Multifunctional Silica Nanoparticles for Covalent Immobilization of Highly Sensitive Proteins

*Xu-Dong Wang, Kersten S. Rabe, Ishtiaq Ahmed, and Christof M. Niemeyer**

The selective immobilization of biological macromolecules, such as proteins or oligonucleotides, on the surface of nanoparticles opens up a wide range of applications in the area of biocatalysis, chromatography, biosensing, or drug delivery.[1–3] Silica nanoparticles (SiNPs) are often used in life-sciences because they are readily available in various sizes, their surface can be functionalized with arbitrary components using established silane chemistry, their interior can be used for encapsulation of numerous compounds including small molecules, proteins, or nucleic acids for targeted application, and importantly, they reveal a very low cytotoxicity. It is therefore not surprising that SiNPs have already been used and are currently refined as intracellular drug delivery vehicles.[4] In addition to encapsulation, mere physicochemical adsorption, or, preferably, covalent immobilization techniques are usually considered for functionalization of SiNPs with biomacromolecules. The adsorption of biomolecules on the surface of nanoparticles via electrostatic and/or hydrophobic interaction is the simplest approach, but it suffers from potential desorption and loss of biomolecules over time. While it is basic knowledge that each protein is different and usually needs very well-tailored conditions for successful immobilization on nanoparticles, beads, or any other substrate, interactions between proteins and the surface might also be strong enough to induce changes of the protein's secondary or tertiary structure, thereby leading to complete loss of bioactivity.[5–7] Encapsulation of biomolecules is also difficult because most types of nanoparticles are synthesized in organic solvents incompatible with biomacromolecules. Hence, direct encapsulating during particle formation usually leads to denaturation and concomitant loss of protein activity. As a consequence, only proteins with high stability, such as horseradish peroxidase,[8] green fluorescent protein,[9,10] or myoglobin,[11] have so far been successfully encapsulated during particle formation. We will demonstrate below that these proteins are inappropriate models for testing biocompatible surface properties and mild immobilization methods due to their robust tertiary architecture. Although stable lipases have been shown to be active after sol–gel immobilization,[12,13] the incorporation of proteins even into the channels of porous nanoparticles[14] suffers from the presence of diffusion barriers as well as potential leakage. Therefore, the covalent immobilization of biomolecules on the surface of SiNPs represents the most promising approach to generate active biohybrid nanomaterials.

Unmodified SiNPs are difficult to be used for covalent immobilization because they often reveal strong interactions with biomolecules, leading to the immediate formation of a dense monolayer, the so-called corona of biomacromolecules. This nonspecific adsorption not only changes the activity of biomolecules but also influences the particle fate in in vivo applications.[15] The minimization of non-specific adsorption is therefore mandatory for engineering of novel bioactive nanomaterials. The installation of oligomeric or polymeric ethylene glycol (PEG) units on the particle's surface, often referred to as PEGylation, is widely used to efficiently suppress non-specific adsorption. However, this approach does not completely eliminate biomolecule adsorption.[16,17] Moreover, when suppression of non-specific adsorption is to be combined with targeted specific immobilization strategies, a dense PEG coating compromises the space for biomolecule immobilization,[18] and the repulsive properties also interfere with efficient interphase coupling reactions. As an alternative for PEGylation, zwitterion-functionalized SiNPs containing both positively and negatively charged functional groups have been synthesized and this modification indeed efficiently suppressed particle aggregation,[19] and non-specific adsorption of proteins.[20,21] However, these particles had an inert surface lacking reactive groups suitable for targeted bioconjugation.

We here report a general and versatile one-pot reaction strategy for the synthesis of multifunctional SiNP, which have tailored densities of three different functional groups on their surface to efficiently suppress non-specific adsorption, and, at the same time, enable efficient specific covalent immobilization of proteins. Through tailored composition of amino, phosphonate, and thiol groups, a zwitterion-stabilized particle surface is created, which not only efficiently eliminates non-specific adsorption, but also provides reactive groups for the desired specific immobilization of biomacromolecules. Using two highly sensitive proteins, an FRET-based glucose-binding protein biosensor and a two-domain cytochrome P450 enzyme, we demonstrate that the surface of the newly developed SiNPs can be tailored to provide a microenvironment, which enables the maximum retention of the immobilized protein's activity. Since these SiNPs are stable and mono-dispersed even after storage in biological buffers for months, we believe that they represent a suitable platform for the generation of a wide range of bioactive nanomaterials.

