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ORIGINAL CONTRIBUTION

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Effects of oligonucleotide adsorption on the physicochemical characteristics of a nanoparticle-based model delivery system for antisense drugs

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Abstract Cationic polystyrene nanoparticles, as a model drug carrier system for nucleic acids, are capable of binding negatively charged oligonucleotides by multiple electrostatic interactions. The effect of the adsorption of phosphorothioate oligonucleotides on the physicochemical properties of the carrier system was investigated for uncoated and sterically stabilized latex particles. Turbidity measurements and photon-correlation spectroscopy indicate that the colloidal stability of the nanoparticle-oligonucleotide conjugates is influenced by the number of oligonucleotides adsorbed on the carrier. Especially in the case of the uncoated material, a destabilizing effect has been observed up to oligonucleotide concentrations of 2.7 μmol/g polymer. Strikingly, at higher concentrations the latexes exhibit colloidal stability similar to the oligonucleotide-free samples. These results were correlated to zeta-potential measurements demonstrating a reversal from positive to negative values of the zeta potential with increasing oligonucleotide concentration. The points of zero charge of the particles are in the region of maximum coagulation. These findings were compared to adsorption studies and calculations based on the random sequential adsorption model. It appears that at first the colloidal stability of the carrier systems is diminished with increasing oligonucleotide adsorption, while higher surface coverages lead to a significant reduction in coagulation. At the saturation level the surface coverage can be considered as a monolayer of "side-on" adsorbed molecules and the conjugates exhibit colloidal stability similar to the bare particles without adsorbed molecules.

Key words Nanoparticles – Drug carrier – Colloidal stability – Oligonucleotides – Zeta potential

Introduction

Antisense technology is based on the application of modified single-stranded oligonucleotides (ONs) which are developed to efficiently inhibit expression of a harmful gene by specific binding to a complementary target sequence located on the mRNA [1–3]. Phosphorothioate oligonucleotides (PTOs) with oxygen replaced by sulfur at a non-bridging position of the phosphodiester linkage represent one of the most commonly used derivatives of natural DNA [4, 5].

Colloidal drug carriers appear to be a promising tool for the delivery of antisense ONs in biological systems and could be valuable for supporting their therapeutic use. Biodegradable poly(alkylcyanoacrylate) nanoparticles have recently been used as drug carriers for unmodified antisense ONs making them capable of specific inhibition of a target gene [6, 7]. ON adsorption was mediated by the formation of ion pairs between the negatively charged

backbone of the nucleic acids and the hydrophobic cations adsorbed on the particle surface. The application of cationic polystyrene nanoparticles for adsorption studies of ONs has been described by Elaissari and coworkers [8, 9]. Due to their biocompatiblity polystyrene nanoparticles are widely used as model drug carrier systems for in vitro and in vivo studies [10–12].

For the evaluation of the suitability and biological compatibility of a drug delivery system detailed information concerning the influence of the adsorbed drug on the physicochemical properties of the carrier system, such as mean particle size, colloidal stability and zeta potential, are required. In recent studies of Fritz et al. [13] a model drug delivery system for ONs based on cationic polystyrene nanoparticles was developed. It was demonstrated that natural and modified ONs exhibit strong affinity to the carrier system mediated by the combination of hydrophobic and multiple electrostatic interactions.

In the present work interactions between cationic polystyrene nanoparticles and a model ON (PTO 16mer) were investigated using uncoated and sterically stabilized latex particles. Detailed studies of the nanoparticle ON conjugates were carried out at various levels of ON adsorption. The results obtained by turbidimetry, photon-correlation spectroscopy (PCS) and zeta-potential measurements were compared to the corresponding adsorption isotherms and theoretical calculations based on the random sequential adsorption (RSA) model.

