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# Bioconjugated Janus Particles Prepared by in Situ Click Chemistry

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Janus silica particles decorated with biotin molecules and poly(ethylene oxide) PEO chains on two hemispheres were prepared by two-step click reactions. Polystyrene (PS) particles coated with azide modified silica particles were used as templates. On the PS surface one hemisphere of a silica particle was exposed to the solvent and the other one was embedded in PS. The alkynated biotin molecules were grafted onto the exposed side of the silica particle by click reaction. After removal of PS, the embedded part of the silica particle was released, and alkynated PEO chains were grafted to the particle by click reaction. FT-IR, TGA, and TEM were employed to characterize the Janus particles. The Janus particles show strong interaction with avidin.

## Introduction

On a Janus particle the surfaces of two hemispheres are different from a chemical point of view, and the two sides of the particle may have different properties.[1] Recently, the preparation of Janus particles has attracted considerable attention because of their huge potential applications in numerous fields, such as the development of switchable devices,[2] optical probes,[3,4] drug carriers,[5] emulsion stabilizers, and so forth.[6−8] Many attempts have been devoted to the synthesis of various asymmetric particles including template-assisted self-assembly,[9] controlled surface nucleation,[10] controlled phase separation,[11] partial masking,[12] and Pickering emulsion-based method.[13,14] Recently, Janus particles which comprise different functional groups or polymer chains on two hemispheres were prepared successfully.[14] Apparently, preparation of polymer chains with different sensitivities on two opposite sides can further diversify the properties of Janus particles. Pickering emulsion assisted synthesis of Janus particles, which was originally proposed by Granick and co-workers, is a promising method in the preparation of functional Janus particles.[15] For example, Stamm and co-workers reported preparation of Janus particles which were decorated with two stimuli-responsive polymer chains on the two hemispheres of micrometer-sized silica particles by using a Pickering emulsion method.[14] In their approach, particles were frozen at the interface of water and wax, and Janus particles were produced after two-step chemical reactions.

In these years, copper (I)-catalyzed azide−alkyne 1,3-dipolar Huisgen cycloaddition has received much attention in the synthesis of a range of functional polymers due to high efficiency and high tolerance to functional groups and solvents, as well as moderate reaction temperatures.[16] Because of their efficiency and simplicity, these cycloadditions were classified as "click" reactions by Sharpless

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and co-workers.[17] Highly efficient and specific click reactions are suitable for introduction of various functional groups onto substrates with highly defined structures. Meldal and co-workers found that click coupling reactions worked well for the reactions on solid-phase.[18] Click chemistry is becoming a powerful tool in the preparation of functional particles. For example, Brittain and co-workers grafted polymer chains on silica nanoparticles based on a combination of reversible addition−fragmentation chain transfer polymerization and click chemistry.[19] Furthermore, copper (I)-catalyzed azide−alkyne click reaction shows excellent compatibility for conjugation of biomolecules to polymers, micelles, and nanoparticles.[20−22] After modification by biological ligands (such as biotin,[23] saccharide,[24] folate,[25] peptides,[26] and other moieties[27]), bioconjugated particles can serve as multifunctional platforms for targeted delivery, biodetection, and diagnosis.[28,29] Although asymmetry is intrinsic in nature and this structure can be found in many living organisms for efficient adaptation and mimicry, until now no report on introduction of biomolecules onto asymmetrical particles can be found.

The target of the present research is to prepare bioconjugated Janus silica nanoparticles on the basis of in situ click chemistry. In this manuscript, we report synthesis of Janus silica nanoparticles decorated with biomolecules on one hemispheres and biocompatible polymer brushes on the other ones. A Pickering emulsion was obtained by adding toluene solution of polystyrene (PS) into an aqueous solution with dispersed azide modified silica particles. After removal of toluene under a reduced pressure, the silica particles were frozen on the surfaces of PS particles. One hemisphere of a silica particle is in aqueous phase and the other one is in PS phase. Janus silica particles were prepared after two-step click reactions. At the first step, biotin moieties were conjugated on exposed sides of the silica particles by a reaction between alkynated biotin and azide groups. After removal of PS, the azide groups embedded in PS phase were released, and the biocompatible poly(ethylene oxide) (PEO) chains were grafted to the other side by click reaction between alkynated PEO and remaining azide groups on the surface after the first click reaction. In this research, the bioavailability of biotin to avidin was also investigated. The outline for the preparation of bioconjugated Janus particles is shown in Scheme 1.