Previous work on the synthesis of SiNPs has indicated that the installation of surface functionalities on SiNPs through successive condensation steps with different organotrialkoxysilane reagents enables suitable control over particle size and prevents

Dr. X.-D. Wang, Dr. K. S. Rabe, Dr. I. Ahmed, Prof. C. M. Niemeyer
Karlsruhe Institute of Technology (KIT)
Institute for Biological Interfaces (IBG-1)
Hermann-von-Helmholtz-Platz
76344 Eggenstein-Leopoldshafen, Germany
E-mail: niemeyer@kit.edu

DOI: 10.1002/adma.201503935

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

ADVANCED MATERIALS
www.advmat.de

www.MaterialsViews.com

aggregation. However, this strategy compromises the amount of functional groups because surface passivation occurs after isolation from the various reaction solutions.[22,23] Likewise, the direct one-pot condensation with different organosilanes is challenging because the sequential addition of reagents can strongly disturb colloidal stability and lead to severe particle aggregation.[22,23] Inspired by seminal work of Bagwe et al.,[19] we reasoned that the use of a microemulsion system should enable the installation of multiple surface functionalities because individual nanoparticles are grown inside micelles, which function as separated reaction compartments (**Figure 1**). Moreover, this approach has the advantage to produce highly uniform particles in the size range of 10–100 nm, which is favorable for biological applications.[24,25] To experimentally investigate this strategy, cyclohexane, 1-hexanol, water, and surfactant Triton X-100 were mixed rigorously to produce a water-in-oil micellar suspension and subsequently tetraethoxysilane (TEOS) was added as a silica source. Ammonia was used as a catalyst to induce hydrolysis and condensation of TEOS and to grow parent silica nanoparticles (SiNP-1, in Figure 1) inside the micelles. Subsequently, a mixture of 3-(trihydroxysilyl)propyl methylphosphonate (THPMP) and (3-trimethoxysilylpropyl) diethylenetriamine (DETAPTMS) organosilanes was added to modify the surface of SiNP-1 with negatively charged phosphonate groups and primary amino groups, respectively, thereby leading to formation of zwitterionic SiNP-2. Subsequent addition of 3-mercaptopropyltrimethoxysilane (MPTMS) led to formation of SiNP-3 bearing amino, phosphonate and thiol functional groups on their surface. The latter served as coupling groups for the installation of PEG units through thiol-maleimide coupling to yield SiNP-4, which were then functionalized with proteins-of-interest taking advantage of the particle's surface amino groups and glutaraldehyde- or NHS-based chemistries.[26]

Initial studies on how different ratios of positively charged amino-silane DETAPTMS and negatively charged phosphonate silane THPMP affected the size, stability, and dispersity, as well as surface charge of SiNP-2 (Table S1, Supporting Information) indicated that a volume ratio of THPMP:DETAPTMS = 4:1 was found to yield monodisperse particles (60 nm diameter, see also TEM images in Figure S1, Supporting Information) with highest densities of amino and phosphonate surface groups. Indeed, the measured amino groups concentration of 108 μmol g^{-1} is significantly higher than that reported for surface modification of SiNPs with amino groups (24 μmol g^{-1}) using (3-aminopropyl)trimethoxysilane (APTMS).[19] We speculate that this difference stems from the increased chain length of DETAPTMS (9 atom spacer between Si and NH_2) as compared to APTMS (3 atom spacer). It is known that the extended chain length of DETAPTMS also increases the particle's stability in aqueous solutions,[27] and we found that SiNP-2 revealed a very high colloidal stability such that the particles remained monodisperse for >2 months in water or PBS buffer.

Following the synthetic route depicted in Figure 1, reactive thiol groups were then introduced in situ on the particle's surface via hydrolysis and condensation of MPTMS to produce trifunctional particles SiNP-3. In initial attempts, MPTMS

