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Site-specific immobilization of proteins at zeolite L crystals by nitroxide exchange reactions[†]

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Site-selective immobilization of dyes and different protein recognizing entities at the surface of zeolite L crystals using mild radical nitroxide exchange reactions is reported. Exposure of these crystals to aqueous protein solutions leads to site-selective immobilization of proteins onto the crystals.

Nano- or microscopic inorganic particles linked to functional (bio)molecules have attracted increased attention during the past years. These biohybrids can be used as components in optoelectronic devices or drug-delivery systems in the fields of engineering and biosciences.¹ Therefore, improvement of existing methodologies and development of new concepts for site-selective chemical surface modification are important. As known procedures often show incomplete surface modification or require comparatively harsh reaction conditions, there is still a demand to develop generally applicable, robust and experimentally easy to conduct surface chemistry.² Little is known about reversible covalent bond formation at inorganic materials which should be useful for the generation of dynamic adaptable organic/inorganic hybrid materials.³ Self-assembly and aggregation behaviour of synthetic hybrid materials, for example surface modified gold nanoparticles,^{4a} dendrimers^{4b} and more generally inorganic/polymer hybrid nanostructures,4c have been investigated. Physical properties like fluorescence⁵ or magnetic behaviour⁶ can be tuned as a function of the hybrid constitution. In the present communication we report mild reversible nitroxide exchange reactions in alkoxyamines which are site-selective covalently bound to zeolite L crystals."

Recently, we successfully achieved dynamic assembly of zeolite L microcrystals by nitroxide exchange reactions.⁸ In these processes, transient C-radicals and persistent nitroxide radicals are generated by reversible thermal C–O bond homolysis of the alkoxyamines (Scheme 1).⁹ Alkoxyamine formation by cross coupling of the nitroxide with the C-radical leads to cross over of the nitroxide moieties.



Scheme 1 Thermal reversible nitroxide exchange.

Zeolite L,¹⁰ a crystalline aluminosilicate, has a defined morphology, is optically transparent, and can be prepared at a defined size ranging from 30 nm to 10 μ m.¹¹ Zeolite L is functionalized readily at the external and internal surfaces¹² and the crystals are biocompatible. Furthermore, it is possible to conjugate either the channel entrances or the whole crystal surface selectively with amine functionalities.¹³

In the present study, zeolite L crystals with a length of 3 µm were site-selectively functionalized with alkoxyamines bearing a carboxylic acid functionality by amide bond formation with amine groups selectively attached at the channel entrances or all over the crystal (for preparation of these zeolites, see ESI[†]).⁸ Scheme 2 shows the general concept of site-selective nitroxide exchange for the immobilization of various interesting molecules at the zeolite. These reactions can be performed either at the pore entrances (hereafter designated as crystals 1a) or at the whole surface (1b) modified zeolite L crystals. Initial studies were conducted at 125 °C for 2-4 h in 1,2-dichloroethane (DCE) with crystals of type 1a and 1b using nitroxides 2 and 3 (large excess) bearing a fluorescent dye (conditions A, see ESI[†]). The sample was then centrifuged and the supernatant solvent was removed. A sample was suspended in toluene, ultrasonicated, and a drop of the suspension was placed on a microscope slide. The reaction success was verified using fluorescence and confocal laser microscopy. The images shown in Fig. 1 clearly prove the success of the site-selective chemical functionalization of the zeolites by thermal nitroxide exchange of crystals 1a and 1b with nitroxides 2 and 3.

We then analyzed the influence of the structure of the alkoxyamine moiety on the exchange reaction. To this end, we prepared two additional types of alkoxyamine zeolite conjugates **7a**, **8a** (Scheme 2) and compared their reactivities with the reactivity of the parent **1a** bearing the 2,2,6,6-tetra-methylpiperidin-*N*-oxyl radical (TEMPO) as the nitroxide moiety. **7a** bears a sterically more hindered 1-*tert*-butyl-3,3,5,5-tetraethyl-2-piperazinone group¹⁴ and in **8a** the TEMPO

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Scheme 2 (i) Nitroxide exchange reaction on entrance functionalized zeolite L crystals 1a for immobilization of various biomolecules at the crystal pore entrances under mild conditions. (ii) Schematic representation of an alkoxyamine modified crystal of type 1b for all over chemical surface modification. (iii) Zeolite conjugates 7a and 8a.



Fig. 1 Red (2, rhodamine) and yellow (3, nitrobenzooxadiazole (NBD)) fluorescent nitroxides. Fluorescence images 4a, 4b (with 3 and 1a, 1b), 6a, 6b (with 2 and 1a, 1b) and confocal laser microscopy images 5a, 5b (with 2 and 1a, 1b) recorded after nitroxide exchange reactions.

alkoxyamine is connected to the surface *via* a tetraethylene glycol linker (see ESI[†]).

