Immobilization of Hexokinase onto Chitosan Decorated Particles†

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Chitosan (CH) decorated polystyrene (PS) particles were synthesized within complexes of CH, a polycation under acid conditions, and tiny amounts of sodium dodecylsulfate (SDS). Particle characterization was performed by means of dynamic light scattering, ζ potential measurements, and transmission electron microscopy. All dispersions were stable in the ionic strength of 2.0 mol L^{-1} NaCl during 2 months. The outstanding colloidal stability was attributed to the presence of a hydrated CH layer around the particles. CH decorated PS particles were attached to atomic force microscopy cantilevers and probed against Si wafers in water and in NaCl 0.01 mol/L. The mean thickness of CH layer amounted to 35 ± 11 and 16 ± 6 nm, when the medium was water and NaCl 0.01 mol/L, respectively. Adsorption isotherm of hexokinase (HK) onto PS/CH particles studied by means of spectrophotometry showed three regions: an initial step; adsorption plateau and multilayer formation. Enzymatic activity of free HK and immobilized HK was monitored by means of spectrophotometry as a function of storing time and reuse. After 3 days, storing HK free in solution dramatically lost its catalytic properties. On the contrary, HK-covered PS/CH particles retained enzymatic activity over 1 month. Moreover, HK-covered PS/CH particles could be reused in the determination of glucose two times consecutively, without losing activity. These interesting findings were discussed in light of the role of water in enzyme conformation.

Introduction

The interest on biomicronano technologies has been growing 10−30% each year.[1] Biosensors belong to one of the most prominent applications of such technologies. A biosensor is an analytical device incorporating a deliberate and intimate combination of a specific biological element that creates a recognition event and a physical element that transduces the recognition event. Enzyme-based biosensors have been largely used in diagnostics, industrial process control, and environmental monitoring.[2] Hexokinase is the first enzyme in the glycolytic pathway, catalyzing the transfer of a phosphoryl group from ATP to glucose to form glucose-6-phosphate and ADP with the release of a proton.[3] Its malfunction has been implicated in a number of diseases in humans.[4] In yeast, two isozymes, PI and PII, are known and they possess 76% overall homology of the amino acid sequence.[5] Hexokinase isozymes exist as dimers of 100 kDa and both of the isozymes share a similar α/β fold, in which the polypeptide chain is distinctly folded into two domains of unequal size, the large and the small domain. These domains are separated by a large cleft forming the active site.[6] The optimum pH for the HK activity is 7.5. Below pH 6.5 it rapidly loses its activity with no enzymatic activity obtained at pH 4.0.[7] Glucose determination with sensors based on immobilized hexokinase (HK) onto solid substrates is potentially interesting for scientific and commercial purposes.[8−11] For instance, Pickup and co-workers reported recently[9] that porous sol−gel based on tetramethylorthosilicate is advantageous for immobilizing HK because it retains its activity and can be used in vivo. Recently, upon adsorbing HK onto silicon wafers its activity was kept even after 48 h storing at room temperature. This outstanding behavior was attributed to specific orientation of HK active site to the solution. A quick survey showed that approximately 30 hexokinase-based sensor patents have been registered in the last 25 years. Despite many advances in developing such biosensors, there is hardly any technology which allows reusing biosensor. One of the biggest challenges in this research field is to provide surfaces, which allow controlling the amount, arrangement and conformation of proteins or enzymes on the surface, so that their functions could be retained.

To our knowledge, emulsion polymerization reactions in complex polyelectrolyte/surfactant have seldom been studied.[12−16] Marie and co-workers[12] obtained stable polystyrene (PS) particles with a very small amount of coagulum using chitosan in combination with cetyltrimethylammonium chloride. Esquena and co-workers[13] obtained stable PS and poly(methyl methacrylate), PMMA, particles using a polyfructose-based surfactant in the polymerization. The synthesis and characterization of hybrid particles of PMMA[14] or PS[15,16] and carboxymethylcellulose (CMC) in complexes formed by CMC and the cationic surfactant, cetyltrimethylammonium bromide (CTAB), have been recently reported. This novel procedure brings the advantage of synthesizing and stabilizing particles with D-glucopyranoside units of CMC on the particle surface in a one-step method using very small amounts of surfactant, a friendly condition for the environment. The present study shows the polymerization of styrene in complexes of sodium dodecylsulfate (SDS), an anionic surfactant, and chitosan (CH), a natural linear

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biopolyaminosaccharide obtained by alkaline deacetylation of chitin, which is the second most abundant polysaccharide next to cellulose.[17−19] CH is a hydrophilic polymer with great potential as biocidal agent[20] for drug delivery due to its biocompatibility, high charge density, nontoxicity, and mucoadhesion.[21] CH microspheres are the most widely studied drug delivery systems for the controlled release of drugs, antibiotics, antihypertensive agents, anticancer agents, proteins, peptide drugs, and vaccines.[21,22] The present study comprises the synthesis of CH decorated PS particles and their characterization by means of dynamic light-scattering, ζ potential measurements, gravimetry, transmission electron microscopy, and adhesion forces measurements. Adsorption behavior of HK onto PS/CH particles was investigated by means of spectrophotometry; last but not least, the enzymatic activity of immobilized HK was monitored by means of spectrophotometry as a function of storing time and reuse.