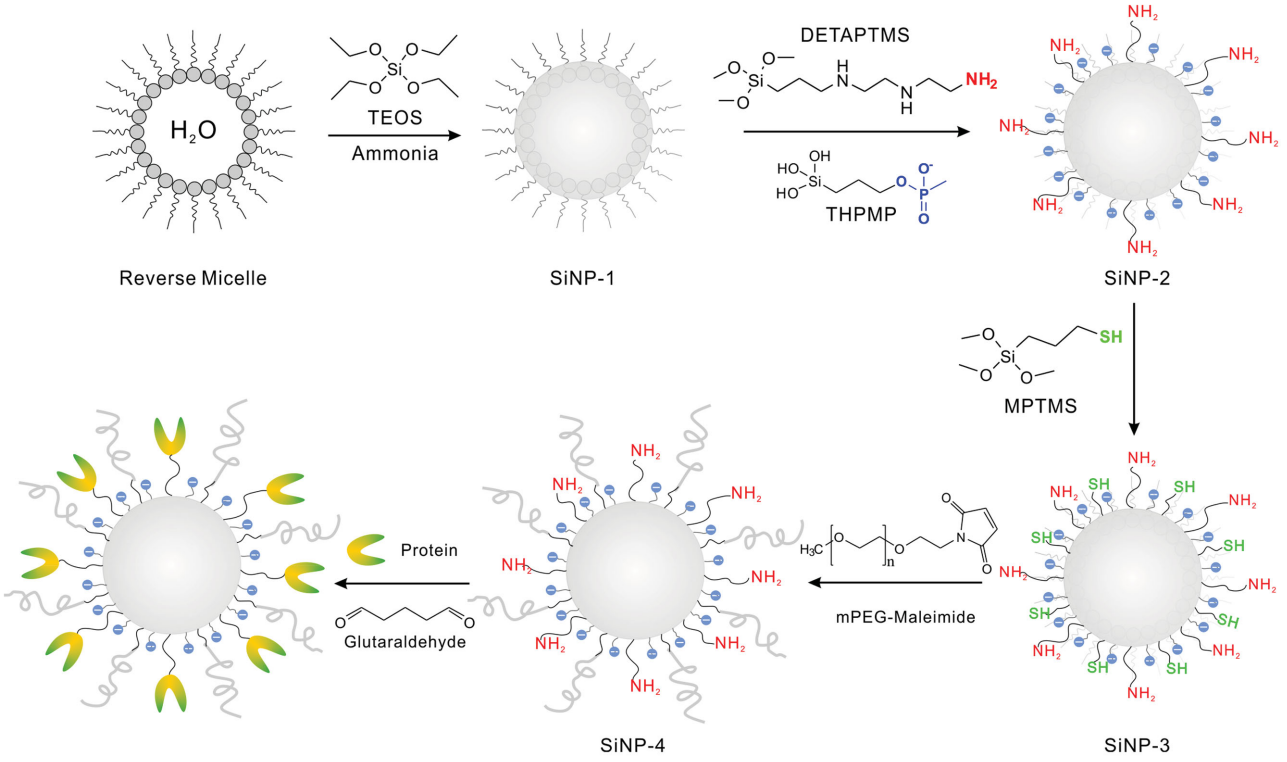

Figure 1. Schematic illustration of the one-pot sequential synthesis of multifunctional SiNPs. Initial formation of bare SiNP-1 was followed by surface functionalization with mixtures of DETAPTMS and THPMP to generate zwitterionic SiNP-2 and subsequent addition of MPTMS to generate SiNP-3 bearing three different functional groups. The partial PEGylation using thiol-maleimide chemistry yielded SiNP-4 to create a microenvironment for specific covalent coupling of proteins through the particle's surface amino groups.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

was added simultaneously with THPMP and DETAPTMS, however, the obtained particles were prone to aggregation and revealed significantly lowered concentrations of surface amino groups (Table S2, Supporting Information), presumably because the hydrolysis rates of MPTMS are higher than that of DETAPTMS under basic conditions.[28] We speculate that, under the given conditions, the competing reaction between the three organotrialkoxysilanes yielded particles with high surface concentrations of thiol groups, which may be prone to oxidation and concomitant interparticle crosslinking through disulfide bonds. Due to these negative results, we then tested whether addition of MPTMS subsequent to condensation of THPMP and DETAPTMS for 3 h leads to improved densities of amino and thiol groups, stability, and dispersity of the desired SiNP-3 particles. Indeed, optimization of the reaction conditions (Table S3, Supporting Information) led to SiNP-3 particles with surface concentrations of reactive thiol groups as high as 127 ± 12 µmol g^{-1}. This density is close to the highest value ever reported for SiNPs.[29] Importantly, the concentration of surface amino groups remained unchanged, as compared to SiNP-2 particles. The obtained SiNP-3 were stable and remained monodispersed in degassed water and buffers but they aggregated over time, most likely due to oxidation by dissolved oxygen. As discussed below, this aggregation could be entirely suppressed by partial PEGylation of the surface thiol groups to yield SiNP-4, which were stable in 100×10^{-3} M PBS buffer for at least 2 months.

