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ORIGINAL CONTRIBUTION

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Synthesis, characterization and potential biomedical applications of *N*-succinimidyl ester functionalized, polypyrrole-coated polystyrene latex particles

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Abstract *N*-Succinimidyl ester functionalized polypyrrole-coated polystyrene latex particles (PS_E-PPyNSE) were prepared by the in situ copolymerization of pyrrole and the active ester-functionalized pyrrole (pyrrole-NSE) in the presence of polystyrene latex particles. Polystyrene microspheres were prepared by emulsion polymerization (PS_E) leading to particles having a diameter of 450 nm. These PS_E particles were precoated with poly(*N*-vinylpyrrolidone) prior to the in situ copolymerization of pyrrole and pyrrole-NSE. The initial comonomer concentration fractions were 25/75, 50/50 and 75/25 for pyrrole and pyrrole-NSE, respectively. The PPy-coated PS_E particles were characterized in terms of morphology, particle size, electrophoretic mobility and chemical composition. The study of morphology by means of scanning electron microscopy showed roughening of the underlying PS_E particles owing to the addition of PPyNSE, the overlayer thickness of which was estimated to be around 7 nm. Moreover, loading PPyNSE overlayers resulted in a shift of the electrophoretic mobility from −5.31 μm cm/V s to a very small but positive value (0.082–0.112 μm cm/V s). X-ray photoelectron spectroscopy and IR spectroscopy permitted the detection of pyrrole-NSE repeat units at the surface indicating that pyrrole and pyrrole-NSE did indeed copolymerize. The PS_E-PPyNSE particles were further evaluated as bioadsorbents of human serum albumin used as a test protein. For this study, PS_E-$PPyNSE_{50}$ particles, synthesized from a comonomer feed ratio of 50/50 in pyrrole/pyrrole-NSE, were used and were shown to attach efficiently human serum albumin macromolecules with a maximum amount of 0.2 mg m^{-2}.

Keywords Polypyrrole · Polystyrene · Latex particles · Surface functionalization · Protein

Introduction

Over the last two decades inherently conducting polymers (ICPs) have attracted a great deal of interest owing to their remarkable physical and chemical properties, such as inherent conductivity, redox, and acid–base properties [1]. Polypyrrole (PPy) is one of the most studied ICPs because it offers reasonably high conductivity and has fairly good environmental stability with regard to air and water. In addition, it is easily synthesized in high yield via oxidative polymerization at room temperature in various common solvents, including water [2]. However, PPy is invariably obtained as a black precipitate which is insoluble in all common solvents.

Since this material is not thermoplastic, it suffers from very limited processability. Nevertheless, recent progress in the synthesis of conducting polymers showed the possibility to prepare, under certain conditions, soluble forms of PPy in either water or organic solvents [3].

Preparation of colloidal dispersions of conducting polymers is originally been sought for improving processability of PPy [4, 5, 6, 7, 8, 9]. These colloids are prepared via dispersion polymerization, using a suitable water-soluble polymer such as poly(*N*-vinylpyrrolidone) (PNVP) [8], poly(vinyl alcohol) [4], poly(ethylene oxide) [7] and poly(2-vinyl pyridine-*co*-butyl methacrylate) [9]. These particles have a conducting polymer core surrounded by an outer layer of adsorbed or chemisorbed, solvated water-soluble polymer.

Another way to prepare conducting polymer particles is to use inorganic oxide substrates for the deposition of conducting polymers [10]. For example, Armes and coworkers [11, 12] discovered a simple route to conducting polymer–silica nanocomposite particles based on the oxidative polymerization of aniline, pyrrole and 3,4-ethylenedioxythiophene, in the presence of ultrafine (20 nm) silica particles.