Experimental Section

Materials. Styrene (S, Aldrich, S497-2), sodium dodecylsulfate (SDS, Aldrich), potassium persulfate ($K_2S_2O_8$, Merck, Germany), and chitosan (CH, Fluka, Switzerland), with nominal mean degree of acetylation (DA) 20% and M_v of 219,500 g mol^{-1}, as determined by means of capillary viscosimetry,[18] were used in the particles synthesis.

Hexokinase type III, from bakers yeast (HK, H5000), β-nicotinamide adenine dinucleotide phosphate sodium salt ($NADP^+$, N0505), adenosine 5′-triphosphate disodium salt (ATP, A3377), β-D(+)Glucose (G5250), glucose-6-phosphate dehydrogenase from bakers yeast (G-6-PDH, G4134), tris-(hydroxymethyl)aminomethane (Tris)-HCl, and $MgCl_2$ were purchased from Sigma and used without any purification. HK solutions were prepared in the concentration range of 0.05−1.00 g L^{-1} in Tris-HCl buffer at pH 7.4 with $MgCl_2$ 10 mmol L^{-1} prior to adsorption onto PS/CH particles.

Interaction between SDS and CH. The interaction between SDS and CH was studied by means of surface tension measurements. First of all solution of CH was prepared in acetic acid 0.10 mol L^{-1} at the concentration of 0.01 g L^{-1}, then SDS was added so that the concentration of SDS varied from 0.001 to 10.00 mmol L^{-1}. As a control experiment, the surface tension of pure SDS was also measured. The surface tension at the air−water interface was measured at 24.5 ± 0.5° C with a Krüss instrument K6 and Du Nouy ring.

Synthesis of CH-Decorated PS Particles. The synthesis of the CH decorated PS particles followed a typical emulsion polymerization recipe,[23] except that instead of water, the medium was solution of CH at the concentration of 0.01 g L^{-1} prepared in 0.1 mol L^{-1} acetic acid. SDS was added to 140 mL of this solution, so that its final concentration amounted to 0.60 mmol L^{-1}. The medium was purged with N_2 during 30 min, while the temperature was brought to 82 ± 2° C. Afterward 0.5 g of $K_2S_2O_8$ (initiator) was added. Two minutes later 10 mL of styrene was added to the system without any particular procedure. The polymerization was carried out under reflux and mechanical stirring (500 rpm). After 2 h the system was cooled to room temperature and dialyzed (dialysis membrane 14000 MW, Viskase Corporation) against water with 4 changes daily during one week, or until the conductivity of dialysis water reached 5 μS/cm. In this process no buffer was used. The dialyzed dispersions presented pH 4 because CH was dissolved in acetic acid, turning CH positively charged to complex with negatively charged SDS monomers. One should notice that chitosan presents pK_a close to 6.8 (see ref 18, pp 204−205). CH decorated PS particles are coded as PS/CH. However, most technological applications require pH near to 7, therefore particle characterization was carried out at pH 6.5.

Particle Characterization. Particles characterization was performed by means of a ZetaPlus- Zeta Potential Analyzer (Brookhaven Instruments Corporation, Holtsville, NY) equipped with a 677 nm laser. The ζ potential value, ζ, was determined from the electrophoretic mobility, μ, in KCl 0.001 mol L^{-1} (an equipment requirement) and Smoluchowski's equation: $\zeta = \mu\eta/\epsilon$, where η is the medium viscosity and ϵ the medium dielectric constant. The particle diameter D was obtained by dynamic light scattering at 90.0°. The dispersions were prepared in 0.001 mol L^{-1} KCl at 3.3×10^{11} particles L^{-1}. The particle size distribution from analysis of quasi-elastic light scattering (QELS) data was performed following well-established mathematical techniques.[24] Transmission electron microscopy (TEM) was performed in a JEM 200C microscope on droplets of dispersions at 3.3×10^{11} particles L^{-1} after drying in the air. A very thin carbon layer was deposited on the dried dispersions prior to the TEM analysis. The solid content and the conversion of monomer into polymer were determined by gravimetry. The mean particle number density (N_p) was calculated considering the particle mean diameter determined by QELS and TEM, solid content in 1.00 mL of dispersion and polymer density as 1.00 g cm^{-3}.

The colloidal stability was tested visually under different conditions: (i) adding 0.3 mol L^{-1} or 2.0 mol L^{-1} NaCl to the stock dispersions at pH 4.0 or (ii) diluting to the half the stocking dispersion at pH 7.4 (Tris-HCl) and adding $MgCl_2$ 10.0 mmol L^{-1}. The latter correspond to the conditions used for adsorption and enzymatic activity experiments.

Adhesion Force Measurements. Multimode Nanoscope IIIa AFM with Picoforce add-on from Veeco/Digital Instruments operating in the force mode was used for the adhesion force measurements. PS/CH particles were glued with epoxy glue (UHU plus) onto the apex of the tipless V-shaped silicon nitride cantilevers (Veeco NP−OW) with the help of a micromanipulator (MMO 203, Narishige Co. Tokyo, Japan) and a Leica Axiotech microscope. The spring constants of the cantilevers (all taken from the same wafer) were determined using the thermal noise method[25] as 0.11 ± 0.02 N/ m. AFM cantilevers with the attached PS/CH particle were mounted in a special fluid cell (Veeco/Digital Instruments) that allows measurements of the interaction forces in liquids. The top part of the liquid cell consists of the cantilever holder made from glass, the sidewalls are formed by an elastic O-ring, and the bottom is given by the sample surface. The cell was filled with about 100μL of water or NaCl 0.01 mol/L solution. Cantilever deflections versus sample position curves were acquired using the AFM software of the manufacturers at a scan rate of 1 Hz. Force measurements were performed at five different locations over every silicon-wafer, with three different probes. About 50 force curves were obtained at each site. Recorded deflection versus piezo position data were converted into force versus distance data using software developed by the Max Planck Institute for Polymer Research. The beginning of the attractive interactions is represented by the jump in event in the approaching curves, while the adhesion force is the value measured at the point of maximum deflection during the colloid probe retraction from the surface. The mean values of jump in distance and adhesion force were determined from a set of at least 150 force versus distance curves taken with three different probes.