We found that in comparison to zeolite 1a, neither immobilization of the sterically hindered alkoxyamine nor the tetraethylene glycol linker resulted in a measurable difference in the nitroxide exchange with 2 at 125 °C.

We also studied the effect of the reaction temperature on the surface exchange reactions on crystals **1a** and **7a**, **7b** at 125 °C, 90 °C, 70 °C and at room temperature (rt) with nitroxide **2**. Pleasingly, exchange at the zeolite L surface was also possible at rt for both systems **1** and **7**. This is in agreement with our previously reported results.^{8,15} As expected, reactions occurred faster at higher temperature and we noted only a small difference for the rt experiments in going from system **1a** to **7a** (see ESI[†]).

We could also show that the nitroxide exchange is a reversible process. To this end, we treated rhodamine conjugated zeolite L crystals (images 5a, 6a in Fig. 1) with an excess of TEMPO for 4 h in DCE at 125 °C and found that the red fluorescence of the dye could no longer be observed at the pore entrances (see ESI†). Exchange reactions worked equally well in H₂O.

As radical chemistry tolerates various functional groups, our approach should allow for site-selective immobilization of different biomolecules and proteins at the crystal. We synthesized nitroxides **9**, **11** and **13** bearing mannose, biotin and the N_{α} , N_{α} -bis(carboxymethyl)-L-lysine hydrate (NTA) linker (Fig. 2). Nitroxide exchange was performed for 2–4 h at 125 °C or rt in DCE or water. Unprotected mannose **9** was immobilized site-specifically at the zeolite L surface. The success was proven by subsequent complexation of the crystals with rhodamine labelled concanavalin A (ConA)^{16,17a} (Fig. 2: 10a, 10b). For complexation, the crystals were treated with an excess of protein in diluted buffer at rt for 1–4 h (conditions B, ESI†). Fluorescence images show a specific red fluorescence either at the channel entrances or at the whole surface depending on the crystal type **1a** or **1b** used for the initial exchange.^{17b}

To exclude non-specific binding of ConA onto the surface, we performed control experiments of non-functionalized as well as alkoxyamine modified zeolites. After extensive washing only little fluorescence was observed (ESI†).¹⁸ We then decided to apply the extraordinarily high and robust biotin–streptavidin binding for selective protein immobilization.¹⁹ Nitroxide exchange with biotin derivative **11**, followed by



Fig. 2 Nitroxides 9, 11, 13 and 15 (left). Fluorescence microscopy images of site-selective protein immobilization (a) with crystals 1a and (b) with crystals 1b: ConA (10), streptavidin (12), eGFP (14) and BSA (16).

interaction with green fluorescence labelled streptavidin (Oyster[®]-488) allowed for site-specific protein immobilization (Fig. 2; 12a and 12b). We further extended this concept using the well established complexation of his-tagged eGFP to nickel bound NTA. After rt nitroxide exchange on **1a** and **1b** with NTA nitroxide **13**, subsequent complexation of Ni²⁺ and addition of his-tagged eGFP, site-selective protein immobilization was achieved (Fig. 2, 14a and 14b).

We also focused on the direct covalent attachment of a protein to a zeolite crystal and chose the reliable reaction of succinimido maleimides with thiols for covalent attachment of a nitroxide to a protein.²⁰ The TEMPO-maleimide derivative 15^{21} was reacted with free thiols of unfunctionalized β-lactoglobulin A (LGA) or of rhodamine tagged bovine serum albumin (BSA). Mass spectrometry analysis revealed successful conjugation of the proteins with 15 (see ESI[†]). For LGA a single product with the correct mass (LGA + 15) was identified. However, for the heterogeneous BSA, as expected, several product peaks were observed. Both nitroxide modified proteins were then reacted with crystals 1a and 1b at rt. Site-specific red fluorescence was detected in the reaction with the BSA-nitroxide (Fig. 2, 16a and 16b). For the non-fluorescent LGA nitroxide, we conducted ζ potential measurements and compared the results with the ζ potentials of the fluorescent BSA-zeolite conjugates. ζ potential measurements were performed at rt at pH 7.4 in NH₄HCO₂-buffer. ζ (mV) were similar (pore entrance modified BSA-zeolite: -31.32; LGA-zeolite: -23.7: all over modified BSA: -2.89: LGA: -5.42). Based on these results and the fluorescence images shown in Fig. 2, we believe that both BSA and LGA were immobilized successfully by nitroxide exchange reactions.

We reported that surface nitroxide exchange reactions are well suited for a site-selective modification of zeolite L microcrystals. We have shown that the nitroxide exchange is a reversible process that can be conducted under mild, physiological conditions at the zeolite surface. Proteins can be immobilized site-specifically to zeolite L crystals by covalent bond formation or by using his-tag, streptavidin tag or concanavalin A-mannose interactions.

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