## Experimental Section

**Materials.** Tetraethyl orthosilicate (TEOS) was purchased from TianJin Institute of Chemical Agents, and it was distilled before use. Propargylamine was purchased from LinQing Chem. Co. Before use it was dried over KOH and distilled under reduced pressure. 3-(Triethoxysilyl)-propylamine (Merck Schuchardt), 2-bromo-2-methylpropionyl bromide (98%) (Sigma-Aldrich), Avidin/4′-hydroxyazobenzene-2-carboxylic acid (avidin/HABA) reagent (Sigma-Aldrich), sodium azide (Sigma-Aldrich), succinic anhydride (Tianjin Institute of Fine Chemicals retrocession), 4-dimethylamino pyridine (DMAP) (Alfa Aesar), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (Alfa Aesar), copper(II) sulfate pentahydrate ($CuSO_4\cdot 5H_2O$, TianJin Institute of Chemical Agents), and sodium ascorbate (Alfa Aesar) were used as received. PS used in this research was synthesized by atom transfer radical polymerization (ATRP). The number-average molecular weight and molecular weight distribution are 4K and 1.06. Synthesis of alkynated biotin was reported in our previous paper.[30] All the solvents were distilled before use.

**Preparation of Silica Particles.** The spherical silica particles used in this study were prepared by using the Stöber method.[31] To a 500 mL flask were added 250 mL of ethanol, 5 mL of water, and 16.8 mL of ammonia solution (25%). After stirring at 40 °C for 2 h, 15 mL of TEOS was added to the solution. The mixture was stirred at 40 °C overnight, and silica particles were formed. The silica particles were collected by centrifugation (10 000 rpm, 10 min) and washed by ethanol and water for at least five times. The particles were dried at 110 °C under vacuum.

**Preparation of Azide Modified Silica Particles.** In a round flask, silica particles (3 g) were dispersed in dry toluene after 30 min of ultrasonication and 3-(triethoxysilyl)-propylamine (5.0 g) was added to the solution, dropwise. The solution was stirred at 95 °C for 15 h. After the reaction, the particles were filtered and washed by ethanol until no blue precipitants were observed upon addition of $Cu^{2+}$ to the upper solution, which indicates that all the excess 3-(triethoxysilyl)-propylamine was removed. The amino group modified silica particles (0.8 g) were dispersed in dry toluene with dry pyridine (0.2 mL). After 30 min of ultrasonication, 2-bromo-2-methylpropionyl bromide (0.3 mL) was added into the solution at 0 °C, dropwise. The solution was stirred at 0 °C for 1.5 h and at room temperature for two days. After the reaction, the particles were collected by centrifugation, washed by mixture of methanol and water (50/50, v/v), and dried under vacuum.

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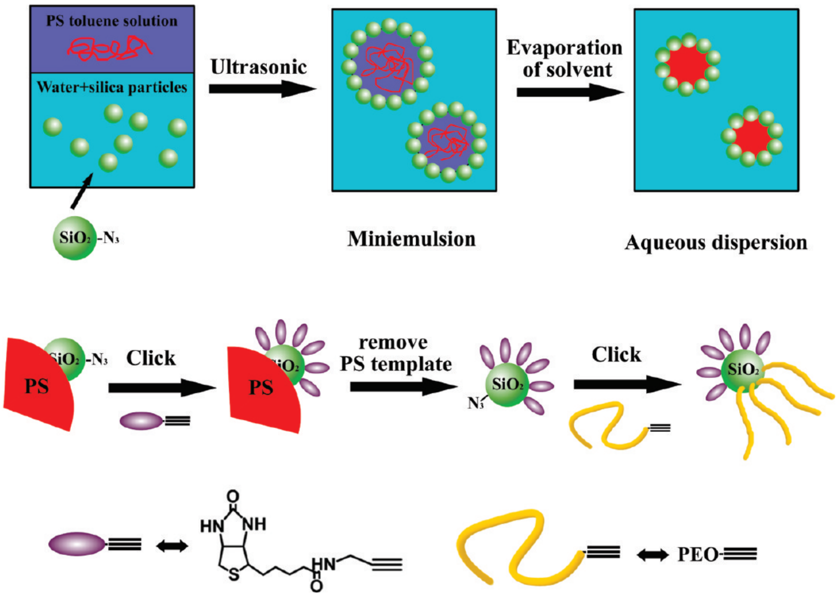
**Scheme 1. Scheme for the Preparation of Bioconjugated Janus Particles**



To prepare azide modified silica particles, the silica particles (0.4 g) were dispersed in 20 mL of dry DMF with sodium azide (0.13 g). The solution was stirred at room temperature for two days. The particles were collected by centrifugation and the cycles of centrifugation and redispersion in water were repeated for three times.

**Synthesis of Carboxylic Acid-Terminated PEO.** A typical procedure for the synthesis carboxylic acid-terminated PEO is described as follows. PEO with average molecular weight of 750 ($PEO_{750}$, 17.0 g, 22.7 mmol), succinic anhydride (3.53 g, 35.3 mmol), DMAP (2.81 g, 23.0 mmol), and triethylamine (3.2 mL, 23.1 mmol) were dissolved in 80 mL of anhydrous 1,4-dioxane, and the reaction was carried out at room temperature for 24 h under vigorous stirring. The solvent was evaporated completely by using a rotary evaporator. The residue was dissolved in methylene chloride, and the polymer was precipitated in diethyl ether. The purified product was dried in vacuum until constant weight was reached. $^1H$ NMR $\delta$ (400 MHz, $CDCl_3$, TMS, ppm): 4.26 ($-COOCH_2-$, 2H), 3.65 ($-OCH_2CH_2O-$, 70H), 3.37 ($CH_3O-$, 3H), 2.63 ($-OCOCH_2CH_2OCO-$, 4H).