Adsorption of Hexokinase onto PS/CH Particles. HK solutions in the concentration range of 0.05 g L^{-1} to 1.50 g L^{-1} in Tris-HCl (0.050 mol/L) buffer and $MgCl_2$ 0.010 mol

L^{-1} were added to dispersions of PS/CH previously diluted in Tris-HCl at the concentration of 3.3×10^{12} particles L^{-1}. After 3 h the mixture was centrifuged at 13 000 rpm for 30 min. The particles were carefully separated from the supernatant. The concentration of free HK in the supernatant was determined by UV-spectrophotometry at 280 nm in a BeckmannCoulter DU600 spectrophotometer and compared to a calibration curve determined previously. The difference between this value and the initial HK concentration yielded the concentration of adsorbed HK. All experiments were carried out at (24 ± 1)° C.

Desorption experiments were carried out by redispersing HK covered PS/CH particles in Tris-HCl (0.050 mol/L) buffer or water, so that N_p was 3.3×10^{12} particles L^{-1}. After 24 h the dispersions were centrifuged at 13 000 rpm for 30 min and the concentration of free HK in the supernatant was determined by UV-spectrophotometry at 280 nm.

Enzymatic Activity of Free and Immobilized Hexokinase. The HK activity assay is based upon the reduction of NADP+ to NADPH, which absorbs at the wavelength of 340 nm, through a coupled reaction with G-6-PDH:

$$\text{D-glucose} + \text{ATP} \xrightarrow{\text{HK}} \text{glucose-6-P} + \text{ADP} \quad (1)$$

$$\text{Glucose-6-P} + \text{NADP}^+ \xrightarrow{\text{G6PDH}} \text{gluconate-6-P} + \text{NADPH} + \text{H}^+ \quad (2)$$

HK activity was determined by spectrophotometry using a Beckmann Coulter DU 640 UV−VIS Spectrophotometer at (24 ± 1)° C by measuring the increase in absorbance at the wavelength (λ) of 340 nm as a function of time, according to the method described by Bergmeyer.[26] Using the Beer−Lambert equation, the amount of NADPH formed can be calculated, considering NADPH molar absorptivity[27] as $\epsilon = 6.22$ $mmol^{-1}$ L cm^{-1} and the optical pathway length as 1 cm.

The final concentrations in Tris-HCl of each reagent mixed in the spectrophotometric cell were the following: $MgCl_2$ 10.0 mmol L^{-1}, G-6-PDH 1.8 mg L^{-1}, $NADP^+$ 0.062 mmol L^{-1}, ATP 1.50 mmol L^{-1} and glucose 50.0 mmol L^{-1}. These reactants were added to the HK-covered PS/CH particles at $N_p = 3.3 \times 10^{12}$ particles L^{-1}, after 1 h the supernatant was taken out and its absorbance was measured. For the activity tests with free enzyme the final volume of 1.0 mL was achieved by adding a HK solution prepared in Tris-HCl, so that the final HK concentration was the corresponding to the adsorbed HK. Solutions of free HK or HK-covered PS/CH particles were stocked during 1 month and the respective enzymatic activity was monitored periodically. In order to check the possibility of reuse, new aliquots of reactants were added to the same HK-covered PS/CH particles. The absorbance of supernatant was measured after 1 h reaction. This procedure has been repeated consecutively four times.

Results and Discussion

Complexes of SDS/CH. The interaction between charged surfactants and oppositely charged polyelectrolytes has been well reported in the literature.[28−33] At constant polyelectrolyte concentration and increasing surfactant concentration, critical surfactant concentrations might be found. The critical aggregation concentration (cac) corresponds to the concentration where the binding of the surfactant to the polyelectrolyte begins. The continuous addition of surfactant leads to polymer saturation with surfactant. Additional surfactant molecules cannot bind to the polyelectrolyte and can therefore lower the surface tension until the formation of pure micelles in the solution at a critical concentration, which corresponds to the critical micellar concentration (cmc). The surfactant forms micelle-like clusters adsorbed to the polyelectrolyte chain, which are smaller than the corresponding free micelles. The interaction depends on the surfactant chain length, on the polyelectrolyte persistence length, on the polyelectrolyte charge density[33] and on the size of the surfactant head group, but it does not depend on the polyelectrolyte molecular weight.[33] In the present system we used commercial CH samples with approximately 922 positively charges per chain, calculated from DA of 20% and M_v of 219,500 g mol^{-1}.

