Covalent-Bonded Reduced Graphene Oxide–Fluorescein Complex as a Substrate for Extrinsic SERS Measurements

Gordana Siljanovska Petreska,* Maitane Salsamendi,*† Alejandro Arzac,*† Gracia Patricia Leal,*† Núria Alegret,* Jadranka Blazevska Gilev,*§ and Radmila Tomovska*‡∥

†POLYMAT and Departamento de Química Aplicada, Facultad de Ciencias Químicas, University of the Basque Country UPV/EHU, Joxe Mari Korta Center, Avda. Tolosa 72, 20018 Donostia—San Sebastián, Spain
‡CIC biomaGUNE, Carbon Nanobiotechnology Group, Miramon Pasealekua 182, 20009 Donostia—San Sebastián, Gipuzkoa, Spain
§Faculty of Technology and Metallurgy, University of Saints Cyril and Methodius, Rudjer Boskovic 16, 1000 Skopje, R. Macedonia
∥IKERBASQUE, Basque Foundation for Science, 48011 Bilbao, Spain

ABSTRACT: When graphene is used as SERS substrates, it contributes to the chemical mechanism (CM) of enhancement of Raman signal, owing to which the detection limit is very low (lower than mM content of probe molecules). The CM of enhancement depends largely on the interactions between the substrate and the probe molecules. Therefore, in this work, we have investigated the possibility of increasing the SERS activity of graphene by improving the interaction between the probe molecule and the graphene substrate by establishing exclusively strong covalent bonding between them. Fluorescein (Fl) was selected as a probe molecule because it is one of the most commonly used fluorophore in bioscience. As a graphene substrate, reduced graphene oxide (rGO) platelets were used. In addition, silver nanoparticles (AgNPs) were added onto the hybrids to further increase the enhancement by electromagnetic (rGO) platelets were used. In addition, silver nanoparticles (AgNPs) were added onto the hybrids to further increase the enhancement by electromagnetic mechanism. Highly enhanced Raman signal of Fl onto neat rGO was achieved for micromolar concentration of the probe molecules. This was attributed to the covalent bonding between them, which introduced hole doping to rGO, decreasing the Fermi level of rGO and bringing it more closely to the LUMO of Fl. This induces aligning of their energy levels, resulting in higher contribution of the nonresonance effect to the charge transfer mechanism of enhancement, which, in this case, occurred intramolecularly. When AgNPs were added onto the rGO substrate, the expected enhancement performance was not observed. On the one hand, this was attributed to small size (∼20 nm) of AgNPs and lack of aggregates and, on the other, due to the unusually high contribution of CM determined.

1. INTRODUCTION

One or few layers of graphene1−5 or graphene oxide (GO)6,7 have been widely used as active substrates for surface-enhanced Raman scattering (SERS) analysis.8 Graphene-based surfaces, similar to the metallic surfaces, quench the fluorescence background of the analyte.9 Besides that, graphene-based substrates provide a large surface area, allowing adsorption of a high number of probe molecules.10,11 The main drawback of graphene-based SERS substrates is that they contribute only to the chemical enhancement (CE) of the signal; hence, the maximum enhancing factors achieved are usually in the range of 103−105.8,10,12,13

On the other hand, fluorescein (Fl) is a xanthene dye with a large variety of technical applications, among them as a fluorophore in bioscience. Owing to its large fluorescence quantum yield, low toxicity, and high photostability, it has been used as a tracer in groundwater, to label biological compounds, to detect corneal abrasions, and as a dye in food, drugs, and cosmetics. However, the fluorescence performance of Fl is sensitive to the environment in which it is used. The fluorescence is repressed in hydrophobic environments, whereas in aqueous (polar) environments (as the most of biological environments), it fluoresces strongly.14 This limits the application of Fl in biosensor devices. The combination of graphene-based substrates with Fl probe molecules is one of the ways to successfully eliminate the problems of both strong fluorescence background of Fl and weak enhancement of graphene.

