Time-depended Quenching Behaviors of Multi-dye Systems by Single-Walled Carbon Nanotubes

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Abstract: A fluorescence quenching phenomenon of fluorophores or dyes could be observed with single-walled carbon nanotubes (SWNTs) suspension. In this study, uranine (Ur) and Rhodamin B (RB), were employed as fluorophores to research time-depended quenching behaviors of multi-fluorophore system by SWNT suspension with single-stranded DNA (SWNT-T30). The effect of "simultaneous quenched" scheme (Ur and RB were quenched at the same time) and "un-simultaneous quenched" scheme (Ur was quenched to equilibrium state, then RB was added; or RB first quenched, then Ur added) to the quenching properties of multi-fluorophore system by SWNT-T30 were compared and researched. The quench equilibrium could be realized in 10 min, for both single and multi-quenching system. Quenching balance and quenching efficiency of each dye basically was not impacted obviously, whether there was another dye existed in the same solution or not. The results or the methodology was important for the future multi-sensor design and application, as it is essential to check the dynamic balance of quenching behavior and confirm that if the quenching performance of target or hunter of sensor (or multi-sensor) was influenced by other constituents.

1 INTRODUCTION

Carbon nanotubes, especial single-walled carbon nanotubes (SWNT) suspensions, have attracted remarkable research interest from first presented in 2003 by Zhang et al., (2003:2). The unique physical, chemical, optical, electrical properties induced by the extremely small size make them suitable for a wide range of biological (Lu et al., 2009: 21), medical (Prato et al., 2008: 41), environmental (Pelencia et al., 2015: 407) and electronic (Chitta et al., 2007:111) applications. The fluorescence quenching phenomenon of dyes/fluorophores (Li et al., 2011: 21; Cao et al., 2009: 21) by the nearby SWNT was found to be universal for a variety of fluorophores or dyes, and is thought to be utilized for biosensor applications (Sgobba et al., 2009: 38).

In our previous work, multi-fluorophore system's quenching properties and competitive adsorption model on SWNT surface were studied. As the succeeding work, here we discussed the time-depended quenching performances of multi-fluorophore system, via constantly fluorescent monitoring. As considering more than one fluorophore existed in the quenching system, "simultaneously quenched" process (two

fluorophores were quenched at the same time) and "un-simultaneously quenched" process (one fluorophore added first and reached to equilibrium state, then another fluorophore added) were designed, then batch assays were carried out. Quenching efficiencies gained from batch assays were also investigated via *T*-test analysis, which is a statistical method to be used to determine if two sets of data are significantly different from each other.

Usually, in the system to be detected, more than one constituent existed which maybe interfere or influent the photosensitizer or aptamer of the SWNT sensor platform. Furthermore, despite enormous number of works focused on fluorescence quenching by carbon nanotubes, there have been few experimental efforts reported in the literature attempting to evaluate the time-depended multi-fluorescence quenching performance. Thus, the methodology and results in this work could be useful to confirm the dynamic equilibrium of quenching behavior of fluorophores and if the quenching performance of target or photosensitizer of sensor (or multi-sensor) was influenced by other constituents, which was necessary and essential for nanosensor design and applications.

2 EXPERIMENTAL

Single-walled carbon nanotubes(SWNT) produced by the high-pressure carbon monoxide (HiPco) method were purchased from Raymor Nanotech (Canada) as purified powders. Single stranded DNA with thymine 30 mers (T30) were purchased from Life Technologies Japan, Ltd., Tokyo, Japan. Acridine orange (AO) solution (1 mg/mL H_2O),

Uranine (Ur, or sodium fluoresecein) and Rhodamine B (RB) were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Hybrids of T30 and SWNT (SWNT-T30): 1 mL of T30 solution of deionized water (1 mg/mL) and 0.5 mg of SWNT powder were mixed and sonicated using a probe-type sonicator in an ice-water bath for 90 min (VCX 130, Sonics & Materials, Inc., CT, USA) with a 2-mm diameter tip at 3 W (Tahahashi et al., 2004: 43; Nii et al., 2013: 106). The hybrid suspension was centrifuged at 12,500 g for 3 h at 8ºC (high speed refrigerated micro centrifuge, MX-150, TOMY, Japan). After centrifugation, the upper 75% of the volume was stored as the SWNT-ssDNA suspension.

