Evaluating the Thickness of Multivalent Glycopolymer Brushes for Lectin Binding

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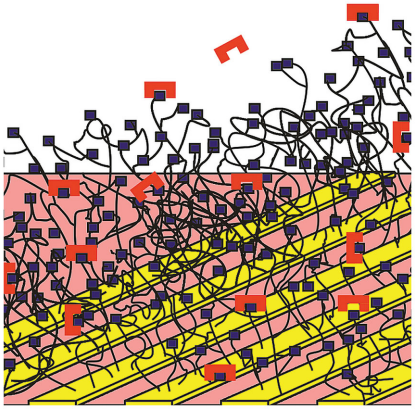
Electrochemical impedance spectroscopy (EIS) is applied for investigating binding of lectins to multivalent glycopolymer brushes grafted from interdigital gold microelectrodes. By variation of the measuring frequency, EIS allows simultaneous analysis of binding at different subnanometer distances from the sensor surfaces. In this way, the binding dynamics along the brushes are quantified, giving an idea about the motion of the lectin through the brush layer. Two different brush lengths are investigated, revealing distinct dynamics of lectin binding due to changing topology of the brushes. Moreover, very low K_D values in the nanomolar range are obtained. This unique platform may be used as sophisticated biosensor for detailed investigation of high-affinity protein binding to polymer layers.



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1. Introduction

Glycans are known to be involved in numerous biological events especially interactions directed by the glycocalix of the cell surface and the extracellular matrix.[1–5] Lectins, glycan-binding proteins, are key players in these recognition events; they bind with high specificity to glycan structures ranging from simple monosaccharides to complex oligosaccharides.[6–9] Although the preferred minimal ligand for a large number of lectins is known[10] and multivalent presentation of the sugar ligands is crucial for enhanced binding,[11–15] dynamics of lectin binding in a multivalent microenvironment such as the cell surface or biomaterials like glycopolymers remain unclear. Here, we present an advanced powerful biosensor platform, based on electrochemical impedance spectroscopy (EIS), for the detailed investigation of the influence of glycopolymer brush thickness on lectin–glycan-binding processes. EIS unique characteristic is the ability of collecting distinct

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kinetic data at different subnanometer distances from the sensor surface simultaneously. Importantly, EIS analysis reveals K_D values in the micro- to nanomolar range emphasizing the superior multivalent quality of our synthesized glycopolymer brushes for highly efficient lectin binding.

Glycopolymers are an emerging class of molecules for studying lectin binding as they provide good solubility, high valency and mostly controlled sizes and shapes. Multivalent glycopolymers have attracted great interest in both fundamental and applied studies of lectin binding.[16–19] The multivalent presentation of sugar moieties in glycopolymer brushes is the key element for higher binding affinities of lectins.[20] Recently, we introduced a novel approach for the synthesis of glycopolymer brushes.[21] Polymer brushes are known to stabilize interphases due to the high osmotic pressure within the brush regime, therefore these materials are prone as anti-fouling, biocompatible materials with low unspecific protein adsorption.[22] By combining the atom transfer radical polymerization (ATRP) technique[23,24] for grafting glycopolymers from the solid substrate and enzymatic synthesis of glycan oligomers,[25] we obtained a glycopolymer-brush-based platform for studying the specificity of multivalent lectin binding.

EIS has been widely used as a detection method for label-free molecular-binding processes such as protein–protein interaction, virus detection, and DNA sensing.[26–33] However, the ability of EIS to kinetically analyze dynamical binding events perpendicular to the sensor surface was to the best of our knowledge not yet demonstrated for molecular interactions. Measuring the electrical current response to an AC potential applied between electrodes renders EIS extremely sensitive to adsorption events onto the biosensor surface. Moreover, utilizing interdigital microelectrodes gives high sensitivity to surface modifications on and in the vicinity of the electrode surface as well as in the inter-electrode space.[34–36] Variation of the measuring frequency of EIS allows monitoring the lectin-binding kinetics to the glycans in different sublayers of the brushes. By calculating the binding constants of receptor–ligand interactions at different frequencies the impact of glycopolymer brush thickness can be quantitatively described. The advantage of the proposed method compared to commonly used SPR (surface plasmon resonance spectroscopy) methods[37–40] is that variation of impedance signals at different frequencies reveals space-resolved kinetic-binding information over the layer thickness. So far, SPR and QCM (quartz crystal microbalance) among others are the well-established methods of choice for investigating the kinetics of lectin–glycopolymer interactions.[41–44] Highly controlled set-ups and syntheses are described for combining glycopolymers with various biosensor platforms.[41,42,45] In contrast to other methods, including fluorescence-based assays or ITC (isothermal titration calorimetry), EIS reveals dynamical binding kinetics and is suitable for sensing the dynamics of binding taking place perpendicular to sensor surfaces. This unique characteristic enables us to observe and quantify space-resolved dynamics of ligand binding along glyopolymer brushes of different lengths. In this way, new insights to binding processes taking place on glycopolymer brushes are gathered and may support for example the quality assessment of such biosensors. Following lectin binding to biofunctionalized surfaces with different topologies is of high interest for creating tailored lectin capturing compounds and modeling the binding behavior of lectins in vivo.

