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ORIGINAL PAPER

Graphene and graphene oxide for biosensing

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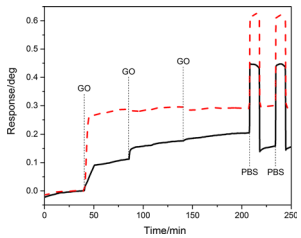
Abstract Graphene-based nanomaterials attract large attention in electrochemistry due to their unique properties. Reliable method to modify electrodes by graphene is necessary to obtain desired improvement. In this work, different sizes of graphite flakes for preparation of graphene oxide (GO) were tested and the final characterization of the resulting GO was focused on a quick and reliable methods such as Raman and UV–Vis spectroscopy, atomic force microscopy, and surface plasmon resonance. Smaller particles resulted in bigger yield with higher stage of oxidation. Although the average thickness of GO was ~1 nm, differences between GO and by ascorbic acid chemically reduced GO were minimal in topography. The binding and stability of reduced GO on gold surface and gold modified by cysteamine were studied by surface plasmon resonance and cyclic voltammetry. The cysteamine provided slightly higher loading capacity compared to bare gold electrode; however, cyclic voltammetry proved that the electrochemical properties are identical, and therefore, cysteamine is not in this case necessary for GO immobilization.

Graphical abstract



Keywords Biosensors · Electrochemistry · UV/Vis spectroscopy · Raman spectroscopy · Atomic force microscopy · Surface plasmon resonance

Introduction

Graphene-based materials have attracted wide attention in the last few years in many scientific fields. Graphene is a two-dimensional single-layered structure of carbon atoms with sp^2 configuration and many derivatives such as graphene nanoribbons, graphene oxide (GO) and its reduced form, as well as doped graphene materials. Graphene has shown many interesting properties such as large surface area (~2630 m^2 g^{-1} for single-layered graphene), high electrical conductivity, and unique optical, thermal, and mechanical properties [1–3].

Although graphene-based materials have many potential applications in capacitors, electronic nanosystems, chemistry, and drug delivery, they also became very popular in biosensing and electrochemistry in general. This is due to their excellent properties, but the relatively low production cost and similar electrochemical potential window as

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graphite [2.5 V in 0.1 M phosphate-buffered saline (PBS), pH 7.0] are also important advantages [2, 4, 5].

Several methods for graphene preparation are known. The most common ones include mechanical exfoliation, epitaxial growth, and various reductions of GO [5]. It is mostly GO which attracted big attention, since it contains several oxygen containing functional groups such as hydroxyl, carboxyl, and epoxide which make GO attractive for further modifications and applications [6, 7].

Graphene oxide was first reported by Schafhaeutl [8] and by Brodie [9]. Since then, many approaches for GO synthesis were developed, mostly based on the Hummers and Offeman method [10]—strong oxidation of graphite flakes. To retrieve graphene, further reduction is usually needed. Many reduction strategies have been reported, such as thermal, microwave, chemical (hydrazine, $NaBH_4$, pyrogallol, KOH, HI, or ascorbic acid), photocatalytic, electrochemical, or multi-step approaches [11, 12]. Reduction of GO results in a material which exhibits similar properties as graphene but lacks its structural perfection. Oxidation and reduction usually create structural defects and some residual functional groups which cause partial loss in electron mobility [5, 11, 13, 14].

Since the most approaches for GO synthesis and reduction are still experimental, further characterization of their product is needed and recommended. It is well known that even small changes in reaction procedures can cause very different results between different batches of produced GO. Moreover, impurities are usually present due to natural occurrence in graphite and some of them might be introduced during purification of GO [15].

In this work, atomic force microscopy, as well as UV–Vis spectroscopy, Raman spectroscopy, and surface plasmon resonance were used to characterize the GO for further biosensing applications. The binding of graphene to gold surface directly and using a cysteamine linker was compared with the focus on amount of bound material and its electrochemical properties.

Results and discussion

The preparation of various types/grades of GO was used improved Hummer's method (IHM) [16] and three different types of graphite flakes −200 mesh (∼74 μm), −100 mesh (∼150 μm), and −20 + 100 mesh (∼150–850 μm).

Table 1 shows the comparison of yields when using different sizes of graphite flakes. Optimal results were achieved when using graphite flakes of −100 mesh (F2), approximately the same size of particles used by Marcano et al. [16]. The total yield of this reaction was 5.6 g from 3.0 g of graphite flakes. On the other hand, when bigger particles were used such as −20 + 100 mesh (F3), the resulted product was mostly graphitic oxide with black-brownish color (Fig. 1), the total yield of this reaction was also very small compared to other two fractions.

