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Fluorescent Nanodiamonds with Bioorthogonally Reactive Protein-Resistant Polymeric Coatings

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The novel synthesis of a polymeric interface grown from the surface of bright fluorescent nanodiamonds is reported. The polymer enables bioorthogonal attachment of various molecules by click chemistry; the particles are resistant to nonspecific protein adsorption and show outstanding colloidal stability in buffers and biological media. The coating fully preserves the unique optical properties of the nitrogen-vacancy centers that are crucial for bioimaging and sensoric applications.

Fluorescent nanodiamonds (FNDs) are a recently introduced class of luminescent probes.^[1–5] FNDs are biocompatible^[5–7] nanoparticles with sizes ranging from about five to several hundred nanometers. FNDs emit bright fluorescence in the red part of the visible spectrum (maximum at ≈ 700 nm), which is well separated from the cell/tissue autofluorescence. Nanodiamond fluorescence originates from so-called nitrogen-vacancy (N-V) centers. Emission from N-V centers is completely resistant to photobleaching and photoblinking.^[8] The absolute stability of N-V emission is unique amongst all fluorescent dyes and is especially crucial in applications that require large laser powers, such as super-resolution microscopy.^[9,10] Furthermore, the electron structure of the N-V center^[11,12] allows for construction of fluorescence-read sensors^[13,14] or for background-free observation of FND probes in vivo.^[15,16]

The use of FNDs without any surface modification as fluorescent bioprobes or biosensors is limited for three major reasons: 1) nanodiamonds (NDs) precipitate in biological solutions,^[17,18] 2) proteins nonspecifically adhere to the ND surface,^[19] and 3) direct surface modifications of the ND surface are limited. The urgent need to overcome these limitations is evidenced by the large number of publications devoted to improving ND particle properties through various surface modifications (for a review, see Ref. [20]).

Coating of NDs with polymeric shells appears to be a promising approach. After coating, ND particles exhibit an improved colloidal stability^[17,19,21–23] and reduced adsorption of blood proteins.^[19] Further functionalization of the polymeric shell has also been demonstrated.^[17] Introduction of azide and alkyne groups onto the ND surface and their subsequent reaction using azide–alkyne cycloaddition (click reaction) was recently reported.^[19,24–27]

Herein, we show simultaneous removal of the three above-described drawbacks of “naked” bright^[28] FNDs by means of a novel surface architecture constructed on a FND surface. This architecture comprises an ultrathin, < 1 nm silica layer (unlike recently published thick silica shells on FNDs^[27,29–31]) and a polymethacrylate copolymer coating that allows further functionalization of the FNDs by click chemistry^[32] (Scheme 1).

The silica shell was grown from a mixture of tetraethoxysilane and 3-(trimethoxysilyl)propyl methacrylate using a modified Stöber procedure. The presence of this coating was confirmed by infrared spectroscopy (Figure S1 in the Supporting Information). Terminal methacrylate groups of the silica coating were used to grow a dense layer of copolymers, which consisted mainly of poly[N-(2-hydroxypropyl)methacrylamide] (poly(HPMA)). Poly(HPMA) is a widely used hydrophilic biocompatible polymer^[33] and has been used to coat various fluorescent probes.^[34] We used a “grafting from” coating method, in which the polymer coating is polymerized from the surface of the FND. This approach usually offers denser and better protecting coatings than “grafting to” methods, in which the polymer is first synthesized in solution and then attached to the surface.^[35,36] Polymer chains were grown using radical polymerization with azobis(isobutyronitrile) (AIBN) as initiator. A small fraction (5%) of HPMA monomer was replaced in the reaction mixture by propargylacrylamide (AlkMA) or 3-(azidopropyl)methacrylamide (AzMA) to introduce azide or alkyne moieties to the polymer, respectively. These groups are suitable for copper-catalyzed click reaction,^[32] enabling attachment of vari-

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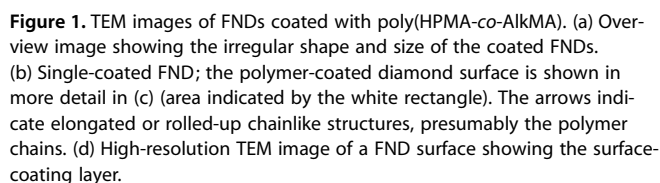
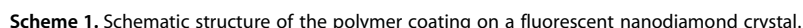


Figure 1 shows bright-field TEM images of FNDs modified by poly(HPMA-co-AlkMA). The FNDs are coated with a thin layer of polymer that evenly covers the nanodiamond surface (Figure 1a,b). The thickness of the layer varies between approximately 2 and 5 nm. The high-resolution images (Figure 1c,d) show that the surface layer is formed by chainlike structures, which are presumably the polymer strands. Thermogravimetric analysis provides additional evidence for the presence of polymers on the FND surfaces (Figure S2). Based on the difference between the silica-coated and silica-poly(HPMA-co-AlkMA)-

The adsorption of blood proteins, which may lead to scavenging of particles into the reticuloendothelial system, was tested with radiolabeled bovine serum albumin (BSA). Serum albumin is the most abundant serum protein (ca. 4–5 % weight concentration in plasma), which makes it the best blood protein representative.^[39] To evaluate the protein resistance of the particles, we mixed noncoated and poly(HPMA-co-AlkMA)-coated FND solutions with ¹²⁵I radiolabeled bovine serum albumin (¹²⁵I-BSA).^[39] After incubation, FNDs were removed by centrifugation, and the remaining radioactivity in the supernatant (corresponding to the BSA concentration) was measured (for details, see Supporting Information). We observed that the introduction of the polymeric film on FNDs caused a nearly four-fold reduction in the total adsorbed amount of BSA. This reduction slightly exceeds the 3.2-fold reduction described for amphiphilic polymer zonyl-coated NDs.^[19]