Experimental

Materials

Styrene was purchased from Merck (Darmstadt, Germany) and distilled under reduced pressure prior to polymerization. 2,2′-azobis(2-(2-imidazoline-2-yl)propane) dihydrochloride (AIBI) was kindly supplied by Wako Chemicals (Neuss, Germany). The steric stabilizer poloxamer 338 (block copolymer of ethylene and propylene oxide, molecular weight: 14 500) was a gift from ICI Chemicals (Manchester, UK). ON synthesis was performed on controlled pore glass (Perseptive Biosystems, Wiesbaden, Germany) as solid support. Chemicals for solid-phase synthesis were purchased from Perseptive Biosystems and Applied Biosystems (Weiterstadt, Germany). Dialysis membranes for the purification of particles (SpectraPor CE membranes, MWCO 10 000) were obtained from Roth. Purified water was provided by a MilliQ185 Plus water purification system (Millipore, Germany).

ON synthesis and purification

The PTO 16mer with the sequence 5′-ACG GAA ACC GTA GCT G-3′ was prepared by standard phosphoramidite chemistry using an ABI 394 DNA/RNA synthesizer. Tetraethylthiuram disulfide (TETD, Applied Biosystems) was used as the sulfurizing reagent. Purification of the crude PTO was carried out with reversed-phase HPLC using an S 1000 solvent delivery system, an S 8110 low pressure gradient mixer (Sykam), a UVIS 205 UV/VIS spectrometer (Linear), and a Nucleosil C18 5 μm column (Grom, Herrenberg, Germany).

Preparation and characterization of the nanoparticles

Polystyrene latexes were prepared by batch polymerization under surfactant-free conditions in aqueous medium and purified by dialysis against deionized water (24 days) as described elsewhere [13]. Before and after dialysis, the polymeric content of the suspension was determined by freeze drying. Prior to use the non-ionic surfactant poloxamer 338 was dissolved in purified water and desalted by dialysis against deionized water (12 days). Sterically stabilized particles were prepared by the addition of poloxamer 338 to the latex suspension (final concentration: 0.1% w/v). The particle size and size distribution of the stabilized and unstabilized latexes were determined by PCS. The surface charge was measured by conductometric titration [14] with 0.01 N NaOH at 25 °C under an argon atmosphere using an LF 2000 microprocessor conductivity meter (WTW, Germany). Prior to titration the polymer suspension was diluted with deionized water to a final solid content of 5 mg/ml.

Physicochemical characterization of the nanoparticle-ON conjugates

Adsorption isotherms

Polymer suspensions (final solid content: 1.5 mg PS/ml) were incubated with the PTO 16mer at 25 °C over a period of 24 h in 10 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7). To determine the amount of ON adsorbed on the latex, the particles were separated by centrifugation (24 000 g, 45 min) and the supernatant was centrifuged again (45 min) to remove residual solid. The ON concentration in the supernatant (c_{eq}, μmol/l) was determined by measuring the optical density at 260 nm according to Eq. (1)

$$c_{eq} = \frac{E_{260nm}}{\varepsilon \cdot d} \cdot \frac{V_a}{V_0} = E_{260nm} \cdot \frac{1000}{(16n_A + 9.6n_T + 7n_C + 12n_G)} \cdot \frac{V_a}{V_0}, \quad (1)$$

where E_{260nm} is the absorption at 260 nm, ε is the extinction coefficient determined by n_A, n_T, n_C and n_G, the number of nucleotides adenosine, thymidine, cyti-

dine and guanosine present in the ON, V_a is the volume of the solution analysed and V_0 is the incubation volume. The amount of adsorbed PTO [$n_{ads}(\mu mol/g_{PS})$] was calculated from the difference in concentration before (c_0) and after incubation (c_{eq}) using Eq. (2)

$$n_{ads} = \frac{V_0 \cdot (c_0 - c_{eq})}{M_{PS}}, \quad (2)$$

where M_{PS} is the amount of adsorbent.

Turbidity measurements

Nanoparticle-ON conjugates were prepared by adding different amounts of PTO 16mer to diluted particle suspensions (final solid content: 1.8 mg/ml). The sample were vigorously shaken and allowed to stand for 1 h. Aliquots of 100 μl were taken, diluted with purified water to a final volume of 3 ml and the turbidity was measured at 400, 600 and 800 nm using a Lambda 5 UV/VIS spectrometer (Perkin Elmer).

