DEVELOPMENT OF DEXTRAN-BASED HYDROGEL LAYERS FOR BIOSENSOR APPLICATIONS

Theses

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1 Introduction

Biofunctional coatings are of high importance in a number of industrial and research fields, including biomedical applications (implants and drug delivery systems), analytical chemistry, biosensors as well as maritime industries. These coatings are intended to endow solid support (substrate) surfaces with special abilities in order to interact with biological systems in a controlled and desired manner. The coatings are usually thin films (thickness is below 1 μm) made of synthetic or naturally derived constituents using various types of surface chemistries. The objective of surface modifications can vary in a wide range depending on the applications. For instance, biomedical implants should be coated to achieve biocompatibility, drug carrier microparticles should be functionalized to reach controlled and targeted drug release as well as submerged surfaces of maritime architectures should be coated to prevent from fouling and subsequent damage by marine organisms. In the field of biosensors, especially in case of label-free measurements (see Figure 1), biofunctional coatings on sensing surfaces play a key role in improving sensing capabilities (e.g. sensitivity) primarily by amplifying the signal of components of interest and minimizing the interfering signal originated from the fouling of surfaces, mostly from the adsorption of protein molecules. Biofouling of surfaces is a very challenging issue that always occurs when biological samples interact with sensing surfaces.

Figure 1 | Labelled and non-labeled (label-free) detection principles in case of optical biosensors
Labelled techniques are primarily based on the measurement of fluorescence intensity generated by associated fluorescent label entities. In contrast, label-free techniques measure signals (e.g. refractive index shift) generated only by the physical presence of analytes on or over the transducer surface.

With respect to all the specified fields, polymers have become one of the most common coating materials owing to their potential for biocompatibility, cost-effective synthesis and the actually immense diversity regarding their structure, chemical modifications and properties. In the field of biosensors, there is a huge need for stable, reproducible and efficiently conjugable biofunctional layers with great protein- and cell-resistant ability. Among the available materials, the surface-related biosensor improvements can be best achieved by using hydrogel

interface layers. Hydrogels are hydrophilic polymers capable of accommodating extreme amount of water. The carbohydrate biopolymer dextran (see chemical structure in Figure 2) can be efficiently used to form hydrogel layers with extended three-dimensional structure on biosensor surfaces, and owing to the high water content of these coatings, they display advanced protein- and cell-repellent abilities. The application of dextran-coated biosensor chips started in the 1990’s, and later on dextran-based layers have become wide-spread in the field of label-free biosensors. Label-free biosensors with dextran-based hydrogel coatings are attractive bioanalytical tools for the exploration of surface-related cellular functions and events such as adhesion, spreading, proliferation, migration as well as signaling processes. For these applications, it is important that dextran layers are able to provide specifically conjugable mechanical support for living cells. Besides supporting fundamental research in cell biology, the understanding of the behavior of cells e.g. on chemically or topographically micropatterned surfaces has of high importance in the development of cell-based biosensors (which apply cells as specific sensing elements, where the cells are usually arranged on the surface according to a specific pattern).

In contrast to the obvious advantages and wide-spread applications, dextran-coated biosensor chips are still not commercially available for basic optical biosensor techniques, such as for optical waveguide lightmode spectroscopy (OWLS). Moreover, dextran layers have remained poorly characterized and technical details about their fabrication processes are missing from the literature. Especially, the lack of available data is more pronounced regarding the layer’s behavior under aqueous conditions, actually it is still an unexplored field. This can be explained by the fact, that the number of analytical methods appropriate for the investigation of heavy hydrated hydrogel layers at the nano-scale level is strongly limited. The comprehensive characterization of such layers demands to deploy various measurement techniques and comb combined data evaluation methods.

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2 Aims and applied methods

In the present research, my aim was to develop covalently grafted, protein- and cell-repellent carboxymethyl dextran (CMD) layers on waveguide-type (SiO$_2$-TiO$_2$) surfaces for biosensor applications and cell adhesion experiments. An essential purpose of my work was the profound characterization of the developed layers in terms of composition, structure and structural alterations under different environmental conditions.