Thus, prepared GO was further characterized by Raman spectroscopy, which is an effective and nondestructive method for characterization of carbon-based materials. Figure 2 displays typical D and G bands for GO with intensity ratios I_D/I_G of 0.95 for samples F1 and F3 and 0.90 for sample F2. The G-band is typically higher in GO, but as reduction proceeds, the D-band becomes more visible with higher relative intensity [13, 17–19]. The intensity ratio (I_D/I_G) is a very simple indicator of disorders, as it is inversely proportional to the average size of sp^2 clusters [20, 21].

UV–Vis spectroscopy was used to monitor direct chemical reduction of GO using ascorbic acid (AA). The position of absorption peak is around 231 nm and it is red-shifted from this value as the reduction proceeds [19, 22, 23]. Basically, the more this peak red-shifts, the more efficient the reductant is. AA was used as an alternative to toxic hydrazine which leads to peak maximum around 268 nm. In our measurements (Fig. 3), GO suspension showed the initial absorption maximum at 225 ± 1 nm, and after the treatment with AA, the chemically reduced GO maximum shifted to 265 ± 1 nm. Whole reduction process was completed within 10 min.

Table 1 Comparison of different graphite samples and resulted GO

Type/size	Amount of graphite flakes used/g	Obtained amount of graphene oxide/g	%	$I_{D\ 1337}$/cps[a]	$I_{G\ 1581}$/cps[a]	I_D/I_G
F1: −200 mesh (∼74 μm)	3.0	4.4	+47	2872	3030	0.95
F2: −100 mesh (∼150 μm)	3.0	5.6	+87	362	401	0.90
F3: −20 + 100 mesh (∼150–850 μm)	3.0	1.2	40	1473	1556	0.95

[a] I_D and I_G—Raman intensity of bands D and G; for further information, see text below and Fig. 2

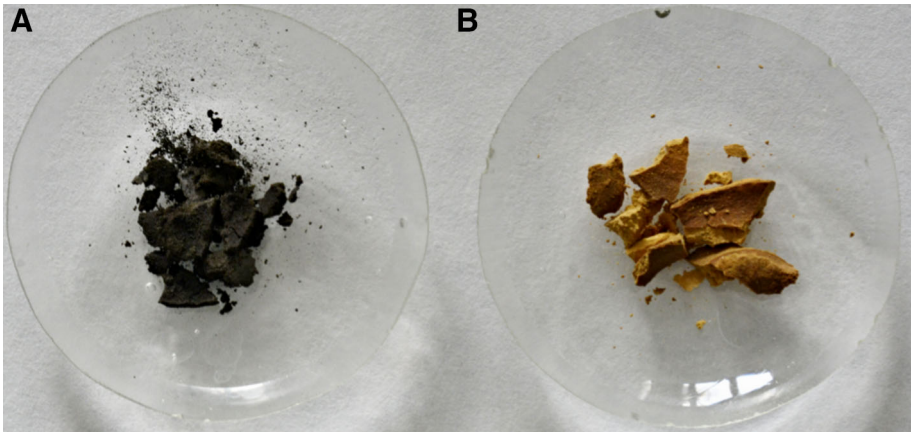

Fig. 1 Graphene oxide—two different products; **a** (F3) from −20 + 100 mesh graphite flakes; **b** (F1) from −200 mesh graphite flakes

The atomic force microscopy is a very powerful method for getting surface topography information about graphene and graphene oxide. The main reason to implement atomic force microscopy (AFM) measurements was to observe the structural changes, size, and shape of graphene nanosheets and to observe the differences between graphene oxide and chemically reduced graphene oxide.

The amount of functional groups varied depending on the method used for preparation of GO. The corresponding ratio C/O within the 4:1–2:1 interval was reported for GO preparations. Even after the reduction, the ratio was usually 12:1. The main problem is that different reduction processes lead to different extents of oxygen functional groups removal [11].

A typical pristine graphene sheet is atomically flat with reported thickness of ~0.34 nm. Graphene oxide, on the other hand, is expected to be thicker due to functional groups, ruptures, and various displacements of sp^3-hybridized carbon atoms above and below the graphene sheets. Typical reported thickness when using AFM in tapping mode is approximately 1.1 nm [11, 13, 16, 24, 25]. It was confirmed using AFM that the size of GO nanosheets significantly varies, although there were sheets neither thinner nor thicker than ~1.1 nm (Fig. 4a, b); multiples of this value are obtained for several overlaid layers.

