BIOFUNCTIONALIZATION OF PATTERED POLY(2-OXAZOLINE) BOTTLE-BRUSH BRUSHES ON DIAMOND

Naima A. Hutter1, Andreas Reitinger2, Jose A. Garrido2 and Rainer Jordan1,3

1 Wacker-Lehrstuhl für Makromolekulare Chemie, TU München, Lichtenbergstraße 4, 85747 Garching, Germany
2 Walter Schottky Institut, TU München, Am Coulombwall 3, 85748 Garching, Germany
3 Professur für Makromolekulare Chemie, TU Dresden, Zellescher Weg 19, 01069 Dresden, Germany

Introduction
The modification of semiconductor surfaces with stable functional organic or polymeric layers has gained an increasing research interest in the past decades. Especially for applications in the field of biomedical microdevices, it is important to create soft biocompatible interlayers between the electrode material and the biological surrounding. Due to a large electrochemical potential window, chemical inertness, mechanical stability, as well as biointertness, boron-doped nanocrystalline diamond (NCD) electrodes are suitable for amperometric biosensing applications.1-2

Poly(2-oxazoline)s (POx) have recently come into focus as a potential alternative to the well established PEG systems for the preparation of biocompatible surface coatings and materials.3-5 It has been shown that hydrophilic POx is non-toxic and that proteins as well as drugs can be coupled to the polymer without losing their activity.6,7 POx have predominately covered the fields of biomembrane design, antimicrobial surfaces, and drug-delivery,8-10 but so far, there are very few reports on the use of this polymer class for biosensing in particular.

The combination of the unique properties of NCD as electrode material and the biocompatibility, as well as the variability of POx may open the way for new and advanced biosensing systems. In contrast to other studies on biofunctionalized self-assembled monolayers (SAMs) on diamond, we were interested in the surface coupling of biomolecules or recognition sites into polymer brushes bearing multiple functions and providing a soft three-dimensional scaffold.

Experimental
Materials. Chemicals were purchased from Aldrich (Steinheim, Germany) or Acros (Geel, Belgium). Chemicals for the LCROP were dried by refluxing over CaH2 under a dry argon atmosphere and were freshly distilled prior to use. The 3,3',5,5'-tetramethylbenzidine (TMB) was purchased as anhydrous sodium carbonate (1.3 eq.) was added to methyl-7-chloro-4-oxo-5-azaheptanoat (1 eq.). At 40 °C the mixture was evacuated until formation of gas was completed. Afterwards, the mixture was heated up to 140 °C and the product was obtained by distillation.

Figure 1. Preparation of a structured P(IPOx) brush gradient on NCD by the combination of carbon templating and SIPGP.

In the perspective of using NCD for biomedical applications, we have investigated the immobilization of GFP in (IPOx) brushes. POx based bottle-brush brushes (BBBs) were prepared analogue to a published protocol11 and 2-ethyl-2-oxazoline (EtOx) was used as monomer for the side chain polymerization (Figure 2). The SI-LCROP grafting was terminated by bifunctional terminating agents glycine-tert-butylester or L-prolin-tert-butyl ester to introduce carboxylic end groups for the ligation reaction with GFP (Figure 3) or HRP. The successful SI-LCROP and the selective termination reaction were characterized by AFM, FT-IR and XPS.11,12

Colorimetric assay. Colorimetric assays were carried out in a Cary 500 Scan UV-Vis spectrophotometer from Varian equipped with a single cell Peltier accessory. To the photocuverte containing buffer and the entirely biofunctionalized surface, different amounts of the TMB kit were added. In order to determine the enzyme activity, light absorption curves were measured over 10 minutes time at different substrate concentrations and constant stirring at 22°C.

Results and Discussion
The preparation of patterned poly(2-isopropenyl-2-oxazoline) (P(IPOx)) brushes on conductive boron doped NCD is outlined in Figure 1. The carbon template gradient was created analogue to previous reports11 by linearly increase of the electron dose from 0 to 100 mC/cm2. After self-initiated photografting and photopolymerization (SIPGP) of 2-isopropenyl-2-oxazoline (IPOx), AFM measurements revealed a selective formation of polymer brushes on the carbon templates. The correlation between polymer thickness and electron dose used for the templating step can be explained by an increase of the polymer grafting density at higher e-beam doses forcing the chains to stretch away from the surface.

