Nanoscaled ZnO films used as enhanced substrates for fluorescence detection of dyes*

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The ability of nanoscaled ZnO films to enhance fluorescence was studied. We found that the fluorescence intensities of Cy5, rhodamine 6G, and fluorescein can be enhanced about 10-fold on nanoscaled ZnO films as compared to that on glass substrates. The lifetimes of all samples were measured, and no obvious change in lifetime was observed for dyes on different substrates. The mechanism for the nanoscaled ZnO film enhanced fluorescence appears to be different from that for the metal–fluorophore systems.

Keywords: nanoscaled ZnO films, lifetime, enhanced fluorescence, dye

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1. Introduction

Fluorescence detection is a central technology in biological research and clinical chemistry. Engineering of advanced substrates is an effective way to improve the fluorescence detection capability and to reduce the cost. Our group has observed that metalized substrates can decrease the lifetime and increase the radiative decay rate through the plasmon coupling between the fluorophore and the metal. Their effect also increases the quantum yield and the photostability of the fluorophore near the metal surface.[1−9] However, the cost of those materials (such as silver and gold) may limit their uses in disposable devices. ZnO nanomaterials are inexpensive, and can be easily processed through many synthetic routes.[8] As a wide band gap (3.37 eV) semiconductor oxide with a large excitation binding energy (60 meV) at room temperature, ZnO nanomaterials have received considerable attention for applications in short-wavelength light emitters, field emitters, luminescence devices, ultravidet (UV) lasers,[10] solar cells,[11] photocatalysts,[12] and superhydrophobic materials.[13−16] Furthermore, ZnO nanomaterials have a very good potential for *in vivo* studies, as they are nontoxic and stable in typical biomolecular environments.[17] Recently, ZnO nanomaterials have been used in biological testing schemes to enhance the fluorescence of dye-labeled proteins.[18−24] Dorfman *et al.* first demonstrated that ZnO structures can serve as ideal fluorescence enhancing substrates for identifying and screening protein–protein interaction.[18] Adalsteinsson *et al.* reported that nanometer-scale ZnO can be used successfully for the ultra sensitive detection of interleukins and tumor necrosis factor, and the detection limit for the cytokine exceeds the sensitivity of the conventional assays by 3–4 orders of magnitude.[21] More recently, Hu *et al.* found that the randomly oriented ZnO nanorods show superior performance in spot morphology, intensity homogeneity, and protein loading density compared to the aligned nanorods, and an nearly 20-fold enhanced fluorescence was found.[23,24] However, even many different kinds of ZnO nanostructures had been synthesized, only prepatterned ZnO nanorods and random ZnO nanorods were used in the previous works on ZnO enhanced fluorescence. The preparation of those substrates required expensive microfabrication techniques or long-time procedures, and also only the dye-labeled proteins were used in most publications, no careful control on the amount of fluorephores on the

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substrates was studied.[18−24] The exact mechanism responsible for the enhanced fluorescence signal of the ZnO nanomaterials is not clear and requires further study.

In this work, we prepared ZnO substrates using simple batch chemical methods and tested their abilities to enhance fluorescence. The ability of ZnO to enhance fluorescence was tested by carefully controlling the amount of fluorephores on the substrates. We gained about 10-fold enhancement for dyes on nanoscaled ZnO films (NZFs) as compared to that on the glass substrate. Lifetimes of all samples were measured. Because there was no obvious change in lifetime, the mechanism might be different from that in metal enhanced fluorescence (MEF).

2. Experimental methods

All reagents and spectroscopic grade solvents were used as received from Fisher or Sigima/Aldrich. The water (> 18.0 MΩ·cm) was purified using a millipore milli-Q gradient system for all experiments. The NZFs were deposited on glass substrates (microscope slides) by alternate dipping into a 0.05 mol/L sodium zincate (Na_2ZnO_2) bath kept at room temperature and hot water maintained near the boiling point for 50 times.[25] The glass substrates were ultrasonically cleaned 3 times with an equivolume mixture of acetone and ethanol before use. The deposited NZFs were subsequently annealed in air at 200 °C for 30 min to enhance their adhesion. All NZFs were ultrasonically cleaned for 5 min to remove the unbound small ZnO nanoparticles before spin-coating. Different dyes, such as Cy5, rhodamine 6G, and fluorescein with a concentration of 0.01 mmol/L in 2 wt.% PVA (polyvinyl acetate) water solution, were then spin-coated on the NZFs and the glass substrates twice for 30 s at a speed of 3000 RPM respectively. After the evaporation of water, dye-doped homogeneous PVA films would be formed on the surfaces of the NZFs and the glass substrates. Smooth ZnO films with preferred orientation along (002) were prepared by using a sol–gel method.[26]

