature should be absolutely above the LCST of the corresponding linear polymer and higher enough in order to favor the precipitation of the oligomers

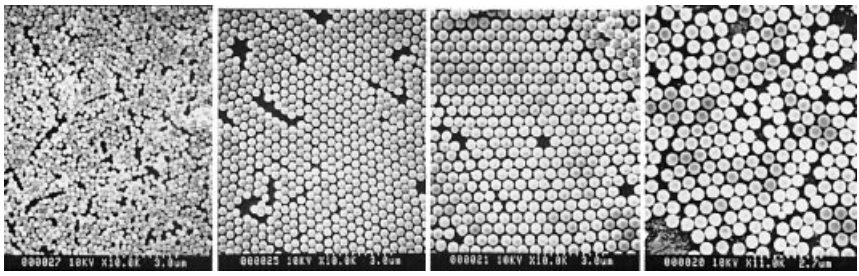
originated in the water phase. Increasing the temperature led to accelerate the decomposition rate of the initiator resulting in the enhancement of the number of polymerization loci as above discussed. In addition, low temperature produced large particle size, whereas small ones were formed at high temperature.

Functionalized poly[NIPMAM] latex particles were also obtained using N-(vinylbenzylimino)-diacetic acid (IDA), a reactive monomer providing ability of immobilization of biomolecules either by covalent binding or by complexation after chelation with metal ions. Such a monomer proved to be very effective in reducing the particle size but the formation of water soluble polymer is significantly enhanced at too a high concentration of IDA[9].

Colloidal properties

Particular attention was paid to the colloidal and physico-chemical properties of the obtained stimuli-responsive particles, especially their particle size and size distribution, the electrokinetic properties, the surface charge density, the colloidal stability behavior as a function of temperature, pH, salinity[10].

Regarding particle size, transmission (TEM) and scanning (SEM) electron microscopy were quite appropriate to reveal the highly narrow size distribution of the latexes as illustrated in the case of poly[NIPMAM] microgels in Figure 3. The same behavior has been observed in the case of batch polymerization of NIPAM/MBA/V50 (2-2'azobisamidinopropane dihydrochloride, V50) as reported by Meunier et al.'[11].



(8 min) (20 min) (50 min) (84 min)

Figure 3: Scanning Electron Microscopy of poly[NIPMAM] particles at various reaction times[7].

AFM in non contact mode has been also used to investigate the morphology of the thermally-sensitive particles as reported by Duracher et al.[5] in the case of cationic amino-containing PS-poly[NIPAM] copolymer latex particles. Two polymerizations processes have been conducted : (i) batch polymerization of styrene, NIPAM using (V50) as initiator and (ii) shot-growth polymerization process (by adding NIPAM, MBA and AEM). As illustrated in Figure 4, the morphology of the two latexes exhibit a raspberry-like structure, that corresponding to the batch reflecting a more compact structure due to the polystyrene core (Figure 4-a), whereas the prepared core-shell particles by the shot- growth process reflects a very heterogeneous and flattened (figure 4-b) surface.

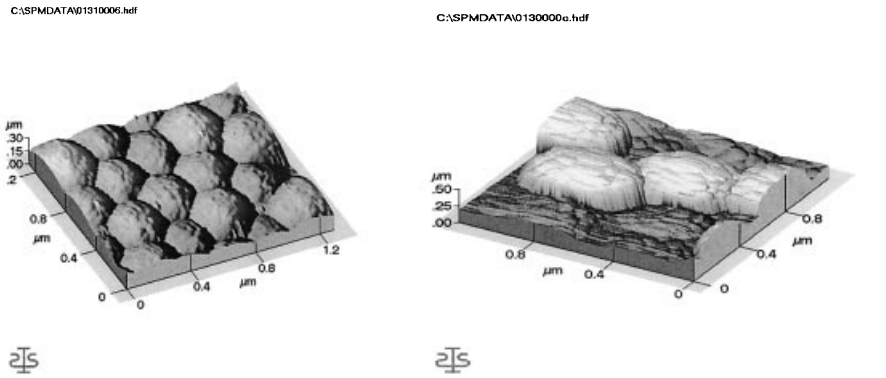


Figure 4: AFM images of (a) batch PS-poly[NIPAM] and (b) shot-growth PS-poly[NIPAM] latex particles[5].