GO chemically reduced by AA exhibited no big differences. In fact, several chemical approaches were tested (data not shown), all resulting in visible color change of the suspension from yellow-brownish GO to dark-grey/black, but still transparent suspension. However, the thickness remained the same (Fig. 4c, d). This supports the previously reported observations [23] of the remaining functional groups and disturbances in thus prepared graphene sheets. Similar effect as mentioned by Si and Samulski was also observed, and small holes and disruptions inside the graphene sheets were probably caused by sonification or reduction process [25].

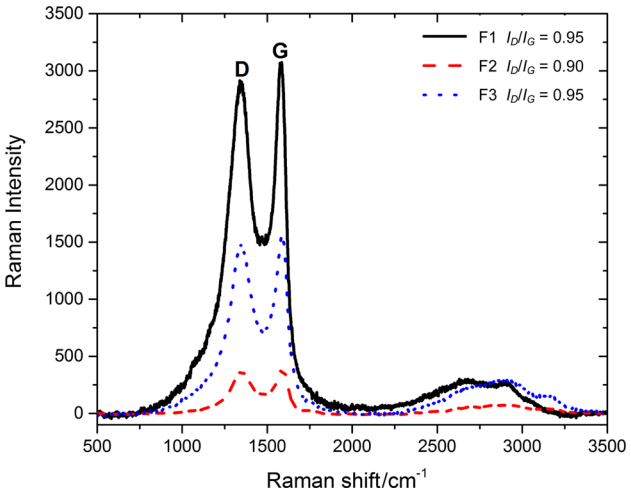

Fig. 2 Recorded Raman spectra. *Black full line* sample F1; *red dashed line* sample F2; *blue dotted line* sample F3. D-band of shift 1337 cm^{-1} and G-band of shift 1581 cm^{-1} (color figure online)

Graphene oxide, when used in electrochemistry, is usually bound to the surface of electrodes covalently using linkers such as cysteamine or APTES ((3-aminopropyl)triethoxysilane). Since GO contains variety of functional groups, remaining question is if such treatment/linker is actually necessary, as it creates another barrier and makes the surface preparation longer and more complex. For this purpose, surface plasmon resonance (SPR) was employed as a reliable method for a real-time observation of GO binding and stability on gold surface in the flow-through conditions.

Figure 5 shows that cysteamine modification improves the kinetics of loading process and that higher amount of GO can be bound compared to the clean gold surface. This is not very surprising, since a strong noncovalent interaction between cysteamine and GO is present. However, what is more important, clean gold electrode without any linker also shows significant covering by

