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Covalent Surface Functionalization and Self-Organization of Silica Nanoparticles**

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The large-scale manufacture of nanometer-scale particles with definite size and shape is a great challenge in materials chemistry.[1] Perhaps even more exciting is the development of versatile methodologies that focus on the covalent functionalization of these particles,[2] and which have an outstanding impact on fundamental research and on chemical and biochemical engineering: functionalized particles play an important role, for example, in pharmaceutical drug delivery systems,[3] in the production of dispersion paints,[4] in the optimization of catalysts,[5, 6] and in processes involving adhesives, varnishes, and lubricants.[7] Silica particles might be particularly useful for applications where toxicity must be excluded. They can conveniently be prepared by the classical method of Stöber et al.[8] Without surface modification these particles tend to coagulate and eventually transform, by polycondensation of their surface OH groups, into (nano)-porous glass or ceramics.[9] Many applications, however, require strictly nonaggregated nanoparticles.

Most of the recent literature concerning surface functionalization is based on the weak adsorption of surfactants or polyelectrolytes.[10] Up to now covalent coating of the hydrophilic SiO_2 surface has mostly been accomplished with silanes that contain lipophilic ligands. This leads to stable dispersions in several organic solvents;[11, 12] for instance, the surface modification of colloidal CdSe clusters by means of organoselenides has been reported.[13] The covalent coating of nanostructured metals with thiol molecules is also well known.[14] All these techniques prevent aggregation by appropriate surface modification. Philipse and Vrij have coated silica spheres with a dense layer of (3-methacryl)oxypropyltrimethoxysilane and these particles can be dispersed in several organic solvents.[15] Hard sphere behavior was obtained by coating the surface with a monolayer of octadecyl alcohol.[16] These coatings make the particles chemically inert, which prohibits agglomeration. Concomitantly, however, further functionalization is hardly possible.[17]

Herein we present a covalent surface functionalization of silica nanoparticles that simultaneously excludes aggregation. Nearly monodisperse silica particles prepared by a modified Stöber synthesis are coated with a silane that contains two carboxylic acid groups. Particles with more than 10000 negative surface charges are generated upon dissociation in polar solvents. On the one hand these strictly nonagglomerated particles, as we demonstrate, exhibit novel colloidal

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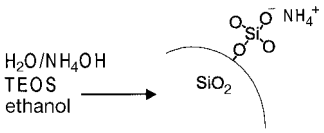
properties that are a result of their stability, monodispersity, low refractive index, and their function as highly charged heterosupramolecular entities. On the other hand these particles can be considered, particular in view of the lack of toxicity, as the basis for biologically or pharmaceutically functionalized systems, for instance, for immunodiagnostics or drug delivery systems.[18, 19]

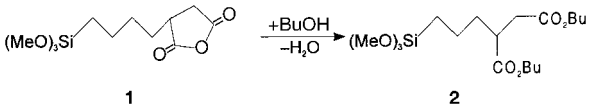
The starting materials of our synthesis are tetraethoxysilane (TEOS), 1-butanol, NaOH, NH_4OH, and, as a coating agent, (3-trimethoxysilyl)propylsuccinic anhydride (**1**; Scheme 1).



Scheme 1. Stöber synthesis of monodisperse silica spheres.

An alcosol is prepared in a mixture of 1200 mL ethanol and 60 mL ammonia (25 %). TEOS (42 mL) is added, and the mixture is stirred at room temperature for one day. The resulting silica particles exhibit a diameter of 90 nm as determined by dynamic light scattering. The dispersion contains 7.7 g silica L^{-1}. Particle sizes of 21, 90, 286, and 440 nm are achieved with other TEOS concentrations. These particles, however, are only stable in the presence of a large surplus of NH_4OH, otherwise they irreversibly aggregate by polycondensation of their surface hydroxyl groups.