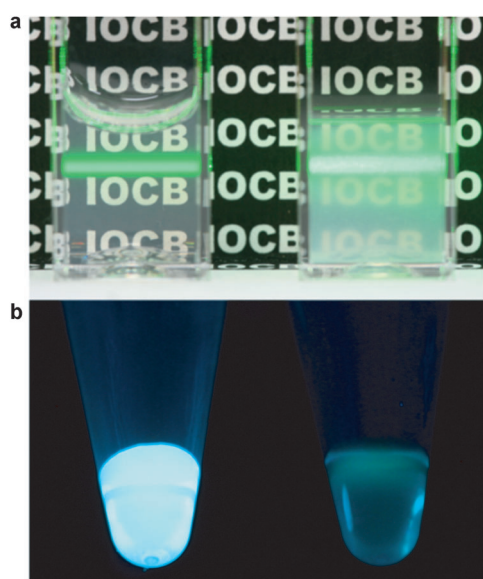


Figure 2. Properties of polymer-coated FNDs. (a) Poly(HPMA-co-AlkMA)-coated FNDs (left) and naked nanodiamonds (right) dispersed in PBS buffer (30 min after mixing; concentration 0.5 mg mL^{-1}). The laser beam is strongly scattered by the precipitating naked FND particles, and the polymer-coated particles form a stable colloidal solution. (b) The reaction of poly(HPMA-co-AlkMA)-coated FNDs (left) and control poly(HPMA)-coated FNDs (right), with the fluorogenic probe coumarin-azide (lighted by UV lamp). The bright fluorescence results from the click reaction.

A copper-catalyzed alkyne-azide cycloaddition reaction (click reaction)^[32] was used to modify the poly(HPMA-co-AlkMA)-coated FNDs with various model molecules. We chose this reaction owing to its excellent bioorthogonality and mild reaction conditions (see below). First, to directly confirm the covalent grafting mechanism, the fluorogenic probe coumarin-azide^[40] was attached to the poly(HPMA-co-AlkMA)-coated particles. The dye itself is nonfluorescent, but a highly fluorescent structure is formed upon cycloaddition to an alkyne. The reaction of coumarin-azide with the particles in the presence of a Cu^I catalyst produced a characteristic bright fluorescent product, whereas the control reaction performed in the absence of Cu^I , remained dark (Figure 2b).

Several molecules were attached to the surface of the HPMA copolymer-coated FNDs to demonstrate the versatility of the conjugation procedure and to quantify the yield of the click reaction (see the Supporting Information). As a representative fluorescent dye we used Alexa488-azide, because its high extinction coefficient allows quantification using absorption spectroscopy. To test the performance of the reaction in the inverse azide-alkyne arrangement, we coupled Alexa488-alkyne with poly(HPMA-co-AzMA)-coated FNDs under the same conditions. As an example of a peptide we used ^{125}I -labeled GGGRGDSGGGY-azide (RGDS-azide),^[41,42] a vectoring molecule used in nanoparticle targeting of tumor cells.^[43,44] The presence of the ^{125}I label enabled the highly sensitive monitoring of the conjugation yield by radioactivity measurements. All of these reactions were performed in aqueous solutions with approximately $100 \text{ }\mu\text{M}$ concentrations of modifying molecules. The reaction time was 2 h. The loads of modifying molecules were

found to be in the range of tens of micromoles per gram of FNDs in all cases. The results show that our surface architecture allows a stable covalent connection of various molecules to the nanoparticles.

In summary, this study describes the preparation and properties of a versatile platform for FND-based bioprobe construction. A new modification of the FND surface using an ultrathin silica shell bearing methacrylate groups as a platform for polymer growth is presented. The methacrylamide copolymeric layer grafted from the silica surface simultaneously overcomes the three current main limits to the bioapplicability of naked FNDs: buffer solubility, nonspecific protein binding, and the possibility of a convenient chemical modification. Moreover, it does not alter the fluorescent properties of the FNDs. The attachment of molecules to FNDs can be performed selectively using click chemistry in aqueous buffers or biological media, while maintaining the excellent colloidal stability of the particles. Our design, based upon the unique photophysical properties of N-V centers in FNDs, provides a versatile biocompatible platform for the construction of bioimaging probes or targeted systems. Experiments on cancer cell targeting and fluorescence imaging are currently in progress.

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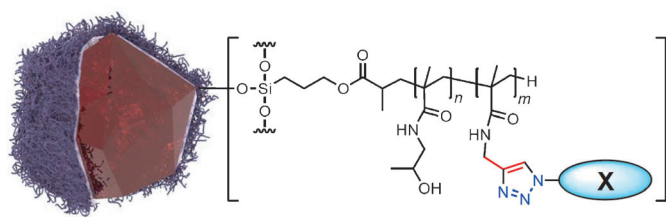
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A shining example: Fluorescent nanodiamonds were coated by a thin layer of methacrylate copolymer enabling high-yield attachment of molecules by click chemistry (see scheme). The particles are protein-resistant and extremely

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Fluorescent Nanodiamonds with Bioorthogonally Reactive Protein-Resistant Polymeric Coatings