When graphene was used as a substrate for SERS, the main mechanisms contributing to the CE of the signal are the vibrational coupling (VC) and the charge transfer (CT) between the probe molecule and the substrate. It was already demonstrated that the interaction of the probe molecules and the graphene substrate determines the extent of the enhancement.1,6 This means that the distance between the probe molecule and the substrate and the positions of molecular orbital (and probability of VC and CT) will be established by the type of the interaction created between the probe molecule...
and the substrate. All of the works using graphene-based substrates for SERS measurements are based on the adsorption of the probe molecule onto the surface of graphene platelets, resulting in low detection limit (lower than mM concentrations) for neat graphene substrates.\textsuperscript{8–12} The attempts to improve the graphene-based SERS are focused on the use of dendrimer morphology polymer chains\textsuperscript{5,15} or the use of Ag or Au nanoparticles in combination with graphene.\textsuperscript{16,17} In the past works, the presence of Ag or Au nanoparticles was likely responsible for the very high enhancement achieved; hence, the detection limits of nanomolar contents of probe molecules were reported.

In this work, we propose a new platform for graphene SERS measurements based on the creation of covalent bonding between the probe molecule and the substrate that is expected to principally improve the interactions between them. In case that the optically active part of the probe molecule is not affected while bonding, it is expected that the change in the interface induced by bonding will increase the probabilities for VC and CT and will result in increased contribution of the chemical mechanism (CM) to the enhancement. For this aim, Fl molecules were covalently attached to monolayer GO platelets by epoxy-ring-opening reaction, followed by reduction. In this way, we have designed composite substrates made of covalently attached Fl on the surface of reduced GO (rGO), with the main idea of creating hybrid SERS substrates that can be used for extrinsic SERS measurements. To increase further the SERS activity by giving rise to electromagnetic (EM) enhancement, we have decorated the hybrid substrate with silver nanoparticles (AgNPs), created in situ from AgNO\textsubscript{3} precursor that was simultaneously reduced during GO reduction.\textsuperscript{18–22} By the synthesis of two types of substrates rGO–Fl and rGO–AgNP–Fl, we created a base for the comparative study of CM versus combined CM and EM enhancements. As a result, the neat graphene substrates with covalently attached Fl have shown unusually higher enhancement than that reported for pristine graphene with adsorbed probe molecules.

### 2. RESULTS AND DISCUSSION

Attachment of Fl onto the GO surface (Scheme 1) was carried out by the reaction of nucleophilic substitution, where the C–O bond in epoxy functionalities present on the basal plane of GO\textsuperscript{23} was broken by the attack of a weak nucleophile—Fl molecule under acidic conditions (pH = 4.3) and increased temperature (60 °C). As a result, covalent bonding of Fl onto the GO surface took place. The synthesis procedure is presented schematically in Figure 1, whereas the synthesis conditions and samples' nomenclature are presented in Table 1.

Covalent attachment of Fl onto the GO platelets was demonstrated by UV and Fourier transform infrared (FTIR) spectroscopic analyses of the thoroughly cleaned samples by 5 + 2 subsequent centrifugations, during which it was supposed that the not attached Fl was removed from the samples. Aggregated and restacked GO platelets were simultaneously eliminated, resulting in exclusively one layer of Fl-modified GO platelets (Fl–GO), as demonstrated later by transmission electron microscopy (TEM) and atomic force microscopy (AFM) analyses. After the Fl attachment, the Fl–GO was reduced to Fl–rGO. A part of the Fl–GO platelets were decorated with AgNPs by simultaneous reduction of GO and AgNO\textsubscript{3} by AsA. It was previously reported that oxygen-containing functional groups of carbon materials adsorb Ag\textsuperscript{+} by means of electrostatic interaction and the adsorbed ions are reduced at the surface of the material.\textsuperscript{24,25} Therefore, the simultaneous reduction of Fl–GO and AgNO\textsubscript{3} was expected to result in the formation of hybrid Fl–rGO sheets decorated with strongly adsorbed AgNPs (Fl–rGO–Ag). The reduction was performed at two different temperatures (25 and 60 °C) to investigate the effect of temperature on the AgNP size, aggregation, interactions with rGO sheets, and morphology.