Ur-H_2O and **RB-H_2O Solution:** 12.4 µg of Ur and 15.8 µg of RB were dissolved in 1 mL of deionized water, respectively to prepared 0.0124 mg/mL (0.033mM) Ur-H_2O solution and 0.0158 mg/mL (0.033mM) RB-H_2O solution.

Buffer Solution: 10 mM *Tris* (hydroxymethyl) aminomethane-HCl (Tris) (pH = 8.0)

Fluorescence Measurement: The fluorescence spectra of dyes, dye/SWNT-T30 were recorded for different concentration by JASCO FP-6500 spectrophotometer with an excitation wavelength at 490 nm (for Ur) and 553 nm (for RB). Fixed amount of dye-H_2O origin solution and SWNT-T30 solution were mixed and diluted with the buffer solution (Tris, 10 mM) up to 2 mL to prepare the fluorescence samples. During the time-depended fluorescent monitoring, a rotator was used for keeping the uniformity of sample system.

Vis-absorption (UV) Measurement: The excitation wavelength for Ur systems and RB systems were confirmed respectively via UV spectra recorded by JASCO V-630 spectrophotometer.

Raman Measurement: Raman spectroscopy was performed to examine the structural conditions of SWNT. To prepare the samples, SWNT-T30 suspension was dropped to a glass slide and then dried at room temperature. NRS-3200 Micro Raman system (JASCO Corporation, Tokyo, Japan) was used for the spectroscopy, with 632.8 nm He-Ne laser excitation sources.

3 RESULTS AND DISCUSSION

3.1 Quenching Equilibrium and Quenching Efficiency of Single- and Multi- Fluorophore System
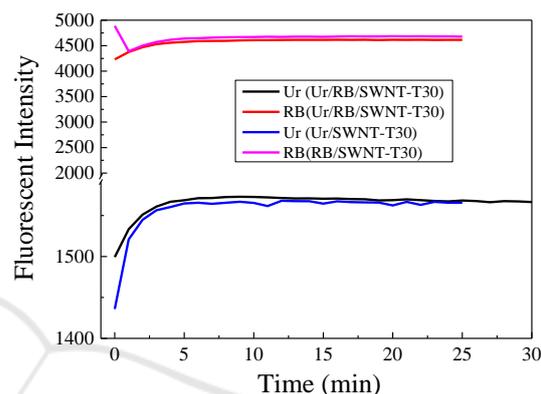

Figure 1: Time-depended fluorescent spectra of **Ur** and **RB** in single-fluorophore(2µL)/SWNT-T30(10µL) system and multi-fluorophore(2µL/2µL)/SWNT-T30(10µL) system, respectively. All the samples were diluted to 2 mL by Tris (10 mM). The excitation and emission wavelength was set as 490 nm and 512 nm for Ur, 553 nm and 572 nm for RB, respectively.

The quenching performance of Ur and RB were confirmed via time-depended fluorescent spectra as soon as the multi-fluorophore sample of Ur(2µL)/RB(2µL)/SWNT-T30(10µL) was prepared. As shown in Figure 1, the fluorescent intensity of Ur (black) and RB (red) remained constantly after 10 min respectively, or the equilibrium state of Ur and RB could be realized in 10 min. As comparison, we also compared the performance of Ur and RB in single-fluorophore quenching system (for instance, Ur(2µL)/SWNT-T30(10µL) and RB(2µL)/SWNT-T30(10µL)). The same proportion of fluorophore to SWNT, the same concentrations of fluorophores and SWNT-T30 were made sure for both single- and multi-quenching systems. Less than 10 min was spent to reach the quenching equilibrium for Ur (blue) and RB (pink), similar with multi-fluorophore system.

