Research Article

On the Complexity of Electrostatic Suspension Stabilization of Functionalized Silica Nanoparticles for Biotargeting and Imaging Applications

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Different means of attaching streptavidin to surface functionalized silica particles with a diameter of 240 nm were investigated with special focus on suspension stability for electrostatically stabilized suspensions. The influence of two different fluorescent dyes covalently linked to the streptavidin on suspension stability was also studied. The results clearly show that the stability of the suspensions is crucially dependent on all functional groups present on the surface. The surface functions may either directly affect the effective surface charge if the functions contain charged groups, or indirectly by affecting the relative concentration of charged groups on the particle surface. Poly(ethylene imine)-functionalized silica particles, where the polymer is grown by surface hyperbranching polymerization, are shown to be promising candidates for bioapplications, as the zeta-potential can remain strongly positive even under biologically relevant conditions.

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1. INTRODUCTION

Nanoparticles have been used in diverse biological applications such as fluorescent markers in vitro and in vivo, clinical diagnosis, and drug delivery [1–3]. Nanoparticles have a comparable size to natural carriers and other biomolecules, which gives them the ability to cross physiological barriers and access different tissues followed by an efficient cellular uptake and intracellular internalization [2]. It has been shown that latex particles are endocytosed by nonphagocytic eukaryotic cells for particle sizes up to 500 nm, but the efficiency of cellular uptake decreased with increasing particle size [4, 5]. The highest uptake was observed for particles having diameters of 100–200 nm or smaller, whereas the uptake of particles with diameters of 1 μ m or larger was negligible [5, 6]. Furthermore, it would be beneficial if the particles carry a positive surface charge, as the cell membrane is normally negatively charged [7]. Targeting specific cells and receptor-mediated endocytosis, however, requires surface functionalization of the particles with suitable ligands,

such as antibodies, for which cells do express specific receptors [2]. The linking chemistry is often relying on peptide (amide) bond formation between carboxylic acids and amines and thus the presence of amine or carboxyl groups on the outer particle surface is of special interest.

Silica submicron- or nanoparticles are emerging as a promising and versatile alternative to polymer-based counterparts, as they can easily be produced with a tunable particle size and pore structure. Furthermore, silica is biocompatible and chemically inert under a wide range of conditions [8, 9]. According to several studies, silica nanoparticles are efficiently endocytosed by mammalian cells [1, 10, 11]. Functionalization of the silica surface is relatively straightforward using well-documented methods, which allows the surface chemistry of the particles to be tailored, and also facilitates further attachment of targeting functions. Pristine silica is negatively charged over the pH range of biological interest, as the isoelectric point (IEP) of silica is in the range 2–3. Functionalization of the silica surface can naturally be used to alter the effective surface charge of silica, to an extent which is

controlled by the nature of the introduced functional groups and their surface concentration. The surface charge is important not only from a biological activity point of view, but also for preventing particle agglomeration in suspensions that are electrostatically stabilized. This is especially important under physiological conditions where the electrolyte concentration is high, which decreases the electrostatic repulsion between the particles. Electrostatic stabilization of a nanoparticulate suspension typically requires an absolute zeta potential value of 30 mV or higher. Introduction of additional surface functions onto the pristine or already surface-functionalized particles will typically also influence the effective surface charge of the particles. This could potentially lead to pronounced particle agglomeration even if the original particle suspensions were stable toward agglomeration. The aim of the present study was therefore to evaluate suspension stability for silica nanoparticles with various surface functionalities relevant to biological applications. As model system, nonporous, monodisperse silica nanoparticles with an average size of 240 nm (SEM) were prepared by the Stöber process [12]. The silica particles were first modified by hyperbranching surface polymerization of polyethyleneimine (PEI) using aziridine as precursor. The highly cationic PEI has in itself been used as a polymer film on cell adhesion substrates (instead of poly-L-lysine) [13], and as a versatile nonviral vector alternative for gene delivery [14–17]. Amino-functionalized silica particles have also been successfully employed as DNA carriers for gene delivery [18–20]. The surface concentration of amino groups is notably higher for PEI-functionalized silica, as compared to corresponding particles functionalized by silanization [21]. This is important, as it has recently been shown that silica particles with amino surface functions introduced by silanization actually strongly aggregate under neutral pH conditions [22]. Furthermore, carboxylic acid groups were introduced to the PEI layer in a second step by reaction with succinic anhydride. The surface-functionalized silica particles were further conjugated to the protein streptavidin, either native or labeled with a fluorophore, following three different routes. This opens up the possibility of later using biotin-antibody complexes for targeting cell receptors of interest, as streptavidin has an exceptionally high affinity to biotin. The surface-bound streptavidin retains its activity upon conjugation, as demonstrated by labeling the conjugated particles with Protein A gold using biotinylated alpha 2 integrin antibodies. The particle size and charge were characterized using dynamic light scattering (DLS) measurements and electrokinetic titrations. Imaging of the particles was performed by confocal fluorescence microscopy as well as SEM and TEM. The results are discussed with special emphasis on the stability of the suspension towards aggregation under different conditions.

