Laser induced fluorescence spectroscopy of various carbon nanostructures (GO, G and nanodiamond) in Rd6G solution

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Abstract: The effect of carbon nanostructures such as graphene (G), graphene oxide (GO) and nanodiamond (ND) on the spectral properties of Rhodamine 6G (Rd6G) emission due to the laser induced fluorescence (LIF) was investigated. It is shown that the addition of carbon nanostructures lead to sensible Red/Blue shifts which depend on the optical properties and surface functionality of nanoparticles. The current theories such as resonance energy transfer (RET), fluorescence quenching and photon propagation in scattering media support the experimental findings. Stern-Volmer curves for dynamic and static quenching of Rd6G molecules embedded with G, GO and nanodiamond are correlated with spectral shifts. Furthermore, time evolution of the spectral shift contributes to determine loading/release rates of fluorescent species with large S-parameter on the given nano-carriers.

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1. Introduction

The major limitations governing the treatment of cancerous tissues by chemotherapeutic drugs are their cytotoxicity and inadequate concentration of drug delivered directly to the tumors [1]. In-vivo studies have shown that only up to % 0.01 of antibodies intravenously administered to the patients reach the targets [2], while it is essential to deliver chemotherapeutic drugs to the tumor at higher concentrations with minimal damage to normal tissues, sustaining the drug concentration to remain above the effective value for an extended period of time [1]. Current researches have therefore focused on developing more efficient local drug delivery or drug-targeted therapies to overcome these obstacles.

Furthermore, (nanoparticle + drug) hybrids enhance the therapeutic efficiency and bioavailability which allow the drug to be specifically directed to the tumor location while limiting the exposure to the normal cells [3–5]. Nanoparticles with small size and high surface to volume ratios resemble to be ideal carrier of the biomedical components such as DNA, proteins and chemotherapeutic drugs to improve in vivo therapeutic treatment especially for early cancerous tumor detection and targeted therapeutic/theranostic treatments [6].

Among the various organic and inorganic nanoparticles, carbon-based materials such as nanodiamonds (ND), carbon nanotubes (CNT), graphene (G) and graphene oxide (GO) show various exceptional properties in aqueous solutions having nearly perfect interfaces useful for bio-chemical sensors and drug delivery applications [7]. G is characterized with less oxygen affinity and abundant unsaturated linkages than GO. The latter is an oxidized graphene sheet decorated mostly with epoxide and hydroxyl groups, in addition to carbonyl and carboxyl groups located at the edges [8,9]. The G surface contains highly delocalized π electrons that can also be easily functionalized through π-π interactions with compounds containing π-electron rich structure. Hence, this sp²-hybrid carbon network has raised tremendous interest not only in the field of drug delivery, but also in various other areas, such as gas storage, sensors [10], nano-electronics, photovoltaic [11] and polymer composites [12]. Functionalized nano-sized graphene has been used as a drug carrier for in-vitro intracellular delivery of anticancer chemotherapy drugs [13–16]. On the other hand, G exhibits very low solubility in common organic solvents while GO is more favorable for applications requiring solution-processing compatibility [8]. Functionalized graphene oxide looks like to be an efficient nanocarrier for cancer therapy [17–20]. Recently, the GO-based fluorescence quenching is shown to be a basis for quantitative DNA analysis [20].
ND as a sp³-hybridized allotrope of carbon is less toxic than other carbon nanoparticles [21]. High rigidity, low chemical reactivity, and good biocompatibility have made it suitable for applications in biomedical imaging, drug delivery and other areas in medicine such as prostatic cancerous tumor treatments [21,22]. In this way, researchers extensively investigate new methods to produce fluorescent nanodiamonds as a non-toxic alternative to semiconductor quantum dots for biomedical imaging [23]. Though the carbon nanostructures do not demonstrate any fluorescence activity, when those are used in the hybrid form with fluorophores (dye molecules), the characteristics of the fluorescence spectra differ due to several mechanisms i.e. energy transfer, charge transfer and dynamic scattering. Characterization of the fluorescence spectral alterations has been subject of interest because it serves as a reliable source of information for the biochemical systems [24–27].

Drug may be loaded on the nanostructures by several mechanisms, such as embedding, surface absorption, hydrogen bonding and strong π-stacking interactions [7]. In order to achieve an efficient drug action, the improvement of the loading efficiency is critical. The loading of several markers and chemodrugs on graphene and graphene oxide such as phthalocyanine [28], porphyrin [29], eosin [30], methylene blue [30], fluorescein [31] Rd6G [32], rhodamine B (RdB) [30, 33], doxorubicin (DXR) [7] pyrene and nile [34,35] have been reported.