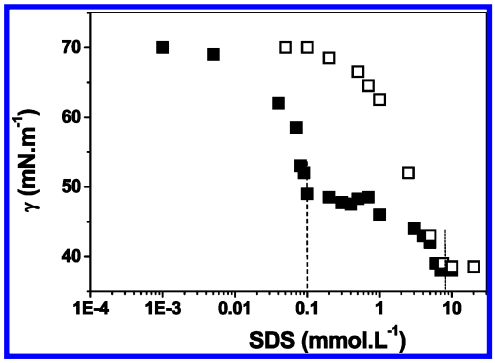


Figure 1. Variation of surface tension as a function of SDS concentration: (■) in the presence of 0.01 g L^{-1} of CH solution prepared in 0.10 mol L^{-1} acetic acid and (□) in water. The dashed lines represent the critical concentrations.

Figure 1 shows surface tension measurements at the air−water interface as a function of SDS concentration in the absence (open squares) and in the presence of 0.01 g L^{-1} CH dissolved in 0.1 mol L^{-1} acetic acid (solid squares). The cmc of pure SDS was determined as 8.0 mmol L^{-1}, which is in agreement with literature value.[34] The surface tension γ decreased from 70.5 ± 0.5 mN m^{-1} (pure water) to 38.5 ± 0.5 mN m^{-1}. In the presence of CH the γ values decreased to 48 ± 1 mN m^{-1} at the SDS concentration of 0.10 mmol L^{-1}. This concentration has been attributed to cac (indicated by dashed line in Figure 1). The γ value remained as 48 ± 1 mN m^{-1} up to 0.70 mmol L^{-1} of SDS. The formation of pure micelles in the solution was evidenced by the γ value of 37 ± 1 mN at 7.0 mmol L^{-1} of SDS (indicated by dashed line in Figure 1). The reduction in the Gibbs free energy ΔG of micelles when bound to a polymer can be calculated by[33,35,36]

$$\Delta G = RT \ln (\text{cac/cmc}) \quad (3)$$

In the present findings the reduction in $-\Delta G$ for the system CH/SDS amounted to 4.4 RT, indicating that the interactions between CH and SDS are strongly favored. For comparison, the magnitude of $-\Delta G$ for the micellization of SDS in the presence of 1.0 g.L^{-1} of poly(vynil pyrrolidone-co-vinyl acetate) is close to 2.3 RT.[37] The complex formation between CH and SDS is driven by attractive electrostatic interactions[38,39] and entropic gain caused by the counter-ions release.[40]

The effect of chain flexibility on the micelle-polyelectrolyte complex was studied by Monte Carlo simulations.[40] Polyelectrolytes are rather stiff due to electrostatic repulsion among their charged segments, but an increase in the ionic strength turns them more flexible due to screening effect. A semiflexible conformation is expected for CH chains in the polymerization medium because there the ionic strength is high due to the presence of cationic surfactant and initiator. Therefore, the polymerization medium might be depicted by the picture of CH chains wrapped around the micelles with the amino groups oriented to the negatively charged heads of SDS. For the

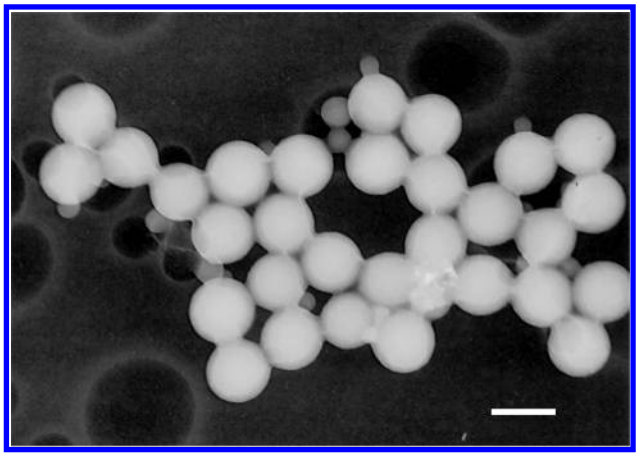


Figure 2. Typical TEM image obtained for dried CH decorated PS particles. The scale bar corresponds to 500 nm.

polymerization of styrene the cac condition was chosen, in order to use SDS/CH complexes as polymerization sites. One should notice that such complexes are seldom used for polymerization process.[14−16] Recently complexes of SDS and poly(ethylene glycol) in microemulsion of pentanol-xylene and water have been applied to grow $BaSO_4$ particles.[41]

Synthesis and Characterization of CH Decorated PS Particles. The use of micelle-polyelectrolyte complexes as polymerization sites has been recently reported.[14−16] This method brings the advantage of synthesizing and stabilizing particles with functional groups on the surface in a one-step method using very small amounts of surfactant, a friendly condition for the environment.

The polymerization of styrene in complexes of SDS/CH (see experimental section for details) led to CH decorated PS particles with mean diameter of (472 ± 40) nm and polydispersity of 0.11 ± 0.03, as determined by QELS measurements. Dried PS/CH particles presented mean diameter of (490 ± 20) nm, as evidenced by TEM images (Figure 3), corroborating with DLS data. The mean ζ potential value, ζ, amounted to − (18 ± 2) mV, indicating that the sulfate groups are partially screened by CH. Generally PS particles synthesized in SDS present mean ζ value of − (60 ± 5) mV. The mean particle number density N_p of (6.6 ± 0.6) × 10^{14} particles L^{-1} was calculated considering the particle mean diameter of 490 nm, polymer density of 1.00 g cm^{-3} and solid content of (40 ± 4) g L^{-1}. In another formulation, the volume of styrene was increased form 10 mL to 20 mL in the reactional medium. The increase in monomer content did not lead to significant changes in mean diameter and mean ζ potential values determined for particles.