Analysis of the particle size versus temperature appears as an appropriate method for reflecting the thermal sensitivity of the various stimuli-responsive particles. As illustrated in Figure 5, a marked change is generally observed around the LCST of the corresponding (i.e. 32°C for poly[NIPAM] and 45°C for poly[NIPMAM]). The particle size decreases with increasing the temperature due to the shrinkage of the thermally-sensitive part of the particle induced by dehydration of the swollen polymer. The phenomenon is found more significant for the core-shell latexes prepared using shot growth process, then exhibiting a large thickness layer and for the poly[NIPMAM] microgel. Such a dehydration process leads to a coil-globule transition as largely reported by different authors. One important feature in the case of latex

particles is that the transition occurs over a broad range of temperature (order of 5 to 10°C) instead of about 0.5°C in the case of thermally-sensitive polymers in free solution. In addition, the swelling ability of these particles can be quantified by measuring the volume ratio $(D_{\text{at } 20°C}/D_{\text{at } 50°C})^3$.

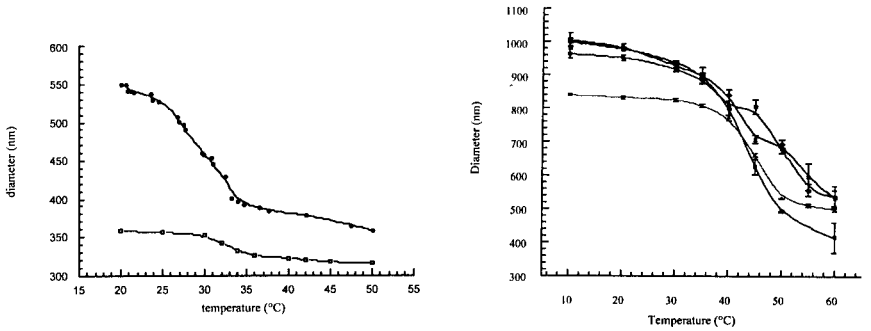


Figure 5: Temperature dependence of the hydrodynamic size of (a) two core-shell (PS (core)-poly[NIPAM] (shell))latex particles[10,13] and (b) various poly[NIPMAM] hydrogel particles[14] at 1mM NaCl.

The electrophoretic mobility, which also gives an indication of the magnitude of the surface charge density, has been investigated as a function of temperature. A general tendency is that the electrophoretic mobility increased when raising the temperature. In fact, when the temperature increases below to above the volume phase transition temperature, the hydrodynamic diameter decreases (by shrinkage of the particle), resulting in an increase in the surface charged density and consequently an increase in the electrophoretic mobility for a constant salinity and pH[5]. As illustrated in Figure 6, such an effect is more significant in the case of core-shell particles having a large hydrogel shell layer (Figure 6-b) and with poly[NIPMAM] microgel particles (Figure 6-a). The effect of salinity on the electrophoretic mobility of thermally-sensitive particles has been scarcely investigated due to the structural complexity of such dispersed systems[12,13].

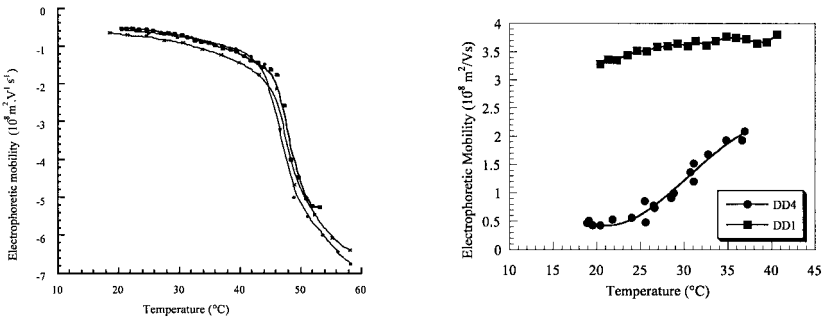


Figure 6: Electrophoretic mobility of thermally sensitive poly[NIPMAM] microgel particles[14] (a) and core-shell (PS (core)-poly[NIPAM] (shell))latex particles[5,10] (b) at pH 6 and 1mM NaCl.