The load and release experiments are mostly characterized by means of AFM, FTIR, TEM and UV–Vis methods [7,30,32,33] and mostly monitored by UV–Vis absorption spectroscopy [7,32,33] and fluorescence quenching measurements [17,32]. Despite these are essential to identify various nanostructures in the solution due to quenching events however, the spectral shift accredits LIF as an efficient technique to determine quantitatively the nanostructure-fluorophore interactions accordingly. Recent studies state the fluorescence spectral shifts due to nanoscatteer additions such as TiO₂, ZnO and Au [36–38].

To our best knowledge, there is no report on the analysis of the spectral shifts due to (G/GO/ND + dye) hybrids and their potential applications. Here, the influence of G/GO/ND on the spectral properties of the LIF emission of Rd6G ethanolic solutions is investigated. It is shown that the optical properties and surface functionalities of carbon nanostructures lead to the sensible red/blue spectral shifts. The dynamic and static quenching of Rd6G molecules due to G, GO as well as ND are discerned by steady state fluorescence measurements. As a potential application, the instantaneous evolution of the spectral shift determines the rate of Rd6G loading on graphene. Furthermore, LIF gives a method to discriminate G and GO as an alternative spectroscopic technique regarding traditional UV-Vis, XPS or Raman spectroscopy. This might be the case for a well controlled experiment where concentrations of both Rd6G and nanostructures in a simple solution are known.

2. Theory

2.1 Stokes shift

One of the distinct characteristics of each fluorophore is the spectral distance between the excitation and emission spectra, known as the Stokes shift that features a notable indicative during the detection of the emitted fluorescence in biophotonic applications. The Stokes shift arises from the shifts of the potential energy surfaces of the ground and excited states at equilibrium condition in the course of an electronic transition. The strength of this anharmonic transition is determined by Huang–Rhys factor (S parameter) which is proportional to the corresponding Stokes shift [39]. Small overlapping of absorption and emission spectra (large Stokes shift) significantly reduces the RET rate between similar fluorophores according to Forster equation [40],

$$k_F(r) = \frac{1}{\tau_D} \left( \frac{R_0}{r} \right)^6. \quad (1)$$
where, \( K_T \), \( r \), \( R_0 \) and \( \tau_D \) denote to be dipole-dipole RET rate, average distance between the donor, D (any excited fluorophore or nanostructure) and acceptor, A (any ground state fluorophore or nanostructure), Forster distance and lifetime of the donor in the absence of the energy transfer, respectively [40]. This attests that the self quenching doesn't affect the fluorescence intensity measurements in the case of large Stokes shift. Conversely, when the dye molecules with small Stokes shift are likely within the Forster distance, RET would be very efficient. It is shown that this event is critical in diversity of multiplex fluorescence applications, because the amount of impurities and the corresponding optical parameters modify the re-absorption rate of emitted fluorescence by fluorophores in the same sample [38].

2.2 Spectral shift of the Rd6G fluorescence due to excessive nanostructures

Based on the experimental studies regarding photon traveling in scattering media, several effects may lead to red/blue shifts of the fluorescence spectra of host molecules due to the addition of guest scatterers. Those contain wavelength dependent scattering, energy-level distortion and self-absorption of fluorescence emission. The latter is known as the salient mechanism to be enhanced in the case of long photon trajectory (random walk) particularly in the hybrid media including nanoscaterrers and dye solutions [36–38].

2.2.1 Re-absorption/re-emission

The significant overlap of the absorption and emission spectra results in the re-absorption of emitted photons by the adjacent molecules such that the emissive lines undergo the red shift in terms of the dye concentration \( C_{\text{dye}} \) [36–38].

Figure 1(a) illustrates the schematics of the absorption-emission spectral overlapping area for a typical 8 µM Rd6G ethanolic solution. The chromophore has symmetric molecular structure such that no dipole moment transition occurs along its long axis which gives rise to the small Stokes shift and large overlap area [40].