With regard to the present work, Yassar et al. [13] have described the synthesis of PPy-coated polystyrene (PS) latex by the polymerization of pyrrole in the presence of 0.1 μm PS particles bearing surface sulfonic or carboxylic groups. These negatively charged groups counterbalanced the positive charges from the PPy backbone. Although the approach of Yassar et al. met some criticisms concerning the colloidal stability of the end particles [14], it nevertheless paved the way towards the general method of thin ICP coatings onto preformed latex particles resulting in "core–shell" microspheres [15, 16, 17, 18]. Barthet and coworkers [19, 20], on the one hand, and Khan and coworkers [21, 22], on the other hand, prepared and characterized micrometer-sized PS latex particles coated with polyaniline and poly(3,4-ethylenedioxythiophene), respectively.

Although conducting polymer particles were intended for overcoming processability problems, it soon became clear, however, that, for example, PPy particles can be suitable carriers for the immobilization of proteins in view of developing various diagnostic assays [23, 24]. The intense optical absorbance of PPy is a valuable property for application of these materials in medical diagnostics. Moreover, the facile preparation of PPy in aqueous media and its surface modification by various specific functional groups makes this polymer particularly suitable for the covalent attachment of proteins. In this regard, the basic requirements that latex particles must fulfill for diagnostic assay applications is the ability to adsorb or covalently immobilize proteins. This can be achieved with microspheres bearing specific groups that bind proteins [25].

Tarcha et al. [23] developed methods for the surface derivatization of poly(vinyl alcohol)-stabilized PPy particles in organic solvents such as *N*-methylpyrrolidone. PPy latex particles were acetylated in*N*-methylpyrrolidone by using bromoacetyl bromide. The bromoacetylated latex particles could be converted into latex particles with carboxylic or amino groups in reaction with thioacetic acid and triethylene tetramine. Latexes obtained in this way were suitable for the covalent immobilization of proteins and the development of diagnostic assays for the human hormone chorionic gonadotropin, HIV antibody, and hepatitis B surface antigen. However, multistep procedures and several transfers between aqueous and nonaqueous solvents are the disadvantages of this method.

Miksa and Slomkowski [26] elaborated a convenient method for synthesis of PPy particles with aldehyde functional groups. These particles were obtained by coating PPy microspheres with the polyacrolein ad-layer formed during polymerization of acrolein in the presence of PPy microspheres. These particles were used for covalent immobilization of human serum albumin (HSA) and γ-globulins. Unfortunately, coating with polyacrolein significantly reduced the conductivity of these microspheres.

The aim of this work is the synthesis of novel PPy-coated PS latex particles bearing surface reactive groups towards proteins. The reactive coatings consist of copolymers of pyrrole and *N*-alkyl substituted pyrrole with *N*-succinimidyl ester (NSE) as easily replaceable leaving groups at the alkyl chain end.

The rationale for choosing the NSE group is that it is known, from peptide chemistry, as an activated ester to react readily with amines under very mild conditions to form the corresponding amides in high yields (Fig. 1) [27].

The latex particles of PPy-coated PS microspheres that had been prepared by emulsion polymerization

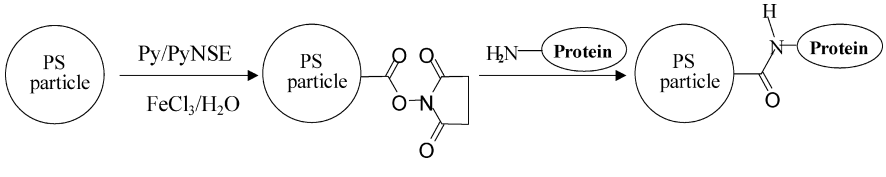


Fig. 1 Schematic representation of the synthesis and the reaction with proteins of polypyrrole(*PPy*)-coated, poly(*N*-vinylpyrrolidone) (*PNVP*)-stabilized polystyrene (*PS*) latex particles bearing surface reactive *N*-succinimidyl ester (*NSE*) groups

(PS_E) were characterized by scanning electron microscopy (SEM), electrophoretic mobility measurements, X-ray photoelectron spectroscopy (XPS) and Fourier transform (FT) IR spectroscopy. Selected batches of particles were further incubated with proteins in order to determine the ability of the functionalized PPy-coated latex particles to attach proteins.