**Preparation of Alkynated PEO.** The alkynated PEO was prepared by a reaction of carboxylic acid-terminated PEO (3.25 g) with propargylamine (0.5 mL) catalyzed by EDC (1.4 g) in chloroform at 0 °C for 2 h and at room temperature for two days. After being washed by saturated aqueous solution of NaCl two times and drying over $MgSO_4$, the solvent was evaporated completely by using a rotary evaporator. The residue was dissolved in methylene chloride, and the polymer was precipitated in diethyl ether. The purified product was dried in vacuum until constant weight was reached. $^1H$ NMR $\delta$ (400 MHz, $CDCl_3$, TMS, ppm): 4.24 ($-COOCH_2-$, 2H), 3.65 ($-OCH_2$-$CH_2O-$, 70H), 3.36 ($CH_3O-$, 3H), 4.0 ($-CH_2-C\equiv C-$, 2H), 2.48 ($-C\equiv CH$, 1H).

**Preparation of PS Particles with Silica Particles on the Surface.** Azide modified silica particles (23 mg) were dispersed in 10 mL of distilled water under ultrasonication for 5 min to give a homogeneous dispersion. To the mixture was added 3 mL of toluene dissolving 0.1 g of PS. After 30 min of ultrasonication, a stable emulsion was obtained. The organic solvent was evaporated carefully under reduced pressure, and a stable dispersion was prepared.

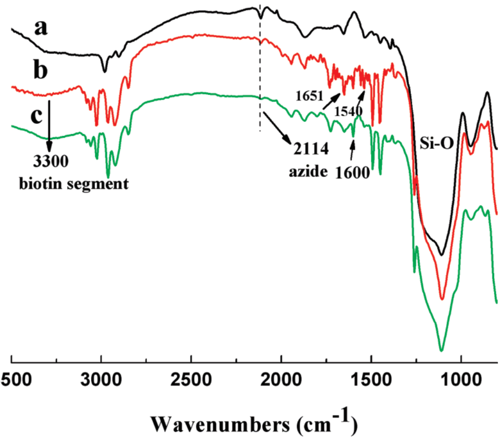


**Figure 1.** FT-IR spectra of (a) azide modified silica particles, (b) biotin-conjugated Janus particles, and (c) biotin/PEO Janus particles.

**Synthesis of Biotin-Conjugated Janus Nanoparticles by in Situ Click Chemistry.** Click reactions between alkynated biotin and azide modified silica particles were performed on the exposed surfaces of silica particles. A typical approach for the synthesis of biotin-conjugated Janus particles was described as follows. To an aqueous solution of PS particles coated with azide modified silica particles (10 mL, $[N_3] = 12$ $\mu$mol) were added *tert*-butanol (1.5 mL), $CuSO_4 \cdot 5H_2O$ (1.5 mg, 6 $\mu$mol), sodium ascorbate (2.4 mg, 12 $\mu$mol), and alkynated biotin (6.7 mg, 24 $\mu$mol, in 1 mL *tert*-butanol). The solution was stirred at 30 °C for 24 h and was then dialyzed against phosphate buffer solution (pH = 7.4, 0.05 M) and distilled water subsequently for 3 days. The products was collected by centrifugation (10 000 rpm, 15 min), washed by THF, and dried under vacuum.

The amount of available biotin on silica particles was determined by avidin/HABA reagent. The avidin/HABA reagent was reconstituted with 10 mL of deionized water. In a 1 mL cuvette, 900 $\mu$L of avidin/HABA reagent was pipetted, and the absorbance was measured at $\lambda = 500$ nm ($A_{500}^{HABA/Avidin}$) by UV−vis. To this solution was added 100 $\mu$L of deionized water containing 0.35 mg of sample, the solution was mixed by inversion, and the absorbance at $\lambda = 500$ nm ($A_{500}^{HABA/Avidin+Sample}$) was read. The