![Fig. 1. Overlapping area of the normalized absorption-emission spectra for (a) 8 µM Rd6G ethanolic solution (Inset: corresponding molecular structure) and (b) Absorption spectra of (Rd6G + G/GO/ND) hybrids.](image)

The chemical interaction of Rd6G molecules with nanostructures could be identified by the UV-Vis spectra of (Rd6G + G/GO/ND) hybrids. According to Fig. 1(b), nearly uniform absorbance in the wavelength region of the fluorescence emission voids the likelihood of significant spectral shift due to re-absorption by small amount of (dye + NP) hybrids. Furthermore, the sufficiently excessive amount of the scatterers dispersed in the medium enhances the number of scattering events to elongate corresponding random walk of the photons. The longer the optical path, the higher the re-absorption rate that subsequently leads to the larger red shift. In a system of dimension \( L \) with identical Rayleigh scatterers, the prerequisite for elongation of photon trajectories due to scattering events is the following criteria \( L >> \frac{\lambda}{\lambda_s} \) [41] where \( \lambda \) and \( \lambda_s \) denote to be the photon wavelength and scattering mean free path (the mean distance of the successive scattering event) respectively. This is determined by diffusion theory [42] namely:
\[ I_s = \frac{1}{\sigma_s \rho_s}, \quad (2) \]

where \( \rho_s \) is number density of scatterers and \( \sigma_s \) ascertains Rayleigh scattering cross section given by [43]:

\[ \sigma_s^{\text{Rayleigh}} = \frac{2\pi^2 a^6}{3\lambda^4} \left[ \frac{n^2 - 1}{n^2 + 2} \right]^2, \quad (3) \]

where \( a, n, \) and \( \lambda \) denote to be particle size, particle refraction index and the incident photon wavelength respectively. For \( l_s \gg L \), the interaction of light with scatterers is so small that the system is sometimes referred to a ballistic interaction. This re-absorption based red shift was previously reported for monotonically increasing TiO\(_2\) scatterer densities in different Rd6G concentrations [36–38]. Similar effect is also observed for the other nanoparticle additives such as ZnO, SiO\(_2\) and Al\(_2\)O\(_3\) [38] in the dye solutions. Figure 2 briefly describes the requisites for the efficient LIF spectral shift spectroscopy of hybrid media.

2.2.2 Chemical interactions

Red/blue spectral shifts due to the chemical interactions consists of the disaggregation of dye molecules by NP additives (blue shift) [44] and (dye + NP) charge transfer states (red/blue shift) [45, 46]. Dye molecules are strongly prone to aggregate at higher dye concentrations which in turn alter the corresponding absorption and emission spectra such that the emission shifts to the longer wavelengths [44,47,48]. Based on the empirical results, these spectral shifts are significant for highly dense solutions (\( C_{\text{dy}} > 100 \) mM for Rd6G) [49]. Conversely, the dimers and polymers decompose to the monomers due to the disaggregation which induces the blue shift particularly at the presence of the quenchers. Moreover, the chemical interaction between the nanoparticle and fluorophore creates (dye + NP) compounds which influence the transition energy between the ground and excited states indicating the spectral shifts [38,40].

2.3 Fluorescence quenching by nanoparticles

Mechanisms evolving the fluorescence quenching include: (i) the formation of weakly emissive ground state complex (static quenching), (ii) the collision of quencher with an excited unbound fluorophore leading to the dissipation of radiation energy (dynamic quenching). Fluorescence quenching follows the well-known Stern-Volmer equation [40].
where $F_0(F)$, $K$ and $[Q]$ ascertain the fluorescence intensity in the absence (presence) of quencher, quenching constant and the concentration of quencher (that is NP here) respectively. Depending on the quenching mechanism, $K$ denotes to be dynamic ($K_D$) or static ($K_S$) quenching constant. Plotting $F_0/F$ as a function of $[Q]$ is expected to yield the linear slope, $K$. Equation (4) emphasizes that a linear Stern-Volmer plot does not indicate that the collisional quenching of fluorescence events certainly occurs and some additional information is required, for instance lifetime measurements to discern the static and dynamic quenching [40].

### 2.3.1 Combination of static and dynamic quenching effects

In many instances, the fluorophore can be quenched both by successive collisions (dynamic quenching) and according to the complex formation (static quenching) with the same quencher. The characteristic feature of the Stern-Volmer plots in such circumstances is a nonlinear curve, which is given by the modified form of the Stern-Volmer equation [40]:

$$
\frac{F_0}{F} = (1 + K_D [Q]) \cdot (1 + K_S [Q]).
$$

(5)

When the multiple static quenching processes take place, the modified Stern-Volmer plot obeys the following equation

$$
\frac{F_0}{F} = \left((1 + K_D [Q]) \times (1 + K_{S1} [Q]) \times \ldots \times (1 + K_{Si} [Q])\right).
$$

(6)

where $i$ denotes the number of static quenching processes [46]. The flowchart regarding the efficient LIF quenching due to addition of nanostructures in hybrid media and subsequent spectral shifts is depicted in Fig. 3.

