A vNAR-Based Hybrid Nanobiosensor for Rapid and Selective HbA1c Detection: A Step Forward in Next-Generation Diabetes Diagnosis

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1. Experimental

1.1 Raman Spectroscopy and Spectral Deconvolution

Raman spectra of graphene oxide (GO) and reduced graphene oxide (rGO) were obtained using an Ocean Optics IDR Raman system equipped with a 785 nm laser source. The spectra were collected in the range of 1100–1800 cm^{-1} to analyze the D and G bands, which are characteristic of graphitic materials. To resolve the overlapping components associated with structural disorder and graphitic domains, spectral deconvolution was performed using Origin software, fitting combinations of Lorentzian and Gaussian functions. The intensity ratio between the D and G bands (ID/IG) was calculated from the integrated peak areas. The average crystallite domain size (La) of sp^2 carbon was estimated using the Tuinstra–Koenig relationship, along with its refinements for nanographitic materials. Additionally, the full width at half maximum (FWHM) values of both bands were extracted to assess structural disorder and the effects of electronic conjugation.

1.2 XPS Measurements of GO and rGO and Spectral Deconvolution

The chemical composition and surface functional groups of the synthesized graphene oxide (GO) and reduced graphene oxide (rGO) were analyzed using X-ray photoelectron spectroscopy (XPS). This technique allowed for the determination of the elemental composition and the chemical states of carbon and oxygen species based on their characteristic binding energies. XPS analyses were conducted using a SPECS® spectrometer equipped with a PHOIBOS® 150 WAL hemispherical energy analyzer, which has an angular resolution of less than 0.5°. The system used an XR 50 X-ray source and a μ-FOCUS 500 monochromator that operates with Al Kα radiation. The binding energies (BE) were calibrated by setting the C 1s peak to 284.8 eV to account for surface charging effects. High-resolution spectra of the C1s and O1s regions were recorded and processed after background subtraction using the Shirley method.[1] Peak fitting was performed through nonlinear least-squares deconvolution employing mixed Gaussian–Lorentzian functions. The C 1s spectrum was resolved into contributions from C–C/C=C (sp^2 carbon), C–O, and C=O species. In the O 1s spectrum, components corresponding to C=O, C–O–C, C–OH, and adsorbed species were identified. Peak positions, full width at half maximum (FWHM), and relative areas were determined to evaluate the chemical state of the surface and the extent of reduction of graphene oxide.

1.3 Morphological Analysis by 4K Digital Microscopy

High-resolution 4K digital microscopy was employed to examine the surface morphology of the rGO sheets. The exfoliated graphene sheets appeared as loosely dispersed flakes with lateral dimensions on the micrometer scale. At higher magnification, the sheets revealed heterogeneous topography with

three distinct height zones: flat regions with an average height of ~6.9 μm (blue areas), moderate elevations of ~20–34 μm (green and yellow areas), and prominent wrinkles reaching up to 45.2 μm (orange areas). These morphological features are consistent with structural deformations caused by oxygen-containing functional groups and defects introduced during oxidation and not eliminated during reduction. The rough texture observed in flat regions is likely due to residual hydroxyl and epoxy groups, while the more prominent wrinkles correspond to topological defects and incomplete removal of oxygen functionalities. Despite the presence of these imperfections, the remaining functional groups are advantageous for subsequent chemical conjugation, particularly for biomolecule immobilization, such as with vNAR domains. Thus, the structural characteristics observed by microscopy confirm that the synthesized rGO maintains sufficient surface reactivity for biosensor applications.