To investigate whether and how the synthesized SiNPs non-specifically interact with proteins, we initially used the glucose biosensor FLIPglu600µΔ13.[30] This protein, in the following denoted as FLIP, is a heterotrimeric fusion consisting of glucose binding protein (GBP) (colored gray in **Figure 2**A), which is genetically fused with an enhanced cyan fluorescent protein (eCFP) (blue) and an enhanced yellow fluorescent protein (eYFP) (yellow) at the N- and C-terminus, respectively. FLIP can be used to detect glucose because the two fluorescent proteins (FPs) form a Förster-resonance energy transfer (FRET) pair. In the absence of glucose, GBP adopts a conformation wherein the FP domains are located in close proximity to each other, and an efficient FRET occurs from eCFP ($\lambda_{em,max}$ = 476 nm) to eYFP ($\lambda_{em,max}$ = 527 nm). Upon binding of glucose, FLIP changes its conformation into a closed form, which separates the FRET pair thereby decreasing the efficiency of energy transfer. Hence, a gradual decrease in fluorescence ratio (I_{527}/I_{476}) is observable

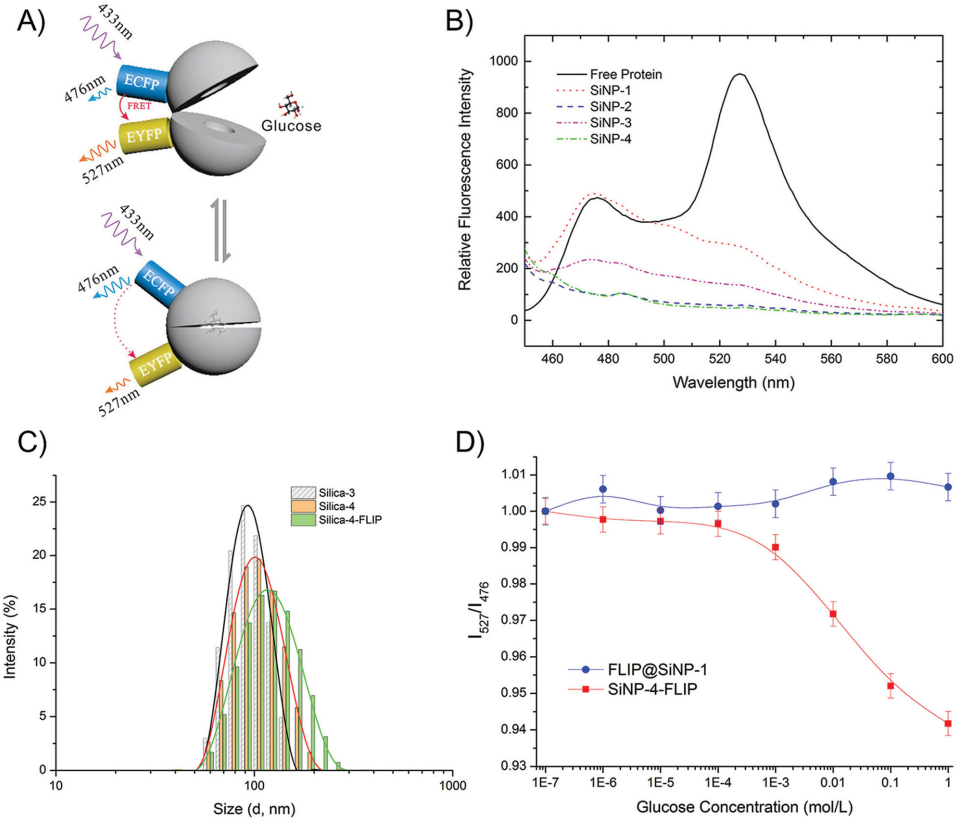

Figure 2. A) Schematic drawing of the glucose biosensor FLIP. Note that binding of glucose leads to the decrease of FRET from the fused eCFP to the eYFP domain of this fusion protein, thereby leading to a decreased fluorescence intensity ratio (I_{527}/I_{476}). B) Fluorescence spectra obtained from the various SiNPs prepared in this study after incubation with FLIP and subsequent washing. For comparison the spectrum of unbound FLIP is shown in black. Note the SiNP-1 (red) and SiNP-3 (magenta) show a significant non-specific adsorption of FLIP, while SiNP-2 (blue) and SiNP-4 (green, prepared as described in entry 2, Table S4, Supporting Information) do not adsorb protein on their surface. C) Changes of hydrodynamic radii of SiNPs as a consequence of partial PEGylation (red) and FLIP immobilization (green). Note that the particles remain monodispersed. D) The functionality of SiNP-4-FLIP (red) as biosensor for glucose is indicated by the marked response to glucose, while FLIP adsorbed onto SiNP-1 (blue) completely lost its binding response capability (see also Figure S7, Supporting Information).

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

with increasing glucose concentrations (Figure S2, Supporting Information). Since the FRET ratio is highly sensitive measure for even slight changes in secondary and tertiary structure of FLIP, this protein is ideal for studying any influences of differently functionalized surfaces on the structure and activity of an immobilized protein. Moreover, FLIP brings with it the additional advantage that its binding onto particles can be sensitively detected via direct measurements of the fluorescence of eCFP.