**Fig. 3.** Flowchart of the optical process for efficient LIF quenching in hybrid media (NP + dye) and subsequent blue and red spectral shifts.

### 2.3.2 Fluorescence quenching of Rd6G by G/GO/ND

Rd6G is a well known dye with high fluorescence quantum yield. This fluorophore is a Xanthene dye with carboxyphenyl as the end group which has no dipole moment parallel to its long axis due to the symmetry of its molecular structure. As a consequence, the Stokes shift is small, and the fluorescence band overlaps strongly the absorption band. On the other hand, the graphene contains largely dislocated $\pi$-electrons that allow energy transfer from the nearby molecules through $\pi-\pi$ interactions, leading to the efficient fluorescence quenching. This event is very useful in the optical detection of biomolecules [50]. Graphene oxide, with
oxygen-containing groups such as -COOH, -CO, and -OH at the surface, gains the advantage of hydrophilicity, high surface activity and significant fluorescence quenching property [51]. Figure 4 illustrates the schematic atomic structure of GO/G/ND.

The binding of Rd6G to G/GO has been reported in the literature based on the several experimental methods such as time-resolved and steady state fluorescence intensity measurements, UV–Vis absorption and AFM method [32,46]. Zhang et al and Kai-li Fan et al. have investigated the fluorescence quenching of Rd6G by G/GO in aqueous solutions using steady-state and time-resolved fluorescence spectroscopy accompanied by the linear absorption measurements. Simultaneous dynamic and static quenching is elucidated. The formation of ground-state complexes by Rd6G and G/GO is shown to be the major mechanism of static quenching [32,46].

3. Materials and methods

Graphene powder with 30-50 layers having ~12 nm average flake thickness and approximate lateral size of 4.5 μm as well as GO powder with ~1 nm thickness and lateral size of 90 nm were supplied from Graphene-Supermarket Inc. ND powder having ~1 nm mean cluster size was purchased from Sigma-Aldrich Co. Rhodamine 6G (C28H31N2O3Cl, Acros Organics, 99% Pure, MW = 479.01 gr/mol) was used as ethanolic solution. Those materials with the suitable analytical grades without excessive purification were ready to immediate use in the solutions. Different ratios of G/GO/ND powders by weight were separately dispersed in the dye ethanolic solutions in various concentrations. Ethanol solvent (C2H5OH, Merck, Purity (GC) ≥ 99.9%, MW = 46.07 gr/mol) was employed in order to minimize the aggregation of dye molecules. Afterwards, 1 CC dye solutions were inserted into a quartz cell having 1cm × 1cm × 4.5cm. A 150 mW SHG of CW-Nd:YAG laser at 532 nm was exploited to excite Rhodamine molecules. Emission spectra was taken in right angle direction using Avantes Ava Spec 2048 spectrophotometer, with spectral resolution of 0.4 nm, 300 lines/mm and NA 0.22 as shown in Fig. 5(a). Here, the right angle set up was arranged to involve the bulk interaction mechanism particularly multiple scattering events instead of the surface effects. Variety of graphene densities were examined for a certain 40 μM dye solution. The suspensions were treated using ultrasonic bath in order to assure that G/GO/ND homogeneously diffuse through the Rd6G solutions. Various concentrations of dye solution and graphene density were considered for LIF systematic measurements including the corresponding spectral shifts as well as Stern-Volmer plots. In order to investigate the effect of re-absorption due to the nanostructures (G/GO/ND) on the spectral shifts, LIF spectroscopy with two cuvettes was carried out based on the arrangement shown in Fig. 5(b). To investigate the loading rate of Rd6G molecules on G/GO/ND nanostructures, corresponding films were made by drying 500 μg/mL ethanolic suspension of each one in a separate beaker and then, films were picked up by a pair of tweezers and immersed at the bottom of a cuvette [33]. Finally, 3 mL of typical Rd6G solution (40 and 100 μM) was added to the cuvettes. As depicted in Fig. 5(c), each cuvette was monitored alongside with another cuvette containing 3 mL of pure Rd6G ethanolic solution (40 and 100 μM) simultaneously using two similar spectrophotometers in right angle setup. All UV-Vis spectra are taken by Shimadzu UV-1800 UV-Vis spectrophotometers with 1 nm resolution.
4. Results and discussions