Experimental

Materials

Styrene (Aldrich) was purified by passing it through a column of activated neutral alumina. Potassium persulfate and PNVP (manufacter's nominal molecular weight 360,000) were obtained from Aldrich and were used without further purification. Pyrrole (Fluka) was purified by passing it through a column of activated basic alumina (Acros) prior to use. 1-(2-Cyanoethyl)pyrrole (Acros) and $FeCl_3{\cdot}6H_2O$ oxidant (Aldrich) were used without further purification.

Monomer synthesis

The conversion of the 1-(2-cyanoethyl)pyrrole to the carboxylic acid was accomplished with good overall yields (70%). The active ester-functionalized pyrrole **2** was obtained directly from the carboxylic acid form by the reaction with *N*-hydroxysuccinimide (NHS, Acros) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC, Sigma) with 75% yield (Fig. 2).

Basically, 0.863 NHS and 1.917 g EDC were dissolved in 50 ml distilled water, then 0.7 g of 1-(2-carboxyethylpyrrole) was added to the mixture. The reaction took place at room temperature and was almost instantaneous; however, it was maintained for 30 min. The white precipitate was collected by filtration, washed with copious amounts of distilled water and dried under vacuum.

The structure of the product was confirmed by the melting point (159 °C) and ^{1}H NMR. ^{1}H NMR ($CDCl_3$, δ): 2,85 (m, 4H), 3.08 (t, 2H), 4.35 (t, 2H), 6.17 (t, 2H), 6.70 (t, 2H).

PS latex synthesis

Anionic PS latex, which provides the core particles, was prepared by the batch polymerization method. For this purpose, 20 g of styrene, 200 g of distilled water and 0.65 g of $K_2S_2O_8$ were added to a 250-cm^3 container and the mixture was purged with nitrogen to eliminate oxygen. The solution was stirred at 75 °C for 28 h. Traces of unreacted styrene were removed by steam-stripping. The microspheres were then isolated and purified by repeated centrifugation/redispersion cycles using deionized water. These PS particles are abbreviated PS_E.

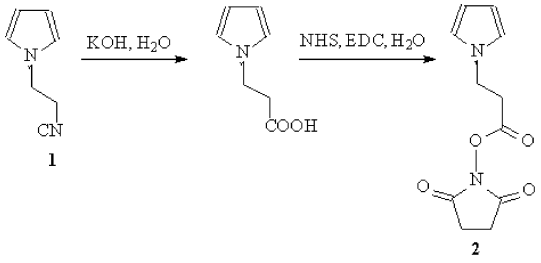


Fig. 2 Preparation of NSE functionalized pyrrole by esterification of 1-(2-carboxyethylpyrrole)

PPy coating protocol

The coating procedure consists of the in situ copolymerization of the monomers in the presence of PS latex. **1** and **2** were premixed in 75:25 (6.5×10^{-3} mol:2.2×10^{-3} mol), 50:50 (4.5×10^{-3} mol each) and 25:75 (2.2×10^{-3} mol:6.7×10^{-3} mol) molar ratios. This comonomer mixture was added to a vigorously stirred solution containing 1 g of dry weight of PS_E, $FeCl_3$ (1.8 g) and PNVP (0.2 g). The solution was stirred at 75 °C for 24 h. The resulting colloidal particles were isolated by five centrifugation/redispersion cycles and redispersed in deionized water.

The PPy-coated PS_E particles (precoated with PNVP) are abbreviated PS_E-$PPyNSE_x$, where $PPyNSE_x$ indicates the PPy functionalized with NSE obtained with an initial molar fraction x ($x = 25$, 50 or 75%).

Immobilization of HSA on particles

Phosphate-buffered saline (PBS, 10 mM phosphate, 138 mM NaCl, and 2.7 mM KCl) was prepared in distilled, deionized water. Immobilization of HSA (Sigma, Cohn fractions V, used as received) was carried out by gently mixing the protein solution in the PBS (pH 7.4) with latex in aqueous suspension. The amount of immobilized protein was evaluated from the protein concentration in solution (before incubation with latex) and in the supernatant after incubation followed by latex isolation by centrifugation. Protein concentrations were determined using Bradford reagent (Sigma) protein assay [28] by measuring the absorption at 596 nm using a Hewlett-Packard 4852A UV spectrometer equipped with a diode array detector.