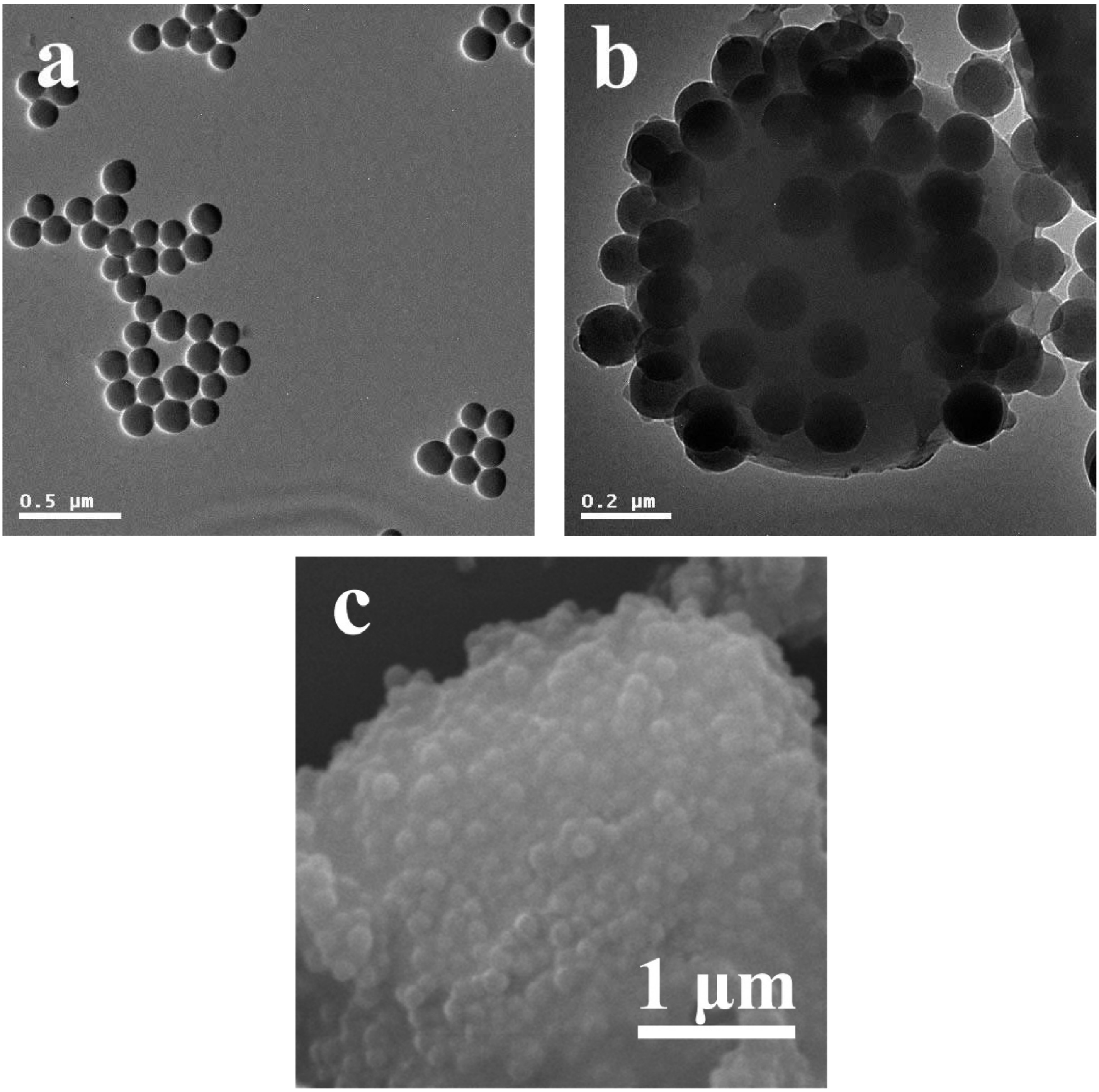


**Figure 2.** (a) TEM image of original silica particles; (b) TEM image of PS particles coated with azide modified silica particles; (c) SEM image of PS particles with azide modified silica particles on the surface.

amount of the available biotin was calculated by the following formula:[29,32] $\mu$mol biotin/mL = $(\Delta A_{500}/34) \times 10$, where $\Delta A_{500} = 0.9(A_{500}{}^{\mathrm{HABA/Avidin}}) - A_{500}{}^{\mathrm{HABA/Avidin+Sample}}$.

**Grafting of Alkynated PEO onto the Silica Particles by in Situ Click Chemistry.** The grafting of PEO to the hemispheres of the Janus silica particles is described as follows. Ten milligrams of biotin-conjugated silica particles, alkynated PEO (13 mg, 17 $\mu$mol), $CuSO_4 \cdot 5H_2O$ (0.3 mg, 1.2 $\mu$mol), and 15 mL of water were combined in a round-bottomed flask. After 30 min of stirring, sodium ascorbate (1.0 mg, 5.1 $\mu$mol) was added to the solution, and the mixture was stirred at 30 °C for 48 h. The particles were collected by centrifugation (10 000 rpm, 15 min), washed by water, and dried under vacuum.