2. EXPERIMENTAL

2.1. Synthesis of silica Stöber particles

The Stöber synthesis [12] was employed to prepare silica nanoparticles with diameters of about 240 nm with a narrow particle size distribution. In this particular synthesis,

2.6 g tetraethyl orthosilicate (TEOS, 98% Fluka) was added to a solution containing 31.6 g ethanol (99.5%, Altia Oyj), 5.0 g deionized water, and 0.88 g NH_4OH (33%, J.T. Baker), which was stirred at 450 rpm for 20 hours at room temperature. The resultant molar composition was 14.04 : 2.6 : 0.35 : 6.45 : 6.45 EtOH: TEOS: NH_3 : H_2O . The silica particles were separated by centrifugation (4500 rpm, 5 minutes), washed with ethanol and water, and dried in air at room temperature for one day.

2.2. Surface polymerization of polyethyleneimine

The silica particles were surface modified by surface hyperbranching polymerization of polyethyleneimine (PEI) using aziridine as the precursor. Aziridine was synthesized from aminoethylsulphuric acid (Sigma-Aldrich, Miss, USA) according to the procedure described by Allen et al. [23]. The polymerization was performed in one step under argon atmosphere with toluene as solvent, in which 0.4 g vacuum-dried silica particles were suspended. Catalytic amounts of acetic acid were added under stirring, after which 100 μL aziridine was added to the suspension. The suspension was refluxed under stirring overnight at 348 K, filtered, washed with toluene, and dried in vacuo at 323 K.

2.3. Succinylation of PEI

Succinic acid groups were formed on the PEI-modified surface by the following procedure according to Schiestel et al. [9]. A suspension of 25 mg well-dried PEI-functionalized nanoparticles, 1.5 mL tetrahydrofuran ($\geq 99, 9+\%$, Sigma-Aldrich), and 25 mg succinic anhydride (97%, Sigma-Aldrich) was shaken for 16 hours at RT. A long reaction time was chosen to maximize the conversion of amine groups. Finally, the particles were filtered, washed with THF, and dried in vacuo at 323 K.

2.4. Binding of glutaraldehyde to PEI

Glutaraldehyde was bound to the amino groups of PEI according to the procedure described by Ashtari et al. [24]. A suspension containing 10 mg PEI-modified silica particles and 5 wt-% glutaraldehyde in 5 mL MeOH was shaken for 2 hours at RT. The particles were subsequently washed twice with phosphate-buffered saline (PBS) (pH 7.4), and separated by centrifugation (2 minutes, 4500 rpm).

3. STREPTAVIDIN CONJUGATION

3.1. Linking streptavidin utilizing carbodiimide chemistry

Carboxylic acid group activation by 1-Ethyl-3-(3-(dimethylamino)propyl)-carbodiimide (EDC) was used to directly conjugate labeled streptavidin (Streptavidin Alexa Fluor 555 conjugate, Invitrogen Molecular Probes) to the amino groups in the PEI layer and to carboxyl acid groups of surface succinic acid groups. The used fluorophore, Alexa555, is a monoreactive, single-isomer succinimidyl ester dye with

an excitation/emission maxima of 555/565 nm and a molar mass of approximately 1250 g/mol. The powder is magenta in color, which facilitates the dye binding recognition visually. In all cases, the particles were intensely pink after the reactions. When indicated, streptavidin conjugated with the DyLight 549 fluorophore (Pierce Biotechnology, Rockford, Ill, USA) was used. The fluorophore DyLight is a photostable orange dye with similar spectra and brightness to Alexa Fluor 555, but with excitation/emission maxima of 550/568 nm in PBS and a molar mass of 982 g/mol.