4.1 LIF spectra taken from Rd6G solutions

Figure 6(a) illustrates absorption and emission (LIF) spectra of various Rd6G ethanolic solutions (1-1000 μM). Increasing dye concentration, both intensity and emission spectra notably alters. Figure 6(b) and 6(c) depict the intensity and wavelength at the peak of emission spectra in terms of the dye concentration. The maximum intensity is obtained for 10 μM Rd6G solution. While the fluorescence intensity increases with dye concentration, the self quenching of dye molecules becomes dominant for dense solutions. It slows down the growth rate of emission intensity to saturate above ~100 μM. The red shift usually occurs where the corresponding dye concentration increases, however the growth rate deviates from linearity near ~10 μM and large discrepancy appears above ~60 μM. This is in agreement with our previous report mainly based on the fact that self quenching affects the emission/absorption events [38]. Notice that these values may be slightly changed in various setup geometries and the experiments were carried out below 100 μM where the self quenching is mostly negligible.
4.2 LIF spectra due to addition of G/GO/ND into the Rd6G solution

Afterwards, carbon nanostructures were dispersed in dye solution in order to investigate the spectral characteristics of hybrid medium. Figure 7 illustrates the sets of LIF spectra due to the addition of G/GO/ND densities (ranging 0-50 μg/mL) in 40 μM Rd6G solution. Adding slight amount of G/GO nanostructures below 50 μg/mL, the emission spectra exhibit a faint blue shift, likely due to the chemical coupling between G/GO and Rd6G molecules as (G/GO + Rd6G) complex \[28,30,51\]. Moreover, Fig. 7(a) and 7(b) depict the fluorescence emission which decreases drastically to be vanished for the G/GO concentrations above 50 μg/mL. Conversely, Fig. 7(c) indicates no significant spectral shift for the Rd6G solution with nanodiamond additives (ND + Rd6G). This nano carbon structure exhibits a loose affinity of transferring electron to Rd6G molecules \[54\]. Notice that the fluorescence emission intensity due to the addition of ND is relatively higher than that of G/GO.

![Fig. 7. LIF emission spectra due to addition of (a) graphene (G) (b) graphene oxide (GO) and (c) nanodiamond (ND) densities (ranging 0.00 – 50 μg/mL) in certain Rd6G solution, typically 40 μM.](image)

Figure 8 illustrates the blue shift of the emissive wavelength due to addition of the various nano carbon structure densities, ranging 1-50 μg/mL, according to the fluorescence spectra. While further amounts of G/GO reduce the fluorescence intensity to a non-measurable level, LIF spectra due to higher ND densities (up to 5000 μg/mL) were measurable. The corresponding emissive wavelengths demonstrate the red shift (~8 nm) as shown in the inset of Fig. 8.

![Fig. 8. Emissive wavelength due to various G/GO/ND additions (ranging 1-50 μg/mL) in 40 μM Rd6G ethanolic solution. Inset: emissive wavelength due to more ND additives (ranging 1-5000 μg/mL). Further addition of G/GO reduces the fluorescence intensity to a non-measurable level.](image)

Due to the dangling bonds as reactive sites, ND particles have shown low chemical reactivity in comparison to G and GO, so their tendency to coupling with Rd6G molecules as (ND + Rd6G) complex resembles to be much weaker than the nanostructures such as G and GO \[55\]. Moreover, G possesses the abundant dangling bonds as the edge structures to create

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chemical bonds with ambient molecules than GO; because oxygen occupies these sites in GO. These functions of chemical reactivity are in accordance with our results given in Fig. 8.