Analytical techniques

The particle morphology and size (D_n) were characterized by SEM using a JEOL 5500LV instrument operating at 30 kV.

The electrophoretic mobility of the latex particles was measured using a Zetasizer 3000 HSa, from Malvern Instruments. NaCl (10^{-3} M) distilled water solutions were used for mobility measurements.

FTIR spectra of PS_E particles and PS_E-$PPyNSE_x$ particles (KBr disks) were recorded using a Nicolet Magna 550 series II instrument. The spectra were typically averaged over 20 scans at 4 cm^{-1} resolution.

^{1}H NMR experiments were performed at 250 MHz using a Bruker AC 200 spectrometer. The chemical shifts (δ) are given relative to external tetramethylsilane (0 ppm).

XPS measurements were performed using a Surface Science Instrument spectrometer, model SSX 100, equipped with a monochromatic Al Kα X-ray source (1,486.6 eV). The X-ray spot size was 1,000 μm. The pass energy was set at 150 and 100 eV for the survey and the narrow scans, respectively. The step size was 1.12 eV for the survey spectrum and 0.096 eV for the high-resolution spectra. Charge compensation was achieved with a flood gun of 3 eV electrons and a nickel grid mounted 1 mm above the sample surface. The pressure in the analysis chamber was around 5×10^{-9} mbar. Data processing was achieved with Winspec software, kindly supplied by the Laboratoire Interdisciplinaire de Spectroscopie d'Electrons (Namur, Belgium). Spectral calibration was determined by setting the N 1*s* component at 399.7 eV, a reference value for PPy [29] and close to that of PNVP [30].

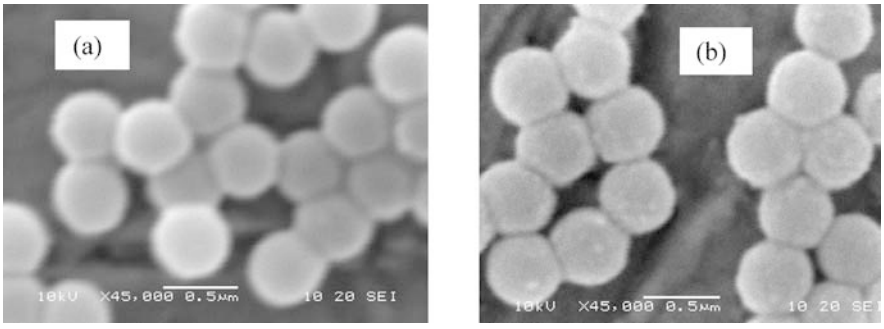


Fig. 3 Scanning electron microscopy pictures of **a** uncoated PS_E and **b** PS_E-$PPyNSE_{75}$

The surface composition was determined using the manufacturer's sensitivity factors. The fractional concentration of a particular element A (%A) was computed using

$$\%A = \frac{(I_A/s_A)}{\sum (I_n/s_n)} \times 100,$$

where I_n and s_n are the integrated peak areas and the sensitivity factors, respectively.

Results and discussion

Scanning electron microscopy

Scanning electron micrographs of the latex particles are displayed in Fig. 3. All particles are spherical in shape. The diameter distribution function of particles is shown in Fig. 4. It is worth noting that the particles have a quasi-monodisperse size distribution and that the number-average particle diameter increases from around 450 nm for PS_E particles to around 465 nm for PS_E-$PPyNSE_{75}$ particles. The difference between these two diameters (15 nm) indicates that the conductive overlayer has a thickness of around 7 nm. However, one has to remember that the accuracy of this evaluation is rather poor since the diameters of the particles are measured with uncertainty around 10 nm. Concerning the surface of the latex particles, one can see that the surface of PS_E particles roughens after being coated with a $PPyNSE_x$ overlayer. It is well known that electrochemically prepared PPy films have a cauliflower morphology when they get thick [31, 32]; this is the morphology of bulk powder PPy [33]. The modification in the surface morphology and roughness of the PS_E latex particles observed in our case is in line with previous results published by Lascelles and Armes [34]. Nevertheless, the surface of PS_E-PPyNSE does not exhibit large globular dots, similar to what was observed for high mass loadings of PPy (25.1%) [19].