At the beginning of my work, the starting purpose was to adopt a CMD synthesis procedure$^8$ in order to produce CMD from native dextran with the required quantity and degree of carboxymethylation for subsequent layer fabrication experiments. Using the synthetized CMDs, two types of layer fabrication methods on silica-based inorganic substrates were applied to obtain CMD layers with varying thickness and mechanical properties. A major part of my work was devoted to the preparation of ultrathin CMD layers (hereafter termed as CMD-ut layers) with a few nanometers in thickness. For the fabrication of CMD-ut layers, two different surface chemistries were applied (Figure 3), including CMD grafting to aminosilane coating using EDC/NHS linking chemistry (see details in figure caption) with the formation of amide bonds (CMD-ut-Am layers) as well as direct grafting to epoxysilane coatings forming ester and ether bonds (CMD-ut-Ep layers). The layer fabrications were performed on SiO$_2$-TiO$_2$-type waveguide surface of OWLS biosensor chips and the SiO$_2$ surface of standard Si substrates.

![Figure 3](image)

**Figure 3** Covalent grafting of CMD to amino- and epoxysilylated surfaces (fabrication of CMD-ut layers)
The CMD was schematically interpreted by a molecule carrying both hydroxyl and carboxyl functional groups. The aminosilane- and EDC/NHS-based linking chemistry (1) resulted in amide bonds between the amino functions and CMD molecules (EDC and NHS refer to the reagents of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride and N-hydroxysuccinimide, respectively). The linking chemistry via epoxide groups (2) does not require additional activating reagents and it forms both ester and ether bonds (hydroxyl groups are also reactive in this pathway).

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In addition, my aim was also to develop CMD hydrogel layers presenting thickness over 10 nm in dry conditions. For this purpose, my plan was to develop a novel CMD layer fabrication method that relies upon the layer preparation processes of chemical crosslinking using sodium trimethaphosphate (STMP) as crosslinking agent and spin-coating as deposition technique (hereafter, the abbreviation of CMD-sc is used for these layers). While the classical (bioassay-type) biosensor measurements demand basically ultrathin layers for sensitive detections (limited penetration depth of the evanescent field), cell-based experiments usually need thicker layers (thickness is several 10 nm or thicker) with greater hydration abilities providing suitable mechanical support for retaining cellular activity. Accordingly, while the CMD-ut layers were intended to use later in classical biosensor measurements for biomolecule detection, the CMD-sc layers were developed for cell-based biosensor experiments.

Besides the preparative work and as a support of the fabrication methodologies, the profound characterization of the fabricated layers was also a major part of my research. For this purpose, a wide range of surface analytical techniques were applied, offering compositional (functional groups, elemental composition), optical (refractive index) and structural (thickness, dominant chain conformation) information about the fabricated layers. For the characterization under the layer’s dry condition, attenuated total reflectance Fourier-transform infrared spectroscopy (ATR-FTIR), x-ray photoelectron spectroscopy (XPS), atomic force microscopy (AFM) as well as spectroscopic ellipsometry (SE) techniques were applied. The wettability was characterized by water contact angle measurements. In order to characterize the structure and structural alterations of CMD-ut layers in aqueous environment and obtain kinetic data on the layer formation, the biosensor-type in situ OWLS and impedance measuring quartz crystal microbalance (QCM-I) techniques were employed.

Parallel measurements with these two label-free methods using chemically identical substrate surfaces offered to gather unique information about the deposited mass, thickness, hydration and viscoelastic properties as well as preferential chain conformation (see the applied OWLS and QCM-I measurement and data evaluation methodology in Figure 5). The analysis of chain conformation using OWLS is enabled by the sensitivity of the determined layer refractive index to optical anisotropy. Since anisotropy is induced by the oriented order of layer constituting chains, the under- or overestimated value of layer refractive index allows to deduce the layer’s nanostructure (Figure 4).

**Figure 4** | Effect of oriented structures on the adlayer refractive index \( (n_A) \) measured by OWLS

Illustration A shows the case of positive birefringence (polymer chains are perpendicular to the surface), while B represents a negatively birefringent adlayer (chains are parallel with the surface). The propagating light in the waveguide layer and the generated evanescent field over the waveguide surface are also depicted. \((n_A, o)\) refers to the ordinary, \(n_A, e\) to the extraordinary layer refractive index, respectively.)
Figure 5 | Schematic representation of the applied measurement and data evaluation methodology for the investigation of the nanostructure of ultrathin, heavily hydrated polymer layers

The parallel QCM-I and OWLS measurements were performed on chemically identical sensor surfaces and the formation of CMD-ut and PLL-g-PEG nanolayers was in situ monitored. Evaluating the raw measurement data, wet mass and viscoelastic parameters (QCM-I) as well as dry mass and anisotropy-related parameters (OWLS) could be obtained and finally combined (composite results) to investigate the conformational alterations in terms of hydration and viscosity.