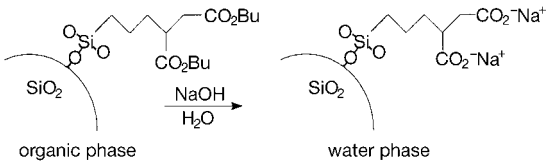
The coating agent **1** has to be chemically modified before use; the bifunctional methoxysilyl substituted succinic anhydride moiety would hydrolyze in the alcosol medium and the resulting carboxylic acids groups on the one end and the methoxysilyl groups on the other end of the molecules would react with the silica particles to form a macromolecular network. So we apply an esterification with butanol (Scheme 2). For that purpose 100 mL of the silane were



Scheme 2. Protection of the silane precursor.

mixed with 20 mL butanol in a second vessel and heated to 50 °C for two hours at reduced pressure (50 mbar) to remove the water from the reaction.

In the first vessel gaseous NH_3 is removed by evaporation and in this way the pH of the alcosol is reduced to pH = 7–8. The protected silane coupling agent **2** is then added. At this neutral pH the diester is stable and only the methoxy groups react with the OH surface groups of the silica particles in a condensation reaction with elimination of methanol. Methanol, ethanol, and water are destilled off at 50 °C and reduced pressure (20 mbar) after stirring the reaction mixture for 12 hours. Under these conditions butanol is not volatile, so the result is a milky dispersion of silica particles with lipophilic surface groups in 1-butanol. After this procedure the mixture is placed in a separating funnel with an equal amount of water. The coated silica particles remain in the (milky) butanol phase, but on addition of solid NaOH and shaking, the esters are hydrolyzed to carboxylic acids by the increase of the pH value, that is, the protection caps are removed, and the now highly charged silica particles disperse in the aqueous phase (Scheme 3). The butanol phase then becomes transparent and



Scheme 3. Removal of the protection caps after coating of the silica spheres.

the water phase weakly turbid. Complete dispersion is a consequence of the mutual Coulombic repulsion of the nanoparticles (charge stabilization).

The phases are separated in the funnel, and the water phase containing the functionalized silica particles is dialyzed in a dialyzing tube against deionized water for three days to remove residual silane and NaOH. FT-IR spectra of the dried particles show bands at 1600 and 3450 cm^{-1}, which clearly prove the existence of carboxylic acid groups in the surface shell of the nanoparticles.

Particle sizes and size distributions of our products have been determined by dynamic light scattering using an ALV photon correlation spectrometer equipped with an ALV-5000E fast correlator. The results for our four samples are shown in Figure 1. The size distributions resulting from the

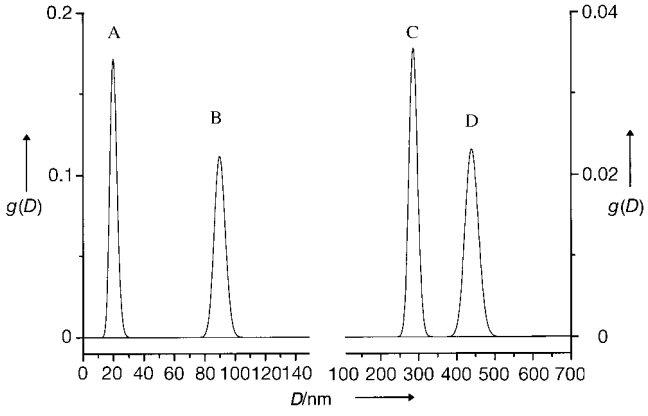


Figure 1. Particle size distribution of four selected batches of silica nanoparticles with average diameters of 21, 90, 286, and 440 nm.

measured autocorrelation functions are analyzed in terms of the log-normal distribution function [Eq. (1)],[20] where the parameters μ and σ describe the median and the

$$g(D) = \frac{1}{\sqrt{2\pi} D \ln\sigma} \exp\left(-\frac{(\ln D - \ln\mu)^2}{2(\ln\sigma)^2}\right) \quad (1)$$

geometric standard deviation of the distribution, respectively; these quantities are listed in Table 1. In addition, we have

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Table 1. Parameters of the size distribution of the silica nanoparticles (results obtained from the dynamic light scattering experiments).

Sample	μ [nm]	σ
sample A	21	1.10
sample B	90	1.03
sample C	286	1.04
sample D	440	1.04

looked at our particles by means of a transmission electron microscope (JEOL 200CX). Two typical micrographs are shown in Figure 2 and confirm the absence of aggregates and the monodispersity of our products.