**Characterization.** The thermal properties of the particles were measured by thermogravimetric analysis (TGA). The samples were heated to 700 °C at a heating rate of 10 K/min under nitrogen atmosphere on a Netzsch TG 209. The number average molecular weight and the molecular weight distribution of PS were determined by gel permeation chromatography (GPC) equipped with a waters 717 autosampler, waters 1525 HPLC pump, three waters UltraStyragel columns with 5K−600K, 500−30K, and 100−10K molecular ranges, and a waters 2414 Refractive Index Detector. THF was used as eluent at a flow rate of 1.0 mL/min. Molecular weights were calibrated on PS standards. Fourier transform infrared (FT-IR) was obtained on a Bio-Rad FTS 6000 system using diffuse reflectance sampling accessories. Ion chromatography (IC) was carried out on DIONEX DX-120 ion chromatograph. $^1$H NMR measurements were run on a Varian UNITY-plus 400 spectrometer

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with deuterated $CDCl_3$ as the solvent. UV−visible absorption spectra were collected on a JASCO V-570 spectrometer. Scanning electron microscopy (SEM) observations were conducted on a SHIMADZU SS-550 scanning electron microscope. The gold-coating samples were used in the measurements. High-resolution transmission electron microscope (TEM) observations were carried out on a Tecnai G2 20 S-TWIN electron microscope equipped with a model 794 CCD camera (512*512). TEM specimens were prepared by dipping copper grids into particles' dispersions and drying in air. To improve the contrast, the biotin was stained by exposing the TEM specimens to $OsO_4$ vapor for 0.5 h. Dynamic light scattering (DLS) was performed on a laser light scattering spectrometer (BI-200SM) equipped with a digital correlator (BI-9000AT) at 636 nm.

## Results and Discussion

The spherical silica particles were prepared by using the Stöber method.[31] In order to introduce bromide groups to the surface, reactions of original silica particles with 3-(triethoxysilyl) propylamine and 2-bromo-2-methylpropionyl bromide were conducted. The IC result indicated that the weight percentage of bromine was about 9.2 wt %. After reaction with sodium azide, azide groups were grafted to the surfaces of silica particles. The asymmetric stretch of the azide group at 2114 $cm^{-1}$ was clearly observed in the FT-IR spectrum (spectrum a in Figure 1). The IC result showed that about 95.2% of bromide groups were converted to azide groups after the reaction. The synthesis of alkynated biotin could be found in our previous paper.[30] Hydroxyl group terminated PEO with number average molecular weight of 750 ($PEO_{750}$) was reacted with succinic anhydride, and carboxylic acid terminated PEO was obtained. The alkynated PEO was prepared by a reaction of carboxylic acid terminated PEO and propargylamine.

It had been demonstrated that polymer particles could be produced by the miniemulsion process.[33] Miniemulsions are stable emulsions consisting of stable oil droplets with a size of 50−500 nm created by shearing a system with oil, water, surfactant, and water insoluble compound. Polymer particles can be obtained by adding a polymer that dissolves in the dispersed oil phase but is insoluble in aqueous phase into a system with dispersed oil droplets in aqueous phase, followed by removal of oil phase. In this research azide modified silica particles were chosen to stabilize toluene droplets in water. So this emulsion is also a Pickering emulsion. PS was dissolved in toluene, and the solution was added to an aqueous solution with azide modified silica particles. After ultrasonication, the polymer toluene droplets stabilized by silica particles in aqueous solution were obtained. PS particles coated with azide modified silica particles can be obtained after removal of toluene at a reduced pressure. Figure 2a shows a TEM image of the original silica particles. The size of the spherical particles ranges from 120 to 180 nm with an average size of 150 nm. Figure 2b is a TEM image of PS particles coated with azide modified silica particles. Silica particles on the surface of PS particles can be observed clearly. For some silica particles almost half of the surface is immersed in the PS phase, however, for some particles only a small part of the surface is in PS phase. Figure 2c presents a SEM image of PS particles with azide modified silica particles. On the image the surface of the PS particles is studded with silica particles.

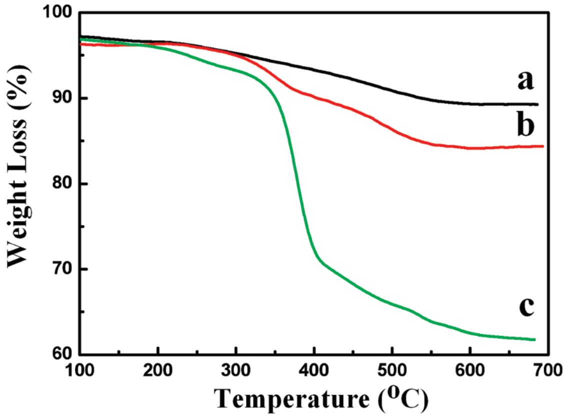

**Figure 3.** TGA of azide modified silica particles (a), biotin-conjugated Janus silica particles (b), and biotin/PEO Janus silica particles (c).