As shown in Figure 2, where the ultraviolet–visible (UV–vis) spectra of the hybrid sheets are presented, the spectra of GO (Figure 2a) display a strong absorption band at 237 nm (attributed to $\pi\rightarrow\pi^*$ transitions of aromatic C=C bonds) and a shoulder band at around 300 nm (attributed to n–$\pi^*$ transitions of C=O bonds). The Fl dye exhibits several absorption bands at 490, 328, 285, and 239 nm (Figure 2b).\textsuperscript{26} The presence of the 490 nm band in the Fl–rGO(60) and Fl–rGO(25) samples after thorough purification clearly indicates the attachment of Fl onto the GO sheets.

The growth of AgNPs onto the graphene sheets is manifested by the presence of the new peak at around 440 nm, assigned to the surface plasmon resonance absorption band of Ag. The observed red shift from 237 to 270 nm and the shoulder band disappearance at around 300 nm in the spectra of Fl–rGO(60) and Fl–rGO(25) samples indicate revival of the conjugated C=C bonds as a result of GO reduction (Figure 2c,d). It is worth mentioning that the spectrum (c) in Figure 2, owing to the appearance of a broad band without clear peaks, was measured few times on repeatedly prepared samples, and the same spectra were observed in all cases. We attribute this band to the irregular AgNP growth and distribution along the graphene platelets and the interactions between them. In addition, there is an overlapping with the 490 nm peak corresponding to Fl.

The indications obtained by the analysis of the UV–vis spectra are confirmed by FTIR spectra, presented in Figure 3, where the characteristic bands of GO are presented: 1725 cm\textsuperscript{-1} attributed to stretching vibration of C=O, 1636 cm\textsuperscript{-1} attributed to skeletal vibration of unoxidized graphene domains, 1395 cm\textsuperscript{-1} attributed to O–H deformation vibrations of C–OH, and 1056 cm\textsuperscript{-1} attributed to the epoxy group (C–O–C).\textsuperscript{25,27} In Figure 3i, the established bonding of GO and Fl is...
confirmed: after the reaction, the C−O−C groups (peak 1056 cm⁻¹, Figure 3i, spectrum a) from GO disappeared, so it is not present in the Fl−GO sample (Figure 3i, spectrum b). It has been demonstrated by theoretical calculations that the epoxy and hydroxyl functionalities are stable on GO up to 70 °C and even contribute toward stabilization of GO,28 which means that the complete loss of the characteristic peak of epoxy functionalities in GO during the reaction demonstrates the chemical bonding. Owing to the aromatic structure of Fl, there is a possibility of establishing a π−π bond with GO. However, no indications for that were observed.

Furthermore, the characteristic peaks for Fl, centered at 1243 and 1211 cm⁻¹ (C−O−C stretch of xanthenes ring),29 were slightly shifted in the spectra of Fl−GO hybrid toward lower wavenumbers, confirming changes in the chemical environment of Fl, likely provoked by the attachment of Fl onto GO. In Figure 3ii, where the samples after simultaneous reduction of GO and AgNO₃ are presented, the same indications are obvious (Figure 3ii spectra c and d).

