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Competitive Adsorption of Cationic Bilayers and Chitosan on Latex: Optimal Biocidal Action

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The competitive adsorption of two biocides, the cationic polysaccharide (chitosan, CH) and some cationic bilayer fragments (composed of dioctadecyldimethylammonium bromide, DODAB), onto oppositely charged polystyrene sulfate (PSS) or poly(methyl methacrylate) (PMMA) particles is described. CH and/or DODAB effects on particle stabilization/flocculation are characterized from the following: (1) DODAB adsorption isotherms from bilayer dispersions onto PMMA particles in the presence or absence of CH or 10 mM NaCl; (2) effects of particle number density, DODAB or CH concentration on mean size, and ζ -potentials of PSS or PMMA particles; (3) visualization of positively stained cationic moieties on PMMA particles by transmission electron microscopy. Driving forces for DODAB bilayer adsorption were the electrostatic and the hydrophobic attraction between latex and bilayer, whereas, for CH, charge neutralization (overcompensation) led to particle flocculation (stabilization). Both cationic species seem to adsorb not only as patches but also as stiff and extended protrusions from the particle surface, e.g. at low particle number densities (Np) and relatively high chitosan or DODAB concentrations, DODAB bilayer fragments attaching on particles also from their hydrophobic edges. At a given Np and low (high) CH concentrations there was DODAB (CH) displacement by CH (DODAB). The entropic penalty paid by CH for adsorption on latex with charge overcompensation did not hold for the also stiff and flat DODAB bilayer fragments which displaced CH and attached via hydrophobic and/or electrostatic interaction to the latex surface. ζ -Potentials range for particles varied from 35 to 40 (DODAB only) up to 65–75 mV (CH only). Together, DODAB and CH had to be added to the particulates in minute doses for preservation and sterilization against bacteria contamination.

Introduction

Mixtures of oppositely charged polymers and colloids, where the colloid component may be another synthetic or natural polymer, an association colloid or vesicle, a solid particle, or even a living cell or microorganism, are found in a variety of important situations involving creation of soluble complexes or viscous liquids,^{1–3} formation of multilayered coatings,^{4–9} particle stabilization/flocculation,^{10–12} gelation,¹³ water purification,¹⁴ mucoadhesion,^{15,16} bioseparation,¹⁷ and bacteria flocculation.¹⁸

Because living cells as well as most biomolecules are negatively charged, cationization has been increasingly explored as a straightforward tool for targeting cells, tissues, and selected organs.^{19–21} Inventive uses for

cationization involving drugs, vaccines, peptides, proteins, or DNA delivery have been proposed: cross-linked cationic polyelectrolytes in the form of microspheres as long-acting drug vehicles,²² cationized amphotericin B for antisense therapy avoiding endosomal degradation,²³ cationic polyelectrolytes grafted on polymeric vesicles,²⁴ phospholipase immobilization on chitosan beads for lowering serum cholesterol concentration,²⁵ synthetic cationic vesicles^{26,27} as antimicrobial drugs at low ionic strength^{28–31} or as interface agents for particle coverage with bilayer membranes,^{32–35} and hydrophobic edges of cationic bilayer fragments for solubilization of hydrophobic drugs.³⁶

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Much recent effort has been devoted to the understanding of interactions between model mixtures of oppositely charged pairs.^{37–45} However, the competitive adsorption of positively charged polyelectrolytes and cationic bilayers onto oppositely charged polymer model particles has not been systematically studied despite its importance.

The second most abundant polysaccharide in nature following cellulose is chitin, a *N*-acetyl-D-glucosamine polymer, which can be deacetylated to yield the cationic polyelectrolyte chitosan, which in turn has been a major asset in many important strategic areas of applied research such as pharmaceuticals, biomaterials, cosmetics, food processing, chelation of heavy metals, and industrial scale flocculation (see ref 45 and references therein). On the other hand, some bilayer-forming cationic synthetic lipids, such as dioctadecyldimethylammonium bromide (DODAB), have also found a myriad of new uses as interface agents or as biocides at low ionic strength (see ref 35 and references therein). In this work, the aim was to evaluate particle flocculation/stabilization of negatively charged latex in the presence of chitosan and synthetic cationic lipid plus biocidal action of the cationic species in the presence of the model particles.

Our strategy was developed in two steps. In the first, the adsorption of the two cationic adsorbents on latex was characterized from the following: (1) DODAB adsorption isotherms from bilayer dispersions onto latex in the presence or absence of CH and in the presence or absence of 10 mM NaCl; (2) effects of particle number density, DODAB, or CH concentration on mean size and ζ -potentials for particles; (3) visualization of positively stained cationic moieties on particles by transmission electron microscopy. In the second step, the biocidal action was characterized from the effect of particulates containing the cationic components on minimal bactericidal concentration against two different bacteria species. The results evidenced the competition between the DODAB cationic bilayer and the cationic polymer for negatively charged sites on latex. The competitive effect could be advantageously used to keep the most effective bactericide,

DODAB, in the bulk solution, thereby sterilizing the particulate dispersion against bacteria.

Experimental Section

Materials. A stock solution of chitosan (CH) (Fluka, 150,000 g·mol⁻¹, 49% acetylation degree, 4 g·L⁻¹ was prepared in dilute acetic acid, yielding a final pH of 3.8. This stock solution was diluted in pure water (pH = 6.3) to yield CH concentrations in the range of 0.1–1.4 g/L. Under these conditions, pH in the final mixtures, which were not buffered due to DODAB sensitivity to ionic strength, varied from pH 5.6 up to 4.5. The proton dissociation constant (K_a) for protonated amino groups in CH will be increased in the presence of similar adjacent groups, thereby decreasing pK_a , but in the literature a very common approximation considers the pK_a as constant at 6.5 (see ref 46 and references therein) so that, in our mixtures (pH = 4.5–5.6), the degree of proton dissociation from the amino groups in CH will vary between 1 and 10%; i.e., chitosan is indeed expected to behave as a polycation under our experimental conditions. Small DODAB (Sigma) bilayer fragments (see ref 36 and references therein), 78–86 nm mean diameter, and 38 ± 2 mV mean ζ -potential,³² were prepared by sonication with a tip in Milli-Q water at 2.0 mM DODAB as previously described.^{26,27} DODAB concentrations were analytically determined by microtitration.⁴⁸ Poly(methyl methacrylate) (PMMA) particles, 113 ± 1 nm mean diameter and -25 ± 1 mV mean ζ -potential, were synthesized by emulsion polymerization using potassium persulfate as initiator, a procedure which generated terminal sulfates as functional groups on latex. The emulsifier used was sodium dodecyl sulfate (SDS). The PMMA particles were cleaned by exhaustive dialysis. Although the actual surface charge density on this latex was not determined, e.g., from conductometric titration, the sessile drop method was used for determining contact angles. For spin-coated PMMA films cast on silicon wafers and prepared from latex (after drying and solubilizing in toluene), the advancing contact angle was $65 \pm 4^\circ$. pH was not controlled during the PMMA particle synthesis so that carboxylate groups are probably present at the PMMA latex surface. Sulfate-charged polystyrene (PSS) particles, 100 ± 5 nm mean diameter, $0.90 \mu\text{C}\cdot\text{cm}^{-2}$ of area per charge group and $571.048 \text{ cm}^2\cdot\text{g}^{-1}$ specific surface area, were purchased as batch no. 10-156–38 from Interfacial Dynamics Corp. (IDC; Portland, OR). For films prepared from PSS latex (IDC), the advancing contact angle was $71 \pm 4^\circ$. Therefore, from the wettability point of view it was difficult to deduce the existence of different functional groups on both latexes. Nevertheless, carboxylic surface groups on PMMA particles could well account for the large differences in behavior toward adsorption presented later on by dispersions of the two latexes.