2. Results and Discussion

As model lectins, we chose the plant lectins from *Griffonia simplicifolia* (GS-II) and *Erythrina cristagalli* (ECL).[46–49] GS-II shows specificity for *N*-acetylglucosamine (GlcNAc) as ligand, whereas ECL binds specifically to the disaccharide *N*-acteyllactosamine (Galβ1,4GlcNAc, LacNAc).

The workflow for fabrication of glycopolymer brushes on the interdigital gold microelectrodes of the sensor chip and the setup for EIS measurements are depicted in Figure 1. Lectin-binding assays with EIS are performed in a microfluidic chip (see Experimental Section and Supporting Information). In total, 24 electrode pairs in eight microfluidic channels were used to study the binding kinetics of GS-II. The electrodes are treated consecutively by buffer and then either GS-II (20 μg mL^{-1}) as binding protein or ECL as non-binder (20 μg mL^{-1}) is applied, finalized by a purge with buffer solution. The normalized impedance amplitude Z/Z_0 over the applied frequency spectrum is shown in Figure 2A for the two different brush lengths (0.5 and 3.45 nm). The frequency spectrum can be divided into three segments (see Figure 2): The first segment with frequencies larger than 100 kHz is mainly dependent on the resistivity of the bulk solution. The middle part between 1 and 100 kHz can be referred to the influence of the double-layer capacity, which increases with decreasing frequency. The impedance signal with frequencies below 1 kHz can be assigned to the influence of the polymer brushes and its sensitivity against lectins. By applying incremental frequency steps in this frequency region changes in the impedance signal correspond mainly to the brush sublayers. The impedance signals obtained from the shorter brushes (10 min polymerization time) show an increased sensitivity compared to the longer brushes (120 min polymerization time) originating from the dense blocking of the sensor surface by the polymer layer. The increased standard deviation of the impedance values obtained from longer brushes in the low frequency range originates in the possible larger