The structure and morphology of the hybrids obtained under different reaction conditions were investigated by TEM. In Figure 4a, the neat rGO platelet deposited from polyvinylpyrrolidone (PVP)-stabilized aqueous dispersions is shown. In Figure 4b, the rGO platelets with attached Fl are presented. The hybrid structures are made of wrinkled and bent platelets decorated with small black hills probably made of some graphitic structures, from which a kind of neuron wrinkles emerge. In addition, the presence of grayish organic matter is noticeable, not presented in neat rGO (Figure 4a), hence attributed to the Fl dye. The same morphology is kept after the addition of AgNPs, which can be noticed as black spherical nanoparticles deposited on the top of the platelets (Figure 4c−f). The influence of reduction conditions on the morphology becomes obvious, by comparing Figure 4c,d (reduction performed at 25 °C/72 h) with Figure 4e,f (reduction performed at 60 °C/4 h). At lower temperature, AgNPs are deposited in higher number and with minor size, observed as black dots with size <10 nm dispersed over the platelets (Figure 4d). The plausible reason for this can be the faster reduction...
reaction at an increased temperature that leads to rapidly growing particles and their clustering before they were reached by the PVP stabilizer. Therefore, as shown in Figure 4f, the AgNP size in the samples reduced at 60 °C is >20 nm. Nevertheless, as theoretical and experimental studies have shown, the optimal particle size for the strongest SERS enhancement is observed for nanoparticles with the size of 20–70 nm.22,31

As the surface properties and the morphology are of extreme interest for SERS activity, the samples were analyzed by AFM, and the results are presented in Figure 5. Lateral dimensions of rGO platelets are within a range of 500 nm to 3 μm, with an observed average of 1.1 ± 0.5 μm. The images show overlapping of the individual rGO layers, which most likely results from the method employed to prepare the samples. The observed single-layer rGO platelets have significantly higher thickness than the one found in the literature for graphene that is close to 0.7 nm32 and higher than that of the neat rGO platelets stabilized with PVP (2 nm31). The average thickness of some of the samples is presented in Table 2.

The layer thickness indicates either a partial reduction of GO or it comes from the presence of PVP on the surface of the platelets, as demonstrated previously.33 The covalently attached Fl additionally contributes to the increased thickness observed. Moreover, it generates agglomerates on the surface of the platelets that increase their roughness as can be observed in Figure 5b, where a phase image of the Fl-coated rGO is presented [sample Fl−rGO(25)]. Owing to the very small dimensions of the AgNPs, it is almost impossible to observe them in Figure 5; although by comparing Figure 5c,d, the effect of reduction conditions on the morphology becomes clear. The hybrids containing AgNPs present slightly lower thickness, indicating that the subsequent reduction reactions performed at different temperatures and in the presence or absence of AgNO3 influence the amount of Fl. To explain this phenomenon, we hypothesize that attachment of Fl was not stoichiometric because of the high tendency of Fl to form aggregates;34 thus, instead of single molecules, these aggregates were covalently attached to the GO surface. After the addition of AsA for the subsequent reduction, there is disaggregation owing to intermolecular hydrogen bonding between Fl and AsA and later on, loss of the disaggregated Fl molecules occurred during purification. The effect of disaggregation is highly accelerated by the presence of AgNPs that results even in lower Fl contents attached to the GO surface, an effect that is always observed and reported.34

Figure 3. FTIR spectra of [i(a)] Fl, [i(b)] GO, and [i(c)] Fl−rGO(25) and [ii(a)] Fl, [ii(b)] GO, [ii(c)] Fl−rGO−Ag(25), and [ii(d)] Fl−rGO−Ag(60).