The stability of stock dispersions of CH decorated PS particles at pH 4 in the presence of 0.3 and 2.0 mol L^{-1} NaCl was observed during periods of 8 months and 2 months, respectively. Dispersions of CH decorated PS particles are stable in Tris-HCl (0.050 mol/L) buffer (pH 7.4) and $MgCl_2$ 0.010 mol/L over 1 year. This outstanding colloidal stability might be attributed to the hydration of CH chains, which promotes electrosteric effects and a hydration cushion around the particles.[42] Fluffy layers formed by highly hydrated CH chains, which cover the particles, probably avoid the aggregation. Therefore, synthesizing particles in the presence of CH chains also favors a stronger interaction between the particle and the electrosteric stabilizer than that commonly achieved by incorporating the stabilizer after the polymerization.

Recently, the thickness of such hydration layer in carboxymethyl cellulose (CMC) decorated particles has been determined by means of atomic force microscopy as being 20 ± 10 nm.[42]

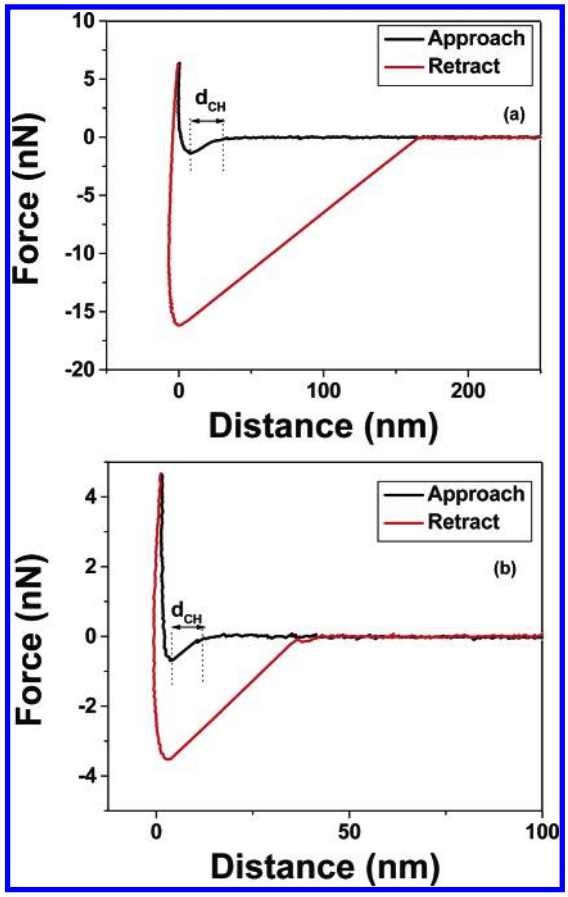


Figure 3. Typical force distance curves obtained for CH decorated PS particles probing onto Si wafers (a) in water and (b) in NaCl 0.01 mol/L NaCl. d_{CH} corresponds to the thickness of CH layer around PS particles.

In order to estimate the mean thickness of CH layers around PS particles, force versus distance curves were taken, where particles attached to cantilevers probed onto Si wafers either in water (pH ∼6.5) or in NaCl 0.01 mol/L (pH ∼6.5). Typical force distance curves obtained from deflection curves (deflections curves not shown, but available under request) under water or NaCl 0.01 mol/L are shown in panels a and b, respectively, of Figure 3. Qualitatively they are similar. When approaching the surface, the cantilever starts to bend downward due to attractive forces and jumps into contact. This jump-in occurs when the gradient of the attractive force between the surface and the CH layer becomes larger than the cantilever spring constant. The onset of the attractive force can be seen as an upper limit for the thickness of the hydrated layer. In water the mean distance corresponding to attractive forces amounted to 35 ± 11 nm and the mean force was 2 ± 1 nN. In NaCl 0.01 mol/L the mean distance corresponding to attractive forces amounted to 16 ± 6 nm and the mean force was 0.6 ± 0.2 nN. Histograms of attractive interaction distance in water and in NaCl 0.01 mol/L shown in panelsa and b, respectively, of Figure 4, evidence larger distances of attractive region in water than in NaCl 0.01 mol/L. These findings indicate that the attraction might be of electrostatic nature, since under pH 6.5 Si wafers are rich in SiO− groups and CH chains carry some positive charges, and salt decreased the mean distance and mean force values due to screening effects. Moreover, CH chains carry many hydroxyl and some carbonyl groups, which can be attracted to Si wafers due to hydrogen bond formation. Therefore, the mean distances determined from force distance curves might be correlated to thickness of CH layer around PS