To investigate the interactions of the synthesized SiNPs (Figure 1), we initially tested their capability to bind FLIP through non-specific adsorption (Figure 2B). To this end, a solution containing 0.6 nmol FLIP was mixed with a solution containing 10.0 mg mL^{-1} SiNPs and the mixtures were incubated for 30 min. The SiNPs were centrifuged, washed to remove unbound FLIP, and subsequently resuspended to facilitate analysis by fluorescence spectroscopy. Bare SiNP-1 revealed a strong interaction with FLIP, and almost the entire protein was absorbed on the particles within 10 min. Extensive washing of the particles with PBS buffer did not remove the absorbed protein from particle's surface. The fluorescence spectrum of the adsorbed protein showed significant differences to that of free proteins in solution (red and black lines in Figure 2B, respectively). This is an indication of severe changes in the protein's structure after adsorption as confirmed by activity tests, which clearly showed that FLIP adsorbed to SiNP-1 had completely lost its binding ability for glucose (Figure 2D, blue line). Similar adsorption studies were conducted with stable proteins, i.e., the enhanced green fluorescent protein (eGFP) (Figure S3, Supporting Information), enhanced cyan fluorescent protein (eCFP) (Figure S4, Supporting Information) and the enzyme horseradish peroxidase (HRP, Figure S5, Supporting Information). All three proteins were readily adsorbed at SiNP-1, however, in contrast to FLIP, these proteins retained their functionality, as judged by characteristic fluorescence properties (eGFP, eCFP) or catalytic activity (HRP). These findings emphasize that proteins with a robust tertiary structure are not well suited for testing biocompatible surface properties.

Protein adsorption studies with SiNP-2 revealed low adsorption of FLIP (Figure 2B, blue), presumably due to their zwitterionic surface properties, which can prevent adsorption of proteins.[20] Installing thiol groups apparently increased the particle's affinity for FLIP, as indicated by the slightly higher amount of adsorbed protein in the case of SiNP-3 as compared to SiNP-2 (Figure 2B, magenta). To omit this unwanted binding, we coupled increasing amounts of methoxypolyethylene glycol maleimide (mPEG-mal) chains to the surface thiol groups using maleimide chemistry (Table S4, Supporting Information). Interestingly, we observed that even the coupling of only 6% of the total numbers of available thiols with mPEG-mal was sufficient to completely suppress the adsorption of FLIP (green spectrum in Figure 2B). Importantly, amino group concentration on the surface of these SiNP-4 particles remained almost unchanged after PEGylation, and both amino- and thiol-groups remained chemically accessible, as indicated by their reaction with fluorescamine and Ellman's reagent, respectively. Notably, the particle's stability also significantly improved upon partial surface PEGylation. SiNP-4 were fully stable and remained monodisperse even after 2 months of storage in PBS buffer with NaCl concentrations up to 500×10^{-3} M.

To test whether the new particles are also suited for specific, covalent coupling of proteins, FLIP was immobilized on the surface of SiNP-4 using glutaraldehyde as a crosslinking reagent. To this end, SiNP-4 were treated with glutaraldehyde to install reactive aldehyde groups on the particle's surface, and after removal of unreacted glutaraldehyde, the particles were allowed to bind FLIP in PBS buffer for 2 h. The reaction between aldehyde groups on the particle and accessible lysine residues of FLIP leads to formation of Schiff bases, which were subsequently reduced to stable secondary amines using $NaBH_3CN$.[26] It is shown in Figure 2C that DLS measurements indicated an increase in hydrodynamic diameter of the resulting SiNP-4-FLIP, thereby providing initial evidence for the successful coupling of FLIP. Specifically, SiNP-3 had an average hydrodynamic diameter of 91 nm, PEGylated SiNP-4 revealed an increased size of 101 nm and the protein tethering further increased this value to about 113 nm. Importantly, SiNP-4 were still monodispersed after protein immobilization (PDI = 0.076). Fluorescence spectroscopy analysis of SiNP-4-FLIP revealed that the fluorescence spectrum of the immobilized protein is similar to that of the free protein in buffered solution (Figure S6, Supporting Information), suggesting that the immobilization did not adversely influence the structure of the protein. Interestingly, we also observed that FLIP cannot be immobilized on highly PEGylated SiNP-4 (prepared according to entry 6 in Table S4, Supporting Information), most likely due to steric hindrance and protein repellence of the densely PEG-coated particle surface (Figure S6, Supporting Information).