Determination of DODAB Adsorption Isotherms. Particles, DODAB dispersions, and CH interacted at 25 °C, following two procedures: (1) particles first interacted with CH and thereafter with DODAB dispersion in the same mixture; (2) particles first interacted with DODAB and thereafter with CH in the same mixture. Details on interaction times, particle number densities, and DODAB and/or CH concentrations are given in the captions of Figures 1 and 2. After interaction, mixtures were centrifuged (14 000 rpm/(1 h)/(10 °C)) in order to separate free from adsorbed DODAB on particles. Controls were performed in order to confirm that the quoted conditions were efficient enough for the separation; namely, particles and DODAB dispersion were centrifuged separately and the supernatants analyzed from turbidity at 280 nm and DODAB concentration, respectively. DODAB concentration was determined in each supernatant as previously described.³² The total surface area on the particles was calculated from the mass fraction and the specific surface area. Adsorption was expressed as the number of DODAB molecules adsorbed per m² polymer.

Determination of Conductance, Mean Diameters, and ζ -Potentials for the Mixtures or Separate Mixture Components. Conductance (μS) at 25 °C for particles and/or cationic components mixed or separate was determined using the

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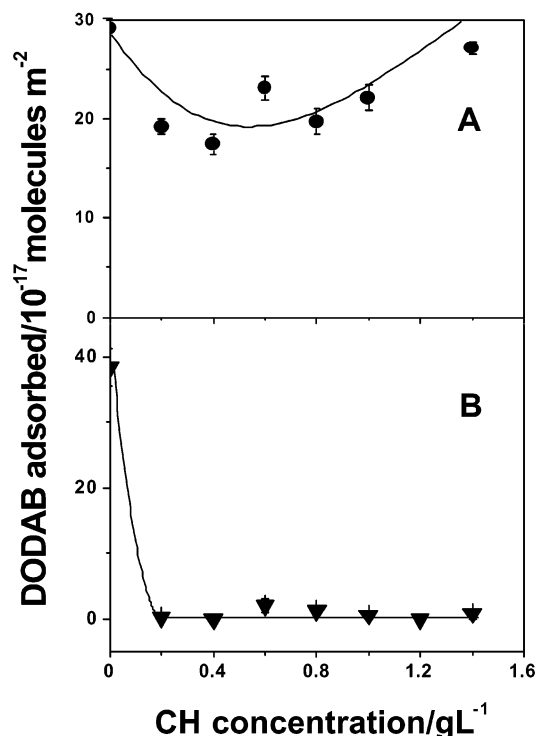


Figure 1. Effect of chitosan concentration on DODAB adsorption from a 1 mM DODAB dispersion onto PMMA particles (3×10^{12} particles/mL). Mixtures of particles and chitosan interacted for 1 h over a range of chitosan concentrations before adding the DODAB dispersion. Thereafter, the mixture interacted over 24 h before centrifuging and measuring free DODAB concentration in the supernatant. In A, the experiment was done in pure water. In B, there was a 10 mM final NaCl concentration added to the mixture.

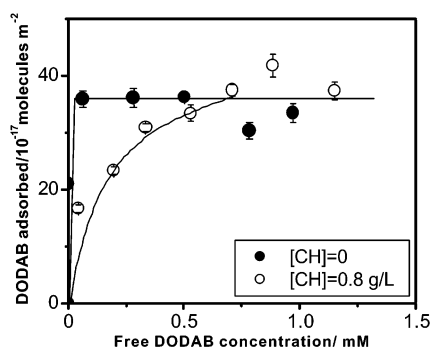


Figure 2. Effect of DODAB concentration on DODAB adsorption onto PMMA particles (3×10^{12} particles/mL) in the presence (○) or in absence (●) of a 0.8 g/L chitosan solution. Particles and DODAB dispersions were left interacting for 1 h before adding either water or the chitosan solution and allowing further interaction for 24 h.

conductance mode of the ZetaPlus- ZetaPotential Analyzer (Brookhaven Instruments Corp., Holtsville, NY). Particle size (mean diameter, D) and ζ -potential in the presence or absence of DODAB, CH, and/or both were determined using the same equipment, which was equipped with a 570 nm laser and dynamic light scattering at 90° for particle sizing. ζ was determined from electrophoretic mobility μ , either in pure water or in 1 mM KCl, and from the Smoluchowski's equation: $\zeta = \mu\eta/\epsilon$, where η is the medium viscosity and ϵ the medium dielectric constant.

Transmission Electron Microscopy. Transmission electron microscopy (TEM) was done using a Zeiss EM 902 microscope. On the copper grid, cationic moieties in samples were positively stained (1% ammonium molybdate/(30 s)) and drained with a filter paper before imaging by TEM. Molybdate is the anion that adsorbs at cationic DODAB or CH sites to yield the electronic

stain that will deflect the electron beam of the electron microscope, generating contrast and visualization of the DODAB and CH layers on the polymeric particles.