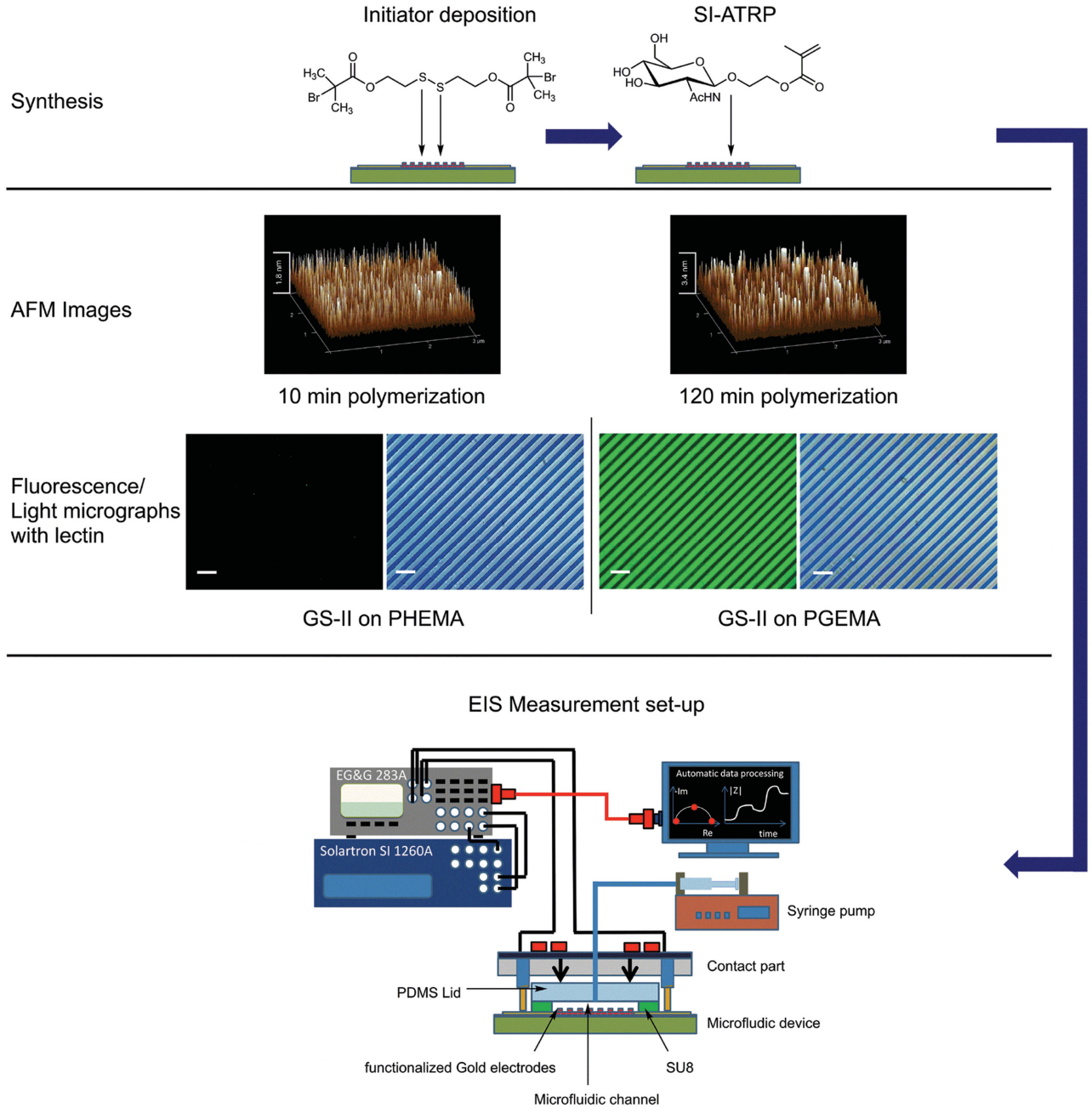

Figure 1. Upper part: Workflow for functionalization of EIS chips. On plasma-treated gold electrodes initiator is deposed followed by controlled SI-ATRP with GlcNAc-monomer for 10 and 120 min, respectively. Middle part: Analysis by AFM shows homogeneous glycopolymer brushes of averaged 0.5 nm and 3.45 nm length. (z-scale bar for 10 min polymerization: 1.2 nm, for 120 min polymerization: 3.4 nm).[21] Fluorescence images with labeled GS-II (green) show specific binding to GlcNAc (PGEMA) presenting chips (right), no fluorescence is detected with GS-II on brushes without GlcNAc (PHEMA) (left). Light micrographs are used for reference and depiction of the electrodes. (scale bar: 40 µm) Bottom: Schematic diagram of the measurement setup.

impact of small differences between the thick polymer layers on different electrodes.

Binding of GS-II to the longer brushes shows variation of the impedance signals over time in dependence of the applied frequencies (Figure 2B). The calculated kinetic parameters (k_{on}, k_{off}) as well as dissociation constants (K_D) (Table 1A) can be related to the different accessibility of sugar ligands due to "mushroom" topology of surface deposited long polymer brushes (Figure S2/S3, Supporting Information). Lectin binding at the top of the polymer brush is followed by dissociation and association along the brushes to reach deeper parts of the brush