Table 1 summarizes the optical parameters regarding the propagation of photons in a typical Rd6G solution (40 μM) with nanodiamond densities 10, 20, 30, 40, 50 and 100 μg/mL. Furthermore, the scattering mean free path, $l_s$ and Rayleigh scattering cross section ($\sigma_s$) are calculated using Eqs. (2) and (3) respectively.

| C(dye(μM)) | $\lambda_{ex}$(nm) | a(nm) | nND | L(cm) | $\sigma_s$(cm$^2$) | $\rho_s$(μg/cm$^3$) | N s(#/cm$^3$)$\times10^{14}$ | $l_s$(cm) |
|------------|--------------------|-------|------|-------|-------------------|------------------|-----------------|----------|
| 10         | 532                | 12    | ~2.4 | 1.0   | 2.86$\times10^{-17}$| 10               | 11290           |
| 50         |                    |       |      |       |                   | 0.15             | 2333            |
| 100        |                    |       |      |       |                   | 0.316            | 902             |
| 1000       |                    |       |      |       |                   | 3.16             | 110             |
| 10000      |                    |       |      |       |                   | 31.64            | 11              |

At low nanodiamond densities, $l_s$ is considerably greater than the sample dimension ($l_s$>>L = 1cm). Therefore, photon travelling path undergoes a single scattering event without elongation. Accordingly, spectral red shift doesn’t take place because there is no enhancement of re-absorption mechanism [38]. Moreover, the absence of sensible spectral shift indicates that the blue shifts do not occur due to the addition of 1-50 μg/mL nanodiamond into the Rd6G solution. In fact, at a certain Rd6G concentration, the higher ND density gives rise to shorten $l_s$ leading to longer optical path. In summary, the Rd6G attachment to G/GO gives rise to the slight blue shift mainly because of affinity to form (Rd6G + G/GO) complex, whereas there is not such an affinity regarding the compound of (ND + Rd6G).

4.3 Double cuvette experiments

A differential measurement was carried out to investigate the nanostructure contribution to the spectral shifts due to the re-absorption of Rd6G fluorescence emission. The fluorescence emission of pure Rd6G ethanolic solution in a dye filled cuvette (#1) was measured after passing dye-free cuvette (#2) as shown in Fig. 5 (b). The first cuvette (#1) contains typical 10 μM Rd6G ethanolic solution without the scatterers and cuvette (#2) includes (i) pure ethanol, (ii) scatterer suspension as a guest in pure ethanol (10 μg/mL) and (iii) 10 μM Rd6G solution in ethanol. The results for G/GO/ND additives are tabulated in Table 2. For all nano-scatterers, the corresponding wavelength of the fluorescence peak transmitted through the dye-free scattering suspension was found to be unaltered whereas the addition of Rd6G molecules in cuvette #2 leads to a significant red shift ~4.1 nm. This emphasizes that re-absorption of the fluorescence by nanoparticle does not contribute to induce any spectral shift. Moreover, $I(\lambda_{max})$, measured after passing through the cuvette #2, shows the significant extinction of intensity due to G/GO and somewhat ND additives.

| Sample in cuvette #2 | 10 μM Rd6G in cuvette #1 |
|---------------------|---------------------------|
|                     | $\lambda_{max}$(nm) | $I(\lambda_{max})$(arb. u.) |
| (i) Ethanol         | 559.5                    | 4560                        |
| (ii) Ethanol + G (10 μg/mL$^{-1}$) | 559.5            | 1818                        |
| Ethanol + ND (10 μg/mL$^{-1}$) | 559.5            | 4284                        |
| Ethanol + GO (10 μg/mL$^{-1}$) | 559.5            | 2067                        |
| (iii) Ethanol + Rd6G | 563.6                    | 4100                        |
Figure 9 displays the spectral plateau of the absorption for G, GO and ND nanostructures respect to that of Rd6G taken by UV-Vis spectrometer. In fact, carbon nanostructures may re-absorb the photons at emissive wavelengths with nearly equal cross section.

Fig. 9. Absorption-emission spectra of Rd6G ethanolic solution and absorption spectra due to G, GO and ND suspension in ethanol.

4.4 Stern-Volmer plots

Figure 10 displays the Stern-Volmer curves due to quenching of 40 μM Rd6G solution by G, GO and ND additives. It lucidly emphasizes that the ratio $F_0/F$ versus the quencher density $[Q]$ for nanodiamond scatterers would be linear.

Fig. 10. Stern-Volmer plots for quenching of various densities of G/GO/ND in 40 μM Rd6G solution. $F_0(F)$ is the R6G fluorescence intensity in the absence (presence) of nanoparticles.

Thereupon, the quenching process is either dynamic or static. In fact, for G/GO additives, there is obviously a frank deviation of $F_0/F$ ratio versus $[Q]$ from linearity. It indicates that the Rd6G fluorescence quenching by graphene is not solely described by either dynamic or static measurements. This is in agreement with the previous reports that a dual dynamic and static effects are demonstrated [46,56]. The origin of static quenching for G/GO, as previously discussed in the literature, is based on the formation of (G/GO + Rd6G) complexes [30,32,46,48,56]. These chemical couplings give rise to the reduction of the number of Rd6G molecules in the solution, implying the reduction of the effective dye concentration.