Determination of Biocidal Action. The minimum bactericidal concentration (MBC) for DODAB or CH or both was determined for *Pseudomonas aeruginosa* (ATCC 27953) and *Enterobacter cloacae* using standard protocols recommended by NCCLS⁴⁹ at final cell concentrations adjusted to ca. 5×10^5 CFU/mL. Each cell suspension adjusted to 0.5 of the McFarland scale (ca. 10^8 CFU/mL) was diluted (1:10) using Milli-Q water. A 0.05 mL aliquot of this diluted suspension was added to 0.95 mL of each biocidal mixture. After incubation ($37^\circ C$, no shaking), mixtures were diluted (1:10, 1:100, 1:1000, 1:10000, 1:100000), plated on Mueller-Hinton agar, and incubated ($37^\circ C$, overnight) for counting of viable cells on each plate. CH or CH on PMMA samples were double-diluted in liquid Mueller-Hinton culture medium, with final CH concentrations in $\mu g/mL$. DODAB or DODAB on PMMA samples were double-diluted in sterile Milli-Q water, with final concentrations in μM . Concentration ranges for biocides on latex are given in Table 4.

Results

Over a range of chitosan (CH) concentrations, there was substantial DODAB adsorption onto PMMA microspheres, the adsorbed DODAB amount varying between 18 and 30×10^{17} molecules/ m^2 (Figure 1A). Thus adsorption remained below the amount expected for bilayer deposition, ca. 33×10^{17} molecules/ m^2 latex surface.³² Chitosan prevented adsorption of DODAB bilayers on latex to a certain extent. However, its effect on DODAB adsorption was milder than the effect of adding 10 mM monovalent salt: complete abolishment of DODAB adsorption onto particles (Figure 1B).

Figure 2 shows two adsorption isotherms for DODAB adsorption from DODAB dispersions prepared by sonication with a tip onto PMMA microspheres at 0 or 0.8 g/L chitosan. In the presence of chitosan, there was a decrease of DODAB affinity for adsorption onto PMMA microspheres, though maximal DODAB adsorption remained equal to the one obtained in absence of chitosan, ca. 33×10^{17} molecules/ m^2 consistently with bilayer deposition onto latex in both cases (Figure 2).

The effect of chitosan concentration on conductance of water solutions containing CH only, PSS/CH, or PSS/CH/DODAB was a linear increase of conductance with increasing CH concentration (Figure 3 A). The largest and smallest slopes for these linear dependencies were those for PSS/CH in the absence of DODAB and for 1 mM DODAB, respectively. This effect was consistent with the very different dependencies of ζ -potentials for the mixtures on CH concentration for PSS/CH microspheres in the absence (Figure 3B) or presence of 1 mM DODAB (Figure 3C). If DODAB was absent, ζ -potentials for PSS particles as a function of chitosan concentration first changed its sign, becoming positive, and then remained very large, attaining a maximum and stable value of ca. 70–72 mV from 0.2 g/L chitosan (Figure 3B). On the other hand, when DODAB was present, the ζ -potential displayed a bell-shaped dependence on chitosan concentration with a 70 mV maximum at ca. 0.2–0.4 g/L chitosan (Figure 3C). The horizontal line in Figure 3C indicated the ζ -potential for the DODAB bilayer vesicle previously determined in pure water, ca. 40 mV.³² Curiously, the ζ -potential for PSS/CH/DODAB mixtures tended to 40 mV at 1.4 g/L chitosan. Possibly, the increase in ionic strength at the higher CH concentrations triggered more extensive DODAB adsorption onto particles with displacement of CH molecules from their adsorption sites (Figure 3C).

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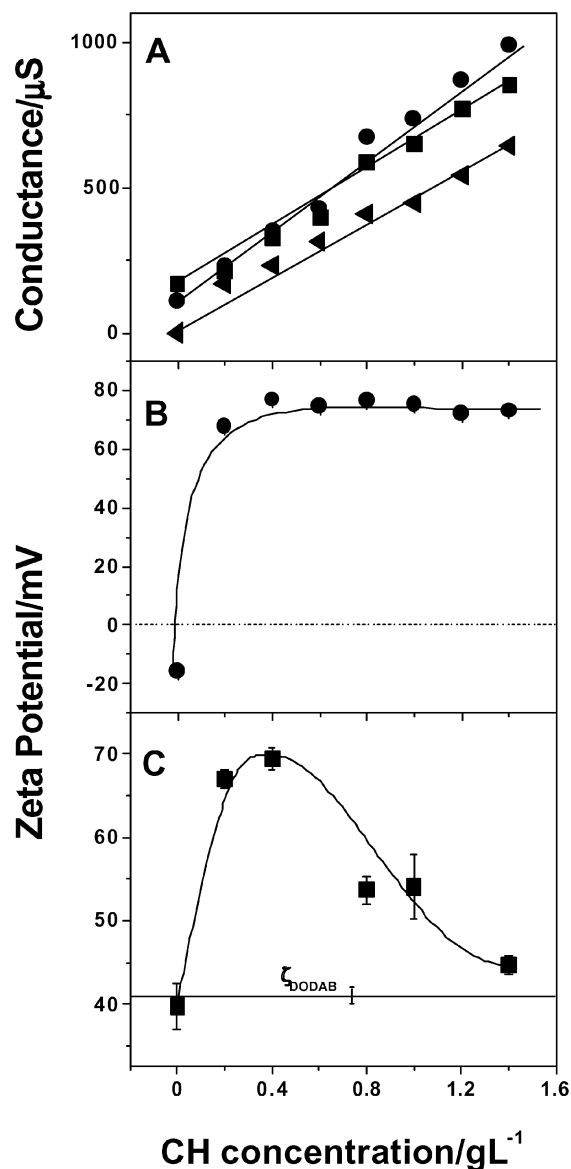


Figure 3. (A) Effect of chitosan concentration on conductance of chitosan solutions (\blacktriangledown), chitosan and polystyrene (PSS) particles (6×10^{10} particles/mL) (\bullet), and chitosan, PSS particles (6×10^{10} particles/mL), and 1 mM DODAB (\blacksquare). (B and C) Effect of chitosan concentration on ζ -potential for particles in the mixtures PSS/CH (1 h interaction) or PSS/CH/DODAB (further 24 h interaction after adding DODAB), respectively.