Figure 4. TEM micrographs of neat rGO (a), Fl−rGO(60) (b), Fl−rGO−Ag(25) (c,d), and Fl−rGO−Ag(60) (e,f).
2.1. SERS Activity of Fl–rGO and Fl–rGO–Ag Substrates. Figure 6 presents the Raman spectra of the blank samples, prepared for comparison with the hybrid substrates. Raman spectrum of GO with its characteristic D band at around 1348 cm$^{-1}$ (originated from the defects in carbon atoms) and the G band at around 1600 cm$^{-1}$ (corresponding to the ordered sp$^2$ in plane vibration of carbon atoms$^{35,36}$) is presented in Figure 6a. Figure 6b shows the Raman spectrum of Fl on Si substrates, where the intensive fluorescence is obvious and the skeletal vibration modes of the xanthene moiety appear in the frequency range from 1000 to 1800 cm$^{-1}$. Raman spectrum of a hybrid substrate made of Fl–rGO presents only the characteristic D and G peaks of GO (Figure 6c). This indicates that GO reduction is necessary to induce SERS activity, as it was reported previously.$^6$ Finally, the simple blend of Fl with rGO was prepared by mixing a solution of Fl with the previously reduced GO (under the same conditions as used in rGO–Fl, with a ratio of 1:1 in weight between Fl and GO). Since, in this case, Fl molecules were expected to be only adsorbed onto rGO platelets, this will demonstrate the importance of bonding between the probe-molecule and the substrate. Figure 6d presents the Raman spectrum of this sample, where the Fl characteristic peaks appear simultaneously with the D and G peaks of the rGO substrate. In comparison to the Fl spectrum on the Si substrate (Figure 6b), the fluorescence is decreased significantly.

In Figure 7, the Raman spectra of the hybrid samples are presented. The slight shift was observed on the Raman signals in all spectra respective to the spectrum of the free Fl molecules in Figure 6b. This shift is attributed to the covalent bonding between Fl and GO, and the shape (sharp and narrow) of the peaks verifies the absence of free Fl molecules, as one may expect broad peaks from the contribution of both Fl free and Fl attached molecules. Furthermore, significant enhancement has been observed for all hybrids, except for Fl–rGO(60), where the rGO characteristic peaks are dominating in the spectrum (Figure 7b). The influence of the synthesis conditions is quite

![Figure 5](https://example.com/figure5.png)

Figure 5. Morphology of the hybrid samples analyzed by AFM: (a) height image of Fl–rGO(25), (b) phase image of Fl–rGO(25), (c) height image of Fl–rGO–Ag(25), and (d) height image of Fl–rGO–Ag(60).

![Figure 6](https://example.com/figure6.png)

Figure 6. Raman spectra of (a) GO (0.5 mg GO/mL dispersion), (b) Fl (3.33 mg Fl/mL dispersion), (c) Fl–GO (3.33 mg/mL), and (d) Fl–rGO blend (3.33 mg/mL).

### Table 2. Average Thickness of the Samples

|          | Fl–rGO(25) | Fl–rGO–Ag(25) | Fl–rGO–Ag(60) |
|----------|------------|---------------|---------------|
| average thickness (nm) STDEV | 3.02 (1.27) | 2.67 (0.59) | 2.86 (0.73) |
noticable. The hybrids obtained by reduction at 25 °C for 72 h (Figure 7a,c), in general, present much better Raman spectra of Fl than the ones reduced at 60 °C for 4 h (Figure 7b,d).

The bad performance of Fl–rGO(60) is attributed to the nonuniform distribution of Fl along rGO platelets as it may be observed in the TEM images (Figure 4b), where neat rGO areas not covered with grayish organic matter is noticeable. In the case of Fl–rGO(25), Fl is well-distributed along the whole platelets, which is in good agreement with the AFM images in Figure 5a,b. In addition, it seems that, in presence of AgNPs, lower amount of Fl was present in the samples, as concluded from the determined thicknesses of the samples by AFM. On the other hand, in the samples containing AgNPs, as presented in TEM images, the AgNPs were smaller in size (<10 nm) and better dispersed throughout the platelets when compared under mild reduction conditions (Figure 4c,d), which obviously is more properose to the SERS activity than the irregular distribution of larger AgNPs. Furthermore, the particle size of AgNPs obtained at 60 °C, which is around 20 nm, is still too tiny for a proper Raman enhancement (Figure 4e,f). With all this in mind, only the Raman spectra of hybrid samples reduced at 25 °C will be taken into account for the discussions of the achieved enhancement.