EDC was used as carboxyl group activating compound to induce the formation of an amide bond between streptavidin and the particle surface. For the particles containing succinic acid groups, EDC was used to form an amide bond between the carboxyl group of succinic acid and streptavidin amino groups. For native PEI-functionalized silica particles, the surface amide bond was formed between PEI-surface amino groups and carboxyl groups of streptavidin (see Scheme 1).

A mixture of 10 mg particles, 5 mL MES ($\geq 99\%$, Sigma-Aldrich)-buffer (0.1 M, pH 5.7), 8.9 μL EDC ($\geq 97\%$, Fluka), and 30 μg labeled streptavidin was prepared. When indicated, unlabeled streptavidin (Streptavidin from *Streptomyces avidii*, Sigma-Aldrich) was used in an otherwise identical mixture. The mixture was stirred for 24 hours at 277 K, in order to retain the activity of the protein. Particles were separated by centrifugation (3 minutes, 4500 rpm) and washed once with MES and twice with PBS.

3.2. Streptavidin conjugation to glutaraldehyde linker

PEI particles with a glutaraldehyde linker were conjugated with streptavidin by preparing a suspension of the following composition: 2 mL PBS, 30 μg streptavidin, and 10 mg particles. When indicated, unlabeled streptavidin (Streptavidin from *Streptomyces avidii*, Sigma-Aldrich) was used in an otherwise identical mixture. The mixture was stirred for 24 hours at 277 K. Streptavidin modified particles were separated by centrifugation (3 minutes, 4500 rpm).

4. CHARACTERIZATION METHODS

4.1. Dynamic light scattering measurements

Dynamic light scattering (DLS) measurements were performed at 298 K using a zetasizer equipment (Model Nano ZS, Malvern, Worcestershire, UK) equipped with a red laser operating at 632.8 nm and the detector positioned at 173° (noninvasive back scattering technology). The data was analyzed using the Malvern Dispersion Technology Software v. 4.20.

4.2. Electrokinetic titrations

The electrokinetic titrations were performed using a Malvern ZetaSizer Nano-ZS coupled with an MPT-2 Titrator unit. The zeta potential was measured as a function of pH by titrating with 0.1 or 0.5 M HCl and NaOH at 298 K. The samples were suspended in deionized water or a saline solution with

the same ionic strength ($\text{NaCl} + \text{KCl} = 150 + 4.2 \text{ mM}$) as PBS-buffer and dispersed by sonication.

4.3. Confocal fluorescence microscopy

Laser scanning confocal microscopy (Carl Zeiss Axiovert 200 M LSM 510 META, Jena, Germany) was performed using a 63x objective, excitation wavelength 543 nm, detection wavelength range $\leq 560 \text{ nm}$, and digital resolution optimized for optical resolution. The images were processed with the BioImageXD software [25]. The measurements were performed on particle suspensions with a concentration of 0.01–0.02 mg/mL in water, which were sonicated before imaging.

4.4. Scanning electron microscopy

SEM images were recorded with a JEOL JSM 6335F, Jeol Ltd, Japan (10 kV) microscope.

4.5. Transmission electron microscopy

A drop of particle-water suspension of concentration 0.5 mg/mL was pipetted onto a copper grid (460 High Trans + coal crimped Formwar-coated grids, 0.3%), whereafter the solvent was allowed to evaporate and the samples were detected by STEM (JEOL JEM-1200 EX Electron Microscope).

4.6. Biotinylation of antibodies

The alpha 2 integrin antibody (MCA-2025, Serotec) was buffered with 91 mM NaHCO_3 . Biotin N-hydroxysuccinimide Ester (Calbiochem) in DMSO was added to the buffered solution to a final concentration of 0.09 mg/mL. The solution was incubated for 18 hours at 277 K (under gentle agitation). Dialysis was performed in a Slide-A-Lyzer Dialysis Cassette 10 K (Pierce Biotechnology) according to the manufacturers' instructions.