According to Fig. 6, the concentration scales down the re-absorption mechanism to attest the slight spectral blue shift.

Regarding linear regression on experimental data depicted in Fig. 10, quenching coefficient, $K_D$, for nanodiamond is determined to be $33.1 \times 10^{-3}$ (μg/ml)$^{-1}$. According to the conventional methods whether the dynamic or static quenching takes place, time resolved fluorescence spectroscopy is required to determine fluorescence decay time, $\tau_0$. However, the spectral shift spectroscopy is carried out based on the data in Table 1. In the range of 0-50 μgmL$^{-1}$, the red shift doesn’t take place with increasing nanodiamond density. Therefore,
zero value for total spectral shift in Fig. 7(b) indicates that no blue shift does occur. This emphasizes that no significant static quenching takes place. The quenching analysis and corresponding spectral shifts are summarized in Table 3.

Table 3. Quenching analysis and spectral shift measurements due to the addition of G/GO/ND nanostructures (ranging 0-50 μg/mL⁻¹) into the 40 μM Rd6G solutions.

| Sample (40μM Rd6G solution) | Spectral shift | quenching   | Stern-Volmer plot |
|-----------------------------|----------------|-------------|-------------------|
| ND (0-50 μg/mL⁻¹)           | -              | -           | linear            |
| G (0-50 μg/mL⁻¹)            | Blue (~10 nm)  | Yes         | Yes               | non-linear        |
| GO (0−50 μg/mL⁻¹)           | Blue (~6 nm)   | Yes         | Yes               | non-linear        |

4.5. Loading rate of Rd6G molecules on G/GO/ND nanostructures

Here, instead of dispersing G/GO/ND nanostructures in Rd6G solution, carbon-based nanostructures were deposited at the bottom of the dye container and emissive wavelength of Rd6G solution was monitored continuously for 20h to investigate the loading rate of Rd6G molecules on G/GO/ND surfaces. As depicted in Fig. 5(c), 3ml of 40 μM Rd6G ethanolic solution was added to each of two similar glass cuvettes. Then, G/GO films were immersed into the first cuvette. The emissive wavelengths due to excitation at 532 nm were subsequently monitored for 20h when fiber probe was adjusted to an upper point of each cuvette in right angle setup. Figure 11(a) represents the time evolution of the emissive wavelengths. The experiments for G/GO nanostructures were repeated for higher concentration of Rd6G, i.e. 100 μM using the same setup as shown in Fig. 11(b).

Fig. 11. Time evolution of emissive wavelength due to excitation of (a) 40 μM (b) 100 μM of Rd6G ethanolic solution, with G/GO, at 532 nm. (insets: real time UV-Vis monitoring of the samples (c) concentration decrease due to the loading Rd6G molecules on G/GO nanostructures.
The fluorescence emission spectra from cuvette with (Rd6G + G/GO) is blue shifted over the monitoring period, while those of (Rd6G + ND) and (Rd6G) almost remain spectrally invariant. The faint blue shift ~2.5 nm/20h (~1.8 nm/20h) induced by G(1GO) is due to the chemical binding of Rd6G molecules to the G/GO sheets. According to Fig. 11(b), larger blue shifts (~3.5 nm/6h for G and ~2.4 nm/6h for GO) occur compared with loading test in lower Rd6G concentration. This implies that Rd6G molecules tend to be loaded on G sheets more than GO sheets. Moreover, the larger loading rate appears for higher Rd6G concentration. In order to determine correlation of the blue shift rate and the reduction of Rd6G concentration, the equations governing the time variation of wavelength at the peak, \( \lambda_{\text{max}}(t) \), and the wavelength at the peak versus concentration, \( \lambda_{\text{max}}(C_{\text{dye}}[\mu \text{M}]) \) are retrieved from the corresponding curves in Figs. 6, 11, and 12, using second order nonlinear regression. The resulting curves, \( C_{\text{dye}}(t) \), are plotted in Fig. 11(c). The retrieved amount of Rd6G loaded on G/GO after 8 and 20h are listed in Table 4. Correlation curves between LIF measurement and UV-Vis monitoring, depicted in the inset of Fig. 11(c), indicates high conformity with that retrieved from the UV-Vis monitoring of the samples according to the insets of Fig. 11(a) and 11(b). It is obvious that G contains relatively higher capacity for loading Rd6G molecules than that of GO which is in good agreement with others [30]. Moreover, loading rates attributing both carbon nanostructures are much higher in the first 6 hours to be in accordance with Ref [33].