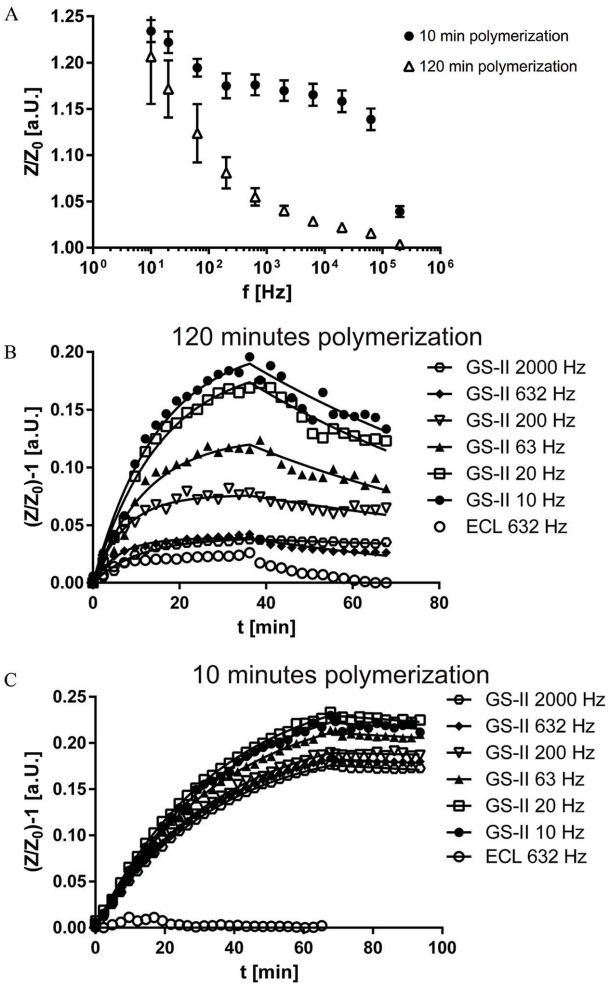

Figure 2. EIS measurements with lectins on glycopolymer brush surfaces. A) Normalized values of maximum impedance amplitude over frequency. Impedance amplitude is normalized to the impedance value directly before application of a GS-II. Impedance signal with respect to time for different applied frequencies, in B) for long brushes (120 min polymerization), in C) for short brushes (10 min). No significant binding of ECL was observed on short as well as on long brushes.

layer with higher glycan accessibility. Kinetic parameters decrease with respect to the localization of lectin binding along the glycopolymer brush layers, e.g., slower association (k_{on}) and dissociation (k_{off}) rates of lectins to glycans occur in brushes which are located closer to the electrode. This results in a threefold shift of the apparent K_D values towards higher values (worse binding) at the inner brush region (Table 1A). Remarkably, the K_D values for binding of GS-II to the multivalent long glycopolymer brush are in the high nanomolar range emphasizing the superior quality of the glycopolymer brushes when compared to previously reported K_D values in the micromolar range found for various natural (multivalent) glycans.[50,51] We conclude that lectin-binding kinetics changes with accessibility of glycan ligands, being better at the top, along the brush. The dynamics of the binding process at long glycopolymer brushes is indicated by the time shift for saturation of the impedance signal for regions closer to the surface (lower frequencies), which is lacking for short brushes (Figure 2B).

Lectin binding on shorter brushes shows a different behavior due to better accessibility of glycan ligands resulting from their topology (Figure S2, Supporting Information and Figure 2C). With similar k_{on} values, only a minor change of K_D values for the applied frequencies (Table 1B) is detected. Remarkably, K_D values for the short brushes are in the lower nanomolar range and thus a magnitude higher when compared to the longer brushes (Table 1B). Besides that, in shorter brushes, a lower overall amount of lectins may bind, the brushes are well perpendicularly oriented to the surface and present optimal ligand accessibility in contrast to the outer zone of long brushes. We conclude that lectins bind more efficiently on shorter brushes due to their topology-dependent presentation of multivalent glycan ligands. Comparing our averaged results to established methods shows that GS-II has a slow binding behavior with k_{on} in the region of 10^{-5} $M^{-1}s^{-1}$. Generally, k_{on} is determined only by the rate of diffusion and is found in ranges of 10^{-6}–10^{-8} M^{-1} s^{-1}. However, slow binding of lectins in ranges of 10^{-5} M^{-1} s^{-1} is a common phenomenon and reflected in various reports.[52–54] Nevertheless, this validates our data collected via EIS for glycan–lectin interactions. Interestingly, k_{off} and K_D values in similar ranges as for the short brushes are reported for a proposed membrane affinity chromatography approach based on glycopolymer brushes and evaluated via SPR.[55] This underlines the high binding affinity of lectins towards glycopolymer brushes. Other glycopolymer systems show values in the higher nanomolar or micromolar ranges gathered by QCM, which has the disadvantage of very long measuring times combined with high substance-need.[56–58] All in all our averaged collected data are comparable to analysis with other methods, validating EIS as novel method for investigating lectin binding to glycans. Thus, so far no method was sensitive to actions taking place at different distances from the sensor surface, which is the unique feature of EIS, together with high sensitivity and short measuring times.

This study introduces a variable polymerization and measuring platform for analyzing detailed dynamical binding kinetics on a biosensor. Although EIS is an established method, the unique ability of investigating binding events kinetically at different distances to the biosensor surface, was not elucidated so far. Combining the high valency of glycopolymer brushes, their good stabilizing and anti-fouling properties with EIS gives the ability to quantify the influence of ligand accessibility, to calculate

Table 1. K_{on}, K_{off}, and K_D values determined from Figure 2B,C. A) 120 min brush polymerization B) 10 min brush polymerization.