Immobilization of biomolecules onto thermally-sensitive particles

The immobilization of biomolecules onto latex particles can be achieved either via adsorption or chemical attachment. Dealing with the second method is quite suitable with the cationic hydrophilic latex particles for two reasons: i) to use the presence of cationic charges in order to favor the approach of biomolecules with opposite charges, possibly above the VPTT of the polymer; ii) to reduce the adsorption of the biomolecules due to the hydrophilic nature of the interfacial layer (especially below the VPTT).

Such a method was extensively applied in the case of oligonucleotides and more recently to nucleic acids and proteins. The adsorption behavior of these biomolecules onto the stimuli sensitive particles was preliminary investigated in order to further adjust experimental conditions (pH, ionic strength, buffer) avoiding adsorption and to carry out covalent immobilization.

A few works have been devoted to the adsorption of nucleic acids onto thermally sensitive hydrophilic latexes. A pioneer work of Elaissari et al.[15] using RNA (16S and 23 rRNA from Escherichia coli) and positively charged crosslinked poly[NIPAM] microgel particles, highlighted that nucleic acids adsorption was principally governed by electrostatic interaction as revealed by examining the effect of pH (Figure 7-a) and salinity (Figure 7-b). In fact, there

is a decrease of the adsorbed nucleic acid amount when the pH is increased. This behavior was attributed to the decrease of surface cationic groups onto the latex particles causing a reduction of the attractive electrostatic interactions. As expected, the adsorption of nucleic acids was also affected by the operating salinity as illustrated in Figure 7-b. The increase of salinity of the medium also leads to lower the attractive electrostatic interactions between latex particles positively charged and the negatively charged nucleic acids. In addition, the desorption of pre-adsorbed nucleic acid molecules onto thermally-sensitive latex particles has been also investigated[15]. Intuitively, since the adsorption process of nucleic acids is principally governed by electrostatic interactions, the desorption of such polyelectrolytes (DNA, RNA) from cationic thermally-sensitive latex particles should be completed by reducing the attractive electrostatic forces by increasing the pH and the salinity of the medium. The combination of both effects (pH and salinity) was found appropriate to complete the desorption after adsorption at acid pH and low ionic strength as shown in Figure 8.

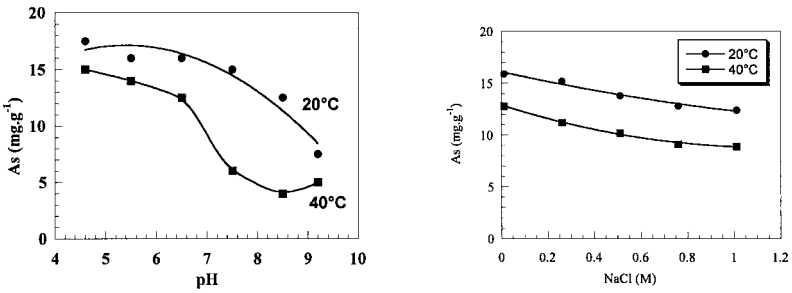


Figure 7: Adsorption of RNA onto cationic poly[NIPAM] latexes[15] as a function of pH and temperature (a) and as a function of ionic strength in phosphate buffer (pH 4.5), adsorption time 2 hours (b).

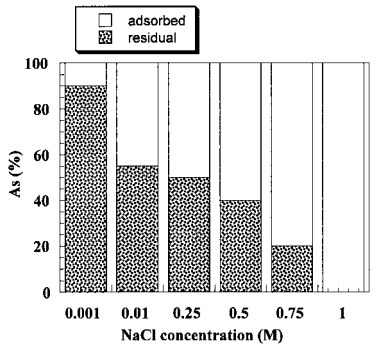


Figure 8: Effect of NaCl concentration on desorption of RNA from cationic poly[NIPAM] latex[15] at pH 9.2, 20 °C. The adsorbed amount of RNA at pH 4.6, 20°C and 10^{-2} M ionic strength was 17 mg/g.