The in situ click reaction between alkynated biotin and the azide groups on the exposed sides of silica particles was conducted in a mixture of *tert*-butanol and water at 30 °C. Alkynated biotin was conjugated to the surfaces of the silica particles via a copper(I)-catalyzed 3 + 2 "click" cycloaddition. The superfluous alkynated biotin and catalyst were removed by extensive dialysis against phosphate buffer solution and water. The products were collected by centrifugation. Silica particles with biotin groups on one hemisphere and azide groups on the other one were obtained after removal of PS by dispersing PS particles in THF and followed by centrifugation. The biotin modified Janus particles were characterized by FT-IR, TGA, and TEM. FT-IR is a powerful tool to verify the click conjugation and the presence of biotin compounds. A reduction of the azide peak at 2114 $cm^{-1}$ was observed concomitant with a new absorption in the triazole peak at 1600 $cm^{-1}$ (spectrum b in Figure 1), suggesting the occurrence of click reaction. In addition, the FT-IR spectrum shows new peaks at 3300 $cm^{-1}$ (N—H stretching mode) and 1651 $cm^{-1}$ (amide I mode of C=O of biotin) and 1540 $cm^{-1}$ (amide II mode of N—C=O of biotin). This result confirmed the conjugation of biotin molecules onto silica particles. The successful bioconjugation of biotin was also evidenced by TGA (Figure 3). After bioconjugation of biotin, the silica particles were found to have 12.26 wt % weight loss, which is due to the loss of the biotin. Moreover, to confirm the location of biotin on silica particles, TEM observation was conducted. Figure 4 presents TEM images of the biotin-conjugated silica particles dispersed in a mixture of *tert*-butanol and water (1:4, v/v). The specimen was prepared by dipping a copper grid in the solution and drying in air. Before TEM observation, the specimen was exposed to $OsO_4$ vapor to stain biotin molecules. In the TEM images, the silica surfaces decorated with biotin molecules are

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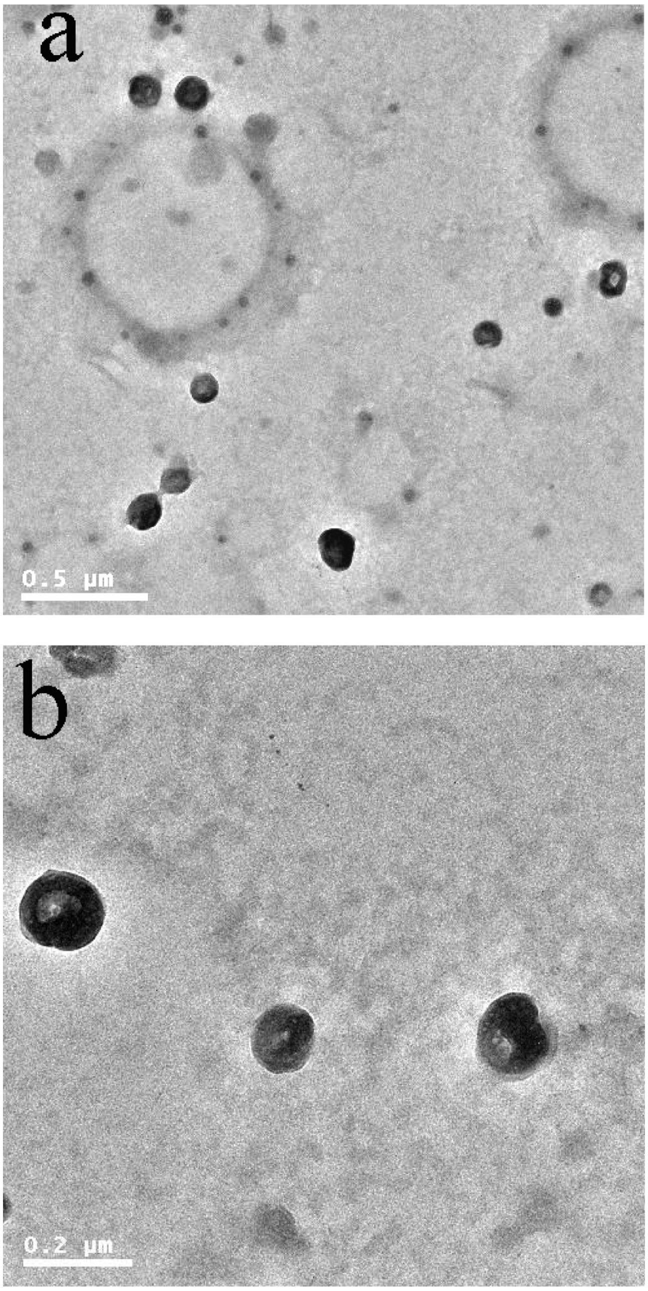

**Figure 4.** TEM images of biotin-conjugated Janus particles dispersed in a mixture of *tert*-butanol and water (1:4, v/v), (a) at low magnification and (b) at high magnification.

darker, and they only could be observed at particular areas of silica particles, which proved that asymmetric surface structure was produced on the silica particles.