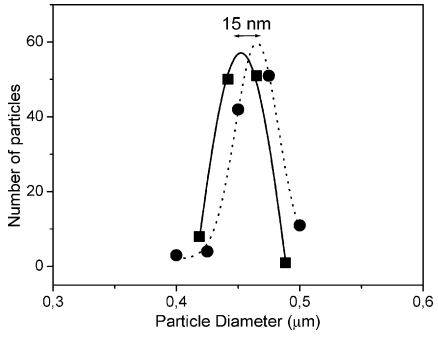


Fig. 4 Size distribution curves of uncoated PS_E (*full line*) and PS_E-$PPyNSE_{75}$ (*dotted line*)

SEM can be used as a semiquantitative method for the determination of the mass fraction of the three PPyNSE copolymers. Indeed, assuming a uniform coating of $PPyNSE_x$ at the surface of monodisperse PS_E particles, one can relate the thickness x of the conducting copolymer overlayer to the main fraction of the PS_E core and the conducting shell using

$$x = r\left\{\left[\left(\frac{M_2\rho_1}{M_1\rho_2}\right)+1\right]^{1/3} - 1\right\}, \quad (1)$$

where M is the mass fraction, ρ the density and r the radius of the core.[1] Subscripts 1 and 2 refer to PS_E and $PPyNSE_x$, respectively. One can rewrite Eq. (1) as

$$\frac{M_2}{M_1} = \left(\frac{\rho_2}{\rho_1}\right)\left[\left(\frac{x+r}{r}\right)^3 - 1\right], \quad (2)$$

where x is estimated from the difference in radii between the uncoated PS_E core and the $PPyNSE_x$ particles. Combining the calculated M_2/M_1 ratio with $M_2 + M_1 = 1$, one can deduce the mass fraction of $PPyNSE_x$ (Table 1), which is around 10%. This value is consistent with the morphology of the three PS_E-$PPyNSE_x$ particles, thus confirming the close relationship between the morphology and mass loading of PPy

[1]The assumed density of PPyNSE is based on the values of 1.53 and 1.45 for PPy and poly(pyrrole-3-acetic acid)[34], respectively. Since the pendent NSE group is much larger than the COOH group, it is in principle expected that pure PPyNSE (100% pyrrole-NSE repeat units) would have a lower density than 1.45. However, since we are dealing with a copolymer of pyrrole and pyrrole-NSE, it follows that the density of the conducting polymer is between 1.4 and 1.53; hence, the assumed value is of 1.5. For PS, the density is 1.05 [35]

Table 1 Properties of uncoated and *N*-succinimidyl ester functionalized polypyrrole (*PPyNSE*)-coated polystyrene (PS_E) latex particles

Latex particles	$PPyNSE_x$ mass fraction[a]	D_n(nm)[b]	Electrophoretic mobility (μm cm/V s)
PS_E	0	450	−5.31[c]
PS_E-$PPyNSE_{25}$	0.089	460	0.112
PS_E-$PPyNSE_{50}$	0.089	460	0.112
PS_E-$PPyNSE_{75}$	0.128	465	0.082

[a]Estimated from scanning electron microscopy and assuming a uniform copolymer coating
[b]Estimated from scanning electron microscopy
[c]Determined for PS_E prior to precoating with poly(*N*-vinylpyrrolidone)

that was observed by Lascelles and Armes for unfunctionalized PPy-coated micrometer-sized PS_E latex particles [34].

Measurements of electrophoretic mobility

Characteristics of PS_E-$PPyNSE_x$ and the reference PS_E particles are reported in Table 1, namely, the number-average diameter, the PS_E-$PPyNSE_x$ mass loading and the electrophoretic mobility.