The fluorescence spectra were recorded by using a Cary Eclipse fluorescence spectrophotometer. The excitation wavelengths used for Cy5, rhodamine 6G, and fluorescein were 600 nm, 500 nm, and 460 nm, respectively. The lifetimes were measured by using a PicoQuant modular fluorescence lifetime spectrometer (Fluo Time 300), for fhodamine 6G and fluorescein, a 485 nm laser was used for excitation, and for Cy5, a 640 nm laser was used. The surface morphology was analyzed by using an atomic force microscope (AFM) (WITec, Alpha300A).

3. Results and discussion

Figure 1 shows the surface morphologies of the NZF (a) and the smooth ZnO film (b). As can be seen in the AFM images, the surface of the NZF is much rougher than that of the smooth ZnO film. The grain sizes in the NZF are also bigger than that in the smooth ZnO film. The rough surface usually scatters light more effectively. In this work, the NZFs are used as emission enhanced substrates, as the smooth ZnO film does not show any significant enhancement.

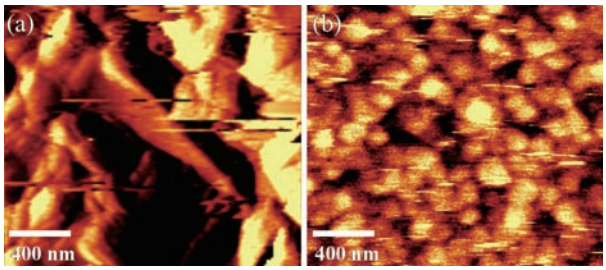


Fig. 1. (colour online) Surface morphologies of (a) nanoscaled ZnO film and (b) smooth ZnO film.

Figure 2 depicts the fluorescence intensity spectra of dyes, such as Cy5 (a), rhodamine 6G (b), and fluorescein (c), on the NZFs and the glass substrates, respectively. It is found that the fluorescence intensities of different dyes can be greatly enhanced on the NZFs as compared to that on the plain glass substrates. After considering the background emission of the NZFs, we find no obvious peak shift in all three dyes on different substrates. However, the fluorescence of Cy5 and fluorescein on the NZFs is enhanced much more than that of rhodamine 6G. This is because of the high quantum yield of rhodamine 6G. For the rhodamine 6G on the NZF, its intensity can easily exceed the limit of the instrument under the same test condition. So a smaller amount of rhodamine 6G was used on the NZF in the spin-coating process for easy comparison with the intensities of the dyes on the glass substrate. The surfaces of the NZFs are rougher as compared to that of the glass surface, and they can scatter light. The scattering emission spectra of the NZFs without dyes were obtained, which are also shown in Fig. 2. The corrected fluorescence intensities of the dyes on the NZFs are obtained by subtracting the scattering emission intensities of the NZFs without dye. After

excluding the scattering light from the NZFs, the fluorescence enhancements of the dyes are still observed, and the enhancement ratios are listed in Table 1.