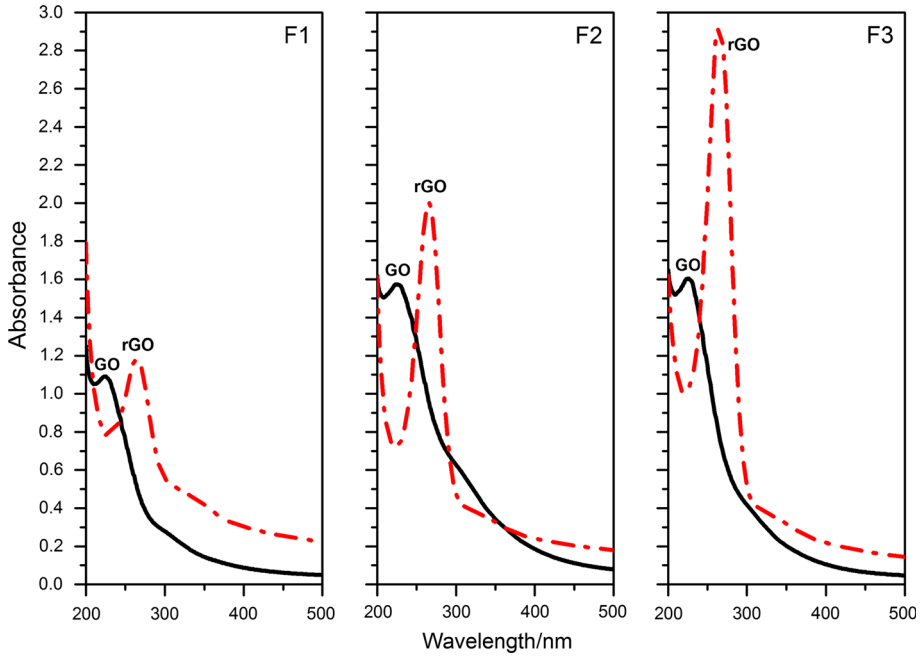

Fig. 3 Absorption spectra of the graphene oxide solutions (GO *full black lines*) with maximum at 225 ± 1 nm and by AA-reduced graphene oxide (rGO *dashed red lines*) with maximum at 265 ± 1 nm. The reduction time was 10 min. Samples F1–3 (color figure online)

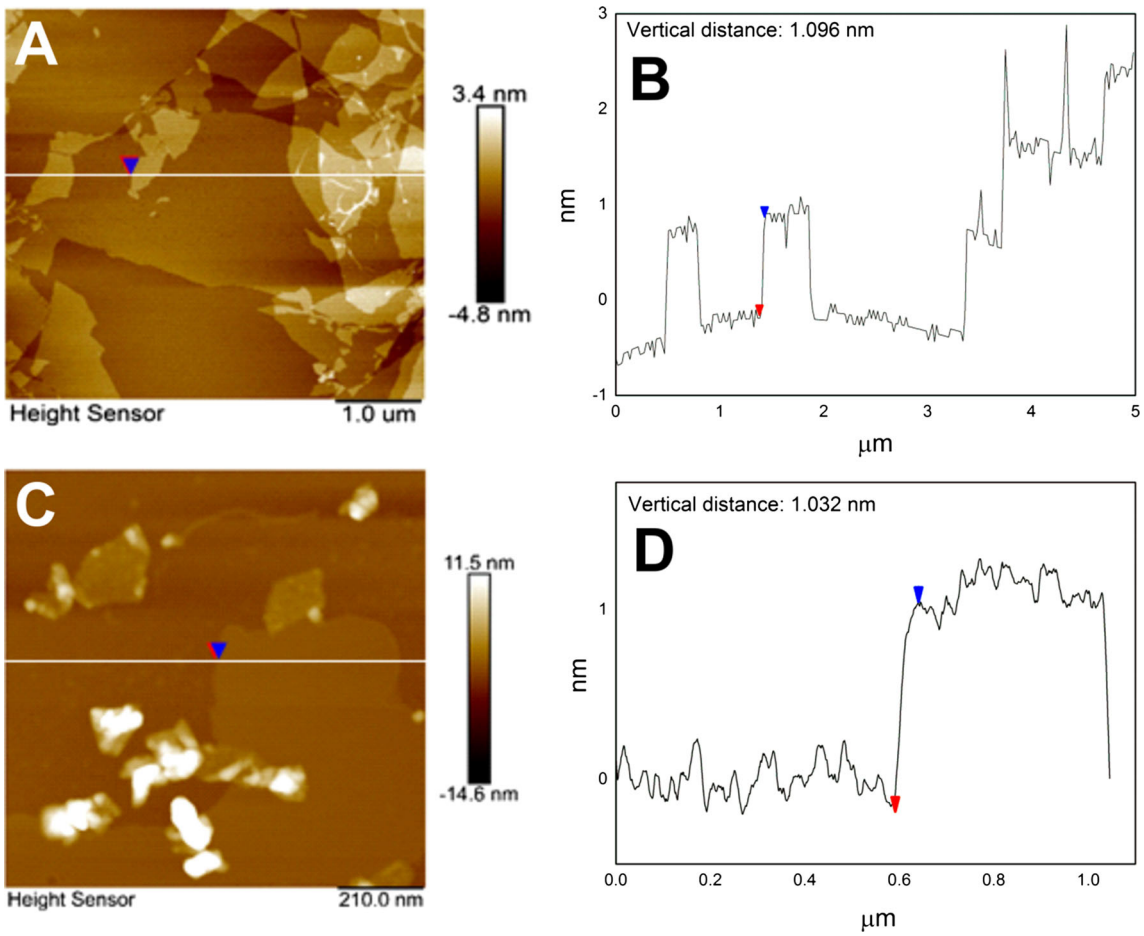

Fig. 4 Atomic force microscopy (AFM). **a** AFM image and **b** cross section of GO (F2) deposited on mica. The average thickness was ~1 nm; **c** AFM image and **d** cross section of GO (F2) reduced by AA (AArGO), deposited on mica. The average thickness was ~1 nm

GO, even after the surface is repeatedly washed by PBS (also Fig. 6a, b). This can help us in future modifications and in understanding of effectiveness of direct electrochemical reduction of GO.