Unfortunately, for PS_E-$PPyNSE_x$, on the basis of electrophoretic mobility measurements, we could not evaluate either surface charge or the ζ-potential, since for conductive particles (it is reasonable to assume that owing to the $PPyNSE_x$ adlayer the PS_E-$PPyNSE_x$ particles are conductive) knowledge of the relative conductivity of the particles and of the continuous medium is needed for determination of the ζ-potential. Nevertheless, since the charges of a solid particle are not fully compensated by bound counterions it is reasonable to assume that electrophoretic mobility measurements, even for conducting particles, provide information on the sign of this charge. Therefore, we could state that the observed difference in the sign of the electrophoretic mobility of PS_E and PS_E-$PPyNSE_x$ particles is in concordance with the formation of the overlayer of the positively charged PPy chains on top of the negatively charged PS_E particles.

Surface analysis by XPS

Survey spectra of PS-$PPyNSE_{25}$ and PS-$PPyNSE_{50}$ are depicted in Fig. 5. The main peaks, C 1*s*, N 1*s* and O 1*s*, are centered at 285, 400 and 532 eV, respectively. In addition, one can observe a very tiny Cl 2*p* peak at 198 eV. The Si 2*p* and Si 2*s* peaks observed at 103 and 152 eV, respectively, are due to some occasional (sometimes unavoidable) contamination from the glass vessel used to prepare the latex particles.

On going from the PS_E-$PPyNSE_{25}$ to PS_E-$PPyNSE_{50}$ particle surfaces, there is a clear increase in the O 1*s* relative intensity. In contrast, the N 1*s*peak intensity slightly decreases. These trends are in line with the chemical structure of pyrrole-NSE in terms of oxygen and nitrogen heteroatoms in comparison with the unfunctionalized pyrrole.

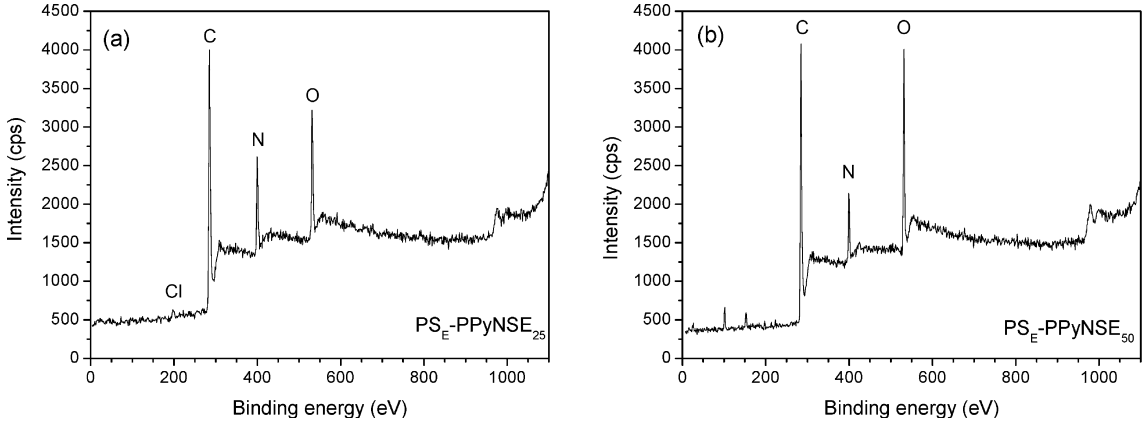
Though XPS is a very surface specific technique, the narrow-region spectra have a very complex structure and it is difficult from this surface analysis to firmly distinguish the pyrrole-NSE C 1*s* components from those of PNVP, for example.

In this regard, FTIR analysis brings complementary results that support the incorporation of the functionalized comonomer in the conducting polymer layer surrounding the PS_E particles, as outlined below.