To study the effect of the CE versus the CE accompanied by EM of the hybrid samples, nondecorated Fl–rGO(25) platelets (Figure 7a) and Fl–rGO–Ag(25) platelets decorated with AgNPs (Figure 7c) were compared. To calculate the enhancement factor, a precisely determined content of attached Fl is needed. Thermogravimetric analyses (TGA) measurements were used for this aim; however, we were able to determine only the Fl content within the samples that do not contain AgNPs. The probable reason for that could be the enhanced combustion of rGO-containing AgNPs. Owing to the high heat conductivity of AgNPs, it acts as a heat sink and makes the transfer of heat within the samples more efficient. It was determined by TGA that Fl concentration in the samples Fl–rGO(25) and Fl–rGO(60) was around 3 × 10⁻⁶ mol/L, with a small difference of 5% between them, which is rather expectable because of the temperature differences in the reduction of GO and the possible manual errors that can be made during the purification step by centrifugation.

In such conditions of lack of the precise values for the attached Fl in all samples, their SERS performance was evaluated by comparison of the normalized values of the intensities of Fl characteristic peak centered at 1492 cm⁻¹ (pointed by black arrows in Figure 7). The intensity of the peaks was normalized using the intensity of the characteristic peak of silicon wafer, centered at about 520 cm⁻¹, as a reference. Values of 2.26 and 9.43 were obtained for Fl–rGO(25) and Fl–rGO–Ag(25), respectively, whereas, for the neat Fl sample (deposited on Si substrate), it was 1.49. Having in mind that the content of the attached Fl onto hybrid samples is at the micromolar level and that of neat Fl is at the millimolar level, obviously very high enhancement by the hybrid substrates was achieved for both.

On the other hand, the CE observed for the Fl–rGO(25) sample in Figure 7a is only four times inferior than the combined enhancement observed for the Fl–rGO–Ag(25) sample, where the additional contribution of the EM is present. Usually, this difference is much higher. For example, as reported for the methylene blue probe molecule adsorbed onto the rGO surface decorated with AgNPs, the Raman intensity was 350 times higher than that obtained onto the rGO surface. On the other hand, the difference was further lowered by the unusually high CE achieved for the Fl–rGO(25) sample, which allows a very clear identification of micromolar Fl contents. To the best knowledge of the authors, this is the lowest concentration of a probe molecule for which an excellent Raman fingerprint spectrum was achieved using neat rGO as a substrate. Reported values of a detection limit of probe molecules on rGO are in millimolar ranges and refer to the identification of the probe molecule. Usually, good spectra are achieved for a much higher probe molecule content. The remarkable Raman enhancement observed for Fl–rGO(25) is associated with the introduced bonding between the probe molecule and rGO substrate, which is the unique difference between our work and the works reported in the literature (referenced previously). It is well-known that graphene substrate exclusively offers CE and that three different effects contribute to it: nonresonance chemical probe molecule–substrate interactions, CT transitions between the molecule and the substrate, and resonance effect of the incident light and molecular transitions (overlapping of the molecular orbital between the molecule and the substrate).

We hypothesized that owing to the covalent bonds between the probe molecules and the rGO substrate, all mechanisms contribute to the significant enhancement achieved. By the covalent attachment of Fl, the proximity of the Fl molecules to the rGO surface and their positioning obviously facilitate the CT, which is the intramolecular CT in this case. By comparison of Raman spectra of Fl in Figures 6b and 7, a blue shift of the Fl characteristic peaks onto SERS substrates is determined, which is a piece of evidence for the electron CT in the direction from Fl to rGO. This is consistent with hole-doped graphene that yields larger Raman-enhanced signal, as demonstrated by Hao et al. As the xanthene ring in Fl connected through the
oxygen bond to the rGO structure is an electron acceptor, one could accept that the proximity of it will introduce a hole-doping in rGO. By comparing the spectra of hybrid samples and that of GO (Figures 6a and 7), the hole-doping of rGO in the hybrids is proven by the upshift of the G peak in the doped samples.\textsuperscript{41} On the other hand, the hole doping induces a decrease in the Fermi level of graphene,\textsuperscript{40} making it aligned with the lowest unoccupied molecular orbital (LUMO) of Fl and giving rise to the contribution of the nonresonance effect to the overall enhancement achieved. Additionally, the resonance effect could be increased if a kind of tuning of molecular resonance relative to the wavelength of the incident light occurred.\textsuperscript{40}

We do not have any further experimental evidence to confirm the hypothesis and to determine the exact mechanism. Further experiments combined with theoretical calculations can help to elucidate it; however, it is beyond the scope of the presented work.