The adsorption of proteins has been largely investigated in the case of plain latex particles, especially with negatively or positively charged polystyrene latexes, however a few works have been dedicated to the case of thermally-sensitive particles. Kawaguchi et al.[16] first reported on the protein adsorption showing that the phenomenon was dependent upon temperature and type of protein. Similar trends were observed in the case of the adsorption of Bovine Serum Albumin (BSA) and a recombinant protein (HIV-1 capsid p24 with molar mass 27500g/mol) onto two core-shell PS-Poly[NIPAM] latexes differing in their hydrophilic layer thickness and charge density (DD4 and DD11). As illustrated in Figure 9 showing the protein adsorbed amount as a function of the temperature, the adsorption drastically increases when temperature crosses the VPTT, with some differences according to the nature of the protein. This behavior has been attributed to the change in the interfacial charge density and the hydrophilic-hydrophobic balance when the temperature was increased. The most driven forces in the adsorption process of proteins onto poly[NIPAM] particles are mainly electrostatic as recently evidenced[17] and below illustrated for BSA and p24 adsorption onto PS/Poly[NIPAM] cationic core shell latex particles as a function of pH (Figure 10) and salt concentration (Figure 11).

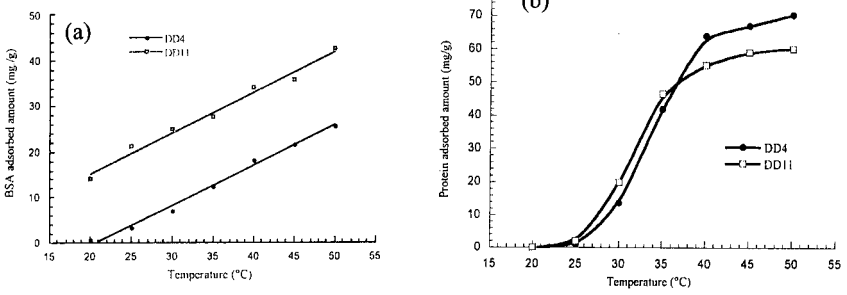


Figure 9: Temperature dependent adsorption of BSA (a) and modified HIV-1 Capsid p24 protein (b) onto core-shell PS-Poly[NIPAM] latex at pH6.1 and 10mM phosphate buffer.

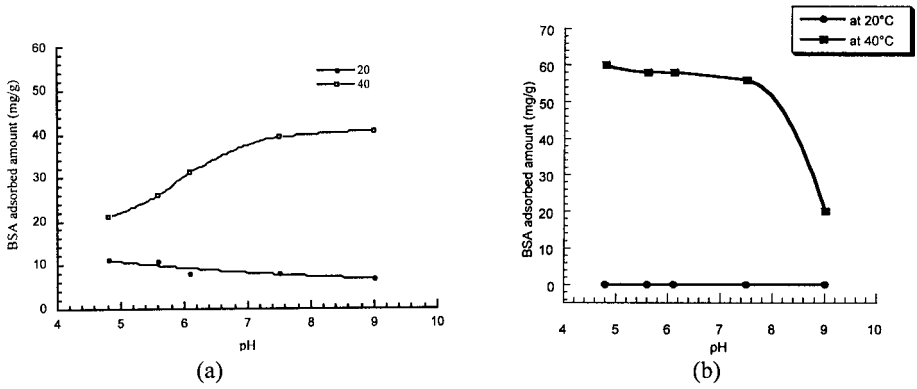


Figure 10: pH dependence adsorption of BSA (a) and modified HIV-1 Capsid p24 protein (b) onto PS-Poly[NIPAM] core-shell latex below and above the VPTT of poly[NIPAM]. Conditions : phosphate buffer at different pH, ionic strength 10mM, adsorption temperature 20°C and 40°C.