At 0.2 g/L chitosan, in pure water or in 1 mM KCl, the effect of particle number density on the mean diameter and the ζ -potential of PSS or PMMA particles was decreasing mean sizes and ζ -potentials in the mixtures (Figure 4; Table 1). At ca. 10^{12} particles/mL, when the total surface area on the particles was sufficiently large, adsorption of chitosan molecules on particles was accompanied by size minimization and occurrence of particle doublets for both particle types (Scheme 1). At 6×10^{10} particles/mL and 0.2 g/L chitosan, a high extent of particle coverage with chitosan yielded the largest ζ -potentials observed, ca. 72–75 mV (Figure 3B, Scheme 1), with particle/chitosan aggregates exhibiting large mean sizes, namely, 600–900 or 600 nm mean diameter for PSS/CH or for PMMA/CH, respectively (Figure 4B,D, respectively, and Scheme 1). At 10^{12} particles/mL, ζ -potentials decreased substantially to ca. 55 mV consistently with the partial particle coverage typical of this large particle

number density and total surface area on particles (Figure 4). The decrease in size upon increasing particle number density was similar for PMMA and PSS and possibly led to formation of doublets and singlets for both particles (Table 1), though slightly lower ζ -potential values were obtained for the PMMA/CH mixtures. CH coverage on PMMA seemed to be less extensive and weaker than coverage on PSS so that final surface potentials attained smaller positive values for PMMA/CH than for PSS/CH mixtures (Table 1). In Figure 4, at 0.2 g/L CH and 10^{12} particles/mL, one should notice that sizes are consistent with a high frequency of particle doublets for both particle types possibly kept as such by patch flocculation.^{11,46}

Figure 5 shows the effect of chitosan concentration on ζ -potential and mean diameter of PSS or PMMA particles (10^{12} particles/mL) in the presence or absence of 1 mM DODAB added 1 h after adding chitosan to the particles and left to interact with the particles/chitosan system over 24 h. In absence of DODAB, the effect of increasing chitosan concentration was a monotonic increase in ζ -potential (Figure 5A,C, empty circles) and particle size that led to sizes expected for PSS or PMMA particle doublets, ca. 200 nm mean diameter (Figure 5B,D, empty circles). However, in the presence of DODAB, there was a bell-shaped dependence of mean particle diameters on chitosan concentration with maximal sizes at 0.2 and 0.6 g/L CH, for PSS and PMMA particles, respectively (Figure 5B,D, full circles). The smallest particle aggregates occurred at large CH concentrations (above 1.0 g/L CH) both for PSS/CH/DODAB and for PMMA/CH/DODAB. The lower range of CH concentration required for maximal aggregation induced by 1 mM DODAB suggested that DODAB affinity for PSS/CH was larger than its affinity for PMMA/CH (Figure 5B,D). Why would DODAB be more efficient as a flocculant toward PSS/CH producing aggregates at smaller DODAB concentrations than those required for the PMMA/CH system? Possibly, there was a stronger hydrophobic interaction between the DODAB dispersion and the PSS particle related to the existence of bilayer fragments with hydrophobic edges that are produced by dispersing DODAB via sonication with tip.^{27,36} This would result in increased affinity between the DODAB bilayer fragment and the more hydrophobic PSS/CH particle surface generating more extensive DODAB-induced aggregation for PSS than for PMMA particles.

Table 2 summarizes the effect of 1.0 mM DODAB over the particle doublets region, doublets kept as such by chitosan, namely, a certain CH displacement with a significant decrease in ζ -potentials. It is interesting to notice that upon addition of 1 mM DODAB on CH-covered particles, the mean size increased for PSS and decreased for PMMA (Table 2), consistently with the interpretation that the hydrophobic interaction between the DODAB bilayer fragment and the PSS surface might have been stronger than the one involving the PMMA surface. Despite the absence of direct evidence of the presence and amount of surface carboxyl (and also probably hydroxyl) on PMMA latex, these groups are very possibly present from established data in the literature for PMMA particle synthesis in absence of pH control.⁵⁰ In the recipe used the pH was not controlled. In some recipes NaHCO_3 is added as buffer to control pH. A side product of persulfate decomposition, produced by reaction of $\text{S}_2\text{O}_8^{2-}$ with water, is HSO_4^- . Since HSO_4^- alters pH, which can drastically reduce initiator efficiency, a buffer might be used. The

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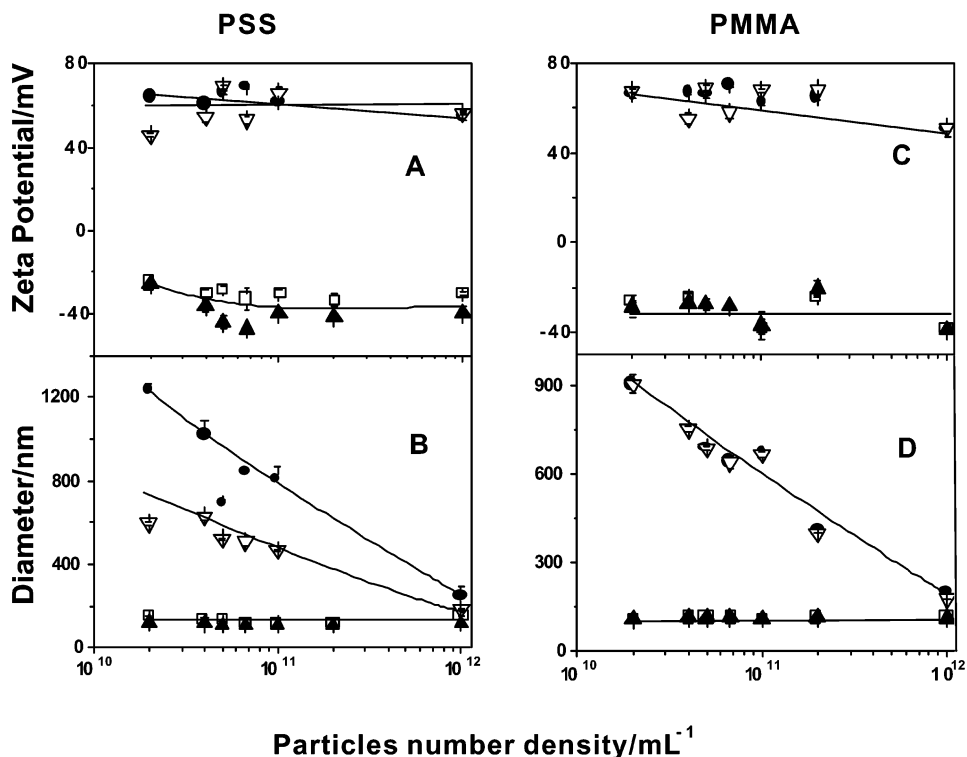


Figure 4. Effect of particle number density on ζ -potential and mean diameter of PSS (A, B) or PMMA particles (C, D) in pure water (\square , \bullet) or in 1 mM KCl (\blacktriangle , ∇), in the presence of 0.2 g/L chitosan (\bullet , ∇) or in its absence (\square , \blacktriangle). Measurements were done after 1 h of interaction for each mixture.

