**Development of a carbohydrate SPR interface based on graphene/sugar coatings**

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**Abstract:**

The paper describes the detection of carbohydrate lectin interaction on graphene-on-metal surface plasmon res onance (SPR) interfaces. Graphene-coated gold-based SPR interfaces were formed through the transfer of large-area graphene grown by chemical vapor deposition (CVD) on polycrystalline Cu foils. The method allowed successful trans fer of single- and double-layered graphene sheets onto the SPRinterfaces in a reproducible manner. Functionalization of the graphene interface with mannose was achieved by simple immersion into a mannose aqueous solution (100 mM), resulting in noncovalent interactions between the aromatic ring structure of graphene and mannose. The utility of the carbohydrate-modified graphene-on-gold interface for the se lective and sensitive detection of carbohydrate–lectin interac tions was demonstrated using model lectins from Lens culinaris (LC) andTriticum vulgaris (TV). While LC lectin binds specifically to mannopyranoside units, TV lectin has an affinity for N-acetyl glucosamine and sialic acid residues.

***Key words:*** graphene, surface plasmon resonance, mannose, lectins

**1. Introduction**

The development of carbohydrate sensors has received much attention over the last years due to essential role of oligosaccharides in the development and maintenances of all living systems [1-7]. Most of the early sensors rely on an end point and thus indirect detection of a labeled protein. Different label-free methods have lately been considered an attractive alternative allowing glycosignatures to be scrutinized more rapidly and more easily. Among the label-free methods available, plasmonic based techniques such as surface plasmon resonance (SPR), SPR-imaging and localized surface plasmon resonance (LSPR) has received the greatest attention to date in high-throughput glycan profiling [1,4,6-9]. The choice of the surface chemistry employed for linking glycans to the sensor surface is one of the important steps in the development of a highly performing glycan sensor. Self-assembled monolayers of thiol-functionalized carbohydrates have found wide use for decoration SPR interfaces. The prerequisite of?are thiol-functionalized carbohydrate analogues that? are however often not straightforward to synthesis and the formation of well-defined monolayers is not guaranteed [10]. Pyrrole [1], azide [2,11] and alkynyl terminated [4] glycans have been used as alternatives to thiolated glycans. Nevertheless, the requirement for prior derivatization of the carbohydrate by one or multi-step sequences presents a significant hurdle for the construction of functional glycan arrays. The field would undoubtedly benefit greatly from alternative conjugation strategies. Among more recent scenarios, photoinduced covalent attachement to interfaces coated with photoreactive groups have been proposed [4,5,12,13].

In this paper, we investigate if graphene coated SPR interfaces could be an alternative for the formation of glycan interfaces in an easy and selective manner without the need of post-functionalization of the carbohydrate. Graphene coated SPR interfaces have been theoretically [14,15] and experimentally [16-23] investigated as alternative SPR surfaces. The advantages of graphene-based SPR surfaces are the high surface-to-volume ratio which has proven to be beneficial for efficient adsorption of biomolecules when compared to gold [17,18] and possible -stacking interactions between the carbon-based ring structures of organic and biological molecules and the hexagonal cells of graphene. The possibility to integrate mannose onto graphene based SPR interfaces in a non-covalent manner and the effectiveness of the resulting interface to assess lectin-recognition abilities in a SPR format is discussed here.

**Experimental Section**

Materials

Mannose, phenol, methanol, sulfuric acid (H2SO4), polymethyl methacrylate (PMMA), hydrogen peroxide (H2O2), hydrochloric acid (HCl), Lens culinaris (LC) lectin, and Triticum vulgaris (TV) lectin were purchased from Sigma-Aldrich and used as received.

Graphene

Growth Graphene was synthesized on polycrystalline Cu foils (50-μm-thick AlfaAesar, purity99.9999 %)inarapid thermal processing chemical vapor deposition (RTPCVD) chamber. A mixture of Ar, H2, andCH4 gases were used with 10 Torr chamber pressure. Cu foils were deoxidized using acetic acid before loading in the CVD chamber. They were annealed at 1070 °C under the flow of H2 andArfor5min.Then,CH4 wasaddedfor 5mintocarry out thegraphenegrowth. Finally, the sample was rapidly cooled below 700 °C with a coolingrate of 40 °C s−1 and further slowly cooled to room tempera ture under H2 and Ar atmosphere.