3. CONCLUSIONS

Two types of hybrid samples based on covalently attached Fl onto rGO platelets with and without AgNPs were developed and tested as SERS substrates for extrinsic SERS measurements. The main idea was to improve the interactions between the substrate (rGO) and the probe molecule (Fl) that may lead toward improving the contribution of rGO, that is, chemical-enhancing mechanism to the overall enhancement.

The Fl attachment to GO was successfully performed by epoxy-ring-opening reaction, followed by GO or GO–AgNO\textsubscript{3} reduction at two different temperatures (25 and 60 °C). The changes were monitored by UV–vis and the attachment was proven by FTIR spectroscopy. The hybrid platelets were characterized by means of TEM and AFM imaging, and the quantity of the dye attached onto the rGO sheets was estimated from the TGA measurements.

It was demonstrated that the reduction conditions, such as temperature or time, were relevant when obtaining rGO substrates with uniformly distributed Fl molecules, since the covalent bonding of Fl to rGO was not stoichiometric.

Almost all hybrid samples presented high enhancement of the Raman signal of Fl, with a superior effect obtained from the substrates reduced under mild conditions (25 °C) during longer time. By comparison of the intensity of the Fl–characteristic peak centered around 1490 cm\textsuperscript{-1}, the performance of the neat rGO and rGO decorated with AgNP hybrids were compared, giving further details about CM versus the combined CM with EM mechanisms of enhancement. The combined mechanism of enhancement was only fourfold higher than CM owing, on the one hand, to the lower than expected EM contribution of the presence of AgNPs because of the very small size of the nanoparticles and absence of aggregates. On the other hand, the small difference observed between CM and combined mechanism was attributed to very high CE induced by rGO owing to the covalently attached Fl, which allow much better interactions between rGO and Fl. Chemical attachment of Fl onto rGO induced the hole doping of rGO, decreasing the Fermi level of rGO close to the LUMO of Fl. This alignment of their energy levels resulted in increased contribution of nonresonance effect to the CT mechanism of enhancement.

The developed hybrid materials create a base for development of unique biosensor because by association of the substrate with biomolecules of interest, the SERS spectrum of the tag (Fl) can be used for sensing and quantification.

4. EXPERIMENTAL SECTION

4.1. Materials. GO aqueous dispersion with concentration of 5 mg/mL composed mainly of 1–3 layered platelets (60% is one layer) was purchased from Graphene supermarket HC. Fl (Fl, C\textsubscript{20}H\textsubscript{12}O\textsubscript{5}, Fluka); PVP (C\textsubscript{6}H\textsubscript{9}NO)\textsubscript{x} with M\textsubscript{w} = 29 000 (PVP, Aldrich); silver nitrate (AgNO\textsubscript{3}, Aldrich); and l-(-)-ascorbic acid, 99%, M\textsubscript{w} = 176, 13 (AsA, ACROS) were used as received without further purification.

4.2. Methods. Schematically, the whole preparation procedure of aqueous hybrid dispersion of Fl–rGO and Fl–rGO–Ag is presented in Figure 1.

4.2.1. Covalent Attachment of Fl onto GO. Methanol solution of Fl (15 mL, concentration 3333 mg/mL) was added into 100 mL of diluted aqueous dispersion of GO (0.5 mg/mL, pH 4.3). PVP (1 wt %) with respect to GO was added as a stabilizing agent to prevent aggregation of the platelets after reduction.