Table 1. Mean Diameter (D) and ζ -Potential for Polystyrene Sulfate (PSS) or Poly(methyl methacrylate) (PMMA) Particles in the Presence or Absence of CH at a High Particle Number Density ($N_p = 1.0 \times 10^{12}$ particles/mL)^a

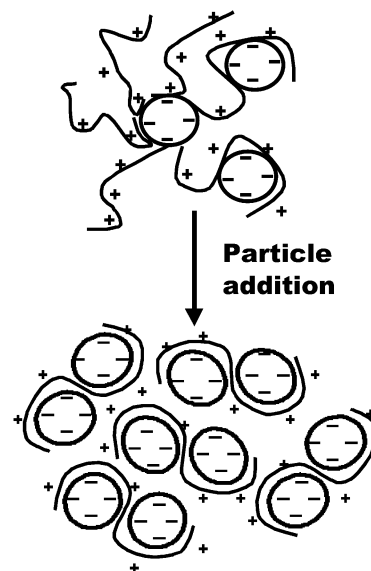
dispersion	CH/(g/L)	D /nm	ζ /mV
PSS in water		116 ± 1	33 ± 3
	0.2	188 ± 6	55 ± 2
PSS in 1 mM KCl		120 ± 1	40 ± 1
	0.2	183 ± 1	56 ± 1
PMMA in water		113 ± 1	38 ± 4
	0.2	198 ± 4	50 ± 3
PMMA in 1 mM KCl		113 ± 1	39 ± 5
	0.2	177 ± 2	51 ± 2

^a PSS and PMMA particle mean diameter was 100 ± 5 and 110 ± 5 nm, respectively, from transmission electron microscopy. Particles and chitosan interacted for 1 h before the measurements were performed.

sulfate group attached to the chain end can suffer hydrolysis to a hydroxyl or, if carbonate ions are present, to carboxylic acid (or carboxylate, depending on the pH); these chains will also have surface-active properties, albeit different from those to which a SO_4^- is attached.

Figure 6 shows the effect of increasing DODAB concentration on ζ -potential (Figure 6A,C) and particle size for the ternary mixtures at 1.0 g/L CH (Figure 6B,D). After 1 h of interaction between PSS/CH and further 1 h of interaction with DODAB both ζ -potential and size dependences as a function of DODAB concentration displayed minimum and maximum values, respectively, so that a minimum of ζ -potential was coincident with a maximum of aggregation and size (Figure 6, open circles). For PSS particles, maximal aggregation was particularly extensive, yielding very large aggregates, mean diameter above 1000 nm (Figure 6B, open circles). However, after 24-h interaction with DODAB, the occurrence of doublets prevailed practically over all ranges of DODAB concen-

Scheme 1. Model for the Interaction between Chitosan and Oppositely Charged Polymeric Particles at Low and High Particle Number Densities



trations, suggesting a high efficiency for chitosan stabilizing effect. ζ -potentials for these doublets, however, also suggested some coverage with the DODAB bilayer; they were intermediate between those for chitosan (60–62 mV) and DODAB (35–45 mV) coverage, namely, ca. 54 mV (Table 3).

Figure 7 offers some transmission electron microscopy views of PMMA mixtures with chitosan and/or DODAB dispersions which are representative of the whole dispersions under each experimental condition. Contrast for visualization of cationic moieties on the samples was obtained by electronic stain with 1% ammonium molybdate, i.e., ca. 6 mM ionic strength due to ca. 1 mM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$.

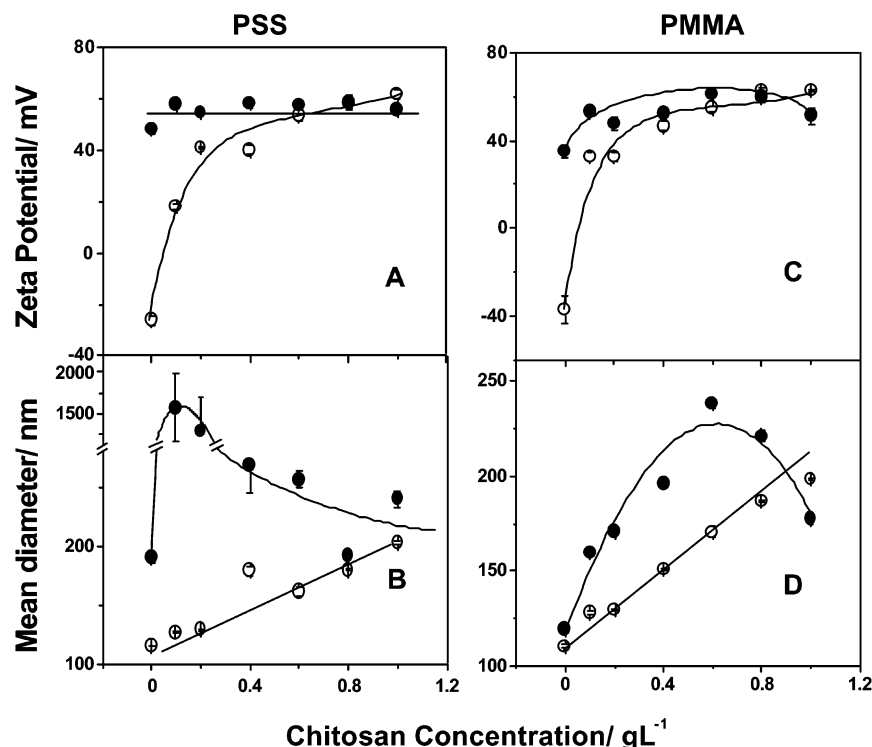


Figure 5. Effect of CH concentration on ζ -potential and mean diameter of PSS (A, B) or PMMA particles (C, D) at 1×10^{12} particles/mL in pure water (○) or in 1 mM DODAB dispersion (●). The interaction particle/chitosan was over 1 h. Thereafter, pure water (○) or 1 mM DODAB was added for interaction over another hour (●).

Table 2. Effect of 1.0 mM DODAB on Mean Diameter and ζ -Potential of PSS or PMMA Particles (1.0×10^{12} Polymeric Particles/mL) Previously Added at 1.0 g/L CH^a

latex type	DODAB/mM	D/nm	ζ /mV
PSS	1.0	202 \pm 2	62 \pm 2
		240 \pm 7	56 \pm 1
PMMA	1.0	198 \pm 1	63 \pm 1
		177 \pm 3	51 \pm 4

^a Measurements were performed after 1 h of interaction time.

Mo₇O₂₄. One should recall the complete DODAB bilayer desorption from the PMMA latex induced by 10 mM NaCl (Figure 1B). Thus, the restricted extent of DODAB bilayer adsorption visualized in Figure 7A is understandable from bilayer desorption induced by 2% ammonium molybdate. Nevertheless, it was still possible to visualize on latex two dark stripes sandwiching a clear one with ca. 5 nm total thickness (Figure 7A).

In Figure 7B, adsorbed chitosan on particles appeared mostly as dark strings, keeping particle doublets together with less frequent images of partially covered single particles. There was a good agreement between TEM images and mean sizes of ca. 200 nm for PMMA/CH mixtures at 10^{12} particles/mL, both evidencing the presence of doublets (Figure 4, Table 2). Thus, the model in Scheme 1 gained further support from TEM data.

In Figure 7C, both structures present in Figure 7A (DODAB dark patches on the particles) and Figure 7B (chitosan dark strings and patches linking doublets) were observed. The occurrence of nonstained, clear regions on particles indicated absence of any cationic moieties on them. In Figure 7D, at 1.4 g/L chitosan and 0.8 mM DODAB, most particles were clear, though some dark patches and strings on particles were still observed. It was interesting that some clear stripes limited by two dark stripes, total thickness of one bilayer, 50 nm, could

barely be seen. They might have corresponded to free DODAB bilayer fragments (Figure 7D, bottom right).

Both DODAB^{28–31} and CH⁴⁴ were previously characterized as antimicrobial agents at low ionic strength. Table 4 shows DODAB and/or chitosan concentration range for evaluation of biocidal action against *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Enterobacter cloacae* (*E. cloacae*). The minimal bactericidal concentration (MBC) for several combinations of DODAB, CH, and PMMA particles are shown in Table 5. PMMA alone did not display any cytotoxicity during 24 h though its presence increased MBC for CH or DODAB (from 125 to 273 μ g/mL; from 13 to 31 μ M DODAB, respectively) due to reduction of biocide adsorption on bacteria caused by its concomitant adsorption onto PMMA. There was a 30 times MBC reduction for CH in the presence of 9 μ M DODAB. Only 8 μ g/mL of CH was required to completely kill *E. cloacae* when 9 μ M DODAB was present. A similar biocidal effect was obtained for the polysaccharide/lipid combination against *P. aeruginosa*. For this bacteria species, the most efficient order of addition was the one where DODAB was the last ingredient added to the mixture, chitosan on PMMA followed by DODAB instead of DODAB on PMMA followed by chitosan. Possibly, DODAB as the most effective bactericide (Table 5), barely adsorbed on CH/latex and remained free to reach bacterial cells in the mixtures thereby acting as a preservative for the particles dispersion.

Discussion

The chitosan molecule used in this work was 49% acetylated and 51% deacetylated, deacetylation originating the amine groups responsible for the polycation character of the molecule. From structural studies, the positive charges on fully deacetylated chitosans were shown to be spaced at 1 nm⁵¹ so that, at 51% deacetylation,

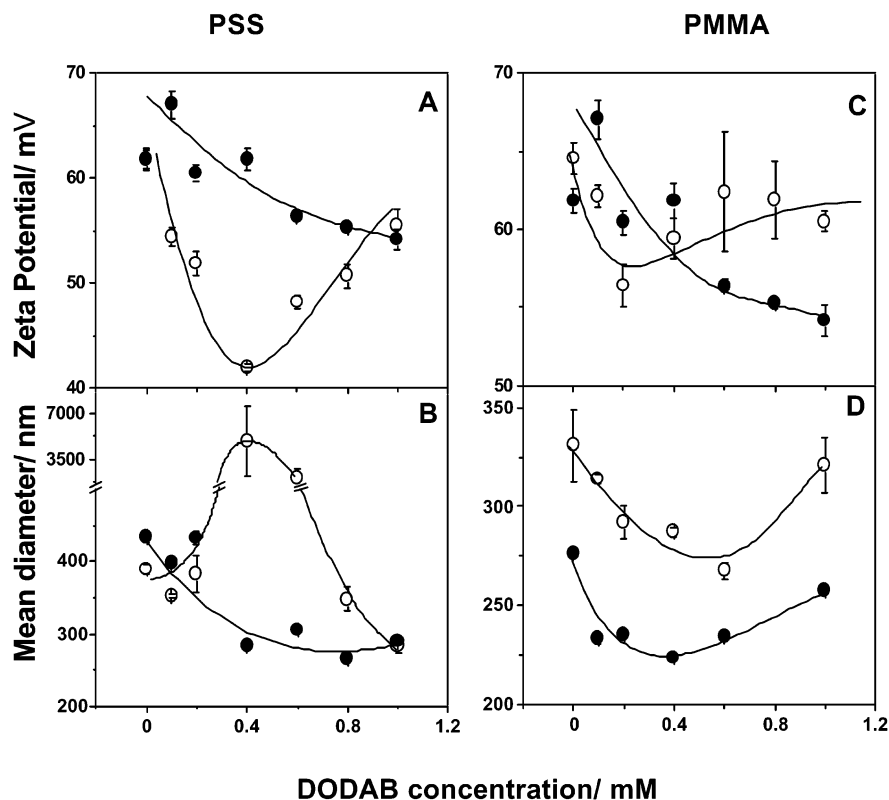


Figure 6. Effect of DODAB concentration on ζ -potential and mean diameter of PSS (A, B) or PMMA particles (C, D) at 1×10^{12} particles/mL and 1 g/L CH with mixtures interacting over 1 h (○) or over 24 h (●) in pure water. The interaction particle/chitosan was over 1 h. Thereafter, DODAB was added for interaction over another hour (○) or over further 24 h (●).

Table 3. Effect of Interaction Time and Presence or Absence of $1 \text{ g} \cdot \text{L}^{-1}$ Chitosan on Mean Diameters (D) and ζ -Potentials of Polymeric Particles at 1 mM DODAB and 1.0×10^{12} particles/mL^a

latex type	interaction time/h	D/nm		ζ/mV	
		[CH] = 0	[CH] = 1 $\text{g} \cdot \text{L}^{-1}$	[CH] = 0	[CH] = 1 $\text{g} \cdot \text{L}^{-1}$
PSS	1	190 \pm 4	285 \pm 11	48 \pm 2	61 \pm 1
	24	190 \pm 4	290 \pm 3	48 \pm 2	54 \pm 1
PMMA	1	119 \pm 1	321 \pm 14	35 \pm 3	56 \pm 2
	24	119 \pm 1	258 \pm 2	35 \pm 3	54 \pm 1

^a Notice the sizes measured for PMMA in the presence of 1 mM DODAB, namely, 119 \pm 1 nm (bare particle size plus one adsorbed DODAB bilayer).

there is ca. 2 nm mean spacing between positive charges at ca. 100% protonation for the CH amino groups. For the PSS latex employed in this work, Interfacial Dynamics Corp. determined ca. 17.7 nm² per sulfate charge group. Assuming uniform sulfate distribution on PSS latex surface, each one positioned at the center of a 16 nm² square, ca. 4 nm would separate sulfate charges on latex. As a polycation, CH energy of adsorption onto the oppositely charged latex surface is high, and it would be expected to adsorb in a relatively flat configuration.³⁸ In fact, charge reversal and overcompensation were observed for the PSS particles at a very small CH concentration, ca. 0.1 g/L, i.e. 0.006 mM CH, leading to large ζ -potentials (Figure 3B). The chitosan molecule is stiff, its lack of flexibility being related to the polyelectrolyte chain having more closely spaced charges than those on the particle.⁴⁶ Very recently, atomic force microscopy imaging has clearly demonstrated that low-charge-density polyelectrolytes tend to adsorb less strongly and in a more loose confor-

mation on oppositely charged surfaces as compared to high-charge-density ones.⁴³ The larger the deacetylation degree, the greater the charge density on CH and the overcompensation leading to large ζ -potentials on particles.