Moreover, we also could see that the fluorescent intensity of Ur at the equilibrium state in both multi-fluorophore/SWNT-T30 system (black or blue) and single-fluorophore/SWNT-T30 system (red or pink)

did not fluctuated obviously, at approximate 4500-4600 (Ur) or 1550-1570 (RB). It was illustrated that under the existence of another fluorophore (for example, Ur), the absorption coverage of one fluorophore (for instance, RB) onto SWNT surface seemed not be dramatically influenced compared with the case of single-fluorophore/SWNT-T30 quenching system.

Quenching efficiency was further calculated and researched via *T*-test analysis. The fluorescent intensity of Ur and RB with and without SWNT-T30 in both single-fluorophore/SWNT-T30 system and multi-fluorophore/SWNT-T30 system were obtained, then the corresponding quenching efficiency was compared. All the intensity was measured after the prepared samples set for 30 min to reach equilibrium state. As *T*-test is a statistical hypothesis test which could be used with small sample sizes (usually less than 30 samples) to determine if two sets of data are significantly different from each other, each experiment here was carried out 10 times for determination.

As listed in table 1, 13.0 ± 1.0 % and 12.0 ± 0.5 % emission of Ur was quenched by same content of SWNT-T30 with and without RB exist, respectively;

as far as RB, about 42.3 ± 1.2 % and 44.9 ± 1.5 % of fluorescence was restrained in single- and multi-quenching systems. The degree of freedom (P value) of *T*-test was calculated via *TTEST* function of EXCEL software based on 10 quenching efficiency values to confirm whether the quenching behavior of Ur or RB in single- and multi-quenching system is different.

In function *TTEST* (array1, array2, tails, type), the data of array 1 and array 2 came from the quenching efficiencies of single- and multi-fluorophore system, respectively; tails was set as 2 (two-tailed test); type was set as 2 (normal distribution). The P value obtained from Ur and RB was 0.0206 and 0.0367, less than 0.05 (usually the statistical significance is set as 0.05), illustrated that the quenching performances of both Ur and RB were not identical in different quenching systems.

We presumed that Ur showed a mild higher quenching efficiency in single-fluorophore system, while RB in multi-quenching system exhibited a little stronger quenching efficiency; although the variances of corresponding efficiency values were seemingly slight.

Table 1: Quenching efficiency and T-test result of Ur and RB quenching system by SWNT-T30.

	Ur		RB	
	Quenching efficiency (%)	P value※	Quenching efficiency (%)	P value※
Ur(2µL)/SWNT-T30(10µL)	13.0 ± 1.1		-	
RB(2µL)/SWNT-T30(10µL)	-	0.0206	42.3 ± 3.4	0.0367
Ur(2µL)/RB(2µL)/SWNT-T30(10µL)	12.0 ± 0.5		45.0 ± 1.5	

Quenching efficiency = [1- (intensity with SWNT-T30)/(intensity without SWNT-T30)] × 100%
※: P value is the degree of freedom of *T*-test, in function *TTEST* (array1, array2, tails, type), the data of array 1 and array 2 come from the quenching efficiencies of samples of single-fluorophore system and multi-fluorophore system; tails was set as 2 (two-tailed test); type was set as 2 (normal distribution), if P value is more than the statistical significance (usually 0.05), the array 1 and array 2 are assumed to be equal.

Table 2: Fluorescent intensity and T-test results of converse-sequent quenching systems.

	Ur		RB	
	intensity	P value※	intensity	P value※
①Ur + RB + SWNT-T30 $\xrightarrow{30\,min}$	1454 ± 57	②③# 0.6388	4656 ± 165	②③0.8818
②Ur + SWNT $\xrightarrow{30\,min}$ + RB $\xrightarrow{30\,min}$	1441 ± 41	①② 0.8279	4105 ± 137	①②1.71E-06
③RB + SWNT $\xrightarrow{30\,min}$ + Ur $\xrightarrow{30\,min}$	1445 ± 20	①③0.7014	4019 ± 105	①③9.39E-08

The exciting wavelength and emitting wavelength of Ur, AO, RB was set as (490 nm and 512 nm) and (553 nm and 572 nm), respectively.
※: P value is the degree of freedom of T-test, in function *TTEST* (array1, array2, tails, type), tails was set as 2 (two-tailed test); type was set as 2 (normal distribution), if P value is more than the statistical significance (usually 0.05), the array 1 and array 2 are assumed to be equal.
②/③# 0.6388: in function *TTEST* (array1, array2, tails, type), the data of array 1 and array 2 come from the fluorescent intensity records of assay ② and assay ③; 0.6388 is the P value calculated from intensities of assay ② and assay ③.