Photon-correlation spectroscopy

The nanoparticle-ON conjugates were analyzed by PCS using a N4 Plus photon-correlation spectrometer (Coulter Electronics, Krefeld, Germany). The samples were diluted with 3 ml purified water (final solid content: around 0.02 mg/ml) and measured at 20 °C (wavelength: 633 nm, angles: 90.0°, 30.1°). The mean particle diameter and the polydispersity index were measured three times and are given as average values.

Zeta-potential measurements

Electrophoretic mobility of the nanoparticle-ON conjugates was measured using a Delsa 440SX (Coulter Electronics, Krefeld, Germany). Determination of the zeta potential was carried out with micro-electrophoresis employing laser Doppler anemometry. To achieve a constant ionic background, the samples were diluted with 0.001 M KCl solution (final solid content: around 0.09 mg/ml).

Results

Physicochemical characteristics of the nanoparticles

Cationic polystyrene nanoparticles (NP) were prepared by emulsifier-free emulsion polymerization using AIBI as the initiator [13, 15]. The crude latex was purified by dialysis against deionized water to remove residual monomer and water-soluble polymer chains. The SEM micrograph of the purified latex shows a rather monodisperse size distribution of spherically shaped particles (Fig. 1). The particles are electrostatically stabilized by positively charged imidazolinium end groups introduced by the cationic initiator during the polymerization process.

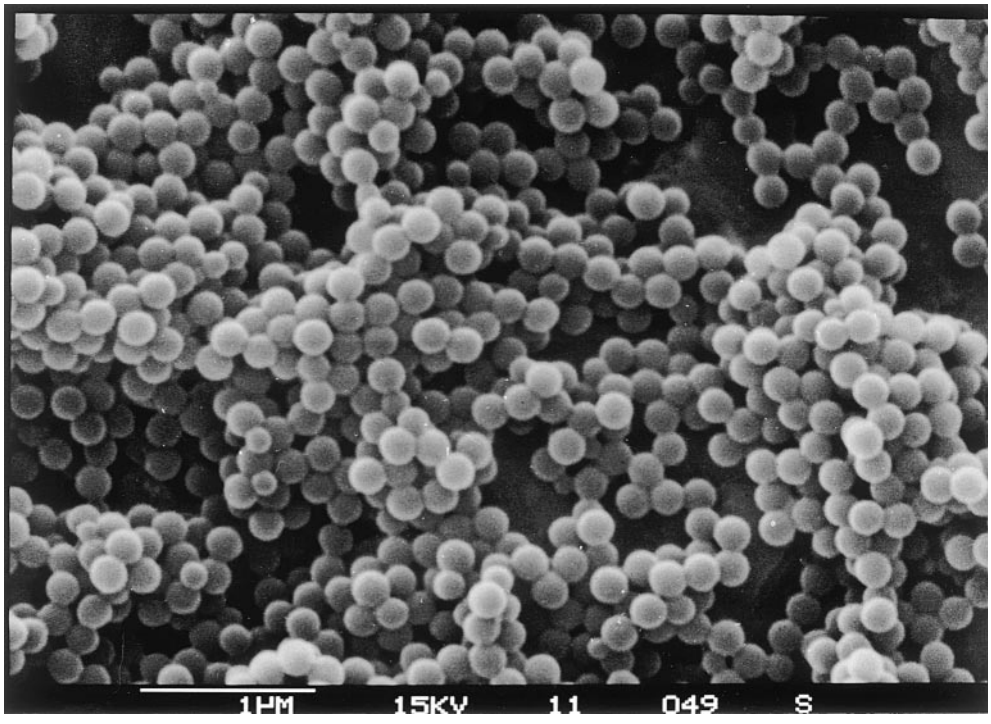

Fig. 1 Scanning electron micrograph of the crude latex (NP)

Sterically stabilized latex particles (NP-338) were prepared by the addition of the non-ionic surfactant poloxamer 338. This block copolymer is capable of adsorbing with its hydrophobic poly(propylene oxide) section on the polystyrene surface while the hydrophilic poly(ethylene oxide) chains protrude into the dispersion medium generating a hydrophilic surface and a steric barrier [16, 17]. Sterically stabilized nanoparticles bearing more hydrophilic surfaces exhibit reduced interactions with plasma proteins compared to unmodified polystyrene latexes [18]. The sequestration of coated particles by the reticuloendothelial system is retarded and, as a consequence, prolonged blood circulation can be achieved. This is advantageous for the application of the nanoparticles as drug carriers [19].

