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Colloidal silica nanoparticles for use in click chemistry-based conjugations and fluorescent affinity assays

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ABSTRACT

Silica nanoparticles (SiNPs) with an average diameter of 25 nm were prepared by a one-pot method that involves the formation of a silica core that is subsequently covered with a shell functionalized with either azido groups or alkyne groups for use in copper(I)-catalyzed click reactions. Respective triethoxysilane reagents are presented. The SiNPs were (a) rendered fluorescent by click conjugation to fluorophores of various colors, and (b) made bioconjugatable by introducing maleimide groups (that covalently bind thiols) and biotin (a widely used bioaffinity reagent that binds streptavidin). Particles were characterized by transmission electron microscopy, infrared spectroscopy, fluorescence, and light scattering. The fluorescently labeled SiNPs carrying maleimido groups were conjugated to the thiol group of bovine serum albumin (BSA) labeled with a fluorophore, and fluorescence resonance energy transfer was shown to occur between the labeled SiNPs and the labeled BSA. This is considered to represent a new approach towards nanoparticle-based fluorescent bioassays.

1. Introduction

Particles of silica (silicon dioxide) at the nano- and microscale are widely used in various kinds of sciences including ceramics, colloids, catalysis [1], nanopatterning, and photonics [2]. One particular application of silica nanoparticles (often referred to as SiNPs) is in bioanalytical methods for gene delivery, drug delivery, immunoassays, scanning probe microscopy-based imaging and sensing techniques [3–8] and in high-throughput-screening for genomics, proteomics, or drug discovery [9,10]. Their versatility is due to the ease of preparation, the possibility of controlling size, a high surface-to-volume ratio, and the biocompatibility of (modified) silica. SiNPs often are rendered fluorescent by the incorporation of fluorophores, partially via pre-activated fluorophores, into the core of the silica matrix [5,11–14]. This makes them a useful alternative to quantum dots which sometimes suffer from their toxicity.

The surface of SiNPs has been functionalized by introducing chemical functions such as amino, thiol, epoxy, carboxy, azido or alkyne groups [15–19]. The latter two allow for the use of such particles in the copper-catalyzed 1,3-dipolar cycloaddition reaction between an organic azido group and an alkyne group that is uniquely suited for linking two species. This so-called click reaction [20,21] and other bio-orthogonal reactions are now widely used in biosciences [22] because they proceed with high efficiency at room temperature, in aqueous solution over a wide range of pH, within short time, do not suffer from major side reactions, and the reagents required can be synthesized in a reasonable number of steps. The click reaction is bio-orthogonal in a sense that azido groups and terminal alkyne groups are hardly occurring in biological systems. Hence, the click reaction, formerly mainly used in the fields of synthesis in homogenous solution, has moved into areas such as surface chemistry with typical applications in controlled drug delivery, diagnostics, fluorescent labeling or material sciences [23–26].

We are reporting here on (a), the preparation of colloidal SiNPs whose surface is functionalized with either azido or alkyne groups using a one-pot method based on the Stöber process [27]; (b), the click conjugation of maleimide and biotin to their surface; (c), click-labeling of such SiNPs with various fluorescent tags; (d), the bioconjugation of fluorescent maleimido-SiNPs to the thiol group of labeled bovine serum albumin (BSA) and a respective fluorescence resonance energy transfer study.

2. Experimental

2.1. Materials

2.1.1. Synthesis of functionalized silica nanoparticles

The azido-modified SiNPs (referred to as **SiNP-1**) and the alkyne-modified SiNPs (referred to as **SiNP-2**) were prepared by a combination of the Stöber method and the seed growth tech-

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nique [27,17]. A mixture of 50 mL of ethanol, 1 mL of distilled water, and 1 mL of ammonia solution (25% in water) was heated to 40 °C. Tetraethyl orthosilicate (TEOS, 1.5 mL, 6.73 mmol) was added and the solution was slowly stirred for 3 h at 40 °C, after which further TEOS (1.0 mL, 4.48 mmol) was added and stirring was continued for another 30 min. In order to obtain azido-modified nanoparticles of type **SiNP-1**, the azide **1** (100 μL, 0.46 mmol; equal to 4 mol% of the total concentration of TEOS) was added to the solution. After another 2.5 h of stirring at 40 °C, the resulting alcosol was cooled to room temperature and matured for at least 1 week to allow for full particle growth. Temperature and the timing of the addition of precursor and silane reagents are important for reproducible particle synthesis. Among others, the temperature has to be kept constant within 40 ± 2 °C. The intervals given for the addition of TEOS and the silane reagents have to be adhered to rather exactly. The time for maturation is between 6 and 9 days.

In order to obtain alkyne-modified nanoparticles of type **SiNP-2**, the reagent *O*-(propargyloxy)-*N*-(triethoxysilylpropyl)urethane (**2**; 140 μL, 0.46 mmol; equal to 4 mol% of the total concentration of TEOS; from ABCR; www.abcr.com) was added, and the solution then treated as described for the azido-modified particles. Click reactions and size measurements were performed with such solutions without further workup.

2.1.2. Click conjugations of SiNPs with fluorophores

In a typical reaction, 2.0 mL of the crude matured alcosol were combined with an ethanolic or aqueous solution of the corresponding alkyne or azido fluorophores **4**–**6** (5 μmol each). After addition of a saturated solution of copper(I) iodide in water (400 μL), the mixture was stirred over night. Excess reagents and the catalyst were removed by size exclusion chromatography (SEC) on a column (Ø 3 cm, height 14 cm) containing Sephadex LH-20 (from GE Healthcare; www.gehealthcare.com) using ethanol as the eluent. The particles clicked with fluorophores were obtained by collecting the colored fraction that leaves the column first, respectively. The second colored band that moves much slower contains unclicked dye and was discarded. A blank sample containing the click reagents but not the copper catalyst was run in parallel in each case to study whether unspecific binding of reagents does occur.