The dispersions were stirred at 60 °C overnight during which epoxy-ring-opening was expected to occur and initiate the reaction with the hydroxyl group of Fl, resulting in a covalent attachment of Fl onto graphene sheets. To remove the unattached Fl, the dispersions were then centrifuged (five times at 10 000 rpm at 20 °C, each lasting 15 min) and washed with de-ionized water.

4.2.2. Reduction of Fl–GO and/or Fl–GO–AgNO\textsubscript{3}. After the covalent attachment of Fl to the surface of GO, 20 mL of the purified dispersions were reduced by adding 1 mL AsA solution (100 mg/mL). In the case of Fl–rGO–Ag, prior to the reduction process, 1 mL of AgNO\textsubscript{3} aqueous solution (6 mg/mL) was added and the reductions of both GO and AgNO\textsubscript{3} proceeded simultaneously. The reduction was carried out by stirring at two different conditions: 4 h at 60 °C and 72 h at 25 °C. The Fl–rGO and Fl–rGO–Ag dispersions were then centrifuged twice at 15 000 rpm for 70 min in total.

4.3. Characterization. Chemical changes in the structure of graphene platelets as a result of the reduction of GO and AgNO\textsubscript{3} as well as covalent attachment of Fl on GO sheets were investigated by the UV–vis Shimadzu UV-2550 spectrometer with the wavelength range from 200 to 900 nm. Successful reduction of GO was demonstrated by the disappearance of the shoulder band at 300 nm (C≡O) and red shift from 230 to 270 nm of the strong absorption band of C=C. Appearance of the new band at about 440 nm in UV spectra is because of the surface plasmon resonance that corresponds to the excitation of AgNPs, which confirms the presence of AgNPs in the samples. FTIR spectra were recorded on an ALPHA coupled to a Golden Gate single attenuated total reflectance unit. The variation in the intensities of characteristic bands of the C–O–C moiety at 1056 cm\textsuperscript{-1} in the spectra of GO and at 1243 cm\textsuperscript{-1} in the spectra of hybrid samples was followed as an indication of introduced chemical modifications.

The TGA was performed under a nitrogen atmosphere (25 mL/min flow rate) using a TGA Discovery 5500 analyzer (TA instruments). The samples were equilibrated at 100 °C for 20 min and then heated at a rate of 10 °C/min, in the range from 100 to 800 °C. For the calculation of the concentration of Fl within rGO in a hybrid sample (Fl–rGO), the TGA of rGO (PVP containing) and Fl samples were measured as blank samples. It is worthy to mention that the blank rGO was obtained following all steps for Fl–rGO preparation, to eliminate the possibilities of influencing the composition of rGO by the procedures. Very briefly, 1 wt % of PVP with
respect to GO was added to 100 mL of diluted aqueous dispersion of GO. The dispersion was stirred at 60 °C overnight and then centrifuged (five times at 10,000 rpm at 20 °C, each lasting 15 min) and was washed with deionized water. Purified dispersion (20 mL) was reduced by the addition of 1 mL of AsA solution (100 mg/mL), and rGO was centrifuged twice at 15,000 rpm for 70 min in total. The attached FL concentration was calculated gravimetrically, using a system of equations in which the relative loss of each compound is taken into account.

The structure of the rGO platelets and the distribution of AgNPs decorated onto rGO surfaces were investigated with a Philips Tecnai G2 20 TWIN transmission electron microscope, using copper grids (300 mesh copper grids, Aldrich) with spin coating at 1500 rpm.

AFM images were obtained by a MultiMode NanoScope IV (Veeco) instrument, working under a tapping mode with the use of TESP tips to determine the thickness and roughness of the sheets of FL−rGO and FL−rGO−Ag hybrid structures. The samples were drop-casted on a mica substrate.

The Raman scattering and SERS activity of GO, FL−rGO, and FL−rGO−Ag nanocomposite structures were investigated using Raman spectroscopy with a Renishaw inVia spectrometer and Fluorescein samples were drop-casted on a mica substrate.

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