The surface charge density of sterically stabilized and uncoated particles was determined by conductometric titration. The results of the physicochemical characterization of both materials are summarized in Table 1 demonstrating that both the mean particle diameter and the surface charge density are only marginally influenced by the addition of steric stabilizer. In contrast, the zeta potential of the coated particles is reduced due to the fact that in presence of the coating layer of non-ionic surfactant molecules the position of the slipping plane is shifted further from the particle surface [20].

Conjugate formation

In order to investigate the interactions between drug and carrier and to obtain information about the binding affinity and the loading capacity of the carrier system, adsorption studies were carried out. The adsorption isotherm of PTO 16mer adsorbed on the sterically stabilized latex (NP-338) is depicted in Fig. 2. The adsorption profile shows an almost vertical slope at low equlibrium concentrations of PTO and a saturation level of 2.83 μmol PTO/g polymer. The data demonstrate that once the saturation level is reached a further increase in the ON concentration does not lead to conformational rearrangement of the adsorbed molecules on the particle surface. These findings are in accordance with recently published results of Fritz et al. [13] who investigated the adsorption characteristics of various cationic polystyrene latexes sterically stabilized with poloxamer 338 using several modified ONs. The observed adsorption profiles indicate strong attractive forces which were reported to be a consequence of multiple electrostatic interactions between the polyanionic backbone of the ONs and the positive surface charges of the particles.

Table 1 Physicochemical properties of the nanoparticles

Latex	$\bar{d}_n^a$ nm	SD nm	Surface charge		Zeta potential mV
			mC/g polymer	μC/cm^2	
NP[b]	210	32.1	1880	6.6	58.2
NP-338[c]	208	32.9	2060	7.2	46.0

[a] Number-average particle diameter
[b] Uncoated nanoparticles
[c] Sterically stabilized nanoparticles

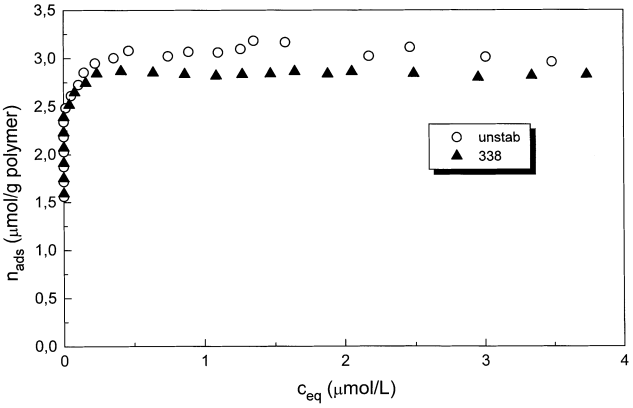

Fig. 2 Adsorption isotherms of phosphorothioate oligonucleotide (PTO) 16mer on NP (○) and on the sterically stabilized latex (NP-338) (▲). Experimental conditions: 10 mM NaH_2PO_4/Na_2HPO_4 (pH 7), 1.5 mg/ml polymer

Notably, the adsorption isotherm of the PTO 16mer adsorbed on the uncoated latex NP has an almost identical shape compared to the coated material NP-338 with a slightly different saturation level of 3.03 μmol PTO/g NP (Fig. 2). Obviously, ON adsorption is only marginally influenced by the coating layer of surfactant molecules.

In Ref. [13] the RSA model was used for the interpretation of the experimental data obtained by the adsorption isotherms. This model describes the irreversible adsorption of objects with random orientation on a surface without overlapping [21]. The maximum surface coverage (jamming limit) was evaluated by considering 2D adsorption of the ONs with the main symmetry axis orientated parallel to the particle surface. The results indicate that the surface coverage at the saturation level can be considered as a monolayer of "side-on" adsorbed molecules.