Fabrication of Graphene-on-Metal SPR Interfaces

Gold-based SPR interfaces were formed by depositing 5 nm of titanium and 50 nm of gold successively onto cleaned glass slides by thermal evaporation. CVD-grown graphene was transferred onto these interfaces as depicted in Fig. 1. PMMA (5 %) was spin coated onto the graphene-coated Cu foil. The PMMA/graphene/Cu sample was baked at 170 °C for 10 min and then slowly cooled to room temperature. Backside graphene was removed by oxygen plasma (power, 50 W; time, 30 s) treatment. Copper was dissolved using a solution of HCl/H2O2/H2O with 1:1:40 proportions. Graphene/PMMA was rinsed with deionized water five to six times to remove any ion contamination. Finally, the graphene was taken up onto the SPR substrate. After waiting for 10 min for natural removal of water underneath the graphene, another backing step (RT→90 °C for 30 min, at 90 °C for 30 min, 90 °C→RT for 30 min) was carried out to remove the trapped water and to increase the contact between graphene and substrate. Subsequently, the PMMA layer was removed by dipping the sample in acetone for 30 min. The substrate with the transferred graphene was then rinsed with methanol and dried by mild nitrogen blow

Noncovalent Functionalization of Graphene-on-Metal SPR Interfaces with Mannose

The graphene coated SPR interface was immersed for 2h into an aqueous solution of mannose (100 mM) and rinsed-three times with water before being used. The amount of integrated mannose was determined as described previously by use of mannose clicked to reduced graphene oxide interfaces [24]. First a standard calibration curve was generated for different mannose concentraitons by mixing aliquotes of aqueous phenolic solution (5 wt%; 60 µL) and concentrate sulfuric acid (900 µL) to a series of 60 µL aliquotes of mannose solutions. After 10 min reaction under stirring the absoprtion spectrum was recoreded (Perkin Elmer Lambda 950 dual beam) using pehnol/H2SO4 mixtures contaning 60 µL of water as blank. The absorption of the solution was recoreded at two wavalengt, 495 and 570 nm, and the absorbance difference (A495-A570) was plotted against the concentration of mannose. To determine the coupling efficienty of mannose to graphene, the modified interface was dipped for 60 min into 2 mL of the phenol/H2SO4 solution and the UV/Vis spectrum was recored.

**Characterization**

Raman

Micro-Raman spectroscopy measurements were performed on a Horiba Jobin Yvon LabRam HR Micro-Raman system combined with 473-nm laser diode as excitation source. Visible light is focused by 100x objective. The scattered light is collected by the same objective in backscattering configuration, dispersed by 1800 mm focal length monochromator and detected by CCD.

Scanning Electron Microscopy

Scanning electron microscopy (SEM) images were obtained using an electron microscope Ultra 55 (Zeiss) equipped with a thermal field emission emitter and a high efficiency In-lens or ESB/SE detector. The SEM measurements were recorded at 3 kVfor a better contrast between the substrate and graphene overlayer.

UV–Vis Measurements

Absorption spectra were recorded using a Perkin Elmer Lambda UV/Vis 950 dual-beam spectrophotometer in quartz cuvettes with an optical path of 10 mm. The wavelength range was 400–800 nm. SPR Instrumentation The surface plasmon resonance instrument used was an Autolab ESPRIT instrument (Eco Chemie, Utrecht, The Netherlands) working at 670 nm. The configuration of this equipment is described elsewhere [24, 25]. The instrument is equipped with a cuvette of about 100 μL. The prism used had a refractive indexofn=1.52. WinSpall 2.0 software (Max Planck Institute for Polymer Research, Mainz) was used to calculate the SPR curves and to approximate the experimental dependences with an optical model within the framework of Maxwell macroscopic approach

**Results and Discussion**

Preparation and characterization of graphene-based SPR interfaces

The graphene-based SPR sensor developed in this work consists of a glass substrate onto which 5 nm titanium adhesion layer and 50 nm gold thin film are deposited, as in the conventional SPR, coated with an additional layer of graphene by a transfer procedure as outlined in **Figure 1A**.

**Figure 1B** shows the Raman spectra of the initial CVD grown graphene on nickel and after the transfer onto the gold substrate……The formation of high quality CVD grown graphene was apparent from the small intenstiy of the D-band in the Raman sepctrum, with a intensity ratio of the 2D to the G bands of appraoximately xxx and the Lorentzian line width of the 2D band ad xxx. After the transfer process….

The SPR signal of the graphene coated gold interfaces with a nprisme =1.52 and a wavalength of =670 is seen in **Figure 2A.** A shift of 0.206° to higher angles is osberved in the presence of graphene which is indictive of the transfer of 1-2 graphene nanosheets. This is in line with the results obtained by Raman spectroscopy. The angle shift is smaller than using dry transfer process based on the use of commercial thermal release tap [16,23] whith an angle shift of ≈1.02 nm (3 layers) and of the ≈2 nm (5-6 layers) by an electrophoretic depositon strategy [22]. SEM image?