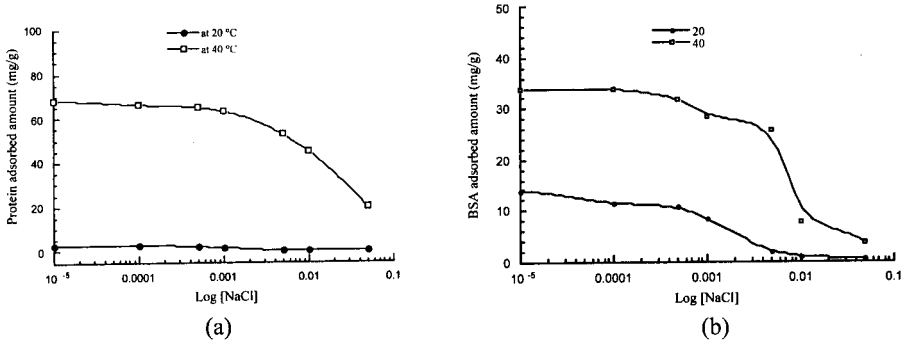
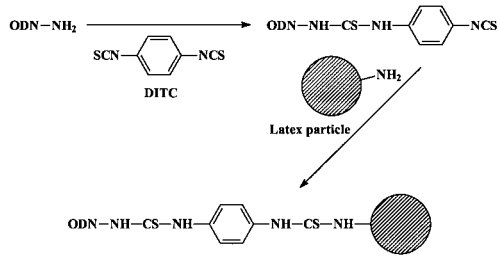


Figure 11: Effect of ionic strength on the adsorption of p24 (a) and BSA (b) onto PS-Poly[NIPAM] core-shell particles at pH 6.1 and 10 mM phosphate buffer.

Taking into account the results reported above in order to favor the covalent immobilization vs physical adsorption, the coupling reaction of biomolecules was generally performed in a basic medium to restore a major part of surface amine groups. For instance, in the case of oligonucleotides (ODN), the covalent immobilization was carried out using a two-step procedure[18] : first the amino-derivatized ODN was activated with an excess of homobifunctional reagent, then the coupling step can take place according to the scheme below described :



Temperature of the reaction medium was maintained at 20°C, ensuring efficient steric stabilization by expanded PNIPAM chains. Ionic strength was found to drastically affect the efficiency of ODN immobilization onto PNIPAM-containing particles. Best covalent binding yields were observed at low salinity.

In the case of p24 protein, an original strategy was conceived for the covalent immobilization onto amino-containing stimuli-responsive particles[19]. It consists in mixing the protein and a reactive copolymer (maleic anydride-alt-vinyl mehtyl ether) above the VPTT for favoring the approach of the entities. The presence of anydride groups allows to bind the p24 (bearing a polylyzine tag) onto the particles. Subsequent lowering of the temperature below the VPTT ensures to desorb the adsorbed proteins.

Biomedical applications

Such stimuli responsive latex particles, especially those based on PNIPAM were used in various applications mostly for diagnostic purposes. One interesting application dealt with the use of amino-containing cationic–ODN conjugates with a view to enhancing the sensitivity of ELOSA tests. Such a genetic test relies on the capture of DNA targets through hybridization with complementary capture ODN immobilized on a solid support. Replacing these ODN probes by formation of self assemblies of ODN-particle conjugates proved to be efficient to increase the capture of DNAs. The concept was successfully demonstrated in the case of the detection of HBV virus[18].

Another application was developed with these particles for the concentration of either nucleic acids or proteins. It was indeed showed that the former can be adsorbed onto cationic hydrophilic particles mainly by electrostatic interactions and desorbed upon raising pH and ionic strength. For proteins, adsorption through electrostatic and hydrophobic forces can be

monitored by raising temperature above the VPTT and inducing the desorption by lowering the temperature and if necessary increasing ionic strength[15].

Finally, the cationic amino-containing particles were used for preparing submicron magnetic particles according to a two-step protocol : i) the adsorption of negatively charged iron oxide nanoparticles onto the oppositely charged latex; ii) the encapsulation of the composite particles by a precipitation polymerization of a mixture of an alkylacrylamide monomer with MBA and a carboxylic monomer (above the VPTT of the polymer). After covalent binding of a specific antibody, the feasibility of the resulting hydrophilic and magnetic particles as solid support in an immunoassay was demonstrated[20].

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