3.2 "Simultaneous Quench" and "Un-Simultaneous Quench"

Considering the competitive adsorption between two kinds of fluorophores, it was admired that if the addition order of two fluorophores alters the quenching equilibrium, because sometimes the multi-detection application cannot satisfy the "simultaneous quench" condition. Here three experiment schemes were designed, e.g., 1) 2 µL Ur and 2 µL RB were mixed and diluted firstly, then 10 µL of SWNT-T30 was added (the final mixture volume was 2 mL), set for 30 min and the fluorescent intensity of Ur and RB was measured respectively; 2) 2 µL Ur and 10 µL SWNT-T30 was mixed and diluted, set for 30 min; then 2 µL RB was added, set for 30 min (the final mixture volume was 2 mL) and the fluorescent intensity was measured; 3) 2 µL RB and 10 µL SWNT-T30 was mixed and diluted, set for 30 min; then 2 µL Ur was added into mixture, set for 30 min (the final mixture volume was 2 mL) and the fluorescent intensity was measured. Each experiment above was carried out in 10 times for determination. The final fluorescent intensity and quenching performance of Ur and RB in three systems were listed in Table 2.

The final Ur intensity after quenched by SWNT-T30 from ① "simultaneous added" model was calculated as 1541 ± 57, less than those from "un-simultaneous added" model (② and ③,1441 ± 41 and 1445 ± 20). In order to confirm if the 3 distributions could be regarded from the same one, T-test analysis was carried out for paired samples of assay ① and ②, ① and ③, ② and ③, respectively. P values (the freedom degree) were extracted as 0.8279, 0.7014 and 0.6388, all above 0.05, the threshold of statistical significance, demonstrated Ur performed similar quenching property in the three quenching situations. Different results were gained for RB, an average intensity of 4656 ± 165 in ① "simultaneous added" model was more than those of 4105 ± 137 in ② "Ur added first" model and 4019 ± 105 in ③ "RB added first" model. The P value of 0.8818 gained via comparing the two "un-simultaneous added" models ②/③, larger than 0.05, suggested that there was not difference for optical properties of RB in multi-fluorophore quenching systems in which whether RB was quenched first or not. However, extremely low P values, 1.71E-06 and 9.39E-08, obtained by comparing ① "simultaneous added" model with "un-simultaneous added" model (② or ③), indicated the independent photo-performance of RB in two models; more RB

molecules attached to the SWNT surface and lower photo-luminescence was observed in the "un-simultaneous added" model.

Thus, Ur showed similar quenching performance in both "simultaneous added" model and "un-simultaneous added" model. For RB, it was seemingly indicated that in "un-simultaneous added" model, SWNT-T30 could not quench RB as much as "simultaneous added" model; while in two "un-simultaneous added" model, the RB added sequence had no effects on the quenching performance of RB. We made a hypothesis that the adsorption of fluorophore onto SWNT surface was a dynamic balance process, equal number of fluorophore molecules attach onto and detach from SWNT surface at the same time. In the "un-simultaneous added" model, for example, before RB added, there had been an equilibrium state of adsorption and desorption of Ur onto SWNT surface; when RB added into the quenching system, several sites of Ur was replaced by RB during the Ur molecules departed from SWNT surface. Perhaps RB exhibited stronger scramble ability over Ur for SWNT surface occupation, which could also be verified by the quenching efficiency result of single- and multi-fluorophore quenching system (see section 3.1 and Table 1), RB showed slighter higher quenching efficiency in multi-system than single-system while Ur appeared a contrary tendency.