Analogously the RSA model has been used for calculations of the theoretical maximum surface concentration of adsorbed ONs in the present study. Assuming the shape of the applied ON to be a weakly elongated cylinder, the jamming limit $\theta_j = 0.53$ has been estimated from previously published simulation data for 2D adsorption of rectangular objects [22]. The surface concentration of a monolayer coverage was calculated for "side-on" adsorbed molecules by

$$A = \theta_j \frac{M}{N_A \cdot S}, \tag{3}$$

where M is the molecular weight of the PTO 16mer, N_A is the Avogadro number and S is the cross-sectional scaling area of the cylindrical adsorbate molecules given by the product of the long axis $a = 5.44$ nm and the short axis $b = 2.0$ nm.

Comparing the experimental data with the calculation based on the RSA model, it appears that the values determined for the maximum amounts of adsorbed ONs are in good agreement with the theoretical value (Table 2). The higher experimental values could be due to interactions between the adsorbate molecules and the interface and among the molecules themselves which are neglected in the RSA model. Considering the available data, however, a partial brush-like orientation or a rearrangement of adsorbate molecules on the particle surface during the adsorption process cannot be excluded completely.

The number of positive surface charges theoretically available for a single adsorbed ON reaches 6.4 and 7.6 mean charges/ON for NP and NP-338, respectively. This corresponds with the assumed multiple electrostatic interactions between ONs and nanoparticles. The lower value calculated for the uncoated particles is due to a higher loading capacity combined with a slightly lower surface charge density compared to the sterically stabilized latex.

Influence of the drug content on the colloidal stability of the carrier system

The application of colloidal systems as carriers for therapeutic agents makes detailed investigation of their dispersive stability necessary. Particle coagulation in biological fluids can lead to undesired side effects such as vascular obstruction and/or irritant tissue reactions [23]. Thus, colloidal stability of the applied drug carrier is one of the basic requirements for their application in biological systems.

On the other hand the dispersive stability of a colloidal system can be significantly influenced by the type and amount of the drug compounds bound to the carrier. Thus, it was intended to investigate the influence of ON adsorption on the coagulation behavior using nanoparticle-ON conjugates with varying surface coverage of PTO 16mer. Additionally the influence of steric stabilzation has been evaluated by comparing the uncoated (NP) and the sterically stabilized latex (NP-338).

Table 2 Loading capacities of the latexes

Latex	Max. amount of adsorbed ON[a]			Charge/ON
	μmol/g	mg/m^2	A[b][mg/m^2]	
NP	3.03	0.55	0.42	6.4
NP-338	2.83	0.51	0.42	7.6

[a] ON: Oligonucletide
[b] Theoretical maximum surface concentration (random sequential adsorption model)

Two different analytical methods, turbidimetry [24] and PCS [25], were utilized to investigate the colloidal stability of both carrier systems. The turbidity of the particle suspensions at various amounts of ON added to the dispersion medium is shown in Fig. 3. In case of the uncoated material a strong increase in the turbidity was observed in the range 1–2.7 μmol ON/g polymer. Strikingly, at higher concentrations the turbidity of the particle suspension decreases to values similar to those of low ON concentrations. In contrast, the colloidal stability of the nanoparticles sterically stabilized with poloxamer 338 seems to be only marginally affected by PTO 16mer present in the samples.

In order to complement the turbidity measurements and to obtain information about the variation of the mean diameters of the nanoparticles bearing different surface concentrations of PTO 16mer, the samples were investigated by PCS at two different angles (90° and 30.1°). The use of multiple-angle analysis makes this method a valuable tool for sensitive monitoring of the agglomeration behavior of colloidal systems [25]. The effect of increasing ON concentration in the dispersion medium on the mean particle diameters of NP is shown in Fig. 4. In accordance with the turbidity measurements a large increase in particle size was observed up to ON concentrations of 2.7 μmol/g polymer, while higher concentrations led to a reduction in the mean particle diameters to values comparable with the initial particle sizes (250 nm).