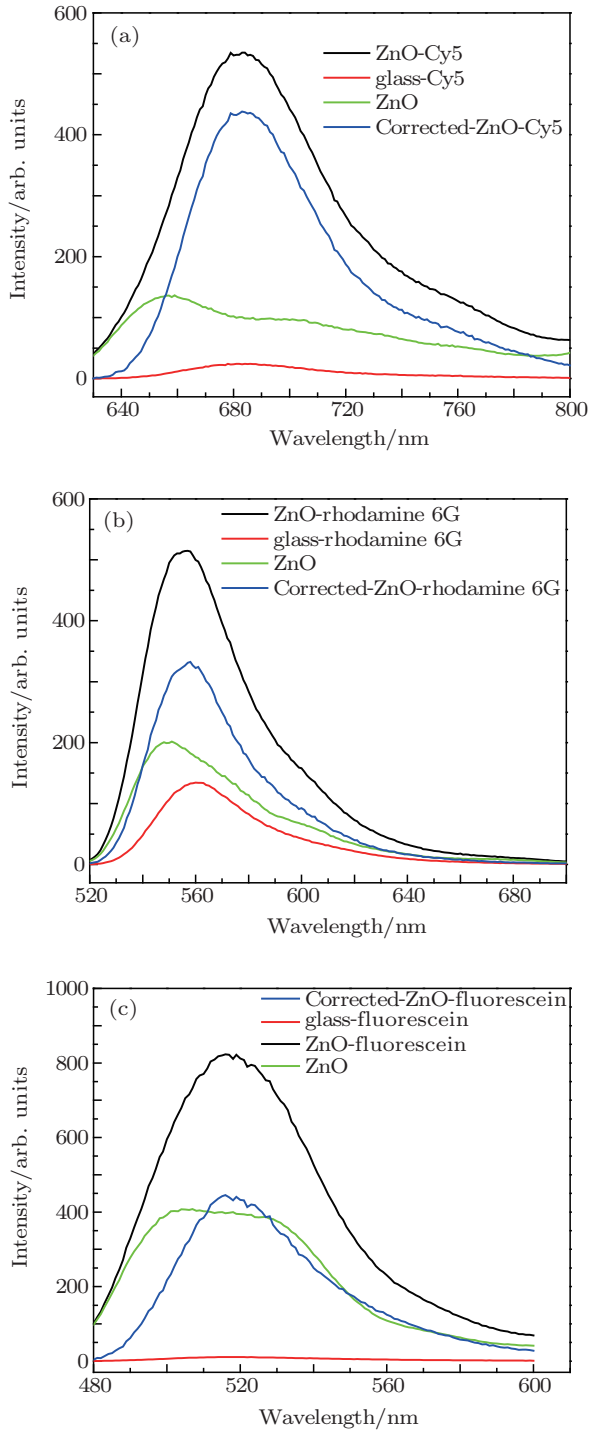


Fig. 2. (colour online) Fluorescence intensity spectra of (a) Cy5, (b) rhodamine 6G, and (c) fluorescein on glass and ZnO nanostructures.

Due to the larger surface areas of the NZFs, more dyes can adhere to the rough surfaces during the spin-coating process, which can result in higher fluorescent intensities. To exclude this factor, ultrasonic washing was used to dissolve the dyes from the NZFs and the glass substrates into equal volumes of water, and then the fluorescence intensities of the solutions were tested respectively. Figure 3 shows the fluorescence intensities of the different dyes obtained from the different samples. The fluorescence intensities of the solutions of the dyes washed off from ZnO (ZnO-dye-solution) are compared to that of the solutions of the dyes washed off from the glass substrate (glass-dye-solution). The different ratios of ZnO-dye-solution intensity as compared to the glass-dye-solution intensity are shown in Table 1. For example, only about twice (2.27) the amount of dyes are found in the Cy5 dissolved solution obtained from the NZFs, as compared to that from the glass substrate. To be careful, after the dyes were washed off from the surfaces, we tested the emissions of the washed NZFs (ZnO-dye-washed) and the washed glass substrate (glass-dye-washed) again. And it was found that the intensities of the washed substrates were nearly the same as the substrates without dye, which indicates that the dyes on the substrates were completely dissolved in the water.

Table 1. Enhancement ratios for Cy5, rhodamine 6G, and fluorescein.

Dye	Cy5	Rhodamine 6G	Fluorescein
ZnO/glass	17.50	2.43	39.607
Dye in water ZnO/glass	2.27	0.25	2.69
Enhancement	7.7	9.7	14.7

To prove that the dyes on the NZFs and on the glass substrates were not deteriorative and the intensity of the dye solution was proportional to the amount of dye on the substrate, equal amounts of dye were put on the NZFs and the glass substrates, and then they were dried naturally under dark conditions. Ultrasonic washing was used to dissolve the dyes into water, and in the meantime the same amount of dyes were dissolved in water directly, then the intensities were measured and compared. It was found that the intensities were more or less the same, as shown in Figs. 3 (d), 3(e), and 3(f). Hence, we can make sure that the NZF substrate can enhance the fluorescence intensities of the dyes. Our result is in accord with the other group's results that the increased fluorescence signal observed using the ZnO nanostructures is not only related to the larger surface areas of the nanomaterials.[19,21]