FTIR analysis

The FTIR spectrum (Fig. 6) of the uncoated PS_E latex is typical of PS with an additional weak feature at

Fig. 5 X-ray photoelectron spectroscopy survey scans of **a** PS-$PPyNSE_{25}$ and **b** PS-$PPyNSE_{50}$ latexes



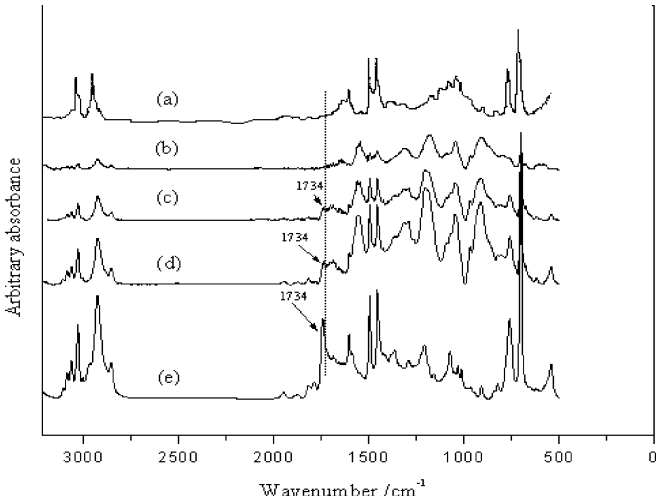


Fig. 6 Fourier transform (*FT*) IR spectra of PS_E (precoated with PNVP) (*a*), PS_E-PPy (*b*), PS_E-$PPyNSE_{25}$ (*c*), PS_E-$PPyNSE_{50}$ (*d*) and PS_E-$PPyNSE_{75}$ (*e*) latex particles

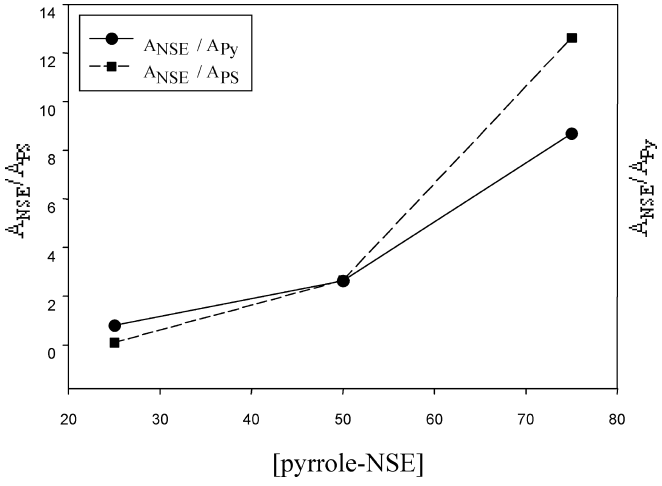


Fig. 7 A_{NSE}/A_{Py} and A_{NSE}/A_{PS} (determined by FTIR spectroscopy) versus the initial fraction of pyrrole-NSE comonomer. A_{NSE}/A_{py} and A_{NSE}/A_{PS} stand for a measure of the comonomer ratios in the conducting coating and a measure of the pyrrole-NSE fraction in the polypyrrole-coated PS_E particles, respectively

1660 cm^{-1} attributable to the PNVP stabilizer. The $PPyNSE_x$-coated PS_E latex exhibits several additional strong bands due to the conducting polymer overlayer and centered at 1559, 1280, 1160 and 900 cm^{-1}. These features are assigned to C–C stretching vibrations in the pyrrole ring, C–H or C–N in-plane deformation modes, N–H in-plane deformation vibrations and C–C out-of-plane ring vibration deformation, respectively. The peak centered at 1734 cm^{-1} is obviously the most important one that arises from the conducting copolymer overlayer and is unambiguously due to the ester C=O stretching of the ester succinimidyl groups. No such feature can be observed in the FTIR spectra of PS_E and PS_E-PPy. It is noteworthy that the intensity of this peak increases monotonically as the proportion of pyrrole-NSE in the comonomer feed ratio is increased. The spectra displayed in Fig. 6 thus provide qualitative evidence for the copolymerization of pyrrole-NSE and pyrrole at the surface of PS_E particles. More importantly, the FTIR analysis clearly shows that the NSE functions have withstood the chemical oxidation conditions and remained intact.