**3.2. Non-covalent coupling of mannose to graphene-on-metal SPR interfaces**

As mentioned above, graphene has attracted considerable attention as a support for divers molecules due of its large surface area (theoretical limit: 2630 m2 g-1) and rich -conjugation structure [23,24,27]. The increased adsorption of biotinylated BSA with a molecular mass of 66.5 kDa on graphene in contrast to gold has been recently shown by us [23]. Based on the assumption generally accepted that 1 RU (response unit) corresponds to 1 ng cm-2 [28], the coverage of biotinylated BSA on gold was approximated at 230 ng cm-2 whereas on graphene it was five times larger (1205 ng cm-2). Lens culinaris (LC) is a lectin of two subunits with molecular weight of 46 kDa,with carbohydrate specificity to D-mannose [29]. The *Maackia amurensis* (MA) lectin, on the other hand, is believed to be multimeric (reference) with molecular weight of 75 kDa, is binding preferential glycoconjugates having galactosyl (-1,4) N-acetylglucosamine structures (ref). Interaction of both lectins (100 nM) with graphene coated gold interfaces resulted in response unit changes of 1215 RU (LC) and 1784 RU (MA), respectively, while on gold the non-specific interaction of the lectins result in RU changes of 237 RU (LC) and 330 RU (MA) (**Figure 2B**). As expected, increasing the molecular weight of the lectin, while keeping the concentration constant, result in an overall increased SPR response. The results also show that graphene has no preferential adsorption to any lectin as was the case in electrophoretically deposited reduced graphene oxide [22].

To obtain an interface specific to Lens culinaris as model lectin, the graphene-on-metal interface was immersed for 2 h into a 100 mM solution of mannose (molecular weight of 180 Dalton) followed by rinsing with water (**Figure 2A**). No significant changes in the SPR signal upon mannose interaction could be recorded. The amount of mannose integrated onto the graphene interface was estimated using colorimetric assay, based on the specific reaction of mannose with phenol in concentrated sulfuric acid [4,24]. The method allowed the amount of mannose to be estimates as **mannose= (2.0±0.6)×1015 molecules cm-2. Corresponding to about 3.6 nmol cm-2. In a control experiment, a gold based SPR was immersed into a mannose solution (100 mM) of the same amount of time. No mannose absorbed could be detected

Sensing of manose-lectin interaction

The biorecognition properties of the mannose modified SPR interface to lectins was subsequently evaluated using SPR by injection of mannose-specific *Lens culinaris* and non-mannose-specific *Maackia amurensis* lectins*.* As it can be seen from **Figure 4A**, an immediate change in the SPR signal is observed upon injection of *LC lectin* as the consequence of its association with the mannopyranoside. In the case of non-mannose specific MAlectin*,* the increase in the SPR signal was considerably smaller. This indicates that -staking interactions of mannose with graphene does not impact on the lectin binding and that the presence of non-covalently bound mannose allows tuning the surface selectivity in an easy manner. The association constant *K*A of the mannose-modified interfaces with LC lectin was estimated to be (3.6±0.6)×106 M-1 and compares to other association constants reported so far in the literature [4,30,31]. In the case of mannose non-specific lectin such as from MA, *K*A of (5.53±0.5)×104 M-1 was estimated. As expected, the SPR signal is dependent on the lectin concentration as seen in **Figure 4B**. The detection sensitivity is dictated by a number of factors of which the most important are the coupling efficiency of the analyte to the chip matrix, strength of recognition and method of detection. With mannose as a test glycan on graphene, the SPR curve levels off at a concentration of 1000 µg ml-1 of LC with an estimated detection limit of 1 µg ml-1 comparable to detection limits of other protein-carbohydrate interfaces using SPR [4,9].

Maybe you may mention of re-use of the mannose modified graphene!

**Conclusion**

In conclusion, we have developed a strategy enabling the transfer of a large amount of single-layered graphene sheets onto a gold-based SPR interface. The newly formed interface was modified with mannose and its ability for the specific detection and investigation of carbohydrate–lectin interactions. While unmodified graphene-on-metal surface showed no pref erential binding to lectins, noncovalent functionalization of the graphene network with mannose allowed the fabrication of a selective carbohydrate interface. The binding event between mannose-specific and mannose-unspecific lectins could be detected, and the magnitude of the signal could be used as a quantitative measure of the lectin concentration at hand. The easiness of the approach and the possibility to use any com mercially available sugar, disugar, or oligosaccharide without any additional synthetic effort to construct a specific carbohy drate interface might be appealing for glycobiologists and other researchers interested in such studies.

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**Figure 1:** Schematic representation of the transfer process of CVD growngrapheneonCuontoglass/ Ti/Au substrates.



**Figure 2:** (A) SPR curve before (black), after graphene transfer (blue) and after modification with mannose (100 mM) for 2. The dotted lines are the experimental date and the solid lines are SPR fits: nprism= 1.52, nTi = 2.36+i3.47 (d=5 nm); nAu = 0.197+i3.67 (d=50 nm); ngraphene = 3.0+i1.216; nmannose=670 nm



**Figure 3:** SPR binding curves of *Lens culinaris* (LC, 500 µg/mL) and *Maackia amurensis* (MA, 500 µg/mL) on gold and graphene-on-metal SPR interfaces

**(A)**



**(B)**



**Figure 4:** (A) SPR binding curves of *Lens culinaris* (LC, 500 µg/mL) and *Maackia amurensis* (MA, 500 µg/mL on graphene-on-metal SPR interfaces modified with mannose (100 mM); (B) Calibration curve for different *Lens culinaris* concentrations