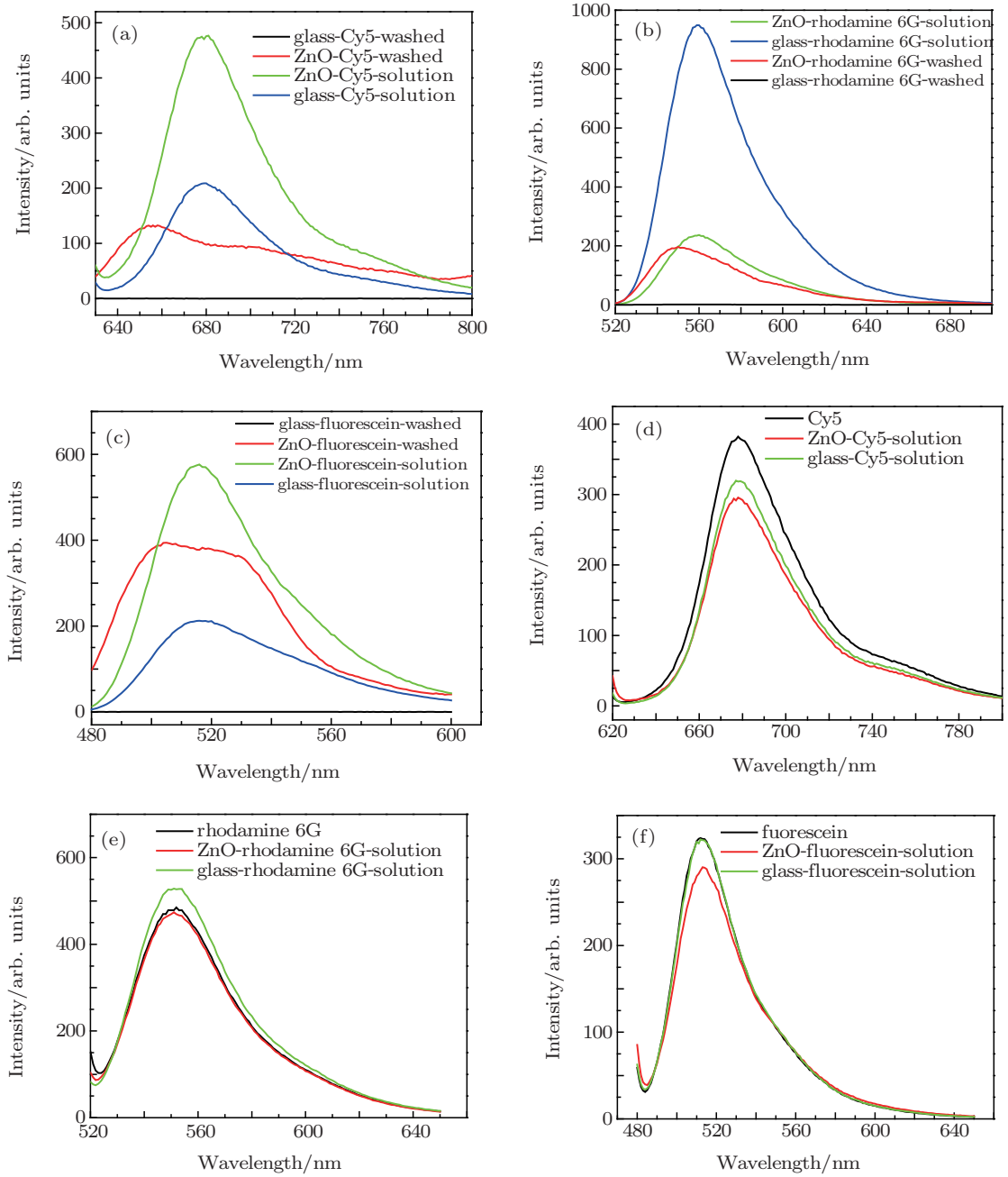


Fig. 3. (colour online) Fluorescence intensities of the washed-off solutions and substrates of (a) Cy5, (b) rhodamine 6G, and (c) fluorescein; equal amount of (d) Cy5, (e) rhodamine 6G, and (f) fluorescein obtained from different sources.