1.4 vNAR R007 ELISA recognition to glycated hemoglobin

To determine the recognition capacity of the purified vNAR antibody, an antigen recognition ELISA was performed. The recombinant glycated hemoglobins used were HbA1c (ExoCell Cat# HbA1), HbGly (ExoCell Cat# GlyHbA0), HbA2 (ExoCell Cat# HbA2), Hb0 (ExoCell Cat# HbA0) as control of non-glycated Hb. The hemoglobins were prepared at a concentration of 50 ng/mL in 1X PBS buffer. In a 96-well plate, 50 μL of each hemoglobin dilution was added in triplicate and incubated at 37 °C for 1 hour. The solution was discarded, and 150 μL of blocking solution (5% BSA in 1X PBS) was added to each well, which was then incubated for 1 hour at 37 °C. The previous solution was discarded, and the sample was incubated for 1 hour with a blocking solution containing 3% BSA in 1X PBS. Next, 1 μg of the purified anti-HbA1c vNAR R007 was fixed by incubating for 16 hours at 4 °C. Subsequently, three washes with 1X PBS-1X tween 0.05 were performed. As a positive control, a protein containing 6-His Tag was added, and as a negative control, 1X PBS was used. Then, the Anti-His antibody diluted 1:1000 in 3% BSA-1X PBS was added and incubated at 4 °C for 12 h. The solution was removed, and three washes with 1X PBS were performed. Finally, 50 μL of the TMB substrate was added to develop color by incubating for 15 min at 37 °C. The reaction was stopped by adding 2N HCl, and absorbance was measured at 450 nm. All assays were in triplicate.

2. Results

2.1 Raman Spectroscopy and Spectral Deconvolution.

The Raman spectra of GO and rGO exhibit the characteristic D and G bands of carbon-based materials (**Fig. S1**). GO exhibits D and G bands at 1318 cm^{-1} and 1594 cm^{-1}, respectively, while reduced graphene oxide (rGO) shows slight shifts to 1322 cm^{-1} and 1595 cm^{-1} after the reduction process. The intensity ratio of the D band to the G band (ID/IG) increases from 1.24 for GO to 1.27 for rGO. This increase indicates the formation of smaller but more numerous sp^2 carbon domains, with the

lateral dimensions (La) approximated at 73.50 nm for GO and 71.76 nm for rGO. Additionally, the G band narrows from 62.21 cm^{-1} to 58.47 cm^{-1}, suggesting a partial restoration of graphitic ordering. Conversely, the D band broadens from 71.58 cm^{-1} to 85.96 cm^{-1}, indicating increased defect heterogeneity. These results confirm the partial removal of oxygen functional groups and the reorganization of the sp^2 carbon network following reduction.

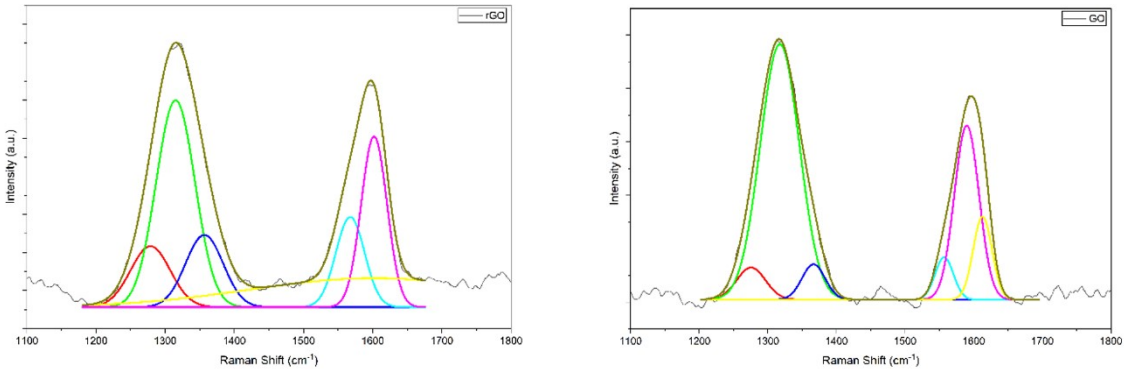

Fig. S1 Shows the deconvoluted Raman spectra of graphene oxide (GO) and reduced graphene oxide (rGO). The D band is located around 1320 cm^{-1}, while the G band appears near 1595 cm^{-1}. The fitted components reveal that the increase in the I_D/I_G ratio and the alterations in the full width at half maximum (FWHM) after reduction indicate a partial restoration of the sp^2 network, as well as changes in the defect density.