4.7. Gold labeling of streptavidin

Labeling of streptavidin-conjugated particles for TEM was prepared as follows. The 0.5 mg/mL particle suspension was added with biotinylated MCA-2025 alpha 2 integrin antibody to a final concentration of 10 $\mu\text{g/mL}$ and the mixture was incubated 2.5 hours on a shaker. The particles were washed twice with ion-exchanged H_2O with centrifugation in between the washes (fast spinning down, 14.1 rcf, 4 minutes), centrifuged once more and diluted in H_2O to an approximate concentration of 0.5 mg/mL. The particles were then incubated with 1:100 diluted Protein A gold (diameter: 10 nm) for 13 hours on a shaker at 277 K. The protein A gold reagent, provided as a kind gift from Dr. Varpu Marjomäki, University of Jyväskylä, was prepared according to the procedure described by Slot and Geuze [26] and the OD_{520} of the 1:100 dilution of the reagent was 0.19. Finally, the particles were washed twice with H_2O with centrifugation in between the washes (fast spinning down, 14.1 rcf, 4 minutes), centrifuged once more, and diluted in H_2O to an approximate concentration of 0.5 mg/mL.

SCHEME 1: Modification pathways for conjugation of the labeled streptavidin to the particle surface.

FIGURE 1: SEM image of polyethyleneimine modified silica nanoparticles.

FIGURE 2: Electrokinetic titration curves for functionalized particles in water. PEI-SiO₂ (▲) IEP 10.3, GA-PEI-SiO₂ (■), IEP 9.0, and Succinic acid-PEI-SiO₂ (●), IEP 5.4.

5. RESULTS

5.1. Prefunctionalization of silica particles

An SEM image of the PEI-functionalized particles is shown in Figure 1, demonstrating that all particles are spherical with uniform size. The total PEI content was 7.7 wt% as estimated from thermogravimetric measurements, corresponding to 1.8 mmol amino groups per gram of silica. The concentration of accessible amine groups was estimated based

on the imine method described by Moon et al. [27] and yielded a primary amine concentration of 0.9 mmol per gram of particles. We note that 50% of the amino groups would be primary amino groups in a fully-branched PEI structure with several generations. The electrokinetic titration curve measured in water for the PEI-functionalized silica particles is shown in Figure 2. The isoelectric point (IEP) of the PEI-functionalized silica was 10.3, which can be

FIGURE 3: PEI-GA-STV(Alexa555) particles (0.01 mg/mL) in water. The scale bar corresponds to 20 μm .

FIGURE 4: PEI-Succinic Acid-STV(Alexa555) in water (0.01 mg/mL). The scale bar corresponds to 20 μm .

compared with the corresponding value of about 2 for the non-functionalized silica particles. The much higher value obtained for the PEI-functionalized particles originates from the presence of amino groups on the surface, which are virtually fully protonated at pH values below 9 as the intrinsic pK_a -value of PEI is 10.6. However, the fact that the PEI-functionalized particles do exhibit an IEP provides evidence for the presence of remaining surface silanols on the particle surface [21]. PEI provides a highly positive charge over a wide pH-range, with a zeta potential of roughly +60 mV in water at neutral pH, which makes the PEI-functionalized silica particles easily dispersible.

As methanol was used as solvent for the reaction of glutaraldehyde (GA) and the PEI-functionalized particles, the functionalization step was performed separately from the streptavidin conjugation step. The isoelectric point decreased from 10.3 to 9.0 after the glutaraldehyde modification (see Figure 2), indicating successful attachment of glutaraldehyde to a fraction of the amine groups. Since the aldehyde group is not a dissociable group, it will not intrinsically contribute to the surface charge density and hence zeta potential, but its effect can be ascribed to the decrease in the effective amino group content of PEI upon glutaraldehyde linking, leading to an increase in the relative concentration of negatively charged silanol groups as compared to positively charged amino groups on the particle surface.

The zeta potential versus pH curve measured for the succinylated PEI-silica particles is shown in Figure 2. The IEP dropped markedly from 10.3 to 5.35 after succinylation, as expected for a successful introduction of carboxylic acid groups. However, the IEP of 5.35 is higher than that expected for a quantitative conversion of amino groups upon succinylation, as the intrinsic pK_a of succinic acid is 4.16. An IEP of 3 has been reported for succinylated amino-functionalized silica particles where the amino groups were introduced by silanization [9]. The higher IEP for the PEI-functionalized particles suggests that some amine groups still remain in the PEI layer after succinylation shifting the IEP towards higher values. The high absolute zeta potential at lower pH also provides evidence for the presence of amino groups remaining from the PEI function.