Polymer analogue hydrolysis. Glycine-tert-butylester or L-prolin-tert-butyl ester terminated P(IPOx-g-ROx) BBBs were prepared as previously reported.11 The side chain polymerization was selectively terminated with a bifunctional terminating agent (glycine-tert-butylester or L-prolin-tert-butyl ester). The LCROP of P(IPOx-g-MOPOx) was carried out accordingly. Piperidine was used as terminating agent.

Figure 2. Above: schematic formation of bottle-brush brushes on NCD; below: synthesis of P(IPOx-g-ROx) BBBs.

Besides the incorporation of proteins via the BBB side chains, a second strategy for protein loading was realized by replacing the non-reactive P(EtOx) side chains with functional poly(2-carboxyethyl-2-oxazoline). The...
pends Oxazoline side chains of P(IPox) were therefore functionalized in a subsequent LCROP with methyl-3-(oxazol-2-yl)propionat (MPO). After saponification, P(IPox-g-CarboxyOx) BBBs were obtained which were further functionalized with GFP (Figure 3).

Figure 3. Generation of pendant carboxylic acid moieties in P(IPox-g-ROx) BBBs and subsequent coupling of GFP.

The resulting GFP-modified BBBs were analyzed by fluorescence microscopy in order to determine the presence and activity of GFP. In this respect, a polymer brush gradient is a convenient approach in order to analyze microscopy in order to determine the presence and activity of GFP. In this unambiguously identified by a strong fluorescence response. Native GFP could be in a single experiment the conjugation of bulky biomolecules within polymer brushes of different thicknesses and grafting densities. Native GFP could be unambiguously identified by a strong fluorescence response. Figure 4 shows the similarity of the polymer brush height profile and the fluorescence intensity which indicates that the proteins were immobilized throughout the entire polymer brush layer, even in the case of the BBBs with high chain crowding. If GFP were only coupled within the upmost brush regions, the resulting fluorescence intensity would be independent of the polymer layer thickness.11

Figure 4. Analysis of 10×50 μm² gradients of P(IPox-g-EtOx)-GFP and P(IPox-g-CarboxyOx) on NCD. Normalized profile plot of the fluorescence intensity and the polymer brush height obtained from AFM and FM section analysis (averaged at the indicated area), AFM image and fluorescence image.

Some of the established colorimetric assays for proteins in solution can be transferred to surface-attached proteins to determine their activity. Here, colorimetry was used as a fast method for determining the presence of native horseradish peroxidase (HRP) in P(IPox-g-EtOx)-HRP and its recognition reaction with hydrogen peroxide. A characteristic color is obtained by the consecutive oxidation of 2,2’,5,5’-tetramethylbenzidine (TMB) and its intensity can be read out as absorbance A. Plotting of the reaction rate, i.e. the observed linear formation of the dye dA/dt, versus the respective substrate concentration provides information about the enzyme kinetics. A typical Michaelis Menten behavior, i.e. diffusion-limited dependence for low substrate concentrations followed by a regime of saturation, was observed in case of P(IPox-g-EtOx)-HRP (Figure 6). However, since the polymer brush-bound biocatalysts are not in solution with a defined concentration, the enzyme surface coverage is unknown and an exact determination of the kinetic constants is not possible.

Figure 6. Colorimetric assay for P(IPox-g-EtOx)-HRP on NCD; plot of the reaction rate versus the relative substrate concentration.

**Conclusions.** The SIPGP-LCROP approach gives access to the design of complex polymer brush architectures of POx on diamond electrodes that allows the incorporation of a broad variety of chemical functions. It was demonstrated by fluorescence microscopy (GFP) and colorimetric essays (HRP) that coupled proteins maintain their native activity after immobilization. The (bio)functionalization of diamond via patterned polymer brushes based on POx enable the study of surface interactions in biological media with the precise variation of the surface topography, local biomolecule concentration and its accessibility.

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**References**


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