The bioavailability of biotin to avidin was evaluated by an avidin/HABA competitive binding assay. It is known that the binding of HABA to avidin gives an absorption maximum at 500 nm. When biotin or a biotinylated entity is added, biotin displaces HABA from the complex with avidin, and the absorbance at 500 nm decreases. The absorption decreases proportionately to the concentration of biotin in the system, and the change in absorbance can be used to calculate the amount of biotin.[29,32] The assay was conducted by measuring the absorbance of the avidin/HABA complex at 500 nm before and after it was mixed with the biotin-conjugated Janus particles. Figure 5 shows UV–vis spectra of the avidin/HABA complex before and after addition of biotin-conjugated Janus particles. Upon addition of the biotin-conjugated Janus particles, the absorbance of the avidin/HABA complex at 500 nm decreased, suggesting that biotin on the Janus particles displaced HABA from the avidin/HABA complex as a result of its higher affinity for avidin. The amount of available biotin to avidin was calculated to be 16.3 nmol biotin/mL of the solution, corresponding to 4.7 $\mu$mol biotin/g Janus particles. This value is much lower than the amount of biotin estimated based on the TGA result. The lower accessibility and bioavailability can be explained by the fact that some binding sites of avidin molecules could not interact with biotin molecules due to the steric hindrance on the silica surface.[29] Additionally, as avidin has four biotin binding sites, it can bind biotin molecules on different particles forming aggregation of silica particles, which would also sterically prohibit biotin molecules from binding avidin. To prove this, a research of the interaction of biotin on the Janus particles with free avidin was conducted.

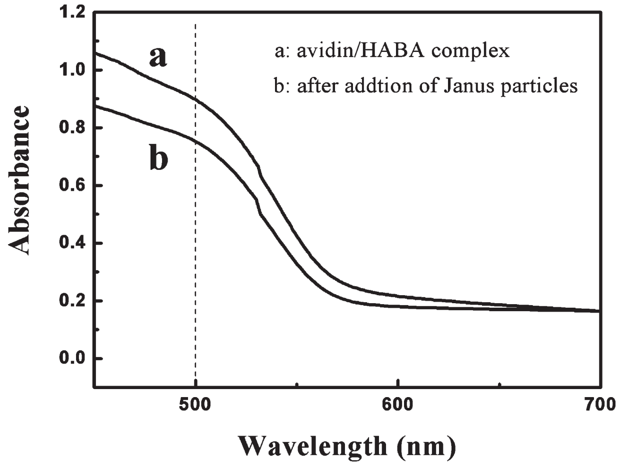

**Figure 5.** UV–vis spectra of the avidin–HABA complex and the avidin–HABA complex added with biotin-conjugated Janus particles, respectively.

A dispersion of biotin-conjugated Janus particles was incubated with free avidin for 5 min. Figure 6 presents TEM images of the dispersion before and after addition of free avidin. Figure 6a is a TEM image of dispersion of Janus silica particles before addition of avidin. A DLS histogram of Janus silica particles is also shown in the inset of Figure 6a. Both results indicate well dispersed Janus particles in the solution. However, after addition of avidin to the dispersion, aggregated structures composed of Janus silica particles could be observed (Figure 6b). As avidin has four binding sites, it can act as a cross-linking agent and induce the formation of particle aggregates.[34] Upon addition of avidin, avidin molecules interacted with biotins on different particles and induced the aggregation of biotin-conjugated Janus particles.

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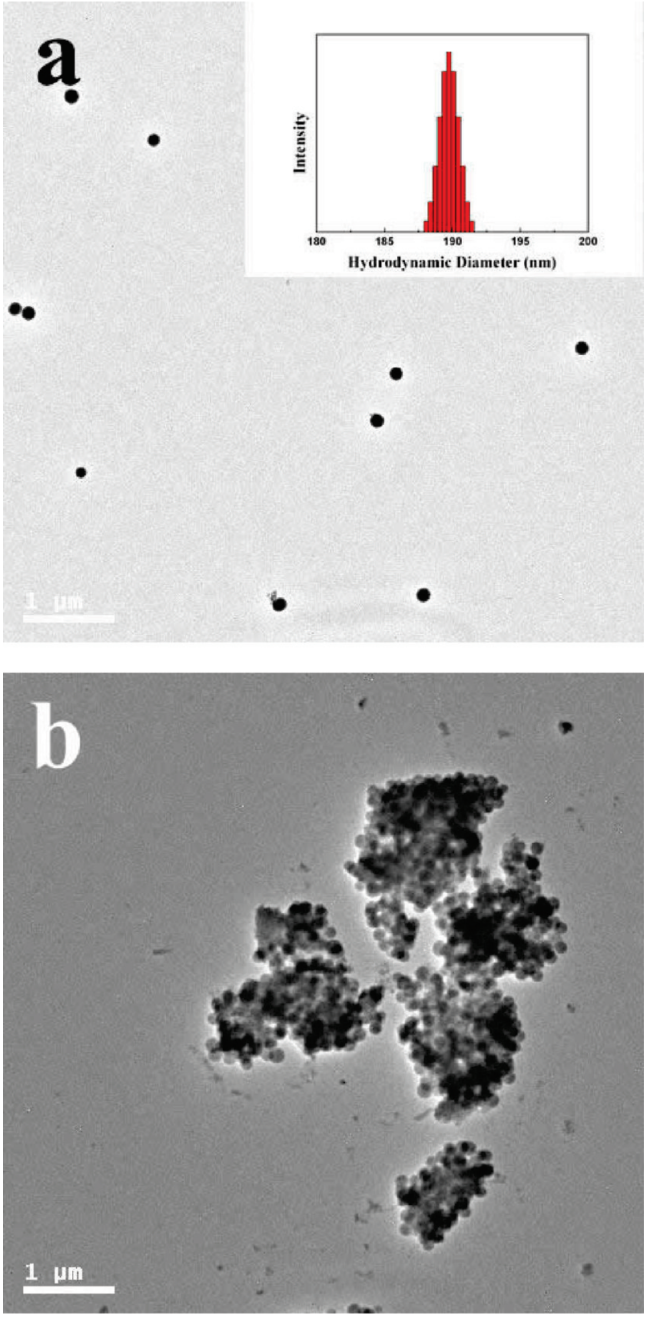