FIGURE 5: PEI-STV(Alexa555) (0.02 mg/mL) in water. The scale bar corresponds to 20 μm .

6. BIOCONJUGATION OF STREPTAVIDIN TO SURFACE-FUNCTIONALIZED SILICA PARTICLES

Electrokinetic titrations were carried out for all streptavidin conjugated particles in both water and a saline solution (0.154 M NaCl + KCl), and the results are summarized in Table 1. We will first focus on the pure water suspensions before returning to discuss the results obtained under saline conditions. When Alexa555-labeled streptavidin was linked to either GA-PEI-SiO₂ or PEI-SiO₂, a clear decrease in the IEPs from 9.0 to 6.1 and from 10.3 to 9.3 was observed, respectively. The decrease in IEP can be understood, as streptavidin has an IEP of 5-6, which is lower than that of the original particles. Furthermore, the linking of the streptavidin to PEI-functionalized particles consumes amino groups, while amino groups in the streptavidin are consumed when linking streptavidin to glutaraldehyde functionalized particles [28, 29]. This could also have an influence on the zeta potential, as could the pH-dependent charging of the Alexa555 fluorophore. For the Alexa555-streptavidin-PEI-SiO₂ particles, the zeta potential value exceeded +40 mV at pH values below about 8 in water, which suggests that these particles are electrostatically stabilized in suspension, while the IEP value

TABLE 1: IEP values for protein-conjugated particles as determined from electrokinetic titrations in water or in saline solution (SS) with the same ionic strength as used in biological media (0.154 M).

	Glutaraldehyde	Succinic acid	PEI
Stv-Alexa555, in water	6.1	5.9	9.3
Stv-Alexa555, in SS	7.1	6.2	8.2
Stv 30 μ g (unlabeled) in SS	8.5	6.2	10.2
Stv-DyLight549, in water	—	—	7.9
Stv-DyLight549, in SS	—	—	6.8

FIGURE 6: PEI-GA-STV(Alexa555) particles, 0.5 mg/mL in water: (a) 3×1000 magnified image and (b) 200×1000 magnified particles where streptavidin is labeled with biotinylated alpha 2 integrin antibody and protein A gold.

FIGURE 7: PEI-Succinic Acid-STV(Alexa555), 0.5 mg/mL in water: (a) magnified image and (b) 200×1000 magnified image of particles where streptavidin is labeled with protein A gold by using biotinylated alpha 2 integrin antibody.

for the Alexa555-streptavidin-GA-PEI-SiO₂ particles is close to 6, and these particles should therefore not form stable suspensions in water. For succinylated particles, a slight increase in the IEP from 5.35 to 5.9 was observed upon linking fluorescently labeled streptavidin to the particles, hence the net effect of the linking of the streptavidin-Alexa555 complex to the succinylated particles results in an increase in the IEP. Again, the IEP of these particles is very close to the pH of water, so flocculation should be observed in water if the suspensions are electrostatically stabilized.

Confocal fluorescence microscopy was used to study the flocculation behavior of the different fluorescently labeled streptavidin particles in water. The corresponding images are shown in Figures 3–5. All particle types are seen to fluoresce under the conditions applied for microscopic imaging, also indicating a successful conjugation of the labeled streptavidin. For the Alexa555-streptavidin-PEI-SiO₂ suspensions, virtually no flocculation was observed, in agreement with the highly positive zeta potential at a pH of 6 for this system (see Figure 5). For the other two sets of particle suspensions, strong agglomeration was observed (see Figures 3 and 4), in agreement with their low absolute values of the zeta potential. However, in addition to a direct charge-related flocculation effect, also linking of different particles via self-conjugation between outer carboxylic acid and remaining

amino groups (for succinylated particles) or between amino groups (for glutaraldehyde-linked particles), or between proteins during the EDC-mediated coupling reaction, can be expected for both the glutaraldehyde and the succinylation-based systems. The conditions during the EDC-coupling (pH = 5.7) are close to the IEP of the succinylated particles, where the spatial proximity due to particle flocculation might further facilitate possible self-conjugation reactions.