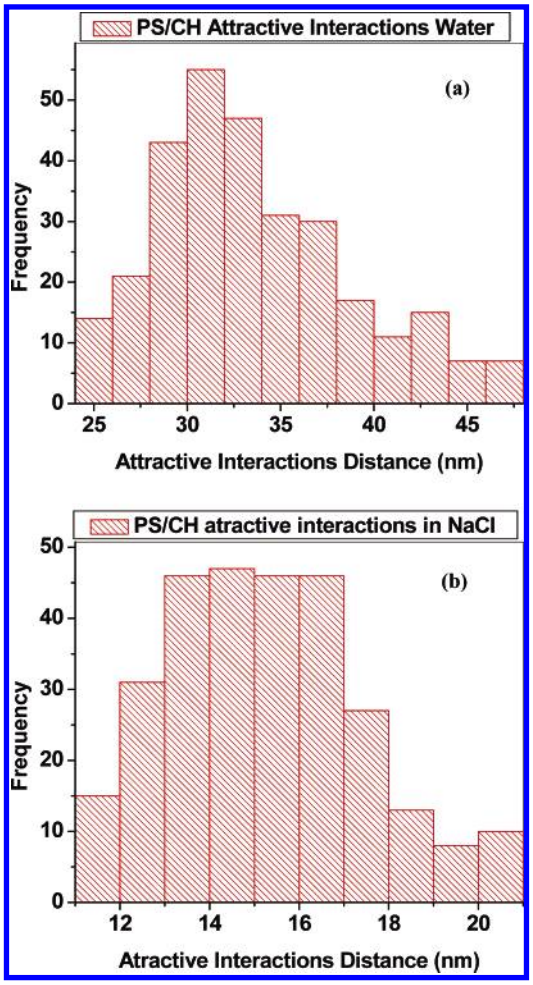


Figure 4. Histograms obtained from force distance curve determined for CH decorated PS particles probing onto Si wafers for the attractive interaction distances (a) in water and (b) in NaCl 0.01 mol/L NaCl.

particles, explaining the high colloidal stability. Another possible effect is some conformational change of CH chains around PS particles upon addition of NaCl 0.010 mol/L to the medium, although CH chains carry just few charges at pH 6.5.

Retract curves obtained in water (Figure 3a) showed mean adhesion force of -15 ± 5 nN, indicating strong adhesion between CH decorated particles and Si wafers surfaces. Upon increasing the ionic strength to NaCl 0.01 mol/L, mean adhesion force decreased to -3 ± 1 nN. These figures confirm screening effects of salt on the interaction between chitosan and Si wafes surfaces.

Synthesis of PS particles have been also carried out using as polymerization sites complexes formed either by SDS and poly(ethylene oxide) or SDS and poly(diallyldimethylammonium chloride). The resulting particles did not show good colloidal stability, they formed aggregates immediately after addition of NaCl 0.1 mol/L. On the contrary, PS particles synthesized in complexes formed by cetyltrimethylammonium bromide (CTAB) and CMC[15] or by SDS and CH showed outstanding colloidal stability. Polymeric particles intimately bound to charged polysaccharide layers as protective coatings serve as stable functional devices.

Adsorption of Hexokinase onto CH-Decorated PS Particles. The adsorption isotherm of hexokinase (HK) onto CH decorated PS particles is presented in Figure 5 and can be divided into three different regions: (i) an initial increase of the adsorbed amount with HK concentration; (ii) an adsorption plateau at $[HK]_{ads} = 0.18 \pm 0.02$ g L^{-1} HK with onset of 0.40

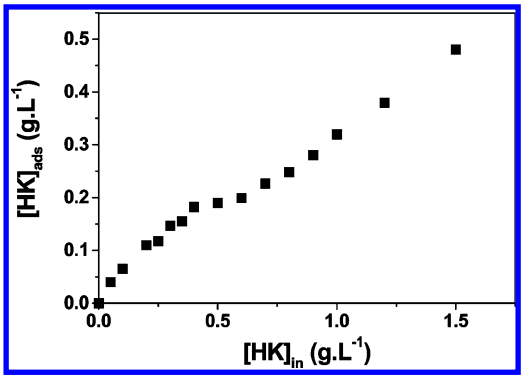


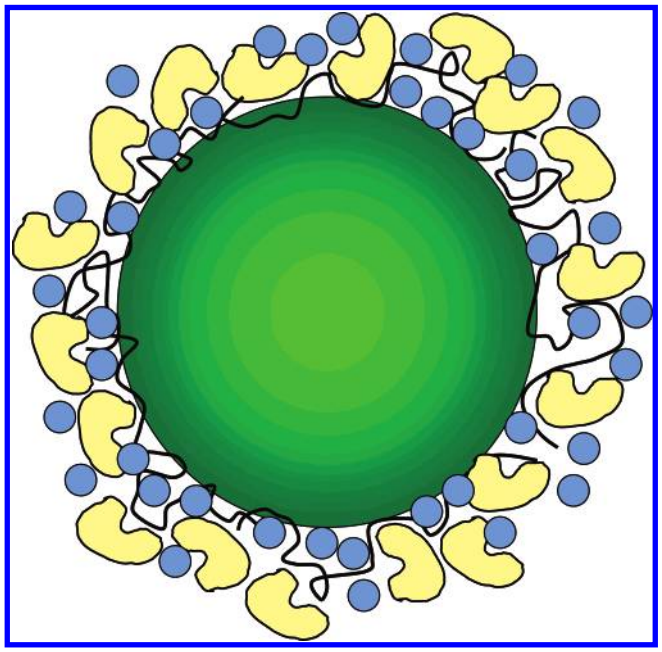
Figure 5. Adsorption isotherm of HK onto CH decorated PS particles.