The finding that the effect of ON adsorption on colloidal stability is significantly lower in the case of the sterically stabilized latex was confirmed by PCS measurements (Fig. 5). Nevertheless, an increase in the mean

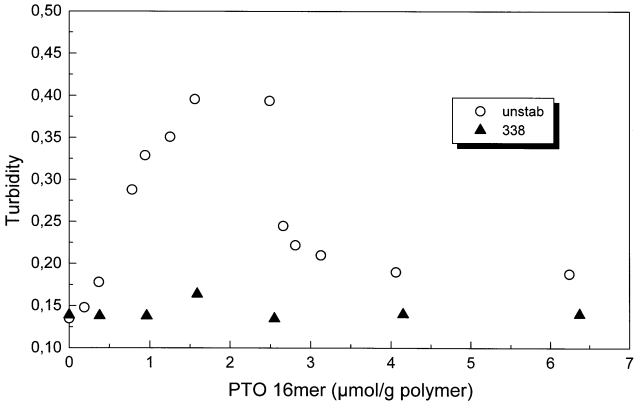

Fig. 3 Dispersive stability of NP (○) and NP-338 (▲) as a function of the amount of PTO 16mer/g polymer measured by turbidimetry at a wavelength of 600 nm

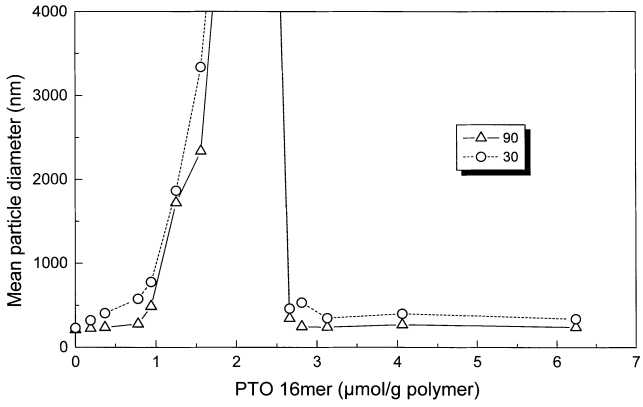

Fig. 4 Mean particle diameter of the latex NP as a function of the amount of PTO 16mer added per gram polymer measured by photon-correlation spectroscopy (PCS) at an angle of 90° (Δ) and 30.1° (○)

particle diameter is visible in the range 1–2.7 μmol ON/gram polymer.

Electrophoretic mobility of the nanoparticle-ON conjugates

Turbidimetry and PCS measurements demonstrated that the coagulation behavior of the carrier systems is strongly influenced by the amount of ON added to the latex. It seems that the coverage of adsorbed ONs changes the surface characteristics and hence the colloidal stability of the particles. In order to explain this phenomenon, the electrophoretic mobility of the nanoparticle-ON conjugates was determined at increasing ON concentration in the dispersion medium. Figure 6 shows the zeta potential of bare (NP) and coated (NP-338) nanoparticles as a function of the amount of PTO 16mer added to the latexes.

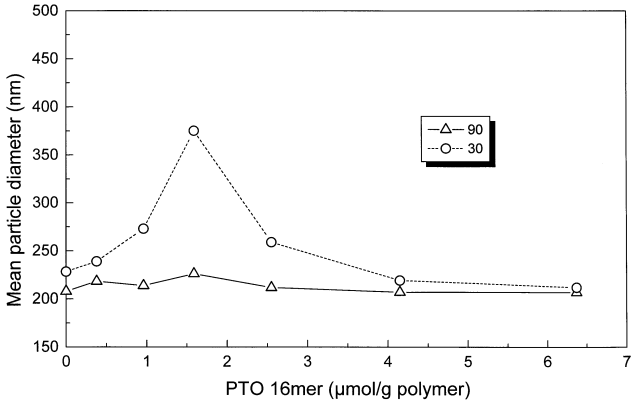

Fig. 5 Mean particle diameter of the latex NP-338 as a function of the amount of PTO 16mer added per gram polymer measured by PCS at an angle of 90° (Δ) and 30.1° (○)