The different extents of particle aggregation can also be observed by TEM, where the samples were prepared by placing a drop of the suspension on a carbon grid followed by evaporation of the water (see Figures 6(a), 7(a), and 8(a)). While the PEI-SiO₂-based particles remain nonaggregated, strong particle agglomeration is seen for the other two systems.

The presence of covalently attached streptavidin on the particle surface and the preservation of the streptavidin activity was ensured in all systems by labeling the streptavidin-conjugated silica particles by biotinylated antibodies in combination with Protein A linked gold particles. The corresponding TEM images of the gold-labeled particles are shown in Figures 6(b), 7(b), and 8(b), where gold particles can be observed on the particle surfaces for the Alexa555-streptavidin functionalized silica particles, regardless of the applied linking chemistry.

FIGURE 8: PEI-STV(Alexa555), 0.5 mg/mL in water: (a) 3×1000 magnified image and (b) 200×1000 magnified picture of particles where streptavidin is labeled with Protein A gold by using biotinylated alpha 2 integrin antibody.

FIGURE 9: Simultaneous electrokinetic titration (■) and particle size determination (▼) (DLS) of SiO_2 -PEI-STV(Alexa555) particles in water, IEP 9.3.

In order to study the influence of the fluorophore on the pH-dependent charging of the particles, and thus also on the suspension stability, another set of PEI- SiO_2 functionalized particles was conjugated with streptavidin, but now using a DyLight 549 labeled streptavidin. The zeta potential curves measured in water for DyLight549-PEI- SiO_2 and Alexa555-PEI- SiO_2 are shown in Figures 9 and 10, together with the mean particle size measured simultaneously by dynamic light scattering. While the Alexa555-PEI- SiO_2 suspension remains fully stable up to a pH of about 8, in good agreement with the highly positive zeta potential of +30 mV or higher in this pH range, the DyLight549-PEI- SiO_2 particles have an IEP value of about 7.9, and agglomerate strongly at pHs higher than about 6.5. This is also clearly seen by fluorescence microscopy (see Figure 11). DyLight fluorophores are synthesized through sulfonate addition, which makes DyLight dyes negatively charged and hydrophilic. DyLight dyes are commercially available as succinimidyl esters, which is also the

FIGURE 10: Simultaneous electrokinetic titration (■) and particle size determination (▼) (DLS) of SiO_2 -PEI-STV(DyLight549) particles in water, IEP 7.9.

FIGURE 11: PEI-STV(DyLight549) (0.01 mg/mL), in water. The scale bar corresponds to $20 \mu\text{m}$.

case for Alexa555. Both fluorophores are hence amine reactive dyes, and are conjugated to free amine groups of the streptavidin tetramer, so the conjugation chemistry of the two fluorophores to the protein molecules is similar. These results indicate that also the charging of the fluorophore has a strong influence on suspension stability.

7. EFFECT OF THE CONJUGATED STREPTAVIDIN

To investigate the effect under conditions relevant to bioapplications, electrokinetic measurements were carried out in saline (0.154 M NaCl + KCl) solutions for both the labeled protein-particle complexes as well as nonlabeled streptavidin-particle complexes at a concentration of $3 \mu\text{g}$ streptavidin per mg of particles. The results are summarized in Table 1 with the corresponding titration curves in Figure 12. The IEP values for the different Alexa555-streptavidin labeled SiO_2 particles are close to, but slightly

FIGURE 12: Electrokinetic titration curves for unlabeled streptavidin-conjugated particles in salt solution. STV-PEI-SiO₂ (▲) IEP 10.2, STV-GA-PEI-SiO₂ (●), IEP 8.5, and STV-Succinic acid-PEI-SiO₂ (■), IEP 6.2.

different from, the values obtained in pure water, which can be ascribed to electrolyte effects on the effective pK_a values of the different ionizable groups present in the different systems. More importantly, the absolute values of the zeta potential are well below 30 mV in all cases at pH = 7.4, which means that the suspensions would be agglomerated under these conditions. Interestingly, the IEP of the PEI-SiO₂ labeled with native streptavidin is very high, 10.2, and the effective zeta potential at a pH of 7.4 is about 20 mV, which suggests that this system could be fairly stable even under high ionic strength conditions. The strong influence of the Alexa555 fluorophore on the zeta potential is connected to the fact that it is conjugated to the free amine groups of the streptavidin tetramer, which decreases the number of positively charged groups in the system.