Finally, simple electrochemical measurements were carried out to test the "non-cysteamine method". All electrodes were tested in the same solution under the same conditions to avoid any imprecisions. Figure 7 shows calibration dependencies of hydrogen peroxide (5, 10, and 15 mM, respectively) for pure gold electrode, electrode modified with electrochemically reduced graphene oxide (rGO) and with cysteamine and rGO. However, the most important are Fig. 7d–f which shows comparison between mentioned electrodes. At the end, there was no real difference between final responses of electrodes with and without cysteamine modification. Even though the cysteamine modification improves the loading of GO onto the electrode surface, it seems to be unnecessary, at least for electrochemical purposes.

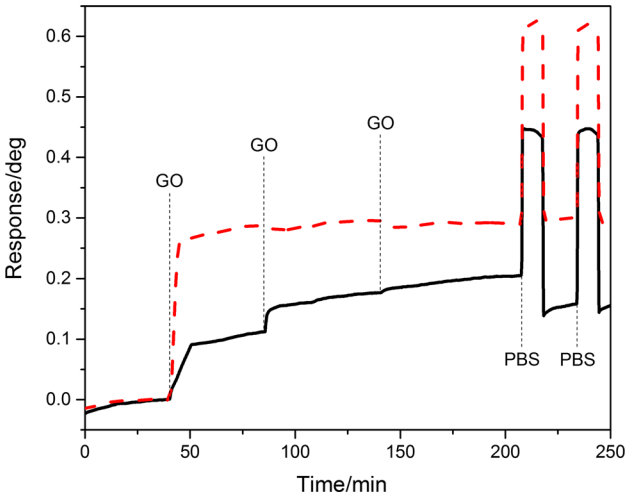

Fig. 5 SPR sensorgram of GO deposition on gold surface from water (pH 3). *Black full line* clean gold; *Red dotted line* gold surface modified by cysteamine monolayer. GO—injections of 50 μg cm^{-3}. PBS—injections of phosphate-buffered saline pH 7.4 (color figure online)

Conclusion

Graphene oxide is definitely very interesting material which provides many modification options. Although in many scientific fields, the pristine graphene layer is demanded, for electrochemistry and biochemistry, the laboratory prepared (reduced) GO seems sufficient for many potential applications.

Disorders in the sheet structure are sources of various functional groups. Even after reduction of GO, several functional groups still remain, which provide possibilities for further modifications. Since the laboratory preparation of GO is still very experimental, for electrochemical methods and biosensing, proper description and characterization of obtained GO is needed and should be always demanded. Even small changes in the conditions lead to different results, not mentioning the purification process which could be source of unattended doping of GO by foreign elements.

As for the application of rGO in electrochemistry, GO seems to have strong ability to attach to the surface of electrodes without any help of, e.g., cysteamine linker, which comes very handy, since one can simplify the deposition procedure and cut down time necessary for preparations.

Experimental

Graphite powders −20 + 100 mesh (∼150–850 μm), −100 mesh (∼150 μm), and −200 mesh (∼74 μm) (Alfa Aesar; www.alfa.com), hydrochloric acid, phosphoric acid,