g L^{-1} of initial HK, $[HK]_{in}$; (iii) a continuous increase of $[HK]_{ads}$ for $[HK]_{in}$ larger than 0.70 g L^{-1}. The initial step is commonly observed in the very dilute range of adsorbate. The adsorbed amount increases up to the substrate saturation. The adsorption plateau often corresponds to the formation of a monolayer. Considering the monomer HK molecular weight of 52 000 g mol^{-1}[15] and the Avogadro's number, at the plateau ($[HK]_{ads} = 0.18$ g L^{-1}) there are approximately 2.1×10^{18} HK molecules L^{-1} adsorbed. Since the mean particle number density N_p used was 3.3×10^{12} particles L^{-1}, at the plateau region there are approximately 6.4×10^5 HK molecules for each PS/CH particle. Taking into account mean radius of PS/CH particles and HK molecules of 245 and 3.2 nm,[6,43,44] the mean surface areas of PS/CH particle and HK can be estimated as ~754 000 and ~128 nm^2, respectively, yielding surface area ratio of ~5900. One should notice that the real particle surface area might be larger than the estimated one, because CH chains might form loops and trains on the particles surfaces, increasing the effective area. Therefore, these rough estimates show that the plateau region might not correspond to HK monolayer adsorbed onto PS/CH particles, due to underestimated surface area. Considering the surface area ratio of ~5900, a monolayer might be expected at $[HK]_{ads} \sim 0.002$ g L^{-1}. The third region in the adsorption isotherm shows continuous HK adsorption with increasing bulk HK concentration, indicating multilayer formation. Desorption experiments showed that HK molecules adsorb irreversibly onto PS/CH particles. Dried HK-covered PS/CH particles presented mean diameter comparable to PS/CH particles, as evidenced by TEM images (not shown). It was expected since HK molecules are much smaller than PS/CH particles.

All adsorption experiments were performed at pH 7.4, which is above the isoelectric point (pI) of hexokinase (5.0).[44] Under such condition HK molecules present predominantly negative charges, while CH is uncharged. Therefore, hydrogen bonding between chitosan NH_2 and OH groups and hydrophilic HK residues might drive the adsorption process. Scheme 1 represents HK (yellow heart-shaped entities) molecules adsorbed onto PS/CH particles. Blue circles depict water molecules bound to HK-covered particles.

Enzymatic Activity of Free and Immobilized Hexokinase. The enzymatic activity of HK was evaluated as NADPH formation as described in the Experimental Section. The catalytic effect of free HK and HK-covered PS/CH particles was studied considering the different regions observed in the adsorption isotherm. Within the initial adsorption stage, $[HK]_{ads}$ was chosen as 0.07 g L^{-1}. For comparison, the concentration of free HK in solution was also set as 0.07 g L^{-1}. NADPH formation in the presence of HK-covered particles was 25% of that observed for free HK after 1 h reaction. Concerning the third adsorption stage, where the adsorbed amount increased continuously with

SCHEME 1: Representation of HK (Yellow Heart-shaped Entities) Molecules Adsorbed onto PS/ CH Particles. Blue Circles Depict Water Molecules Bound to HK-Covered Particles[a]



[a] The sizes of scheme elements are not to scale.

bulk HK concentration, $[HK]_{ads}$ was chosen as 0.34 g L^{-1}. For comparison, the concentration of free HK in solution was also set as 0.34 g L^{-1}. Curiously, NADPH formation in the presence of HK-covered particles was 25% of that observed for free HK. In summary, below and above plateau region, immobilized HK retains only 25% of enzymatic activity.

The most interesting results were found for $[HK]_{ads}$ within the adsorption plateau. HK-covered particles $[HK]_{ads}$ and solution of free HK were freshly prepared at the concentration of 0.17 g L^{-1}. In the presence of HK-covered particles NADPH formation corresponded to approximately 50% of that observed for free HK, under the same conditions (Figure 6). HK-covered particles $[HK]_{ads}$ and solution of free HK were prepared at the concentration of 0.17 g L^{-1}, but only after 24 h the reactants were added and the enzymatic activities were measured. HK-covered particles retained their activity, but free HK lost 50% of activity. All well-established protocols[26] recommend using freshly prepared solutions of HK, since activity loss by aging has been already noticed. Storing HK-covered particles $[HK]_{ads}$ and solution of free HK at the concentration of 0.17 g L^{-1} for longer period of time (up to 1 month), the enzymatic activity of free HK was completely lost after one week (Figure 6). However, after 1 month immobilized HK retained 50% of initial activity and 25% of activity of freshly prepared solution of free HK. This finding is very important because it shows that the enzymatic activity reduction observed upon adsorbing HK onto CH decorated PS particles can be compensated by the possibility of storing HK-covered particle dispersions over long term.