To investigate whether the immobilized FLIP has indeed retained its activity, we investigated the binding ability of SiNP-4-FLIP to function as a biosensor for glucose. As shown in Figure 2D, the immobilized FLIP (red line) showed a binding response to different concentrations of glucose, which is similar as the free protein in buffer solution. In comparison, FLIP adsorbed on SiNP-1 by non-specific interactions (FLIP@SiNP-1) completely lost its response capability (blue line in Figure 2D, see also Figure S7, Supporting Information). The spectral response capability of SiNP-4-FLIP confirmed that the activity of FLIP is preserved after immobilization on SiNP-4, suggesting that the immobilization on the tailored surface did not significantly affect the conformational freedom of this highly delicate fusion protein. This result is remarkable because the glutaraldehyde-mediated immobilization can be considered as a relatively crude method where covalent cross-coupling occurs unselectively with accessible lysine residues of the protein. Therefore, the protein can be attached to the surface in a variety of conformations even through multiple fixation points and all these different attachment modes will more or less impair the protein's secondary and tertiary structure as well as its conformational flexibility necessary to maintain its activity.

To avoid the aforementioned drawbacks of statistical cross-coupling methods, directional binding approaches offer a much higher control on the orientation of immobilized proteins on solid surfaces because the protein is coupled only through a distinctive site, e.g., a single amino acid or a small epitope, thereby prohibiting damage of active sites and/or restrictions

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

in conformational freedom.[31] Many of such techniques take advantage of fusion proteins bearing self-ligating protein tags, such as the so-called SNAP-[32] or Halo-tag.[33] The latter is a variant of a bacterial dehalogenase, which specifically forms a covalent bond with a small molecule chlorohexyl (CH)-ligand. To explore whether SiNP-4 are suitable carriers also for the directional immobilization of enzymes, we coupled NHS-activated CH-ligands to the particle's amino groups (**Figure 3**A) and the resulting SiNP-4-CH were tested for specific binding of the bacterial cytochrome P450 enzyme BM3 genetically fused with a Halo-tag protein. The monooxygenase P450 BM3 (CYP102A1) from *Bacillus megaterium* is a 118 kDa protein composed of two domains, the reductase domain BMR, bearing FAD and FMN prosthetic groups, and the porphyrin domain BMP, containing the catalytic heme (protoporphyrin IX) group. This two domain protein is widely used in biocatalysis and has been applied, for instance, for the synthesis of drug metabolites and precursors of artemisinin and paclitaxel.[34] Here, we genetically fused the BM3 variant A74G/F87A[35] with a Halo-tag to obtain a 153 kDa enzyme capable to convert the fluorogenic substrate 12-(4-trifluoromethylcoumarin-7-yloxy)dodecanoic acid. To facilitate site-selective immobilization of BM3 with SiNP-4-CH, the enzyme was allowed to bind to the particle's surface for 2 h. Subsequent to centrifugation and washing, the amount of covalently linked BM3-Halo was determined as 5.4 μg mg^{-1} of particles, being in the same range as FLIP (SiNP-4-FLIP, 3.6 μg mg^{-1}). For comparison, the amount of physically adsorbed protein on SiNP-1 particles was slightly higher (6.6 μg mg^{-1} and 4.5 μg mg^{-1} for BM3-Halo and FLIP, respectively), presumably due to the absence of PEG chains on the surface. The enzymatic activity of SiNP-4-CH-Halo was then tested by measuring the produced 7-hydroxy-4-trifluoromethylcoumarin fluorophore. Indeed, the observed increase in fluorescence clearly indicated that the immobilized BM3 exhibited high activity, which was similar to that of free proteins in solution (Figure 3B). Notably, almost no activity was observed in controls where the BM3 was physically adsorbed onto SiNP-1. Furthermore, immobilization of BM3 on SiNP-4 by statistical glutaraldehyde crosslinking led to rapid inactivation of the enzyme (data not shown). These results proved that the nanoparticles SiNP-4 developed in this study provide a suitable microenvironment for covalent attachment of highly sensitive proteins.

In conclusion, we here describe a simple and efficient method for the preparation of multifunctional SiNPs of about 60 nm in diameter using a reverse micellar system in a one-pot reaction. Specifically, parent core particles were synthesized in a first step within reversed micelles and, in the second step, an initial zwitterionic surface modification was installed by in situ co-condensation of amino- and phosphonate-derivatized alkoxysilanes. In the third step, condensation of a thiol-derivatized alkoxysilane was added in situ to the micellar suspension to install reactive thiol groups on the surface of the nanoparticles. In the final step, fractions of the reactive thiols were used for maleimide-based coupling of poly(ethylene glycol) chains. The resulting nanoparticles not only revealed uniform size, high colloidal stability, and low non-specific adsorption properties for proteins, but they also contained accessible and thus reactive amino groups to enable covalent immobilization of proteins on their surface. We note that FTIR analyses of our particles revealed a very strong surface adsorption of water, which prohibited the direct detection of amine, thiol, or ethylene-glycol groups as well as amide I and amide II bands of immobilized proteins (data not shown).