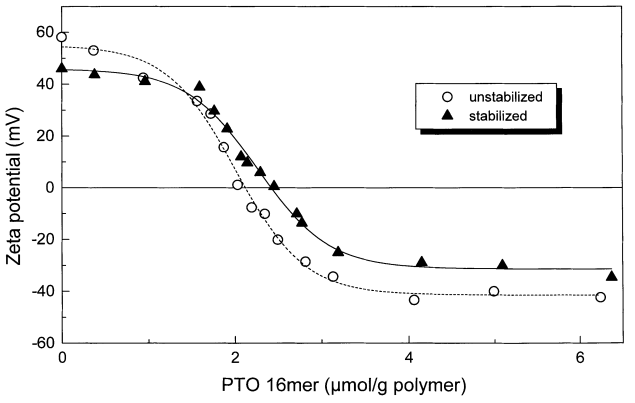

Fig. 6 Zeta potential of uncoated NP (○) and sterically stabilized NP-338 latex (▲) as a function of the amount of PTO 16mer/g polymer present in the samples. Analysis was carried out with an ionic background of 0.001 M KCl

The potential profiles of both materials have a similar shape. With increasing amounts of ON added, a reversal of the zeta potential from positive to negative values is observed. In the case of the sterically stabilized latex, however, the range of the zeta potential variation is diminished in comparison to the uncoated material. This can be explained by the influence of the coating layer of surfactant molecules which induces a shift of the slipping plane away from the particle surface.

The point of zero charge is reached at 1.9 and 2.4 μmol PTO 16mer/g polymer for NP and NP-338, respectively. The values obtained for both materials are similar indicating that the adsorption of ONs influences the surface charge of the particles in a comparable manner.

Discussion

Comparing the experimental data obtained by turbidimetry and PCS with the results of the zeta-potential measurements, it appears that the variation of the surface charge mediated by ON adsorption is responsible for the observed coagulation behavior of the carrier systems.

In the case of the uncoated material, addition of ON leads to a strong increase in turbidity and in the mean particle diameters indicating a decrease in colloidal stability which can be attributed to a reduction of the zeta potential. The point of zero charge is reached around 1.9 μmol ON/g polymer and this lies within the observed region of strong coagulation ranging from 1–2.7 μmol ON/g polymer. Notably, the presence of higher amounts of ONs was shown to have a stabilizing effect on the carrier system. This can be explained by an increase in the corresponding zeta-potential values and electrostatic repulsion of the particles with reversed sign.

The results obtained for the sterically stabilized latex, NP-338, indicate that the presence of poloxamer 338 has a considerable effect on the colloidal stability of the particles. Only the PCS measurements at 30° demonstrate coagulation by an increase in the mean particle diameters in the range 1–2.7 μmol ON/g polymer which is around the point of zero charge near 2.4 μmol ON/g polymer. Therefore, the influence of ON adsorption on colloidal stability cannot be neglected completely.

Considering the available data there is no evidence that the presence of surfactant molecules has a significant effect on the surface coverage with ONs. The colloidal stability as well as the zeta potential and the corresponding adsorption isotherms of PTO 16mer of the uncoated and the coated latex exhibit a comparable dependence on the amount of ON added.

In Fig. 7 the surface coverage as a function of the amount of ON added to the suspensions is given as a percentage of the maximum surface concentration determined from the adsorption isotherms. As expected, the profiles obtained for NP and NP-338 have a similar shape. As the maximum of coagulation cannot be determined exactly by turbidimetery and PCS, a region of coagulation has been defined where an increase in turbidity and mean particle diameter was unequivocally detected (lined area). The range between the points of zero charge of both materials are indicated in the diagram as a cross-lined area. It can be seen that the extent of coagulation reaches significant values around a surface coverage of 30% where the particles still exhibit a positive zeta potential. Further addition of ON leads to an increase in surface coverage and a growing destabilization of the nanoparticle-ON conjugates. The point of zero charge is reached at 63% and 83% surface coverage for NP and NP-338, respectively.