*2.1.3. Competitive click conjugations for the preparation of **SiNP-1-5a/7***

Both species (**5a**, **7**) were clicked to **SiNP-1** in a competitive way. The fluorophore **5a** (1 μmol in 0.5 mL ethanol) and the maleimide reagent **7** (0.5 μmol in 0.5 mL ethanol) were added along with 400 μL of a saturated copper(I) iodide solution in water to 5 mL of colloidal **SiNP-1** and the mixture was stirred over night at room temperature. The solution was purified by SEC like described above for click conjugations to single fluorophores. A blank sample containing no maleimide **7** was prepared in parallel to exclude unspecific binding of the protein to the particles in the following experiments.

*2.1.4. Competitive click conjugations for the preparation of **SiNP-1-5a/8***

The fluorophore **5a** and reagent **8** were competitively clicked to **SiNP-1**. Therefore, fluorophore **5a** (25 nmol in 0.5 mL ethanol), the biotin reagent **7** (12.5 nmol in 0.5 mL ethanol) and 400 μL of a saturated copper(I) iodide solution were added to 5 mL alcosol of **SiNP-1**. The mixture was stirred over night at room temperature and the particles purified by SEC. A blank sample containing no biotin **8** was prepared in parallel to exclude unspecific interactions in the following experiments. A 40-fold lower concentration of **5a** and **8** was used compared to the preparation of **SiNP-1-5a/7** since this was enough in this particular study.

*2.1.5. Preparation of **SiNP-1-5a/8-PEG***

The crude alcosol of **SiNP-1** in ethanol was run through a SEC column (Sephadex LH-20) with a water-ammonia mixture (50/1 v/v) as eluent to exchange the solvent. The ammonia is necessary to preserve the colloidal state of the particles while the solvent is exchanged. In parallel a mixture of silane **1** (21.0 μL) and [methoxy(polyethylene-oxy)propyl]-trimethoxysilane (**3**; 229 μL; PEG-Silane; purchased by ABCR, www.abcr.com) was prepared. In a sonication bath 3.0 μL of the silane mixture were added to 3.0 mL of the obtained **SiNP-1** in water (**3**/**1** = 5/1) and the solution kept there for 4 h at 50 °C under sonication. The particles were purified by SEC with water-ammonia as eluent (50/1 v/v). The competitive click reaction to fluorophore **5a** and reagent **8** was done like described above with water as the solvent. The blank sample was prepared in the same way except the addition of reagent **8**.

2.1.6. Fluorescent labeling of bovine serum albumin and avidin

Chromeo-642 NHS ester (**C-642**) was purchased from Active Motif Chromeon GmbH (www.chromeon.de). The lyophilized powder (1 mg) was dissolved in 30 μL of dry dimethylformamide, bovine serum albumin (5.0 mg) was dissolved in 1 mL bicarbonate buffer (50 mM, pH 8.3). The protein solution was treated with 5 μL of the fluorophore solution and stirred at room temperature over night. Unreacted dye was removed by SEC using Sephadex G25 and phosphate buffered saline (150 nm, pH 7.4) as the eluent. The fast running blue fraction contained the labeled protein which was collected. The procedure yielded a blue solution of the labeled protein in a concentration of 26 μM and a dye-to-protein ratio (DPR) of 0.7 as determined by photometry.

Avidin (3.5 mg) was labeled with **C-642** (0.5 mg) according to the same protocol yielding the labeled protein in a concentration of 15 μM with a DPR of 0.7 as determined by photometry.

*2.1.7. Fluorescence resonance energy transfer studies of **SiNP1-5a/7** with labeled BSA*

The colloidal solution containing **SiNP1-5a/7** was used as prepared (see Section 2.1.3, ca. 5 mg/mL) and first diluted with water (1:10 ethanol/water). Then, 50 μL of a 26 μM solution of the labeled BSA (see Section 2.1.6) in buffer was added to 3 mL particle solution. The reaction between protein and thiol causes a significant change in the fluorescence emission spectra of both fluorophores due to the induction of FRET. Fluorescence was excited at 480 nm, and spectra were acquired in intervals of 15 min between 610 and 730 nm. A blank sample (containing no maleimido groups on the surface) was submitted to the same protocol.

*2.1.8. Fluorescence resonance energy transfer studies of **SiNP1-5a/8** and **SiNP-1-5a/8-PEG** with labeled avidin*

The colloidal solutions containing **SiNP1-5a/8** or **SiNP-1-5a/8-PEG** were used as prepared (see Sections 2.1.4 and 2.1.5). The nanoparticles of type **SiNP1-5a/8** were diluted with water (1:10 ethanol/water). Then 0.5 μL of the 15 μM solution of labeled avidin (see Section 2.1.6) was added and the mixture incubated under stirring for30 min at room temperature. Afterwards, the fluorescence spectrum was collected between 610 and 730 nm after excitation at 480 nm. This procedure was repeated until a total amount of 3.0 μL of labeled avidin was added. A blank sample (containing no biotin groups on the surface) was submitted to the same protocol respectively.

2.2. Instrumentation

Fluorescence spectra were collected on a Jasco FP6300 luminescence spectrometer, ^{1}H- and ^{13}C NMR spectra on an Avance 300 MHz NMR spectrometer (Bruker-BioSpin; www.bruker-biospin.com). Conventional IR spectra were acquired on a Varian