8. SUMMARY AND OUTLOOK

Suspension stability is important in many bioapplications, including drug delivery and targeting of cells. As shown above, the suspension stability in a given electrostatically stabilized nanoparticulate system is affected by the linker chemistry applied for surface functionalization related to targeting and imaging, by the surface charge behavior of the biological molecules, and by any additional function introduced, in our case a fluorescent dye. PEI-functionalized silica particles are shown to be very promising candidates for biological applications due to their high positive effective surface charge even under conditions of high ionic strength, provided that the introduced additional surface functions do not decrease the IEP of the particles to values close to neutral. Furthermore, as it should be possible to control the thickness of the PEI layer by variation of the aziridine to silica ratio used in the surface functionalization step, it should be possible to extend the stabilization from electrostatic to electrosteric stabilization without changing the functionalization chemistry.

Such studies are underway in our laboratory. Thus, it should be possible to develop completely stable nanoparticulate suspensions that can be used for targeting and imaging in biological systems based on PEI-functionalized SiO₂.

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REFERENCES

- [1] Y.-S. Lin, C.-P. Tsai, H.-Y. Huang, et al., "Well-ordered mesoporous silica nanoparticles as cell markers," *Chemistry of Materials*, vol. 17, no. 18, pp. 4570–4573, 2005.
- [2] J. K. Vasir, M. K. Reddy, and V. D. Labhasetwar, "Nanosystems in drug targeting: opportunities and challenges," *Current Nanoscience*, vol. 1, no. 1, pp. 47–64, 2005.
- [3] P. Sharma, S. Brown, G. Walter, S. Santra, and B. Moudgil, "Nanoparticles for bioimaging," *Advances in Colloid and Interface Science*, vol. 123–126, pp. 471–485, 2006.
- [4] J. Rejman, V. Oberle, I. S. Zuhorn, and D. Hoekstra, "Size-dependent internalization of particles via the pathways of clathrin- and caveolae-mediated endocytosis," *Biochemical Journal*, vol. 377, pp. 159–169, 2004.
- [5] M. M. Amiji, "Nanotechnology—improving targeted delivery," in *Drug Delivery*, pp. 53–56, Touch Briefings, London, UK, 2007.
- [6] I. I. Slowing, B. G. Trewyn, S. Giri, and V. S.-Y. Lin, "Mesoporous silica nanoparticles for drug delivery and biosensing applications," *Advanced Functional Materials*, vol. 17, no. 8, pp. 1225–1236, 2007.
- [7] Z. P. Xu, Q. H. Zeng, G. Q. Lu, and A. B. Yu, "Inorganic nanoparticles as carriers for efficient cellular delivery," *Chemical Engineering Science*, vol. 61, no. 3, pp. 1027–1040, 2006.
- [8] C. Barbé, J. Bartlett, L. Kong, et al., "Silica particles: a novel drug-delivery system," *Advanced Materials*, vol. 16, no. 21, pp. 1959–1966, 2004.
- [9] T. Schiestel, H. Brunner, and G. E. M. Tovar, "Controlled surface functionalization of silica nanospheres by covalent conjugation reactions and preparation of high density streptavidin nanoparticles," *Journal of Nanoscience and Nanotechnology*, vol. 4, no. 5, pp. 504–511, 2004.
- [10] D. R. Radu, C.-Y. Lai, K. Jeftinija, E. W. Rowe, S. Jeftinija, and V. S.-Y. Lin, "A polyamidoamine dendrimer-capped mesoporous silica nanosphere-based gene transfection reagent," *Journal of the American Chemical Society*, vol. 126, no. 41, pp. 13216–13217, 2004.
- [11] I. I. Slowing, B. G. Trewyn, and V. S.-Y. Lin, "Effect of surface functionalization of MCM-41-type mesoporous silica nanoparticles on the endocytosis by human cancer cells," *Journal of the American Chemical Society*, vol. 128, no. 46, pp. 14792–14793, 2006.
- [12] W. Stöber, A. Fink, and E. Bohn, "Controlled growth of monodisperse silica spheres in the micron size range," *Journal of Colloid and Interface Science*, vol. 26, no. 1, pp. 62–69, 1968.
- [13] S. Lakard, G. Herlem, A. Propper, et al., "Adhesion and proliferation of cells on new polymers modified biomaterials," *Bioelectrochemistry*, vol. 62, no. 1, pp. 19–27, 2004.