2.2 GO and rGO XPS analysis

The high-resolution C 1s spectrum reveals a dominant peak at 284.8 eV, which is associated with C–C and C=C bonds, indicating the presence of an sp^2 carbon network (**Fig. S2**). Additional peaks observed at higher binding energies (around 286–289 eV) correspond to oxygen-containing functional groups (C–O and C=O). The relative decrease in the intensity of these oxygenated components, along with the prominence of the sp^2 peak, suggests that successful chemical reduction has occurred, leading to a partial restoration of the conjugated graphene structure. In the O 1s spectrum, multiple contributions can be observed from carbonyl (C=O), epoxy/ether (C–O–C), and

hydroxyl (C–OH) groups. The distribution of these components indicates a partial removal of oxygen functionalities while retaining residual groups that allow for further surface functionalization.

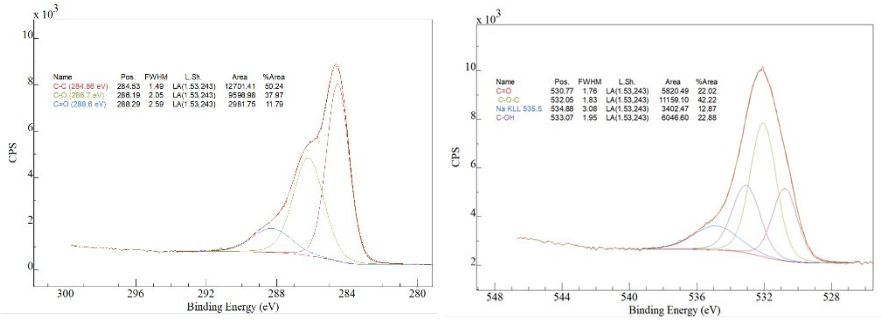

Fig. S2 High-resolution X-ray Photoelectron Spectroscopy (XPS) spectra and deconvolution results for reduced graphene oxide (rGO). On the left, the C 1s spectrum is deconvoluted into three components: C–C/C=C at 284.8 eV, C–O at approximately 286–287 eV, and C=O at around 288–289 eV. On the right, the O 1s spectrum is broken down into contributions from C=O, C–O–C, C–OH, and adsorbed species. The predominance of the sp^2 carbon peak and the reduced presence of oxygen-containing groups confirm the effective reduction of graphene oxide and the partial restoration of the conductive carbon network.

The characteristic peaks found are related to the negative conductivity of the nanomaterial (FWHM C=C). Specifically, a lower FWHM of the C=C band corresponds to a higher conductivity. Table S1 shows the FWHM C=C results for the synthesized GO and rGO nanomaterial samples.

Table S1. Peak positions and intensities of C1s and O1s for reduced graphene oxide (rGO).

C1S PEAKS	POSITION (EV)	%GO AREA	FWHM (CONDUCTIVITY)
C-C/C=C	284.8	56.10	1.64
C-O	286.6	34.74	2.0
C=O	288.7	9.16	1.95

O1S PEAKS	Position (eV)	%GO Area	FWHM (Conductivity)
C=O	530.97	23.56	2.05
C-O-C	532.24	49.51	1.95
C-OH	533.29	19.60	1.74

After GO reduction, there is a decrease in the C=C conductivity (FWHM), which indicates that the conductivity corresponds to the recovery of sp2. This observation, along with the decreased peak in the C-O region of the rGO sample spectrum, also indicates an increase in conductivity. In addition, the relative C/O atomic ratios of GO and RGO highlight the different degrees of reduction. Notably, the high-temperature process can significantly increase the C/O ratio.[2] Taken together, these results show a decrease in the peaks corresponding to the functional groups on the graphene surface after chemical reduction, confirming the elimination of oxygen in the film during the reduction process. The reduction in functional groups is indicated by the increase in conductivity, as reflected in the FWHM value. Finally, because the remaining oxygen groups on the rGO sheet enable chemical functionalization, the synthesized nanomaterial is suitable for hemoglobin biosensing.

2.3 4K Digital Microscopy of GO and rGO

High-resolution 4K digital microscopy was employed to examine the surface morphology of the rGO sheets (Fig. S3). The exfoliated graphene sheets appeared as loosely dispersed flakes with lateral dimensions on the micrometer scale. At higher magnification, the sheets revealed heterogeneous topography with three distinct height zones: flat regions with an average height of ~6.9 μm (blue areas), moderate elevations of ~20–34 μm (green and yellow areas), and prominent wrinkles reaching up to 45.2 μm (orange areas). These morphological features are consistent with structural deformations caused by oxygen-containing functional groups and defects introduced during oxidation and not eliminated during reduction. The rough texture observed in flat regions is likely due to residual hydroxyl and epoxy groups, while the more prominent wrinkles correspond to topological defects and incomplete removal of oxygen functionalities. Despite the presence of these imperfections, the remaining functional groups are advantageous for subsequent chemical conjugation, particularly for biomolecule immobilization, such as with vNAR domains. Thus, the structural characteristics observed by microscopy confirm that the synthesized rGO maintains sufficient surface reactivity for biosensor applications.