It is well known that fluorescence intensity changes are usually related to the changes in lifetime and quantum yield. For example, the fluorescence enhancement on a metal surface is believed to occur through increases of the intrinsic decay rate and the quantum yield.[1] In order to investigate the mechanism for the ZnO enhanced fluorescence, we measured the lifetimes of different samples. The emission intensity decays were collected by using a single photo counting spectrometer, and the data were analyzed in terms of double exponential decays for all samples, except for the fluorescein on glass, for which the three exponential decays were used to obtain more accurate data. The multiexponential intensity decays are described by

$$I(t) = \sum_{i=1}^{n} \alpha_i \exp(-t/\tau_i),$$

where τ_i is the decay time, and α_i is the amplitude. The amplitude weighted average lifetime is represented by

$$\langle \tau \rangle = \sum_i \alpha_i \tau_i.$$

Figure 4 shows the emission decay curves of Cy5 (a), rhodamine 6G (b), and fluorescein (c) respectively. The lifetime of Cy5 in water is 1.853 ns, on glass is 2.939 ns, and on NZF is 2.571 ns, as shown in Table 2. The lifetime in water is different from that on the substrates, which is caused by the different environments. Only a small decrease in lifetime is found for Cy5 on NZFs as compared to that on glass. The situations of rhodamine 6G and fluorescein are more or less the same as that of Cy5, no obvious change in lifetime is observed, and only a small decrease is found.

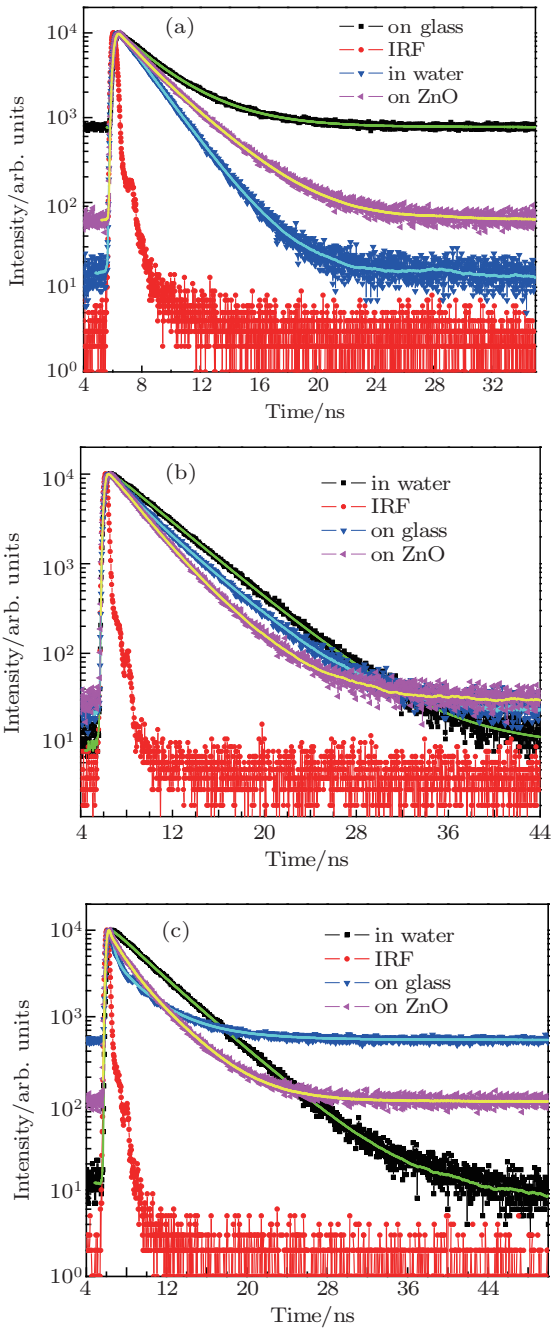


Fig. 4. (colour online) Lifetime spectra of (a) CY5, (b) rhodamine 6G, and (c) fluorescein.

It appears that the ZAFs do not cause any significant modification in the decay rates on the radiative pathways. Therefore, the mechanism for the ZnO enhanced fluorescence appears to be different from that for the MEF, in which the enhanced fluorescence is usually accompanied with a remarkable decrease in lifetime.[1] Furthermore, for the MEF, when the fluorophores are placed less than 5 nm away from the metal surface, the fluorescence can be quenched.[26] However, we do not observe any quenching of the dyes when they are placed directly on the NZFs, which is accord with Adalsteinsson's results.[21]

Table 2. Calculated lifetime data of Cy5, rhodamine 6G, and fluorescein.