Long glycopolymer brush (120 min polymerization)				
	K_{on} [$M^{-1}s^{-1}$]	K_{off} [s^{-1}]	$K_{off}/K_{on} = K_D$ (M)	Average K_D (M)
10 Hz	3.09E+5	1.17E–2	3.79E–8	Inner zone 3.90E–8
20 Hz	2.89E+5	1.32E–2	4.57E–8	
63 Hz	3.84E+5	1.28E–2	3.33E–8	
200 Hz	7.08E+5	7.91E–3	1.12E–8	Outer zone 1.21E–8
632 Hz	8.45E+5	1.57E–2	1.86E–8	
2 kHz	5.06E+5	3.38E–3	6.68E–9	
Short glycopolymer brush (10 min polymerization)				
	K_{on} [$M^{-1}s^{-1}$]	K_{off} [s^{-1}]	$K_{off}/K_{on} = K_D$ (M)	Average K_D (M)
10 Hz	1.51E+5	1.19E–3	7.88E–9	Inner zone 7.49E–9
20 Hz	1.52E+5	1.20E–3	7.89E–9	
63 Hz	1.51E+5	1.01E–3	6.69E–9	
200 Hz	1.57E+5	3.47E–4	2.22E–9	Outer zone 4.98E–9
632 Hz	1.59E+5	9.17E–4	5.77E–9	
2 kHz	1.58E+5	1.10E–3	6.96E–9	

and depict the dynamics of protein binding as well as standard binding kinetics. Additionally, the microfluidic device reduces the used volumes and sample amounts to few µL and µg of protein.

3. Conclusions

In conclusion, we here demonstrate for the first time the topology-dependent binding kinetics of GS-II lectins in PGlcNAcEMA polymer brushes by space-resolved EIS. Applying incremental frequency steps up to 1 kHz impedance signals differ according to the binding of GS-II at short and long glycopolymer brushes. We demonstrate that the calculated binding rates and constants change due to lectin binding along the long brushes whereas in short brushes no significant change is observed. We relate the better lectin binding on shorter brushes to their topology with better accessibility of the multivalent glycan ligands. EIS gives for the first time insight into lectin binding to multivalent glycopolymer surfaces of different thicknesses and will assist to develop advanced glycan-functionalized material surfaces.

So far our chips contain six interdigital electrodes, which may all be covered differently and measured at once; by multiplexing the microfludical system high-throughput low sample amount screening of detailed kinetical data is accessible. Together with the broad scope of possible ligands tailored either by enzymatic reactions or by substituting the glycan by other biomolecules prior to polymerization, we present a variable, stable, and powerful biosensor platform technology with a broad scope of applications in bioanalytics.

4. Experimental Section

Gold interdigital electrodes (gap 10 µm, width 10 µm, length 3885 µm, count 100) on oxidized silicon chips were used as sensor elements for all EIS experiments. The fabrication process is described in detail in Supporting Information. Three elements were subsequently addressed within one microfluidic channel by switching a custom-made relay multiplexer. EIS measurement (10 mV, 10 Hz–200 kHz) was performed during lectin assay consisting of buffer pretreatment (application till stable signal), lectin application, and buffer purge. The chips were integrated in a microfluidic channel system (see Supporting Information). To prevent diffusion limitation of the applied analyte, the flow rate through the channel was set to 0.5 mL h^{-1}, which corresponds to 1 mm s^{-1} over the sensor surface.[59] Self-assembled initiator monolayers were formed on the gold surfaces by immersion of the chips into 2×10^{-3} M solutions of bis[2-(2′-bromoisobutyryloxy)ethyl]disulfide (Sigma–Aldrich, Steinheim, Germany) using ethanol as solvent at room temperature. The polymerization of GlcNAcEMA from the electrode surfaces was performed as described in our previous work.[21]

Unconjugated lectins were from Vector Laboratories (Burlingame, CA, USA), purified by gel chromatography and diluted with 10×10^{-3} M HEPES (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) –NaOH, pH 7.5, 150×10^{-3} M NaCl, 0.1×10^{-3} M $CaCl_2$ to give a protein concentration of 20 µg mL^{-1}. For fluorescence images FITC-labeled lectins (Vector Laboratories) treated

the same way were used. The electrodes were submerged in FITC-lectin solution without previous blocking steps and washed three times with PBS (phosphate buffered saline) containing 0.05% (v/v) Tween-20. Fluorescence images were taken with a Zeiss Axioplan microscope.

Supporting Information

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

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