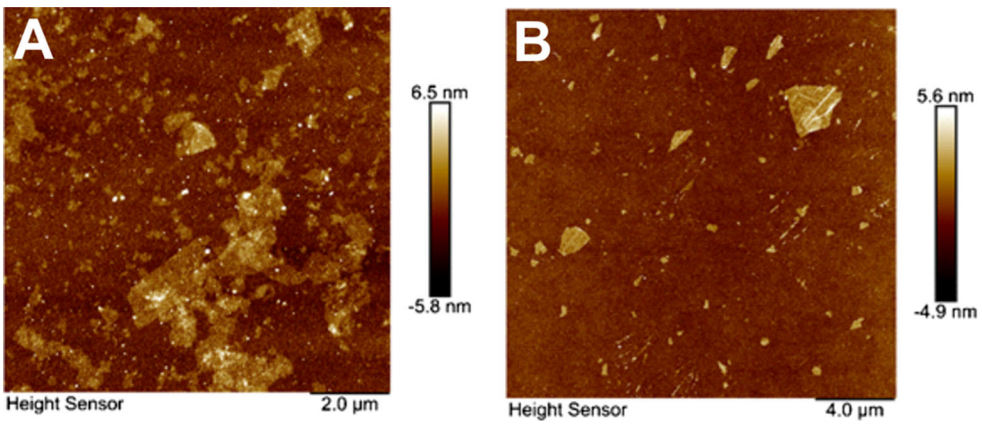

Fig. 6 Atomic force microscopy (AFM) of GO on surface of the SPR102 Au chip

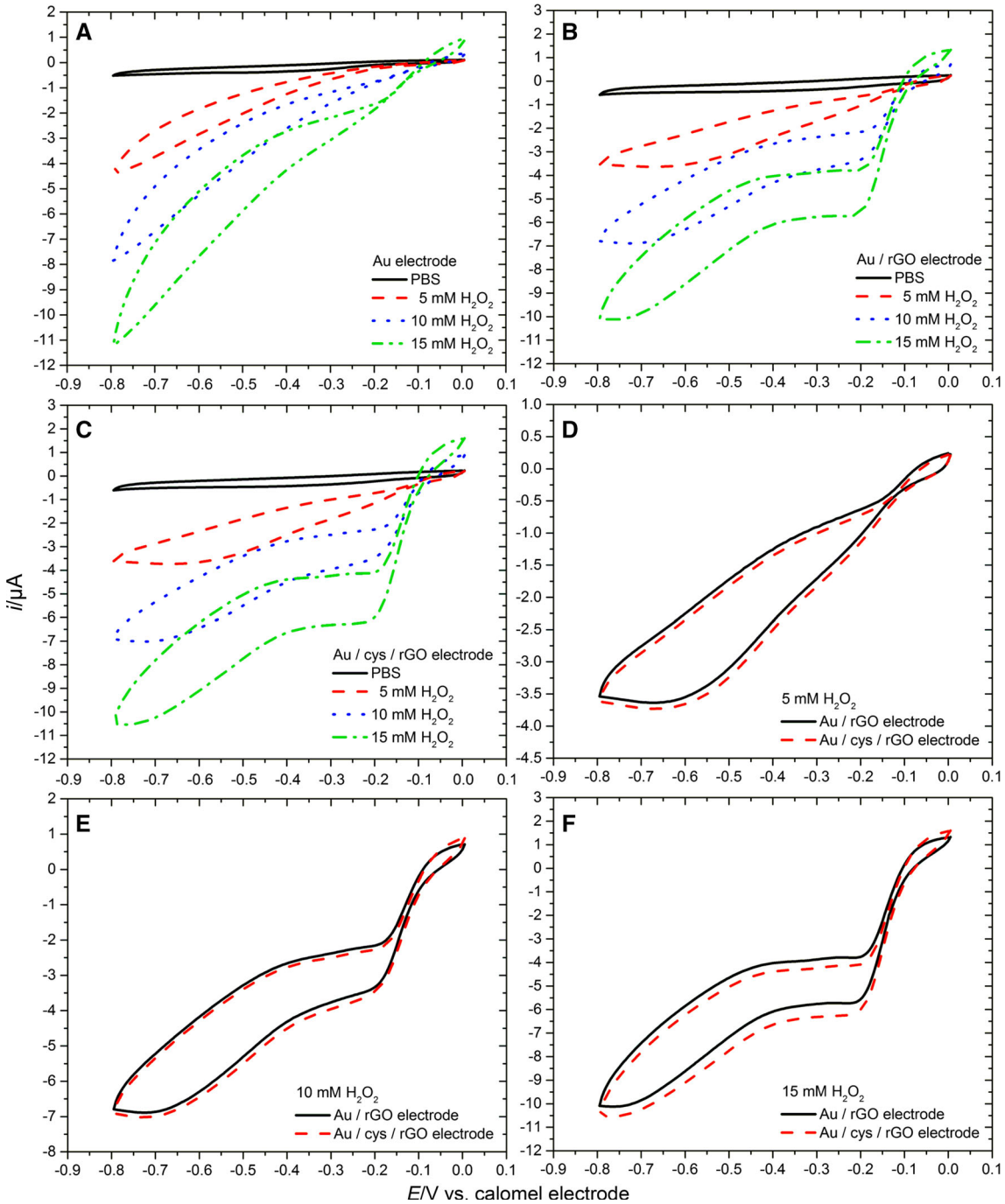

Fig. 7 Electrochemistry of graphene on gold surface with and without cysteamine. **a** Clean Au electrode without any modifications, **b** Au electrode covered with electrochemically reduced GO, **c** Au electrode covered with cysteamine self-assembled monolayer (SAM) and electrochemically reduced GO; **d–f** different concentrations of H_2O_2 (5, 10, and 15 mM); *Black full lines* Au electrode covered with electrochemically reduced GO; *Red dashed lines* Au electrode covered with cysteamine SAM and electrochemically reduced GO (color figure online)

potassium permanganate, ethanol, diethyl ether, hydrogen peroxide, sulfuric acid, ascorbic acid (Lach:ner; www.lach-ner.com), cysteamine hydrochloride (Sigma-Aldrich; www.sigmaaldrich.com), mica grade V-1 muscovite were purchased from SPI Supplies (www.2spi.com).

Preparation of graphene oxide

Samples of GO were synthesized according to the improved Hummers method as reported by Marcano et al. [16]. Graphene oxide was then diluted in acidified water pH 3.0, sonicated for

2 h, and centrifuged for 10 min at 4000 rpm to remove larger particles and stored in the fridge when not in use.

AFM

Atomic force microscope Dimension FastScan (Bruker, USA) was used to measure the topography of GO. The FastScan-A probe (Bruker) with spring constant 18 N/m and resonance frequency of cantilever 1400 kHz was used. The mica (1.5 cm × 1.5 cm) was cleaned with adhesive tape and remaining microelements were removed by compressed air. For imaging, a droplet (5 mm^3) of diluted GO was deposited on the surface and let to dry.

Raman spectroscopy

Raman spectra were recorded using micro-Raman spectrometer Horiba Labram HR Evolution with a 532 nm laser as excitation source. Samples were deposited in dry state on microscope slides.

Chemical reduction

Chemical reduction of GO using ascorbic acid (AA, 2 mM) was carried out at pH 9–10 (25% ammonia) water [19, 23], and concentration of GO was 0.5 mg cm^{-3}. The whole mixture was then incubated under stirring at 90 °C. The reduction process was monitored using UV–Vis absorption spectroscopy.

UV–Vis spectroscopy

For UV–Vis measurements, the microtiter plate reader Synergy 2 BioTec (Winooski, USA) together with 96-UV-transparent ELISA plates MTP PUREGRADE (Brand) was used. All measurements were recorded as a function of time. An aliquot of 300 mm^3 was used for recording spectra.

Surface plasmon resonance

The SPR experiments were performed on BioNavis 210A using gold chips SPR102 Au (BioNavis, Finland). The sensor surface modifications were performed outside the system, cysteamine (10 mg cm^{-3}, 2 h) was immobilized to one channel, while the other channel remained unmodified. The GO was diluted in weak HCl solution (pH 3) to represent the conditions for GO immobilization to electrodes, and the HCl solution was used as the running buffer. The experiments were performed with flow rate of 20 mm^3 min^{-1}.

Electrochemistry

For all electrochemical measurements, 0.05 M PBS with 0.1 M KCl, pH 7.4 at room temperature was used. Electrochemical measurements were carried out using PalmSens potentiostat/galvanostat (www.palmsens.com) in the standard 3-electrode setup. The modified Au electrode was used as a working one. Platinum wire and calomel electrode (3 M KCl) were used as the counter and the reference electrodes, respectively. All solutions were purged with nitrogen before measurements. Cyclic voltammetry was performed with 100 mV s^{-1} scan rate in the range from −0.8 to 0 V vs. calomel reference electrode (3 M KCl).

Electrodes (Au disk, 0.5 mm in diameter, embedded in glass) were cleaned prior to use, polished to mirror-like finish using 0.3 and 0.05 μm alumina slurries, and finally sonicated for 10 min in ethanol to remove any remaining particles from polishing.

Electrodes were further modified with cysteamine or/and GO solution. For cysteamine modification, 10 mM aqueous solution of cysteamine hydrochloride for 2 h was used, and then, the electrodes were transferred into 0.5 mg cm^{-3} solution of GO (pH 3.0) and incubated for 4 h.

After modification by GO, the electrodes were electrochemically reduced using cyclic voltammetry. Three scans were used in the range from 0.0 to −1.5 V vs. calomel reference electrode (3 M KCl), scan rate 0.03 V s^{-1}, and 0.5 M KCl which was used as an electrolyte.

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