We applied two highly sensitive proteins to test the functionality of our new particles: the FRET-based glucose-biosensor FLIP, which requires distinctive conformational changes to be active, and the two-domain cytochrome P450 enzyme BM3, which requires multiple substrate binding and interdomain electron transport to be active. By taking into account controls with the aforementioned rock-stable model proteins and by applying two different coupling chemistries (statistical glutaraldehyde crosslinking and directional Halo-tag-based coupling), we could establish that our new particles provide a suitable microenvironment for covalent immobilization of

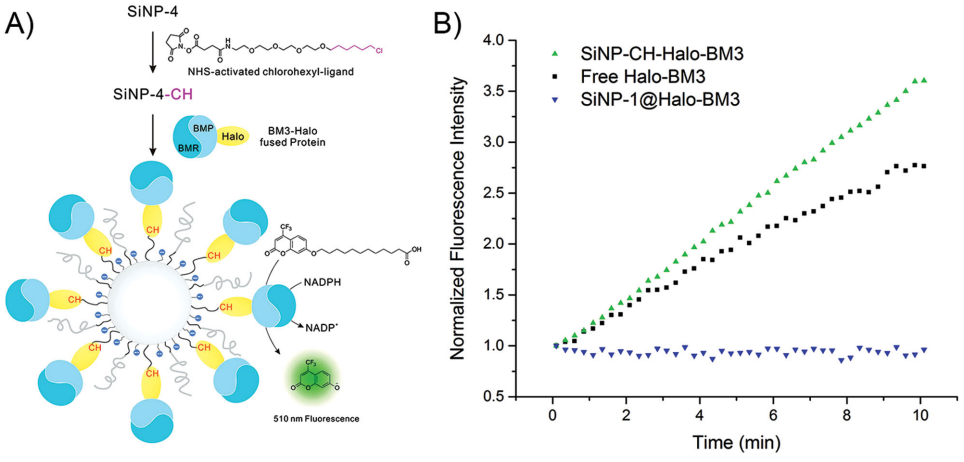

Figure 3. Directional immobilization of Halo-tagged BM3 to SiNP-4. A) Schematic illustration of the immobilization chemistry. NHS-activated chlorohexyl (CH)-ligands were initially coupled with the amino groups on SiNP-4 to yield SiNP-4-CH, which was then allowed to bind the Halo-tagged BM3. The BM3-catalyzed conversion of 2-(4-trifluoromethylcoumarin-7-yloxy)dodecanoic acid shown on the left leads to formation of the fluorescent product (excitation 410 nm, emission 510 nm). B) Enzyme activities of free, unbound, or SiNP-4-bound BM3-Halo (black and green line, respectively). Note that BM3 adsorbed on SiNP-1 (blue) shows no enzymatic activity.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

very delicate functional proteins. We therefore believe that these particles will be a useful platform for applications in biocatalysis or even biomedical diagnostics and therapy.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

Acknowledgements

This work was financially supported in part through GRK 2039 founded by German Research Foundation (DFG) and the Helmholtz Association programme "BioInterfaces in Technology and Medicine". The authors thank Anke Dech and Cornelia Ziegler for their help with the protein preparation, and Jens Bauer for help with TEM and DLS measurements.

Received: August 12, 2015
Revised: September 30, 2015
Published online: November 17, 2015