The possibility of consecutive reuse of HK-covered particles for glucose determination was tested with $[HK]_{ads}$ at the concentration of 0.17 g L^{-1}. The enzymatic activity level was kept after two times. After three times usage the activity level fell to approximately 50% of the initial value. Considering the high cost of enzymes based biosensors, these findings show that adsorbing HK onto PS/CH particles brings practical and economical advantages. Enzymatic activity of enzymes depends on their conformation. HK active site involves polar Thr234,

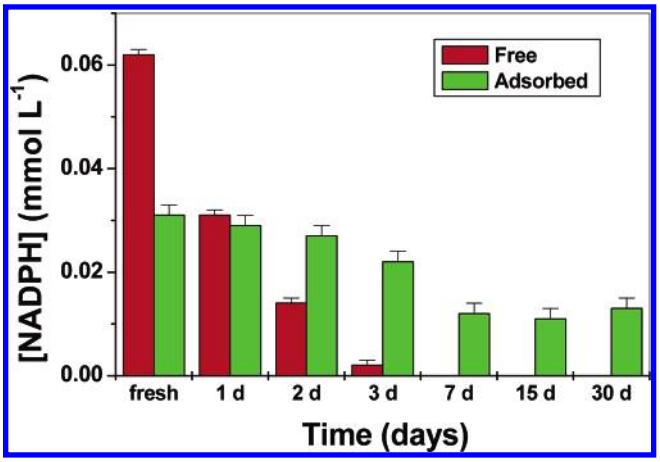


Figure 6. NADPH formation as a function of storing time determined after 1 h of reaction in the presence of stocked solution of free HK (red column) or stocked dispersion of immobilized HK (green column) and proper reagents (see experimental part for details).

Ser419 residues and acidic Asp 211 residue.[6] Upon adsorption, HK might undergo conformational changes, exposing its active site partly to chitosan rich particle surface. Hydrogen bonding between chitosan hydroxyl groups and Thr234, Ser419, Asp 211, and other hydrophilic HK residues might drive the adsorption of HK onto PS/CH particles. HK concentration at the isotherm plateau led to higher enzymatic activity level, than the HK concentrations below and above the plateau. Below the plateau, the enzymatic activity might be lower due to the small amount of adsorbed HK. Above the plateau, multilayer formation of adsorbed HK onto PS/CH particles might increase HK denaturation. Therefore, the enzymatic activity results indicate that at the plateau, a HK monolayer is adsorbed onto PS/CH particles, confirming that the nominal areas are underestimated.

Another issue raised from these results concerns the forces that keep the conformation of immobilized HK on PS/CH, so that the enzymatic activity is retained for long terms and after continuous usage, while free HK lost its activity. A possible explanation for such behavior is based on hydration forces,[45,46] which might be different for free HK and adsorbed HK. Kim and Cremer showed by means of sum frequency generation that water structure at the interface silica/water changes upon adsorbing bovine serum albumin (BSA) as a function of pH. At pH 5.6 the "ice-like" structure was slightly decreased, while the water-like structure showed a substantial reduction in intensity.[47] By lowering pH to 3.8, the intensity of the ice-like mode increased while that of the water-like mode decreased. The activity of phosphatases was preserved under extreme pH, when they were entrapped in sol−gel matrices. The hydration level inside the pores seems to cause the enzyme preservation.[48] Similarly, the activity behavior of creatine phosphokinase (CPK) immobilized onto Si wafers under different pH conditions showed that under alkaline conditions, the contribution of strong hydration forces might weaken the CPK unfolding upon electrostatically driven adsorption.[49] Upon immobilizing HK onto Si wafers, hydrophilic substrates, its activity level was kept even after 48 h storing at room temperature. Such effect has been attributed to specific orientation of HK active site to the solution and to the strong hydration forces,[11] which preserved native HK conformation on the substrate. Hydration forces are not only due to the structure water layers but also to the osmotic effect, which can be expressed as the changes in the number of water molecules associated with the single molecule undergoing conformational changes.[50] Rand and co-workers showed that in solution water activity affects both glucose equilibrium and

HK turnover in such a way that the affinity for glucose increases with decreasing water activity.[51] The preservation of enzymatic activity of HK adsorbed onto PS/CH particles might be associated to the interface hydration, which avoids HK denaturation after usage or upon storing.

As a control experiment we tried to adsorb HK onto bare PS particles, however, they precipitate in the presence of Tris-HCl (0.050 mol/L) and $MgCl_2$ 0.010 mol/L, evidencing the crucial role played by chitosan outermost layer in the colloidal stability of PS/CH particles. Even when colloidal stability is not affected, bare PS surface is rather hydrophobic for enzyme immobilization, because their hydrophobic surfaces often induce denaturation. There are two interesting reports by Caruso and coworkers,[52,53] which show that bare PS particles are not appropriate for enzyme immobilization. They modified PS latex particles using layer-by-layer polyelectrolyte adsorption technique to facilitate subsequent enzyme adsorption. Four alternating poly(allylamine hydrochloride), PAH, and poly(styrenesulfonate), PSS, layers were deposited onto the PS lattices (first layer PAH), resulting in negatively charged particles with PSS as the outermost layer. The enzyme beta glucosidase (beta-GLS)[52] or glucose oxidase[53] was then adsorbed onto the outermost PSS layer. The enzyme multilayer-coated particles were enzymatically active, with the general finding that the total activity of the particles increased with increasing enzyme layer number.

Conclusions

Stable CH decorated particles of PS were polymerized in complexes of SDS (in very small amounts) and CH chains. The outstanding colloidal stability was attributed to the presence of thick CH hydrated layers surrounding the particles, which are strongly bound to the particle surfaces. Adsorbing HK onto PS/CH particles led to glucose responsive particles, which can be stocked for long terms and can be re-used at least three times. Improved preservation of enzyme activity on PS/CH can also be attributed to the presence of CH hydrated layers around the particles (schematically depicted in Scheme 1). From the practical and economical point of view the activity loss of 50% in comparison to the free HK molecules is compensated by the unique possibility of storing and re-using HK-covered particles.

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