The surface charges were characterized by measuring the electrophoretic mobilities with a Zetasizer III (Malvern Instruments) at pH 7 and a temperature of 25 °C. From the electrophoretic mobility the zeta potential is calculated. The resulting reduced inverse Debye–Hückel screening length, taken together with the particle radius, allows the determination of the effective, electrophoretic surface charge z_{eff}. We obtain $z_{eff} = 28\,530$ for the particles with $\mu = 286$ nm and $z_{eff} = 10\,230$ for those with $\mu = 90$ nm. These values even exceed those of highly charged polymer based colloids.[21]

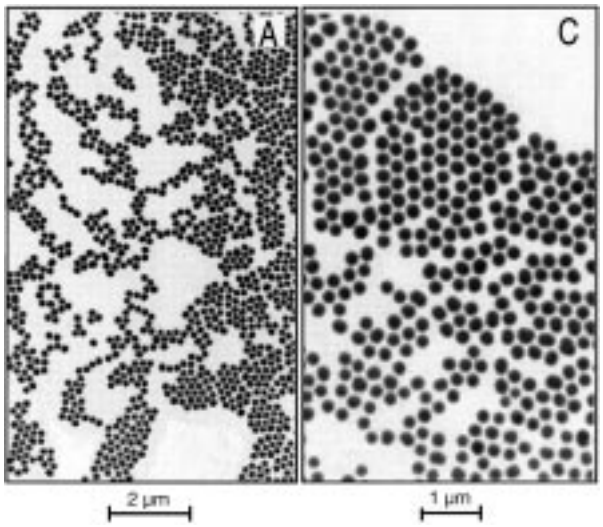

Figure 2. Transmission electron micrographs of samples A and C of Figure 1.

By means of a mixed-bed ion exchanger (Dowex 50W, Fluka) we completely removed the excess ions and thus reduced the ionic strength such that the conductivity reaches values as low as 13 µS. Under these conditions the Coulomb potential is hardly shielded anymore, and the repulsive interaction of the functionalized particles becomes long ranged. This leads to the formation of colloidal supercrystals in a polycrystalline form. The long-range ordering in the respective supercrystallites of sample B (with $\mu = 90$ nm) is evident from the opalescence phenomena, as demonstrated in Figure 3, which originate from Laue-backscattering of white visible light. Thereby the human eye acts as an energy-dispersive detector. From the well-known Bragg equation $n\lambda = 2d\sin\theta$ under backscattering conditions ($\sin\theta = 1$), with the index of refraction of water $n = 1.33$, and with the wave

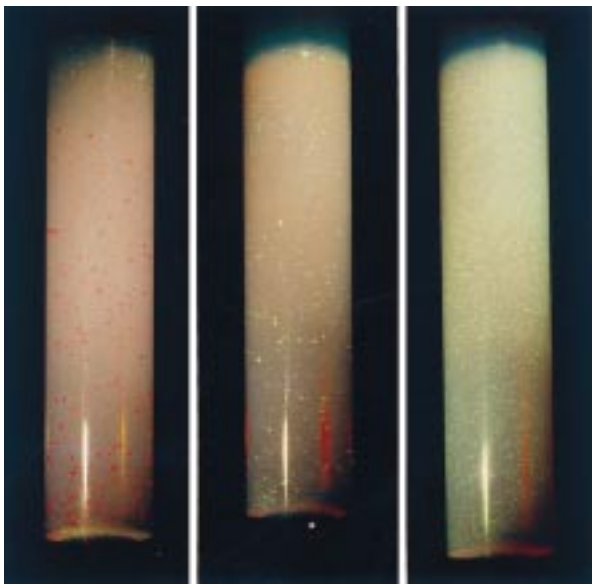

Figure 3. Colloidal supercrystals (in polycrystalline form) of 90 nm diameter silica nanoparticles with covalent surface funtionalization; the red, yellow, and green speckles arise from Laue-backscattering of white visible light on suspensions of the same particles at different concentrations.

lengths $\lambda_{red} = 700$ nm, $\lambda_{yellow} = 590$, and $\lambda_{green} = 520$ nm, we estimate the following interplanar distances:

left tube, red reflections:	480 nm
middle tube, yellow reflections:	390 nm
right tube, green reflections:	330 nm.

For demonstration purposes the volume fractions had been chosen such that interference effects occur just in the visible range of electromagnetic radiation; the reciprocal third roots of the particle number densities yield the interparticular distances: left tube 550 nm, middle tube 403 nm, right tube 325 nm, which are in satisfactory agreement with the above estimation done just by eye.

In summary, we have prepared highly charged inorganic/organic colloidal core/shell particles. They are, for all practical purposes, monodisperse and perfectly dispersed. On the one hand these coated particles can be considered as a basis for a variety of applications since by an appropriate chemical reaction with their surface carboxylic acid groups further more complex functionalization can easily be accomplished. On the other hand, optical matching is easily possible as a consequence of the particles' low refractive index. This enables fundamental light-scattering studies of different phenomena, for instance of order–disorder phase transitions or hydrodynamic interactions at large concentrations without the problem of multiple scattering; investigations of this kind are in progress now in our laboratory.[22]

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Real-Time Characterization of Ribozymes by Fluorecence Resonance Energy Transfer (FRET)**

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Ribozymes are increasingly applied to the inhibition of gene expression at the level of protein-encoding mRNAs.[1, 2] The possibility of transferring ribozymes endo- or exogenously into living cells opens up a broad potential for application in gene therapy,[1, 3-5] functional genomics,[6] and biotechnology.[7] The ribozyme most commonly used for these purposes is the hammerhead ribozyme (HHR), a small catalytic RNA that is able to cleave other RNA molecules in an intermolecular fashion.[8] The specificity of this cleavage process is determined by substrate binding sites that are variable in sequence and length. As a result, the cleavage activity can be directed against almost any mRNA sequence. Despite their potential for universal application there are a number of factors that have to be taken into account when developing therapeutic ribozymes, for example, the accessibility of ribozyme binding sites on the target RNA in vivo,[9] the selectivity of substrate recognition,[1] or the cleavage efficiency in an intracellular compartment.[10, 11] Although computer-assisted predictions can be made to address some of these points[12] they still have to be verified experimentally by characterizing the respective HHR-constructs under various conditions. Therefore, there is currently a high demand for technologies that allow a high-throughput screening of ribozymes for a rapid qualitative evaluation of their kinetic parameters, especially as current conventional methods hardly provide sufficient solutions to this problem.

Here we report a novel approach based on FRET-oligonucleotides[13] (FRET = fluorescence resonance energy transfer[14]), which allows the real-time kinetic analysis of ribozymes within hours. The FRET-principle has previously been applied to various problems such as determination of phosphodiesterase activities,[15] structural elucidations of RNA molecules,[16, 17] and for quantification of PCR reactions.[18] Intermolecular FRET-measurements with single-labeled substrates and hammerhead ribozymes have also been applied to measure the association and dissociation of substrate and product.[19, 20]

In our FRET-substrate the fluorescence of a fluorophore (for example, 6-carboxyfluorescein (FAM)) is intramolecularly quenched because of the close spatial proximity of a fluorescence-quenching molecule (for example, 6-carboxytetramethylrhodamine (TAMRA); Figure 1a, b). Upon cleavage of such substrates by the ribozyme a fluorescence-signal is generated which can be quantified by an appropriate read-out system in real time (Figure 1c). As the increase in fluorescence is directly correlated with the rate of cleavage, this system is well suited for the sensitive, nonradioactive, rapid analysis of ribozyme activities.

Based on a published HHR/substrate complex (Figure 1a) we have constructed a FRET substrate (SL1), which was used for real-time determination of the activity of the ribozyme HHR1. An inactive HHR-mutant ($HHR1_{mut}$) with substrate binding sites identical to those in HHR1 was used as a reference and negative control. The Michaelis–Menten parameters of HHR1 could be obtained in a single experimental setup by measuring the increase in fluorescence as a result of cleavage at different substrate concentrations. The result of this experiment is shown in Figure 2b in the form of an Eadie–Hofstee plot for the determination of the k_{cat}/K_M values. To investigate whether the FAM/TAMRA label affects the cleavage efficiency we determined the kinetic parameters by conventional methods[22] with a ^{32}P-labeled

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