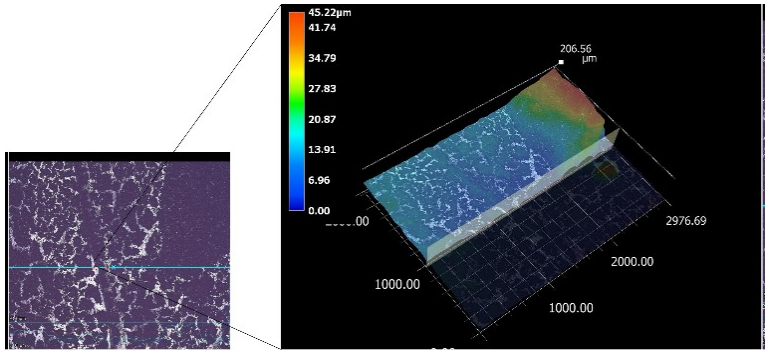

Fig. S3 4K digital microscopy of rGO sheets. High-resolution digital microscopy revealed that the synthesized rGO sheets possess an average thickness of approximately 6.9 µm. These morphological characteristics confirm successful reduction and structural integrity of the rGO material.

2.4 Anti-glycated Hb recognition ELISA

The results for vNAR recognition of glycated hemoglobin, including its variants, are presented in (**Fig. S4**). When comparing the recognition of glycated hemoglobin types (HbA1c, HbAGly, HbA2) with non-glycated hemoglobin (HbA0), the absorbance measurements indicate that vNAR strongly recognizes hemoglobin glycated at the N-terminus and lysines. Specifically, the absorbance values for HbA1c, HbGly, and HbA2 are higher than for HbA0 (non-glycated hemoglobin), demonstrating that the vNAR antibody preferentially recognizes glycated forms of hemoglobin rather than the non-glycated form (*P < 0.05).

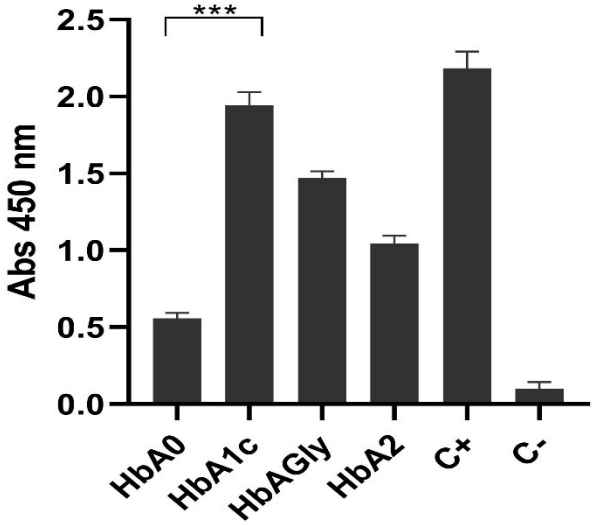

Fig. S4 ELISA for vNAR recognition against glycated hemoglobin. Detection of glycated hemoglobin, in its different variants, by the vNAR R007 domain.

In blood, total glycated hemoglobin includes all glycated variants of hemoglobin; however, their proportions vary (HBA1c 5.8%; HbAGly 3%; HbA2 1.6%) depending on the patient's glycemic status. HbA1c is the glucose-bound hemoglobin, which is found in greater proportion in the bloodstream and increases in hyperglycemic diabetic patients.[3] Therefore, the results obtained demonstrate that the anti-glycated Hb vNAR has the capacity to specifically recognize glycated hemoglobin in a sample of reconstituted Hb and in a greater proportion of HbA1c with respect to native Hb. Therefore, the protocol to generate the graphene-based hybrid biosensor was standardized.

References

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