Most mechanisms proposed to explain CH-induced aggregation of oppositely charged particles are related to the charge density on the polyelectrolyte, although the chitosan capacity for hydrogen bonding toward cell debris related to bridging flocculation of *Escherichia coli* at 0.1 M ionic strength was recently pointed out.¹⁸ Highly charged polymers cause aggregation by way of charge neutralization, and their efficiency is independent of molecular weight while weakly charged polymers adsorbing in loops and tails conformation flocculate predominantly by bridging. A third suggested mechanism is patch flocculation due to the attraction between oppositely charged domains on particles partially covered with adsorbed polymer.⁵² Under our experimental conditions, ionic strength was very low and measured ζ -potentials in the presence of CH were high. Therefore, the most probable mechanism accounting for chitosan-induced particle stabilization/flocculation should be related to charge overcompensation/neutralization, respectively. In the absence of DODAB, charge overcompensation upon chitosan adsorption at high particle number densities had a stabilizing effect (Figures 4 and 5), particles barely doubling their sizes at the highest CH concentration employed of 1 g/L (Figures 4 and 5). Charge overcompensation represented an important mechanism of particle stabilization which prevented flocculation despite the fast collision rates at high particle number densities. At low particle number densities, however, chitosan was very effective as a flocculant (Figure 4, Scheme 1).

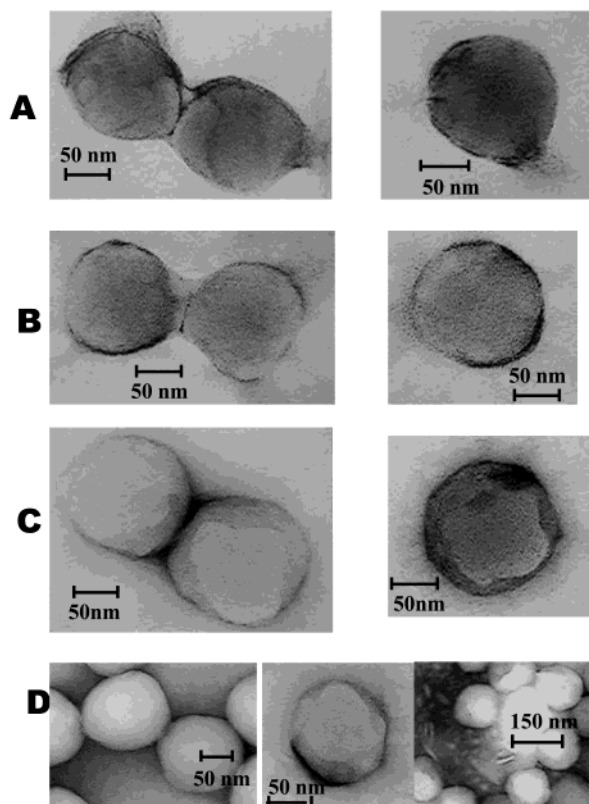


Figure 7. Transmission electron microscopy for samples positively stained on cationic moieties by using 1% ammonium molybdate ($(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$) at a final PMMA particles number density of 10^{12} particles/mL. Mixtures were left interacting with the electronic stainer over 30 s before drainage and observation under the electron microscope. (A) Particles and DODAB bilayer fragments (0.8 mM of final DODAB concentration) which interacted over 24 h. (B) Particles and 0.5 g/L chitosan mixtures (1 h interaction). (C) Particles and 0.5 g/L chitosan mixtures were added to DODAB bilayer fragments (0.8 mM final DODAB concentration) and left to interact over 24 h. (D) Same as in C but at 1.4 g/L final chitosan concentration.

Table 4. DODAB (μM) and CH ($\mu\text{g/mL}$) Concentration Range Used To Evaluate Biocidal Action against Bacteria (5×10^5 CFU/mL)

biocide	conn range	
	chitosan/ $(\mu\text{g/mL})$	DODAB/ μM
CH	100 to 3	
CH on PMMA	545 to 1	
CH and/or DODAB on PMMA	500 to 0.5	600 to 0.6
DODAB		3300 to 6.4
DODAB on PMMA		990 to 1.0
DODAB and/or CH on PMMA	500 to 0.5	600 to 0.6

On the other hand, cationic DODAB bilayer fragments (see ref 36 and references therein) are also stiff and flat due to their rigid gel state at room temperature.⁵³ They are composed of open, charged, and electrostatically stabilized bilayer pieces in bilayer-forming synthetic amphiphile dispersions prepared in water by sonication with a tip—a high-energy-input procedure which not only disperses the amphiphile powder in water producing bilayer vesicles but also disrupts them thereby originating bilayer fragments. The main evidence for their existence is as follows: (1) osmotic nonresponsiveness of the dispersion indicative of absence of inner vesicle compartment; (2) TEM micrographs with electronic staining; (3)

Table 5. MBC (24 h) Presented in $\mu\text{g/mL}$ or μM for Cationic Biocides on PMMA Latex As Compared to the Isolated Action of DODAB or Chitosan Dispersions^a

biocidal mixture	for given organism			
	<i>E. cloacae</i>		<i>P. aeruginosa</i>	
	$\mu\text{g/mL}$	μM	$\mu\text{g/mL}$	μM
chitosan	125 ± 10		250 ± 40	
chitosan/PMMA	273 ± 12		273 ± 40	
chitosan/DODAB/PMMA	8 ± 1	9 ± 1	16 ± 1	19 ± 2
DODAB		13 ± 1		26 ± 2
DODAB/PMMA		31 ± 1		31 ± 2
DODAB/chitosan/PMMA	8 ± 1	9 ± 1	8 ± 1	9 ± 1