Sample	τ_i/ns	α_i	$\langle\tau\rangle$/ns	χ^2_R
Cy5 in water	1.583	0.945	1.583	0.967
	1.829	0.055		
Cy5 on glass	3.346	0.749	2.939	1.071
	1.724	0.251		
Cy5 on ZnO	2.833	0.845	2.571	1.005
	1.146	0.155		
Rhodamine 6G in water	10.370	0.019	4.186	1.021
	4.065	0.981		
Rhodamine 6G on glass	3.939	0.852	3.587	1.106
	1.562	0.148		
Rhodamine 6G on ZnO	3.643	0.540	2.928	1.143
	2.091	0.460		
Fluorecein in water	4.337	0.912	4.145	1.006
	2.167	0.088		
Fluorescein on glass	5.348	0.335	2.970	1.681
	2.567	0.428		
	0.335	0.237		
Fluorescein on ZnO	3.504	0.773	2.967	1.091
	1.137	0.227		

The separation of the dye molecules by the large surface areas of the NZFs may reduce the resonance energy transfer between the fluorophores themselves and prevent self-quenching. However, the large surface areas of the NZFs usually absorb more dyes, as shown in Table 1. The distances between the dyes should not be changed much as compared to that on the glass substrate. On the other hand, for rhodamine 6G, fewer dyes are absorbed on the NZFs compared to that on the glass substrate, so the dyes are better separated, however no further enhancement is found. Thus, the segregation of the dyes by the large surface areas of the NZFs is not the main reason for the fluorescence enhancement.

Recently Zhang *et al.* reported a new way to greatly enhance the fluorescein isothiocyanate (FITC)

fluorescence intensity by directly doping FITC into a ZnO nanoparticle matrix. They demonstrated that the interaction between the dye carbonyl group and the Zn^{2+} ions red shifts the fluorescence and narrows the fluorescence band gap, thus increasing the excitation rate of the fluorophores and leading to the higher quantum yield.[28] However, the fluorescence of Cy3 used by Hu *et al.* was enhanced by ZnO nanorods, and Cy3 did not contain a carbonyl group.[23] Our results also show that the fluorescence of dyes such as e Cy5 without a carbonyl group can be enhanced by the NZFs. So that mechanism is not reasonable for dyes without carbonyl group. But that work shows us a way to understand the mechanism for the ZnO enhanced fluorescence. There should be other unknown interaction between dyes and ZnO, which induces the enhanced fluorescence. The ZnO enhanced fluorescence comes from the ZnO intrinsic fluorescent enhancement, as the other materials, such as silicon nanorods and polystyrene thin films, do not show the enhanced fluorescence property.[18]

Even though the ZnO enhanced fluorescence is not due to the larger surface areas of the nanomaterials, the roughness surfaces of the NZFs indeed influence the enhancement ratio, as we did not get as much enhancement as that of the NZFs when a smooth ZnO film was used. For the smooth ZnO film, we only observed less than 3 times enhancement (not shown here). The results suggest that the roughness of the NZF plays a very important role in the fluorescence enhancement effect. Adalsteinsson *et al.* demonstrated that the waveguiding property of the ZnO nanomaterials and their ability to enhance the intensity of evanescent field may explain the ZnO enhanced fluorescence.[21] The predicted decay distance of the evanescent wave field on the metal oxide nanorods for visible wavelengths is much longer than 20 nm.[29] The average diameter of the grains in the NZFs is commensurate to the predicted dimensions of the ZnO nanorods to guide the visible light effectively. Moreover, with the rough surfaces, the NZFs should be more effective than the smooth ZnO films in guiding the visible light and enhancing the intensity of evanescent field. Herein, the NZFs can serve as efficient evanescent waveguides enhancing the absorption and the emission processes of the fluorophore, which in turn enables the extremely high detection sensitivity of the dyes. We also find that the NZFs are more effective in scattering light in the emission peak wavelength of fluorescein (520 nm) than that in the emission peak wavelength of Cy5 (680 nm). So, a larger enhancement ratio is found for fluorescein (14.7) than that for Cy5 (7.7), as shown in Table 1. However, further work is still needed to verify this result. Experimental variables, such as the different emission wavelengths of dyes, the physical dimensions and the surface roughnesses of the ZnO nanostructures, can be changed to systematically investigate the fluorescence enhancement effect.

4. Conclusion

In conclusion, the NZFs were prepared by using an simple method. We found that the fluorescence intensities of dyes, such as Cy5, rhodamine 6G, and fluorescein, can be enhanced about 10-fold on the NZFs as compared to those on the glass substrates. We also tested the lifetimes of all samples and investigated the mechanism for the ZnO enhanced fluorescence. Even though the exact mechanism for the ZnO enhanced fluorescence is still unknown, what we have done promotes the use of ZnO nanostructures as enhanced substrates for fluorescence detection and expands the applications of ZnO nanostructures in biotechnology.

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