**Figure 6.** TEM images of the biotin-conjugated Janus dispersion before (a) and after (b) addition of free avidin.

The click reaction between alkynated PEO and the remaining azide groups on biotin-conjugated Janus silica particles was performed in water. The alkynated PEO was grafted to the surfaces of the silica particles by a 3+2 "click" cycloaddition. After the reaction, the products were collected by centrifugation and thoroughly washed by water to remove the excess PEO and catalyst. On the FT-IR spectrum of the Janus silica particles with biotin molecules on one hemisphere and PEO on the other one (spectrum c in Figure 1), the stretching peak of azide groups at 2114 $cm^{-1}$ disappeared after the reaction, indicating the successful coupling reaction. However, the characteristic bands of PEO at 1100 $cm^{-1}$ due to the ether stretch modes were immersed into the asymmetric and symmetric stretching of the Si−O bonds at 1000−1300 $cm^{-1}$. TGA result also proved the grafting of PEO chains to the silica particles with evidence of an additional 22% weight loss due to the decomposition of the grafted PEO (Figure 3).

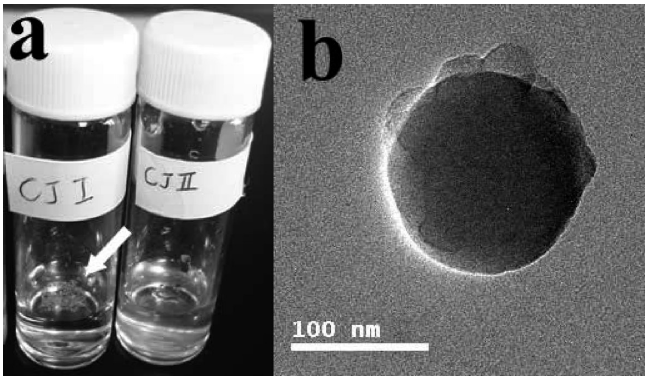

**Figure 7.** (a) Photograph of dispersions of biotin/azide Janus particles (CJ I) and biotin/PEO Janus nanoparticles (CJ II) in water; (b) TEM image of a biotin/PEO Janus particle.

It is worth note that after grafting of PEO the dispersibility of the Janus particles in water was significantly improved. Figure 7a shows a photograph of two dispersions of Janus particles. The Janus particles with biotin and azide groups on the two sides could not disperse well in the aqueous solution; they only stayed on the surface of solution as indicated by an arrow on the image. However, the Janus particles with PEO on the surface could disperse well in water, and a homogeneous solution was obtained. Figure 7b presents a magnified TEM image of a Janus particle with PEO on one side and biotin on the other side. The specimen for TEM observation was not stained. On the image the grafting PEO brushes could be observed on the half rim of the particle due to the asymmetric surface structure.

## Conclusions

In summary, we have developed a simple versatile two-step approach for the synthesis of biconjugated Janus silica particles based on in situ click chemistry. This is the first report on the synthesis of a Janus particles decorated with biomolecules on one hemisphere and biocompatible polymer chains on the other one. In this approach, a Pickering emulsion was prepared to freeze the azide modified silica particles on the surfaces of PS particles. Biotin moieties and PEO chains were conjugated on the surfaces of the silica particles by two-step click chemistry. The avidin/HABA assay proved the bioavailability of the Janus particles to avidin. The Janus particles could find potential applications in the fields of targeted drug delivery, biodetection, and so forth. In the future this fabrication approach can be extended to various biomolecules and also extended to different functional polymer chains. On the basis of this facile approach more novel bioconjugated Janus particles will be prepared. Some of this research is being investigated in this laboratory.

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