^a PMMA final particle number density was ca. 3×10^{10} particles/mL.

cryo-TEM micrographs; (4) quasi-elastic light scattering and electron paramagnetic resonance spectroscopy; (5) solubilization of hydrophobic drugs such as amphotericin B at the borders of DODAB bilayer fragments which does not occur for DODAB closed bilayer vesicles (see refs 27 and 36 and references therein). Therefore, in the case of DODAB bilayer fragments, another important component of the interaction latex/cationic species has to be considered, the extraattractive interaction between the hydrophobic latex surface and hydrophobic borders of the bilayer fragment. Previously, other types of hydrophobic defects leading to vesicle fusion upon salt addition had been identified in DODAB dispersions composed only of large and closed bilayer vesicles which decreased their colloid stability.^{54,55} Consistently, at high particle number densities such as 10^{12} particles/mL, DODAB was more effective in flocculating (increasing mean particle size) the latices than was chitosan, a flocculant agent for which the hydrophobic attraction between macromolecule and surface is absent (Figures 5 and 6). Again, consistently, particle flocculation kinetics by DODAB was attenuated upon increasing the hydrophilic character of the latex surface by using PMMA instead of PSS (Figure 5B,D, Figure 6B,D).

At 10^{12} particles/mL, considering the PSS particles from IDC with 110 nm mean diameter, a total surface area of 4×10^{16} nm² is available on the latices in 1 mL of latex dispersion. This surface area would require ca. 0.2 mM DODAB in the form of bilayer fragments to attain complete particle coverage with a bilayer, assuming that each DODAB molecule occupies 0.6 nm² in the bilayer assembly in water.⁵⁶ If only one adsorption mode for the bilayer fragments was available, namely, pairing opposite charges on latex and bilayer, upon interaction with 0.2 mM DODAB, 10^{12} latex particles should attain a very large colloid stability with final particle sizes close to $(110 + 10)$ nm mean diameter (the bilayer thickness is ca. 5 nm). We have previously shown that this is not so, at least, for the PSS particles.^{57,58} Upon increasing DODAB concentration, in the absence of chitosan, DODAB adsorption increased far beyond bilayer deposition and this was interpreted as due to the hydrophobic attraction between adsorbed DODAB and free DODAB bilayers in the bulk.^{57,58} From the understanding that bilayer fragments had been present in the mixtures with the PSS latex throughout,

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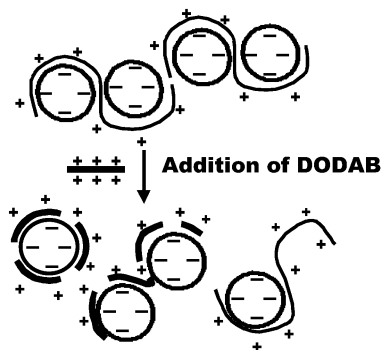
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Scheme 2. Competitive Adsorption for DODAB Bilayer Fragments and Chitosan onto Oppositely Charged Polymeric Microspheres



possibly the second adsorption mode for the bilayer fragment on latex, the attachment from its hydrophobic edge, might have yielded not only DODAB adsorption beyond bilayer deposition but also colloidal destabilization with particles aggregation.

In the present work, for the PMMA latex, which is more hydrophilic than PSS, the bilayer fragments apparently covered the particle surface with one DODAB bilayer at maximal adsorption and this was exactly so without further increases in adsorption or sizes beyond ca. 119–110 nm, the diameter expected for one-bilayer deposition, (Figure 1A and Table 2). Thus, on PMMA particles, bilayer fragments might have adsorbed as flat patches which could either be sealed or nonsealed. Because complete DODAB desorption from PMMA took place in the presence of 10 mM NaCl over a range of chitosan concentrations (Figure 1B), the bilayer coverage on latex from DODAB bilayer fragments did not correspond to one sealed and continuous bilayer; rather, disconnected DODAB bilayer patches remained as such upon adsorption on particles. If this were not the case, NaCl and/or chitosan should not have caused DODAB bilayer desorption, since closed DODAB bilayers—thin, continuous and closed sheets of hydrocarbon layers with low dielectric constant—surrounding the particle would not allow NaCl passive permeation as previously shown.⁵⁹ Scheme 2 showed chitosan displacement from PMMA latex doublets upon adding DODAB bilayer fragments to the dispersion at high particle number densities.

Not only salt caused DODAB desorption: in a competition between DODAB bilayer fragments and chitosan at low chitosan concentration, chitosan also displaced adsorbed DODAB bilayer fragments, yielding a lower DODAB affinity for adsorption on particles (Figure 2). This situation was reverted, upon increasing DODAB concentration, the same maximal DODAB adsorption

being achieved. Increasing salt concentration, however, readily displaced both cationic species from the latex surface. In fact, both CH and DODAB were previously reported to be prone to desorption upon screening of the electrostatic attraction between cationic species and its oppositely charged pair.^{43,60}

Interestingly enough, at low ionic strength, the competition between DODAB and CH for adsorption on latex could be exploited for a new application, preservation of the latices against bacteria at very minute concentrations of both microbicide agents (Table 5).

Conclusions

Driving forces for DODAB bilayer adsorption were the electrostatic and the hydrophobic attraction between latex and bilayer. For DODAB, the hydrophobic attraction was in control of the very extensive DODAB concentration range where particle flocculation transiently occurred. Charge neutralization by CH led to particle flocculation and charge overcompensation, to particle stabilization. Both cationic species adsorbed on latex, forming patches of cationic moieties, but other adsorption modes were also required to explain the results. CH, as a stiff and extended string, could also protude outward from the latex surface at low particle number densities. DODAB bilayer fragments might also have adsorbed from their hydrophobic edges perpendicularly to the latex surface upon addition of excess DODAB to the particles. At a given particle number density, once established the competition between these two cationic species for sites on latex, chitosan easily displaced DODAB at low CH concentrations, whereas DODAB easily displaced CH at large CH concentrations. Mean ζ -potential of the particulate could be used as a tag identifying either CH or DODAB adsorption since the cationic bilayer intrinsically had a much lower surface charge density than the one obtained from CH adsorption on latex under CH excess. Although transient differences in colloid stability for PSS and PMMA dispersions in the presence of DODAB and CH did occur as observed at 1 h of interaction time, at 24 h, a higher colloid stability for the mixtures was achieved as seen from the high frequency of singlets and doublets in the mixtures at high particle number densities. The competition between both cationic species could be advantageously used to preserve latex against bacteria contamination upon addition of minute CH and DODAB doses, better conditions being those where CH remained adsorbed on latex and DODAB was free for adsorbing on the bacterial cells.

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