The combination of the peak area of the signal centered at 1734 cm^{-1} (A_{NSE}) (fingerprint of pyrrole-NSE) with that centered at 1448 cm^{-1} and due to PS_E (A_{PS}), can be used as a quantitative measure of the incorporation of pyrrole-NSE repeat units at the surface of the PS_E-$PPyNSE_x$ latex particles. Similarly, from the determination of the peak area of the pyrrole-NSE signal relative to that centered at 1160 cm^{-1} and due to the –N–H vibration of unfunctionalized PPy (A_{Py}), one can also deduce a measure of the fraction of pyrrole-NSE repeat units at the surface relative to that of unfunctionalized pyrrole. Plots of A_{NSE}/A_{Py} and A_{NSE}/A_{PS} are depicted in Fig. 7 as a function of the initial pyrrole-NSE fraction prior to copolymerization in the presence of the PS_E particles. Obviously, pyrrole-NSE repeat units are loaded at the surface in greater amounts as a function of the initial fraction, and similarly the pyrrole-NSE/pyrrole repeat unit ratio increases with that of the initial fraction of monomers prior to copolymerization.

Protein attachment

The PS_E-PPyNSE particles were further evaluated as bioadsorbents of HSA. For this study, we focused on the PS_E-$PPyNSE_{50}$ particles which are supposed to provide a reasonable number of reactive groups in order to immobilize proteins covalently. The dependence of the surface concentration of the attached protein, (Γ), on the concentration of protein in solution was studied in a series of experiments with a constant concentration of latexes (3 g l^{-1}) and different initial concentrations of protein (0–0.4 g/l^{-1}). The incubation time was 20 h. The graph plotted in Fig. 8 was determined by the depletion method, which consists in measuring the bulk protein concentrations before and after immobilization. The immobilization curve exhibits two distinct portions: a steep initial increase of Γ, when all added protein molecules find enough space for attachment, followed by a horizontal part. The latter occurs at conditions when apparently the whole surface of the latex particles becomes saturated with protein macromolecules. The plateau value determined from the immobilization plot, Γ_{HSA}(max), is 0.2 mg m^{-2}. This value is consistent with that obtained for polyacrolein latex particles [23], where covalent coupling occurs between surface latex groups and proteins.

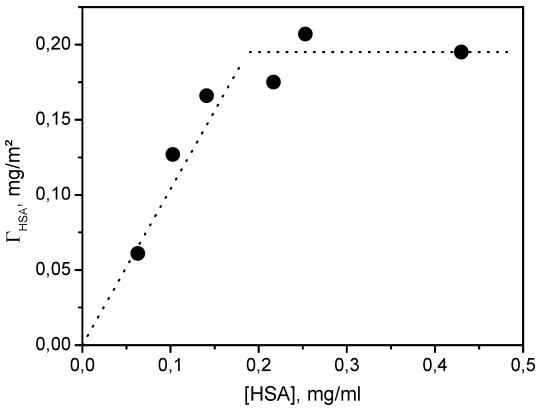


Fig. 8 Attachment of human serum albumin (*HSA*) onto PS_E-$PPyNSE_{50}$

Conclusion

Surface-functionalized PPy-coated PS_E particles bearing reactive NSE groups were prepared and characterized in terms of size, electrophoretic mobility, morphology and chemical structure. The ratio of pyrrole to NSE-functionalized pyrrole monomers was varied and its effect on the surface and bulk properties of the end products investigated. The particles had a rougher surface when the initial concentration pyrrole-NSE was increased. XPS and FTIR spectroscopy indicated the presence of pyrrole-NSE repeat units at the surface of the particles. Furthermore, these latex particles, bearing reactive groups, are found to be effective in binding proteins. These results show that these novel NSE functionalized latexes with appropriate attached proteins may have potential in view of developing visual agglutination tests.

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