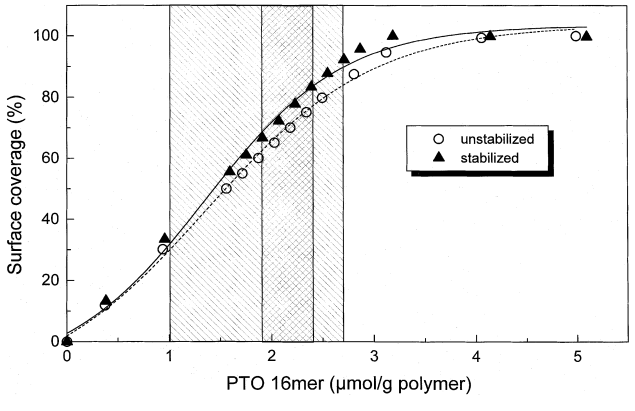

Fig. 7 Percentage surface coverage of PTO 16mer on NP (○) and NP-338 (▲) as a function of the amount of oligonucleotide added. The *lined area* indicates the main region of coagulation determined by turbidity and PCS measurements. The range between the points of zero charge of both materials determined by zeta potential measurements is depicted by the *cross-lined area*

At about 2.7 μmol PTO 16mer added to the polymer suspensions, the extent of coagulation is significantly diminished and the conjugates exhibit a colloidal stability similar to the ON free samples. As already mentioned, the variation in the coagulation behavior is much more dramatic in the case of the uncoated material. The upper margin of the coagulation area corresponds to a surface coverage of about 85–90% which is still on the increasing part of the adsorption profiles. In summary, the decrease in turbidity and mean particle diameter observed at higher surface concentrations can be attributed to an enhancement of colloidal stability due to a growing zeta potential with reversed sign and hence an increase of the electrostatic repulsion of the particles. The maximum surface concentration is reached at 4.1 and 3.2 μmol PTO 16mer added per gram polymer for NP and NP-338, respectively.

Conclusion

In order to evaluate the suitability and biological compatibility of a model drug carrier system for antisense ONs the mean particle size, colloidal stability and zeta potential of the nanoparticle ON conjugates were investigated under the aspect of drug loading. The findings were correlated to the corresponding adsorption studies.

The adsorption profiles of the uncoated latex and the sterically stabilized latex have a similar shape indicating that the coating layer of non-ionic surfactant only marginally influences ON adsorption. Comparing the adsorption studies with calculations based on the RSA model, it appears that the surface coverage at the saturation level can be considered as a monolayer of side-on adsorbed molecules.

The colloidal stability of both latexes – as a function of the amount of ON added – was monitored by turbidimetry and PCS. The results demonstrate that the amount of ON present in the dispersive medium has a considerable influence on the coagulation behaviour of the materials. The dispersive stability decreases with increasing amount of ON added, reaches a minimum and strikingly increases again at the higher ON concentrations. Obviously, the coating layer significantly enhances the colloidal stability of the nanoparticles. However, a small increase in the mean particle diameter was detected in the same range as for the uncoated material.

The coagulation behavior was found to be dependent on the zeta potential of the latexes. For both latexes, the profiles of the zeta potential as a function of the amount of ON present in the samples show a reversal from positive to negative values. The points of zero charge are within the region of main coagulation, whereas the enhancement of the colloidal stability at higher ON

concentrations corresponds to negative values of the zeta potential.

The findings of the colloidal-stability and the zeta-potential measurements were correlated with the results of the adsorption studies. The extent of coagulation reaches significant values at a surface coverage where the particles still exhibit a positive zeta potential. Comparison of the adsorption profiles of both materials with the zeta-potential data indicates that the bottom plateau of the zeta potential correlates with the maximum surface coverage.

In conclusions, the observed coagulation behavior of the carrier system is a consequence of conjugate formation between nanoparticles and ONs. The growing surface coverage causes a reversal of the zeta potential. Thus, the observed stabilization of the conjugates at high ON concentrations can be attributed to an increase in the electrostatic repulsion of particles with reversed sign.

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