Moreover, RB itself seemed attached onto SWNT surface easier in the "un-simultaneous added" model than "simulaneous added" model. It was try to be explained that in "simultaneous added" model, Ur and RB molecules collided more intensely, so that the attaching probability on SWNT surface decreased steadily. Also in section 3.1 and Table 1, RB showed slight higher coverage onto SWNT surface in competitive system (or multi-system) than un-competitive system (or single-system), we thought perhaps it was a little difficult for RB molecules detach from SWNT surface because they were blocked by the Ur molecules surrounded.

As far as the unchanged Ur appearance in all the three quenching situations, it was proposed that perhaps there was tiny differences, but the violation could be ignored, as Ur exhibits a strong quench-restrain ability to SWNT surface, based on our previous work.

3.3 Raman Feature

Raman spectra of SWNT-T30 (black), SWNT-T30/Ur (blue) and SWNT-T30/RB (red) were measured for evaluating the effects of fluorophores

to SWNT surface. The volume ratio of SWNT-T30 origin solution to fluorophore origin solution was set as 10:1. The G-band and D-band (see the arrows) were clearly observed, the shapes were similar in all the samples. As shown in the features, SWNT's G band shift of both SWNT-T30/Ur (blue) and SWNT-T30/RB (red) located at 1580 cm^{-1}, the same as that of SWNT-T30 (black), maybe indicated that the fluorophore-wrapping did not affect the vibrations of SP^2 hybrid orbital of carbon atoms. Usually the frequency of D band is sensitive to the lattice defects of carbon atoms, here D band assigned to 1332 cm^{-1} for both SWNT-T30 (black) and SWNT-T30/Ur (blue), however, 2 split peaks (1336 cm^{-1} and 1361 cm^{-1}) appeared in the case of SWNT-T30/RB (red). Perhaps some lattice deformation occurred during RB attached onto SWNT surface compared with Ur.
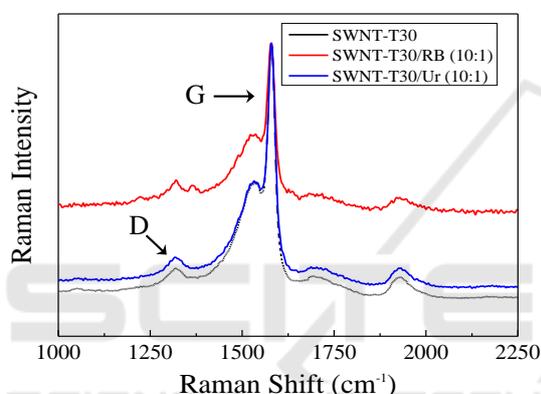

Figure 2: Raman features of SWNT-T30, SWNT-T30/RB and SWNT-T30/Ur.

4 CONCLUSIONS

Our analytical assays present time-depended quenching behaviors of alone-fluorophore and multi-fluorophore by SWNT-T30. All the quenching equilibrium could be realized in 10 min. Ur showed a mild higher quenching efficiency in single-fluorophore system, while RB in multi-quenching system exhibited a little stronger quenching efficiency.

The effects of "simultaneous quenched" and "un-simultaneous quenched" on the quenching properties of Ur/RB/SWNT-T30 multi-quenching system were researched. Ur showed similar quenching performance in both "simultaneous added" model and "un-simultaneous added" model; RB itself seemed attached onto SWNT surface easier in the "un-simultaneous added" model than "simultaneous added" model. In "un-simultaneous added" mode,

the RB quenching sequence had no effects on the its quenching performance. As shown in Raman results, fluorophore-wrapping did not influent the vibrations of SP^2 hybrid orbital of carbon atoms in SWNT; some lattice deformation occurred during RB attached onto SWNT surface compared with Ur. The fundamental methodology and results in this work were necessary and essential for multi-nanosensor applications, and could be useful for the further and extended application of fluorophores/dyes detection and quantification in aqueous solution.

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