[1] C. S. S. R. Kumar, *Nanotechnologies for the Life Sciences*, Wiley-VCH, New York **2011**.
[2] A. Verma, F. Stellacci, *Small* **2010**, *6*, 12.
[3] S. T. Yang, Y. Liu, Y. W. Wang, A. Cao, *Small* **2013**, *9*, 1635.
[4] J. L. Vivero-Escoto, I. I. Slowing, B. G. Trewyn, V. S. Lin, *Small* **2010**, *6*, 1952.
[5] M. Lundqvist, I. Sethson, B. H. Jonsson, *Langmuir* **2004**, *20*, 10639.
[6] J. C. Cruz, P. H. Pfromm, J. M. Tomich, M. E. Rezac, *Colloids Surf., B* **2010**, *79*, 97.
[7] J. C. Cruz, P. H. Pfromm, J. M. Tomich, M. E. Rezac, *Colloids Surf., B* **2010**, *81*, 1.
[8] X. Cao, J. Yu, Z. Zhang, S. Liu, *Biosens. Bioelectron.* **2012**, *35*, 101.
[9] A. Cao, Z. Ye, Z. Cai, E. Dong, X. Yang, G. Liu, X. Deng, Y. Wang, S. T. Yang, H. Wang, M. Wu, Y. Liu, *Angew. Chem. Int. Ed. Engl.* **2010**, *49*, 3022.
[10] Z. Cai, Z. Ye, X. Yang, Y. Chang, H. Wang, Y. Liu, A. Cao, *Nanoscale* **2011**, *3*, 1974.
[11] D. Ma, M. Li, A. J. Patil, S. Mann, *Adv. Mater.* **2004**, *16*, 1838.
[12] M. T. Reetz, A. Zonta, J. Simpelkamp, *Biotechnol. Bioeng.* **1996**, *49*, 527.
[13] P. Tielmann, H. Kierkels, A. Zonta, A. Ilie, M. T. Reetz, *Nanoscale* **2014**, *6*, 6220.
[14] Y. Wang, F. Caruso, *Chem. Mater.* **2005**, *16*, 1838.
[15] P. Aggarwal, J. B. Hall, C. B. McLeland, M. A. Dobrovolskaia, S. E. McNeil, *Adv. Drug Delivery Rev.* **2009**, *61*, 428.
[16] R. Gref, M. Luck, P. Quellec, M. Marchand, E. Dellacherie, S. Harnisch, T. Blunk, R. H. Muller, *Colloids Surf., B* **2000**, *18*, 301.
[17] H. R. Kim, K. Andrieux, C. Delomenie, H. Chacun, M. Appel, D. Desmaele, F. Taran, D. Georgin, P. Couvreur, M. Taverna, *Electrophoresis* **2007**, *28*, 2252.
[18] Y. Y. Luk, M. Kato, M. Mrksich, *Langmuir* **2000**, *16*, 9604.
[19] R. P. Bagwe, L. R. Hilliard, W. Tan, *Langmuir* **2006**, *22*, 4357.
[20] Z. G. Estephan, J. A. Jaber, J. B. Schlenoff, *Langmuir* **2010**, *26*, 16884.
[21] Z. G. Estephan, P. S. Schlenoff, J. B. Schlenoff, *Langmuir* **2011**, *27*, 6794.
[22] J. W. Park, Y. J. Park, C. H. Jun, *Chem. Commun.* **2011**, *47*, 4860.
[23] L. Wang, K. Wang, S. Santra, X. Zhao, L. R. Hillard, J. E. Smith, Y. Wu, W. Tan, *Anal. Chem.* **2006**, *78*, 646.
[24] F. Alexis, E. Pridgen, L. K. Molnar, O. C. Farokhzad, *Mol. Pharm.* **2008**, *5*, 505.
[25] B. Haley, E. Frenkel, *Urol. Oncol.* **2008**, *26*, 57.
[26] G. T. Hermanson, *Bioconjugate Techniques*, Academic Press, San Diego, CA, USA **1998**.
[27] C. Graf, Q. Gao, I. Schutz, C. N. Noufele, W. Ruan, U. Posselt, E. Korotianskiy, D. Nordmeyer, F. Rancan, S. Hadam, A. Vogt, J. Lademann, V. Haucke, E. Ruhl, *Langmuir* **2012**, *28*, 7598.
[28] M.-C. Brochier Salon, P.-A. Bayle, M. Abdelmouleh, S. Boufi, M. N. Belgacem, *Colloids Surf., A* **2008**, *312*, 83.
[29] G. Irmukhametova, B. Fraser, J. Keddie, G. Mun, V. V. Khutoryanskiy, *Langmuir* **2012**, *28*, 299.
[30] K. Deuschle, S. Okumoto, M. Fehr, L. L. Looger, L. Kozhukh, W. B. Frommer, *Protein Sci.* **2005**, *14*, 2304.
[31] P. Jonkheijm, D. Weinrich, H. Schroeder, C. M. Niemeyer, H. Waldmann, *Angew. Chem. Int. Ed.* **2008**, *47*, 9618.
[32] A. Keppler, S. Gendreizig, T. Gronemeyer, H. Pick, H. Vogel, K. Johnsson, *Nat. Biotechnol.* **2003**, *21*, 86.
[33] G. V. Los, K. Wood, *Methods Mol. Biol.* **2007**, *356*, 195.
[34] S. T. Jung, R. Lauchli, F. H. Arnold, *Curr. Opin. Biotechnol.* **2011**, *22*, 809.
[35] K. Neufeld, S. M. Zu Berstenhorst, J. Pietruszka, *Anal. Biochem.* **2014**, *456*, 70.

© 2015 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim