DEVELOPMENT OF A FLEXIBLE CELL TARGETING SYSTEM BASED ON SILICA NANOPARTICLES

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ABSTRACT

Commercially available and synthesized silica particles were fluorescently labeled with FITC and modified to get a wide variety of particle systems with defined size and surface charge. By a variation of reaction conditions particles with diameters of 10 and 80 nm determined with TEM and with zetapotentials between -50 to +30 mV under physiological conditions (pH: 7.4, PBS-buffer) were available.

A further molecular shell consisiting of avidin was obtained by binding the molecules to negatively charged particle surfaces through electrostatic interactions. The amount of avidin coupled to the silica particles was 1.7 µg per mg particle. Starting with particles with an hydrodynamic diameter determined with PCS of 260 nm, the size increased to 500 nm, while the zeta potential was altered to -8 mV under physiological conditions.

Biotinylated wheat germ agglutinin (bio-WGA) can be bonded to such particles through avidin / biotin complex formation. Up to 2.8 µg lectin per mg particles could be coupled to the particle surface. This leads to a further increase of hydrodynamic diameter to 650 nm. It could be shown by hemagglutination test, that the bonded lectin is still active. No toxic effects of the silica particles were found at 1 wt.-% particle concentration with various cell types (Caco-2, L132). The binding of lectin-particle complexes to cells was increased by a factor of 4.4 in comparison to uncoated particles.

In addition it was found that WGA can directly be coupled to the particle surface. An amount of 1.8 µg Lectin per mg particle was determined. The hydrodynamic diameter increases from 260 nm to 432 nm, while a zetapotential of -28 mV was found under physiological conditions.

It could be shown, that negatively charged silica nanoparticles are suitable systems to couple various biomolecules retaining their biological function.

INTRODUCTION

In pharmceutical research there is need for drug carrier systems for various applications. Such systems are not only under consideration for the safe transport of drugs sensitive against biodegradation, the accumulation of drugs in targeted tissue would also allow a reduction of the dose. For these applications the use of different drug carrier systems of colloidal size (liposomes, nanoparticles) are under investigation. Most of the described systems consists of organic polymers [1]. A multitude of synthesis methods and materials used resulted in a variety of nanoparticles formulations that differ in size, surface charge and hydrophilicity [2].

But these organic nanoparticles possess several limitations. Until now the synthesis of really small nanoparticles (< 50 nm) failed and the particle size distribution is usually very broad. For lack of surface functional groups surface modification is normally carried out by adsorption of various molecules. A systematic variation of particle size and surface chemically properties is not possible.

There are only a few approaches to use inorganic nanoparticles as drug carrier systems (e.g. insulin and antibodies were coupled to brushite [3] and these systems were tested in-vivo). For medical applications the body distribution of organically modified silica particle systems is described only with much bigger sizes and surfactant modification [4]. In this interdisciplinary study very small silica particles are investigated for their potential as drug carrier systems and therefore various biomolecules were coupled to the particles. From material science point of view, the preparation [5,6] and surface modification [7] of silica particle systems are well descri-bed, but they have to be adapted for this special application. With this flexible particle system a broad spectrum of different particle properties will be potentially available.

Two strategies are considered for the coupling of biomolecules. The avidin / biotin systems will be tested as a flexible linker system. Avidin is a glycoprotein with a molecular weight of 66 kD and a pI of 10. Therefore it is positively charged under physiological conditions and bonded to negatively charged surfaces by electrostatic interactions. Avidin can form strong and selective complexes with four molecules of biotin (K_D: 10^{-15}M). So e.g. biotinylated lectins can be coupled to avidin. The lectin wheat germ agglutinin (WGA, 36 kD, pI: 8) can specifically recognize N-acetylglucosamine and N-acetylneuraminic acid which are structures located on various cell sur-faces [8,9]. WGA was chosen to modify silica particles and to bind such WGA modified particle systems to cells. In addition, it should be investigated, if lectins can enhance endocytosis of mo-dified particles. Besides experiments with the above described linker system, the direct coupling of WGA to the particle surface will be tried.

EXPERIMENTAL

A reagent for the fluorescent labelling of silica particles with FITC was synthesized by the co-valent attachment of FITC to N-aminopropyltriethoxysilane (APS) according to [10].

Commercially available silica particles (IPA-ST, Nissan Chemical Industries Ltd.) were fluorescently labeled by stirring 30 g IPA-ST, 10 g H_2O and 100 µl of an ethanolic APS-FITC solution (15.6 mg/ml) for 12 h and dialysing against deionized water.

For the formation of bigger silica particles, a variation of the so-called Stöber method [5] was carried out. 100 µl of an ethanolic APS-FITC solution (15.6 mg/ml) was added to a mixture of 8 g tetraethoxysilane, 250 ml ethanol and 13 g ammonia (25 wt.-%). After 16 h of hydrolysis and condensation at room temperature the particles were cleaned and isolated by several washing and centrifugation steps.

These particles could be modified according to the following procedure: 100 ml of an aqueous silica particle suspension (5 wt.-%) was added to a solution of 15 ml acetic acid in 75 ml ethy-lenglykol. After the addition of 1 and 100 wt.-%. resp. APS the mixture was stirred for 16 h at 80°C. Water and acetic acid was distilled of and the resulting glycolic suspension was dialysed against deionized water.

For the methylation of the aminosilane particle surface, 7.5 ml of the glycolic suspension was stirred with 4 g MeI for 24 h at room temperature. The excess of MeI was reacted with ammonia and the suspension was dialysed against deionized water.

For the coupling of avidin, 1 ml of a 2 wt.-% particle suspension in PBS was incubated with avidin (60 µg/ml) for 1 h at 37°C. Avidin modified silica particles were separated by centrifuga-tion. Biotinylated WGA (bio-WGA) were bonded to the avidin modified silica particles by incu-bation of 1 ml of a 2 wt.-% particle suspension in PBS with bio-WGA (75 µg/ml) for 1 h at 37°C. For the direct coupling of WGA 1 ml of a 2 wt.-% particle suspension in PBS was incuba-ted with WGA (100 µg/ml) for 1 h at 37°C.

Gelelectrophoresis was carried out as described in [11]. Lectin activity was tested with a hemagglutination assay. Therefore 50 µl of the particle suspensions (1 mg/ml) were incubated for 1 h at 37°C with 50 µl of an erythrocyte suspension derived from human blood. For determination of cytotoxicity cell proliferation test WST-1 (Boehringer Mannheim GmbH) was used. Nanoparticle suspensions (10 mg/ml) were incubated with the desired cell cultures (Caco-2, L-132) for 48 h (37°C, 5 % CO_2, 95 % humidity). Then the WST-1 reagent was incubated for 1 h and the absorption was measured at 450 nm. Cell binding and endocytosis studies were carried out with L-132 (pulmonary epithelium) cells. 20 µl of different particle suspensions (1 mg/ml: unmodified, avidin modified, lectin modified, lectin modified with the lectin inhibitor methyl-α-D-mannopyra-noside) were incubated at 4°C and 37°C for 1 h. After washing with PBS, the fluorescence activity was determined. After cytolysis with 1 % Triton X-100, fluorescence was determined again.

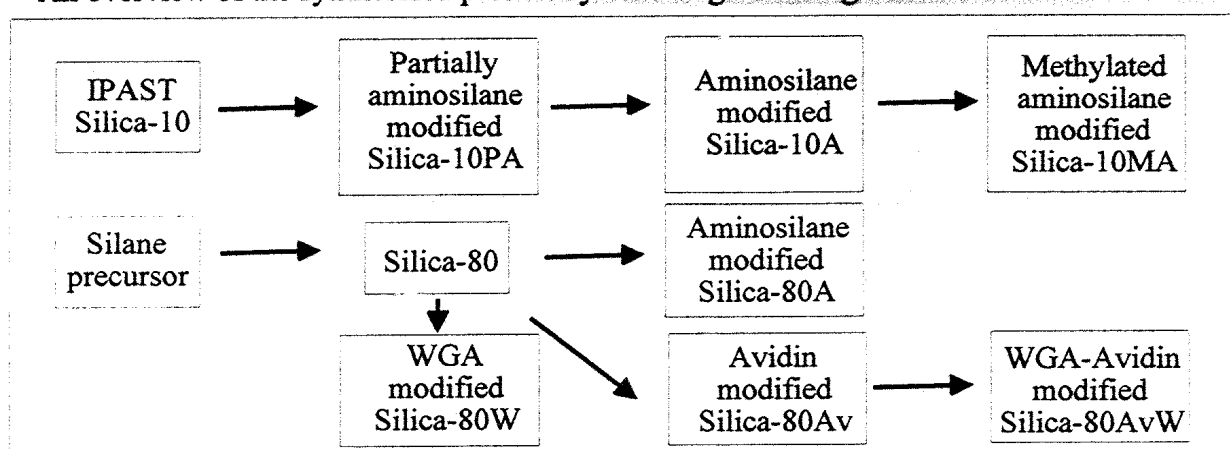An overview of the synthesized particle systems is given in fig. 1.

Fig. 1: Overview of the synthesized particle systems.

RESULTS AND DISCUSSION

Unmodified silica particles

The particle size was investigated by TEM, laser back scattering and PCS. TEM pictures of silica-80 show single particles with particle sizes between 70 and 90 nm (see fig. 2). The mean hydrodynamic diameter determined with laser back scattering was 104 nm for silica-80 and 8.3 nm for silica-10 (see fig. 3). With PCS 260 nm were found for silica-80, while silica-10 show 29.7 nm.

Surface chemical properties were studied by zetapotential titration (see fig. 4A for silica-10). The curves measured are nearly identically for silica-10/-80. The point of zero charge is found at pH between 2.7 and 2.9 , which can be explained by acidic Si-OH groups on the particle surface. Under physiological conditions a zetapotential between -55 and -45 mV was measured. The negatively charged silica particles form stable suspensions under physiological conditions with solid contents of up to 10 wt.-%. Under physiological conditions the particles should be suitable for the electrostatic coupling of positively charged biomolecules.

Aminosilane modified silica particles

The particle size was investigated by TEM, laser back scattering and PCS. No significant change of particle size could be obtained by TEM pictures (for silica-10 see fig. 5). Only in the case of silica-80 the mean hydrodynamic particle diameter is altered to 87 nm (back scattering) and 141 nm (PCS) respectively.

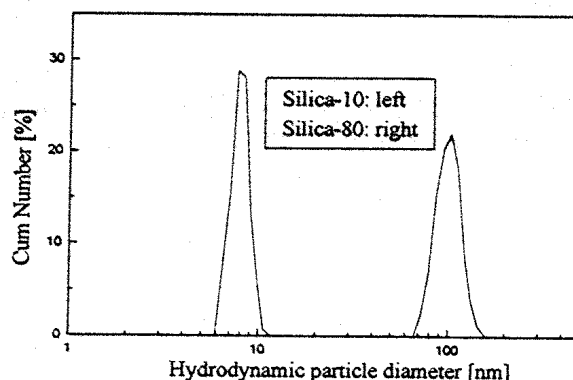Fig. 2: TEM picture of unmodified silica particles (silica-80).

Fig. 3: Laser back scattering experiments with silica-10 and silica-80. Mean hydrodynamic diameter found: 8.3 nm for silica-10 and 104 nm for silica-80.

For surface chemical properties, a dramatic change is observed. In the case of a modification with small amounts of APS (1 wt.-%), the IEP is shifted from 2.9 to 5.4 (see fig. 4B). The high influence of such a small amount of APS could be explained by the preferred reaction of APS with the most active and therefore most acidic silanol groups. The IEP is determined by a mixture of Si-OH and -NH_2 groups on the particle surface.

If the surface is completely covered by aminosilane molecules, the surface chemical properties are only determined by the pK_b of the -NH_2 groups. As shown in fig. 4C, an IEP in the range of 8.7 to 9.0 is found. As mentioned above, no significant difference in zetapotential curve were found for silica-10 and silica-80. Under physiological conditions, these particles possess zetapotentials between 30 and 35 mV. They are positively charged and could be used for the electostatic coupling of negatively charged biomolecules.

With an excess of methyliodine a methylation of surface aminogroups is possible. The IEP is shifted above 10 (see fig. 4D). A permethylation and therefore the independence of zetapotential from pH is obviously not reached under the used reaction conditions.
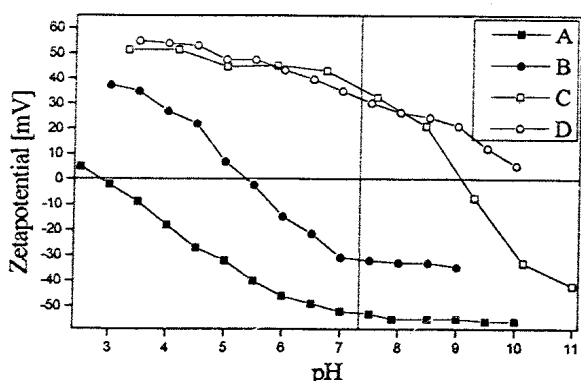

Fig. 4: Zetapotential as a function of pH for various particles (all silica-10): A unmodified silica, B partially APS modified silica, C APS modified silica, D methylated APS modified silica.
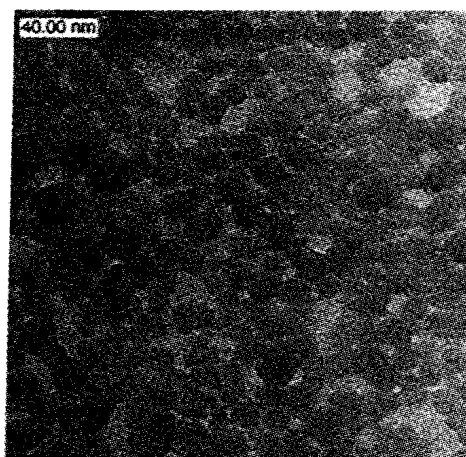

Fig. 5: TEM picture of APS modified silica particles (silica-10A)

Coupling of biomolecules via a linker

Positively charged avidin can be coupled to negatively charged silica particles (silica-80). Using fluorescent labelled avidin, the amount of bonded avidin was indirectly checked by measuring the avidin concentration in the supernatant. An amount of 1.7 ± 0.14 µg Avidin per mg particles were calculated, this means that 56 % of the avidin is bonded to the particle surface. The hydrodynamic particle size determined with PCS increases from 260 nm for unmodified silica particles to 500 nm for avidin coated particles. The zetapotential changes from -53 mV to -8 mV by this modification. The reaction with bio-WGA leads to a further increase of hydrodynamic particle sizes to 650 nm. The amount of WGA coupled to the silica particles is determined by gelelectrophoresis (SDS-page). 2.8 ± 0.22 µg WGA per mg silica is found corressponding to a yield of 75 %.

A rough calculation shows that 8 avidin molecules and 24 WGA molecules are bonded to a single silica particle (assumptions: particle diameter 80 nm, particle density 2 g / ml, molar mass Avidin 66 kD, WGA 36 kD).

To check the activity of the bonded lectin a hemagglutination test was carried out with the unmodified, the avidin and the WGA modified particles. A strong aggregation of the erythrocytes was found only in the case of the lectin modified particles. It was concluded that these molecules are still active while bonded to the particle surface.

An important criteria for the choice of materials for medical applications is their toxicity. To determine the cytotoxicity of silica nanoparticles the cell proliferation assay WST-1 was carried out. With unmodified silica particles no significant cytotoxic effects were determined for the cell lines L-132 and Caco-2 in a time range of 48 h.

Cell targeting

To investigate the cell targeting effect of modified silica particles cell binding and endocytosis studies were carried out. The results with L-132 cells are shown in fig. 6. If the particles are modified with WGA the binding of particles to cells at 37°C is increased by a factor of 4 in comparison to uncoated silica particles (see fig. 6b 1,3). Addition of an inhibitor leads to a blocking of the lectin binding sites and to a significant decrease of binding (fig. 6b 3,4). In comparison to binding only a tenth of the lectin modified particles show endocytosis (see fig. 6b 3). But internalisation is increased by a factor of 10 through lectin modification (see fig. 6b 1,3 grey). At 4°C no energy in form of ATP is produced, which is necessary for an active endocytosis. Because no endocytosis is observed at 4°C it can be considered that the lectin coated silica particles are internalized by an active process.

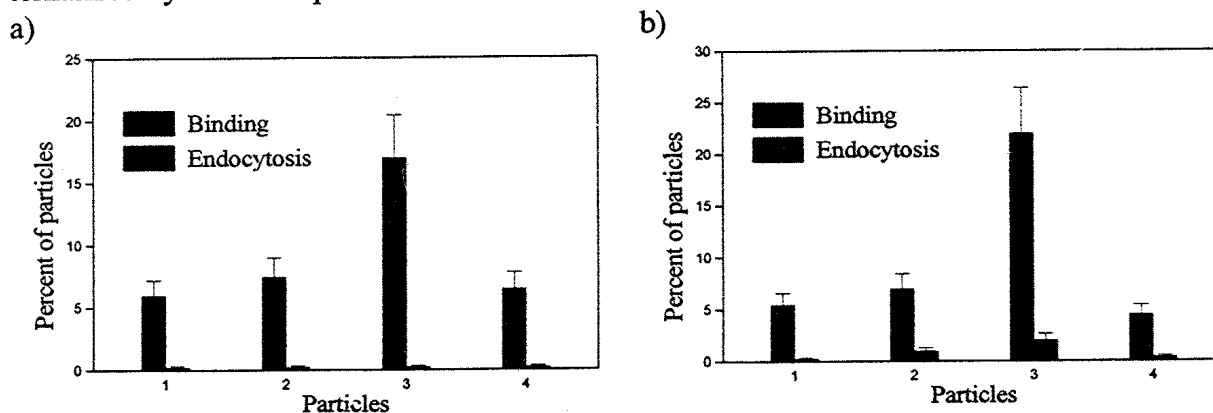a)   b)

Fig. 6: Binding and endocytosis of nanoparticles silica-80 to L-132 cells at 4°C (a) and 37°C (b). 1: uncoated, 2: avidin-coated 3: WGA modified, 4: presence of inhibitor.

It could clearly be demonstrated with these experiments that lectin modification increases the cell binding and endocytosis. WGA modified silica particles may be suitable for the targeting of the tested cells.

Direct coupling of biomolecules

WGA (pI: 8) is positively charged under physiological conditions. Therefore it can be coupled to negatively charged silica particles (silica-80). Using fluorescent labelled WGA, the amount of bonded WGA was indirectly checked by measuring the WGA concentration in the supernatant. An amount of 1.8 ± 0.15 μg WGA per mg particles was calculated. This means that 36 % of the WGA is bonded to the particle surface. The hydrodynamic particle size, determined with PCS, increases from 260 nm for unmodified silica particles to 432 nm for WGA coated particles. The zetapotential changes from -53 mV to -26 mV by this modification. A rough calculation shows that 16 WGA molecules are bonded to a single silica particle (assumptions see above).

Fig. 7 shows TEM pictures of silica-80 particles contrasted with lead ions. 7a is an uncoated silica particle that shows almost no contrast. No lead can be found on the particle by EDX analysis. 7b / c is the same WGA modified silica particle, photographed directly (7b) and after 5 min (7c) under the e⁻ beam. In comparison with 7a there is much more contrast and therefore adsorbed lead on 7b, which was controlled with EDX. A microstructure of black dents can be recognized on the particle surface which can be contributed to coupled WGA molecules (expected size: 4 nm). Electron beam leads to the denaturation and destruction of protein structures. After 5 min black spheres can be seen, which are crystalline and the lattice parameter can be assigned to lead. The coupling of biomolecules is proven with these investigations.
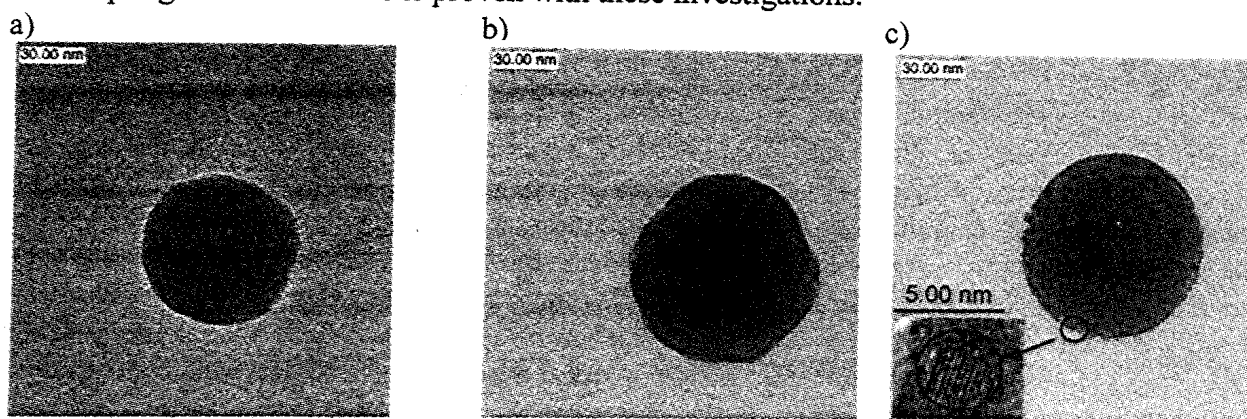

Fig. 7: TEM picture of uncoated (a) and WGA coated (b,c) silica nanoparticles (1 % lead-citrate as contrast agent).

Both physico-chemical and biological methods were used to characterize lectin modified silica nanoparticles. TEM pictures, particle size and zetapontial investigations showed the presence of WGA on silica particle surface, while cell binding and endocytosis studies demonstrated the bioacticity and the suitability of surface modified silica particles for cell targeting.

CONCLUSION

It was shown, that silica nanoparticles can be synthesized with a wide variety of size and surface chemical properties. By a surface modification with aminosilane the zetapotential can be controlled in the range from -50 mV to +30 mV under physiological conditions.

A linker system based on avidin / biotin complex was used to couple the lectin WGA to the particle surface. Because various biotinylated biomolecules are available, this linkage is very

flexible. Avidin theoretically can bind four molecules of biotin. In our study it was found that 8 avidin and 24 WGA molecules were bonded per particle. But the binding of three large WGA molecules per avidin molecule could be sterically problematic. In addition it was shown that the direct binding of WGA to silica particle surface was possible. So it could not be definitely clarified whether there is any unspecific binding in the case of the avidin / WGA modified particle.

The bioactivity of biomolecules after the coupling to silica particles was shown and the cell binding and endocytosis of lectin modified silica particles was enhanced. It could be shown that WGA modified silica particles are in principle suitable for cell targeting.

The use of positively charged silica particles for the coupling of negatively charged biomolecules will be shown in future work.

ACKNOWLEDGEMENTS

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