While the optical biosensor OWLS measures the deposited mass of a polymer layer without bound or trapped solvent molecules („dry” mass), the mechanical detection principle of QCM-I enables to measure the solvated mass of polymer chains („wet” mass). In order to analyze the QCM-I data in terms of viscoelasticity, my aim was to develop an evaluation code that is based on the widely used Voinova’s Voigt-based equations\(^9\) as well as to develop a graphical interface for the evaluation code in MATLAB\(^8\) programming environment. My plan was to validate the code both by comparing its outputs with literature values and by performing reference QCM-I measurements on well-characterized poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) nanolayers. My plan was also to use these measurement data for validating the newly commercialized QCM-I device (manufactured by MicroVacuum Ltd.) that was employed in the experiments. In addition, I proposed to use the results on PLL-g-PEG layers as comparative

basis for the analysis of heavily hydrated CMD-ut layers. Since the stability of CMD layers was a significant requirement for the applications, my plan was to test and measure the effect of the applied washing processes.

My aim was also to demonstrate the protein- and cell-repellent ability of the developed CMD layers. For this purpose, in situ OWLS measurements were performed to determine the adsorbed amount of proteins and the adhesion of living cells was observed by phase contrast microscopy.

3 Results

The ATR-FTIR and XPS measurements proved the presence of CMD-ut layer on Si substrates by detecting the signal of carboxylic groups and obtaining the elemental composition of the specimen surfaces. According to the results of XPS depth analysis, the separate thickness of aminosilane and CMD layers was 2.30 nm and 0.73 nm, respectively, correlating well with the AFM result that provided 4 nm as a collective thickness of the aminosilane and CMD layers. AFM images about a sample coated with CMD-ut layer, as well as the details of AFM-based thickness determination can be seen in Figure 6. It was shown that the grafting of CMD-ut layer on aminosilylated surfaces accompanies with significant decrease in the water CA data (65 - 80° → 20 - 40°), proving the presence and hydrophilic characteristic of CMD-ut coatings.

![AFM images captured on CMD-ut surface with aminosilane undercoating](image)

**Figure 6** | AFM images captured on CMD-ut surface with aminosilane undercoating

A. AFM image taken on a 10 × 8 µm (in x and y lateral dimensions) area of a Si wafer partially covered with CMD. The gold lithography allowed to establish a sharp step between the substrate and CMD-covered area. The gold cover was deposited just on the one half of the sample, thus the substrate surface was not accessible for aminosilane and CMD molecules. Peeling off the gold, an aminosilane- and CMD-free SiO₂ surface could be acquired right next to the CMD-covered area. B. The 0.5 × 0.5 µm AFM image revealed the topography of the CMD surface. The z height values of graph C were measured along the profile line of image A, providing a height cross-section of the sample.
Supported by two main observations, it was shown that the CMD-ut layer was covalently grafted to the surface. These observations were the followings: (i) the XPS-measured thickness of CMD-ut layer on Si substrates without silane coating (control sample) was one order of magnitude lower (0.07 nm) than in case of using silylated substrate for grafting (0.73 nm), (ii) and based on in situ OWLS measurements, it was also proven that the CMD molecules formed stable layer which could not be washed off even by a concentrated solution of NaCl.

Revealed by the analysis of deviations between the OWLS apparent layer refractive index data and realistic CMD refractive index values (quasi-isotropic analysis), it was found that there is the possibility to tune the nanostructure of CMD-ut layers both by the composition of the silane coating and the pH of CMD grafting solution. In case of the CMD-ut-Ep coatings, the layer structure was strongly pH-dependent: while the dominant chain conformation was parallel with the surface in neutral and basic conditions (significant negative birefringence), at acidic pH, the structure was found more open with larger number of loops perpendicular to the surface. In case of CMD-ut-Am layers, an intermediate structure was observed which could be characterized by a structure that was between the two pH extremes of CMD-ut-Ep layers. The pH-dependent nanostructure of CMD-ut-Ep layers was explained by the varying efficiency of the grafting reaction at different pH values resulting in a significant variation in the number of surface grafting points. The corresponding OWLS results are shown in Figure 7.

Figure 7 | Apparent refractive index ($n_A$) of CMD-ut adlayer as a function of deposited CMD areal mass density ($M_A$), obtained from in situ OWLS grafting experiments at different experimental conditions.

The shown arrows indicate the direction of experiments in time (regarding the arrows, mark $G$ refers to the grafting, $W$ to the washing phase). In the inset table, Am refers to the aminosilylated, Ep to the epoxysilylated surfaces. The changes in the CMD layer structure during its formation was schematically drawn. As the refractive index ellipsoid (a diagram intended to illustrate the orientation and relative magnitude of ordinary ($n_{A,o}$) and extraordinary ($n_{A,e}$) refractive indices in a birefringent medium) demonstrates at the top left corner, the overestimated $n_A$ values suggested a negatively birefringent CMD layer ($n_{A,o} > n_{A,e}$).
Figure 8 | In situ QCM-I measurement results on the formation of PLL-g-PEG and CMD-ut nanolayers. Determination of the mass of bound/trapped water by complementing the QCM-I mass data with OWLS results.

The evaluation was performed by the Voinova’s Voigt-based viscoelastic model using the developed evaluation code.

A, B. Areal mass density data obtained from in situ QCM-I (solid black line) and OWLS (solid red line) on PLL-g-PEG (A) and CMD-ut layers (B). The flow-cell experiments were performed in three consecutive steps, indicated by the given numbers in graph headers: the polymer-free solution (buffer (PLL-g-PEG) or water (CMD)) was first driven through the flow-cell to have a stable baseline (1); then the solvent was exchanged to polymer solution (2), which was flowed until it was replaced by the solvent (3) in order to wash away the loosely adsorbed polymer chains. Two types of QCM-I mass curves are plotted, represented by thicker lines (mass data calculated by the Voinova’s Voigt-based model) as well as thinner lines (3rd overtone Sauerbrey mass (S3), calculated from a simple model that does not consider viscoelastic properties). In the deposition section of CMD experiment, the calculated Voigt mass was too high for a proper interpretation, therefore only mass values up to 3200 ng/cm² are shown. The amount of bound/trapped water in the polymer layers are indicated by $M_w$ and the highlighted mass difference.

E, F. Shear viscosity (black) and shear elastic modulus (red) plots of PLL-g-PEG and CMD-ut layers.

G, H. The drawings demonstrate the assumed final structure of the formed polymer layers (besides the polymer chains, the bound/trapped water molecules are also indicated).
The parallel OWLS-QCM measurements with the application of the self-developed and validated evaluation program provided viscoelastic and hydration-related parameters such as shear viscosity (1.43 ± 0.27 mPa-s), shear elastic modulus (0.03 ± 0.01 MPa), hydrated thickness (11.0 ± 4.9 nm) as well as hydration degree values for CMD-ut-Am layers (see result graphs of Figure 8). It is worth highlighting that regarding the CMD layers the resulted data are unprecedented in the literature. It was found that compared to PLL-g-PEG layers, although a smaller amount of CMD molecules were deposited on the sensor surface, the extreme hydration of CMD-ut layers (φ_A (hydration degree) = 89%) resulted in a significantly higher wet mass (in case of PLL-g-PEG, φ_A = 59%).

The developed OWLS-QCM data analysis methodology (quasi-isotropic analysis supported by QCM data) was successfully applied for revealing the time-dependent nanostructure and formation mechanism of CMD-ut-Am layers in terms of hydration and viscosity. According to the results, the washing induced remarkable conformational rearrangement in the layer, involving the slight extension of chains from lain down conformations to loops as well as consecutive decreasing and increasing levels of layer hydration. The resulted final structure was composed of predominantly lain down chains with moderate number of perpendicular loop conformations.

Besides the ultrathin coatings, CMD-based chemical hydrogel layers with increased thickness values (CMD-sc) were also prepared. The developed methodology – that has not been applied for the fabrication of dextran layers before – is based on the spin-coating and chemical crosslinking of CMD molecules using STMP as crosslinking agent. To control the thickness, the layers were prepared using different rotational speeds. For the evaluation of the recorded ellipsometric spectra, an ellipsometric optical model was developed considering the structure as well as optical properties of the examined samples. As a part of the comprehensive SE analysis, the parameter describing the wavelength dispersion of the bulk CMD refractive index (Cauchy B parameter) was also determined (B = 4.5 × 10^{-3} ± 7.4 × 10^{-4}). The dispersion function can be seen in Figure 9. According to the SE results, whilst the thickness of the unwashed layers was well controlled by the applied rotational speed in the range of 100 - 200 nm, and the thickness followed the expected tendency, the thickness values of washed, stably bound (remained) layers at different rotational speeds were found to be uniform (values between 10 and 50 nm). According to the CA measurements, the CMD-sc layers lowered the CA of water droplets form 65 - 80° (aminosilylated surface) to the range of 10 - 30°. The effect of the intensive washing phase applied as a final step of layer fabrication process was analyzed by XPS. The XPS analysis also enabled to estimate the crosslinking degree in CMD-sc layers. It was proven that the detected phosphorous atoms in the washed specimens originated from covalently bound crosslinks, and based on the determined phosphorous content, the crosslinking degree was estimated to be 5%. Additionally, the dynamic CA measurements revealed that the layers can absorb a large amount of water inducing a pronounced change in the CA values. This finding confirms the hydrogel nature of CMD-sc layers.
The in situ OWLS-monitored protein adsorption experiments, performed using bovine serum albumin, fibrinogen and lysozyme protein molecules, showed that the CMD coatings suppressed the amount of adsorbed proteins by more than one order of magnitude compared to protein amounts measured on uncoated OWLS chip surfaces (control measurements). This observation clearly demonstrates the protein-repellent ability of the developed layers. Relying on the cell adhesion experiments, it was demonstrated that the CMD-sc surfaces were resistant to the adhesion of living cells (see Figure 10)