Finally, the amount of Rd6G molecules to be loaded, exhibit significantly larger at higher Rd6G concentrations. Increasing Rd6G concentration, PH of solution decreases which leads to more efficient hydrogen bonding. In comparison, Yang et al. have investigated the loading capacity of doxorubicin (DXR) on a given density of GO. They have also shown that with the increase of the initial DXR concentration, the loading capacity linearly increases [7]. It is likely due to the fact that increasing Rd6G concentration, leads to the reduction of PH and subsequently the enhancement of hydrogen bonding. The latter is known as one of the main loading mechanisms [7,54].

| Carbon nanostructure | Initial Rd6G concentration(\( \mu \text{M} \)) | Loaded Rd6G (\( \mu \text{mol.L}^{-1}/8h \)) | Loaded Rd6G (\( \mu \text{mol.L}^{-1}/20h \)) |
|----------------------|---------------------------------|-------------------------------|-------------------------------|
| G                    | 40                             | 9.39                          | 14.5                          |
|                      | 100                            | 34.72                         | 39.49                         |
| GO                   | 40                             | 7.18                          | 9.37                          |
|                      | 100                            | 22.35                         | 22.51                         |

In summary, when spectral overlap sensibly exists mainly due to large S-parameter (Huang-Rhys factor) for a certain fluorophore such as Rd6G, then the dye concentration \( C_{\text{dye}} \) and nanoscatteer density \( \rho_s \) affect competitively to induce blue or red spectral shifts. For slight G/GO additives, the photon’s random walk does not elongate such that no spectral shift occurs. However, the emission spectra exhibit a faint blue shift, likely due to the chemical coupling between G/GO and dye molecules to form the non-emissive compounds, which is revealed by the static quenching of the fluorescence intensity based on Stern-Volmer plots. This event leads to the slight reduction of the Rd6G concentration which gives rise to decrease the re-absorption rate regarding Forster energy transfer (RET), leading to the subsequent blue shift.

5. Conclusion

Here, the influence of carbon nanostructures on the spectral properties of LIF emission of Rd6G ethanolic solutions is investigated regarding the significance of (dye + NP) hybrid compounds in nano-oncology. It is shown that the addition of G/GO/ND nanostructures lead to sensible red/blue spectral shifts, accompanying a notable reduction in the fluorescence intensity. It depends on the surface functionality of the nanostructures. While the slight amount of nano G/GO additive, up to ~50 \( \mu \text{g/mL} \), induces the blue shift of the spectra mainly
because of strong chemical interactions, no sensible spectral shift occurs for excessive addition due to the strong quenching. Larger spectral blue shift takes place by G additive (~9.5 nm per 50 μg/mL) compared to that of GO nanostructure (~5.4 nm per 50 μg/mL). This indicates that the former has relatively more capacity to create chemical bonds with Rd6G molecules. On the other hand, despite the trace ND additives exhibit low affinity to bond with Rd6G molecules which gives rise to negligible spectral shift, however a slight red shift is induced by larger amounts of ND. It provides longer photon trajectory to enhance the re-absorption process due to large S-factor of Rd6G. The current theories such as RET, fluorescence quenching and the photon propagation (diffusion) in the scattering media attest the correlation between Stern-Volmer curves for dynamic/static quenching and the corresponding spectral shifts. This in vitro investigation supports as an empirical database for further in-vivo experiments using biocompatible chemotherapeutic drugs with large S-parameters. It also fulfills the demand to find a simple and reliable technique for G/GO identification in diverse biomedical investigations. We have studied G/GO as drug carriers by using new real time monitoring method based on the LIF spectral shift measurements. Rd6G fluorophores are well known probes that are used as a drug model with large S-parameter. Time evolution of the spectral shift indicates that loading capacity of Rd6G on G (39.49 μmol.L⁻¹/20h for 100 μM Rd6G) is much higher than that of GO (22.51 μmol.L⁻¹/20h for 100 μM Rd6G), mainly due to its abundant unoccupied bonding sites. It is also deduced that the loading rate enhances in higher Rd6G concentration. The results are in good agreement with real time UV-Vis measurements.