- [14] O. Boussif, F. Lezoualc'h, M. A. Zanta, et al., "A versatile vector for gene and oligonucleotide transfer into cells in culture and in vivo: polyethylenimine," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 16, pp. 7297–7301, 1995.
- [15] W. T. Godbey, K. K. Wu, and A. G. Mikos, "Poly(ethylenimine) and its role in gene delivery," *Journal of Controlled Release*, vol. 60, no. 2-3, pp. 149–160, 1999.
- [16] A. von Harpe, H. Petersen, Y. Li, and T. Kissel, "Characterization of commercially available and synthesized polyethylenimines for gene delivery," *Journal of Controlled Release*, vol. 69, no. 2, pp. 309–322, 2000.
- [17] K. Kunath, A. von Harpe, D. Fischer, et al., "Low-molecular-weight polyethylenimine as a non-viral vector for DNA delivery: comparison of physicochemical properties, transfection efficiency and in vivo distribution with high-molecular-weight polyethylenimine," *Journal of Controlled Release*, vol. 89, no. 1, pp. 113–125, 2003.
- [18] Z. Csögör, M. Nacken, M. Sameti, C.-M. Lehr, and H. Schmidt, "Modified silica particles for gene delivery," *Materials Science and Engineering C*, vol. 23, no. 1-2, pp. 93–97, 2003.
- [19] C. Kneuer, M. Sameti, E. G. Haltner, et al., "Silica nanoparticles modified with aminosilanes as carriers for plasmid DNA," *International Journal of Pharmaceutics*, vol. 196, no. 2, pp. 257–261, 2000.
- [20] M. N. V. Ravi Kumar, M. Sameti, S. S. Mohapatra, et al., "Cationic silica nanoparticles as gene carriers: synthesis, characterization and transfection efficiency in vitro and in vivo," *Journal of Nanoscience and Nanotechnology*, vol. 4, no. 7, pp. 876–881, 2004.
- [21] J. Rosenholm and M. Lindén, "Wet-chemical analysis of surface concentration of accessible groups on different amino-functionalized mesoporous SBA-15 silicas," *Chemistry of Materials*, vol. 19, no. 20, pp. 5023–5034, 2007.
- [22] R. P. Bagwe, L. R. Hilliard, and W. Tan, "Surface modification of silica nanoparticles to reduce aggregation and nonspecific binding," *Langmuir*, vol. 22, no. 9, pp. 4357–4362, 2006.
- [23] C. F. H. Allen, F. W. Spangler, and E. R. Webster, "Ethyleneimine," in *Organic Syntheses*, N. Rabjohn, Ed., vol. 4, pp. 433–435, John Wiley & Sons, New York, NY, USA, 1963.
- [24] P. Ashtari, X. He, K. Wang, and P. Gong, "An efficient method for recovery of target ssDNA based on amino-modified silica-coated magnetic nanoparticles," *Talanta*, vol. 67, no. 3, pp. 548–554, 2005.
- [25] P. Kankaanpää, K. Pahajoki, V. Marjomäki, J. Heino, and D. White, "BioImageXD — New open source free software for the processing, analysis and visualization of multidimensional microscopic images," *Microscopy Today*, vol. 14, no. 3, pp. 12–16, 2006.
- [26] J. W. Slot and H. J. Geuze, "A new method of preparing gold probes for multiple-labeling cytochemistry," *European Journal of Cell Biology*, vol. 38, no. 1, pp. 87–93, 1985.
- [27] J. H. Moon, J. W. Shin, S. Y. Kim, and J. W. Park, "Formation of uniform aminosilane thin layers: an imine formation to measure relative surface density of the amine group," *Langmuir*, vol. 12, no. 20, pp. 4621–4624, 1996.
- [28] S. O. Molin, H. Nygren, and L. Dolonius, "A new method for the study of glutaraldehyde-induced crosslinking properties in proteins with special reference to the reaction with amino groups," *Journal of Histochemistry & Cytochemistry*, vol. 26, no. 5, pp. 412–414, 1978.
- [29] A. F. S. Habeeb and R. Hiramoto, "Reaction of proteins with glutaraldehyde," *Archives of Biochemistry and Biophysics*, vol. 126, no. 1, pp. 16–26, 1968.