Figure 9 | Ellipsometric spectra of a spin-coated CMD layer used for the determination of refractive index dispersion of the bulk CMD (Cauchy B parameter)
Ψ' and Δ spectra (A) measured on a CMD layer spin-coated at 3000 rpm rotational speed on top of a gold substrate. The dispersion parameter was determined by fitting the measured spectra. The refractive index dispersion function can be seen in graph B.

Figure 10 | Phase contrast microscopy images about an adhesion test performed with HeLa cells on CMD-sc-coated and uncoated glass slide surfaces
Image A was taken on a sample that was partially covered with CMD-sc layer. The CMD-covered part and uncovered part of the glass substrate are indicated by the labels. The edge of CMD layer is indicated by the shown arrow. The cells could not adhere to the CMD layer, they were congregated on the glass surface, where then they started adhering (demonstrated by the enlarged image (B)). Image A and image B were taken after 4 and 6 hours of incubation in cell culture.
4 Theses

I. The nanostructure of ultrathin carboxymethyl dextran (CMD-ut) layers covalently grafted onto silylated SiO$_2$-TiO$_2$-based surfaces was characterized by in situ optical waveguide lightmode spectroscopy (OWLS) measurements and the data were evaluated by the quasi-isotropic optical model. Analyzing the deviations of the determined apparent layer refractive indices from the realistic values, I found that the nanostructure of CMD-ut layers is dependent on the chemical composition of the applied silane coating and the pH of grafting solution. In case of epoxysilylated surfaces, the layer structure was found to be strongly pH-dependent, which was explained by the pH-dependence of the grafting reaction’s efficiency. While the dominant chain conformation was parallel with the surface under neutral and basic conditions, at acidic pH the structure was found to have larger number of chains with orientation perpendicular to the surface and have therefore a more open structure. In case of the aminosilylated surfaces at neutral pH, an intermediate structure of the above two extremes was observed. [T1]

II. Based on parallel OWLS and viscoelasticity-sensitive quartz crystal microbalance (QCM) measurements, I presented data on the hydration ability, hydrated thickness, shear viscosity and shear elastic modulus of CMD-ut layers for the first time. The layers were prepared on aminosilylated SiO$_2$-TiO$_2$-based waveguide-type surfaces and the QCM measurements were performed by a new impedance measuring QCM-I instrument. The data obtained by this QCM-I instrument and the used evaluation code were validated by employing self-assembled poly(L-lysine)-graft-poly(ethylene glycol) layers as reference material. I found that the CMD-ut layers have great hydration abilities demonstrated by the determined hydration degree of 89%. [T2][T4]

III. a. I developed a novel OWLS-QCM combined data analysis methodology for the kinetic evaluation of nanostructural alterations in ultrathin hydrated polymer layers based on the quasi-isotropic analysis of OWLS data in terms of hydration degree and viscosity. [T2] b. Based on the developed methodology, I found that the aqueous washing of CMD-ut layers prepared on aminosilylated surfaces induces the conformational rearrangement of CMD chains that results in significant alterations in layer hydration and viscosity. I proved that the resulted structure at the end of the washing was composed of predominantly lain down chains with a moderate number of loop conformations perpendicular to the surface. [T2]

IV. For the first time in the literature, I presented spectroscopic ellipsometry (SE) measurement results on CMD layers spin-coated onto waveguide-type (SiO$_2$-TiO$_2$-based) surfaces. Based on separate, high-sensitivity SE measurements performed on spin-coated CMD layers on gold substrates, I determined the parameter describing the wavelength dispersion of the bulk CMD refractive index for the first time. Supported by the developed optical model and calculated dispersion parameter value, the thickness of CMD layers spin-coated at various rotational speeds were determined by SE measurements. [T3]
5 Publications

5.1 Publications related to the thesis


5.2 Other publications


5.3 Oral presentations


5.4 Poster presentations


