Dyes and Pigments 95 (2012) 18–22

Contents lists available at SciVerse ScienceDirect

Dyes and Pigments

journal homepage: www.elsevier.com/locate/dyepig

FRET – based technique for the characterization of contour lines

Yana Milyutin [a], Nurit Shaham-Waldmann [a], Sima Yaron [b], Yaron Paz [a,*]

[a] Department of Chemical Engineering and the Russell Berrie Nanotechnology Institute, Technion, Haifa 32000, Israel
[b] Department of Biotechnology and Food Engineering and the Russell Berrie Nanotechnology Institute, Technion, Israel

ARTICLE INFO

Article history:
Received 28 June 2011
Received in revised form
3 November 2011
Accepted 5 March 2012
Available online 3 April 2012

Keywords:
FRET
Composite particles
Adsorption
Contour line
FITC
TRITC

ABSTRACT

Catalysts that are made of composite particles may comprise several compounds, where each compound utilizes its own specific properties, thus providing synergistic effects that cannot be found when the constituents are used separately. Developing and quality control of such particles require measuring the shape of the domains and the length of the contact line between domains made from different compounds.

In what follows, the feasibility of using Fluorescence Resonance Energy Transfer (FRET) in order to gain information on the length of contour lines between neighboring domains is demonstrated. The principle of the method is to selectively adsorb the donor on one type of domain while selectively adsorbing the acceptor on the second type of domain. The requirement for a short distance between the acceptor dye molecule and the donor dye molecule in order to obtain fluorescence is then translated into a linear dependence between the emission intensity and the contour line length. This facilitates, upon using calibrated standards, the calculation of the average length of the contour between domains.

1. Introduction

Solid state catalysts are used in a large variety of applications, reflecting the advantages of heterogeneous catalysis among which is easier separation between the catalyst and the product stream, which facilitates continuous operation. In addition, heterogeneous catalysts are typically more tolerant of extreme operating conditions than their homogeneous analogs [1]. Catalysts made of composite particles, comprised of several compounds, each with its own specific properties, may provide synergistic effects that cannot be found when the constituents are used separately [2]. Altering one of the components provides a way to control the catalytic properties, thus making these composites ideal for a large variety of applications [3,4]. Of particular interest are composite catalysts designed for multi-step catalysis. Here, each domain catalyzes a specific different reaction and the products of these specific reactions react at the interface between the domains to give the final product (Fig. 1).

For such catalytic particles to operate, an intimate contact between the various components is a necessity. Likewise, the relative amount of each constituent on the surface of the particles may affect activity beyond the effects of surface area, porosity and acidity found in non-composite catalysts. The use of different phases or compounds within a single catalytic particle raises questions regarding not only the relative mass ratio between the components but also about the interfacial surface area. Since the performance is governed by the external surface, it is not the interfacial surface areas that is important here, but rather the length of the surface contact line (SCL) between the various constituents. Unfortunately, measuring the length of the SCL is a very difficult task. To the best of our knowledge, there is no method that can provide the length of SCLs, apart from direct observation by microscopic means (SEM or AFM for example). These means, if applicable at all, are relatively expensive and sophisticated and may require well-trained personnel. Moreover, the data gathered by these imaging techniques represent, at most, a very small number of particles, therefore obtaining the averaged properties can be quite cumbersome, time consuming and expensive.

Fluorescence Resonance Energy Transfer (FRET) is a distance-dependent physical process, by which energy is transferred non-radiatively from an excited molecular fluorophore (the donor) to another fluorophore (the acceptor) by means of intramolecular or intermolecular long-range dipole–dipole coupling. The efficiency of FRET is proportional to one over the sixth power of the distance between donor and acceptor [5]. Accordingly, in order for the process to be efficient, the distance between the donor and the acceptor fluorophores has to be within a few nanometers, as the typical Forster radius (the distance at which half the excitation

* Corresponding author. Fax: +972 4 8295672.
E-mail address: paz@tx.technion.ac.il (Y. Paz).

0143-7208/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.dyepig.2012.03.007

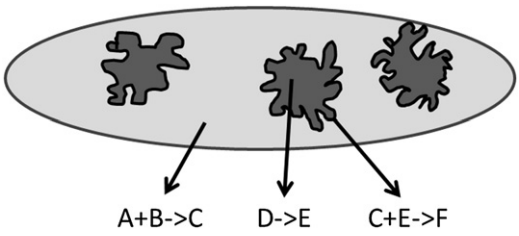

Fig. 1. A schematic view of a composite bi-catalyst particle, performing multi-step catalysis.

energy of the donor is transferred to the acceptor) is around 3–6 nm. Other requirements include substantial overlap (>30%) between the emission spectrum of the donor fluorophore and the absorption spectrum of the acceptor (Fig. 2) and similarity in the orientation of the donor emission dipole moment and the acceptor absorption dipole moment. Along this line, control over the FRET efficiency can be achieved by altering the donor–acceptor pairs [6], which changes absorption characteristics, emission characteristic, overlap characteristics, and the extent of parasitic energy loss.

At present, the FRET phenomenon is utilized quite extensively in life science [7,8]. One mode of operation is based on intramolecular transfer, where both flurophores are located on the same molecule [9]. This operation is widely used to measure distances in proteins or to monitor protein conformational changes. By virtue of specific adsorption, the FRET technique is used in this case to indicate the shape and locus of the underlying substrate [10]. A different mode of operation utilizes two or three different molecules, one molecule serves as a donor while the other(s) as an acceptor [11]. Here, the two probe molecules are adsorbed on two different substrates, so that the emitted light indicates the proximity between the adsorption locations of the fluorophores. This operation is used to study protein–protein interactions or the interaction of molecules (proteins, DNA, etc.) with substrates or ligands in live biological samples [12].

In what follows, the feasibility of using the technique of fluorescence energy transfer to measure the length of interdomain surface contact lines will be demonstrated, evaluated and analyzed. The principle of the method is to selectively adsorb the donor on one type of a domain while selectively adsorbing the acceptor on the second type of domain. The requirement for short distance between the acceptor and donor is then translated into a linear dependence between the emission intensity and the SCL. This facilitates, upon using calibrated standards, to ability to easily calculate the length of the contour line between the domains.

2. Experimental

The application of FRET for the measuring of SCL was demonstrated with model systems comprised of well-defined domains, whose SCL was pre-designed, and measuring the FRET signal as a function of SCL length. The model-systems contained silicon substrates over-coated with bare domains of TiO_2 and phosphoric acid-treated TiO_2 domains.

2.1. Sample preparation

Fig. 3 presents a schematic view of the sample preparation process. The titanium oxide thin film was prepared by a sol gel process, previously reported elsewhere [13]. The precursor solution was prepared by mixing 4.5 mL $Ti[OCH(CH_3)_2]_4$ (97% in propanol) with 10.0 mL n-propanol and 1.6 mL acetylacetone (acac) to provide a Ti: propanol: acac molar ratio of approximately 1:9:1.05. A coating solution was prepared by mixing 1.0 mL of the anhydrous

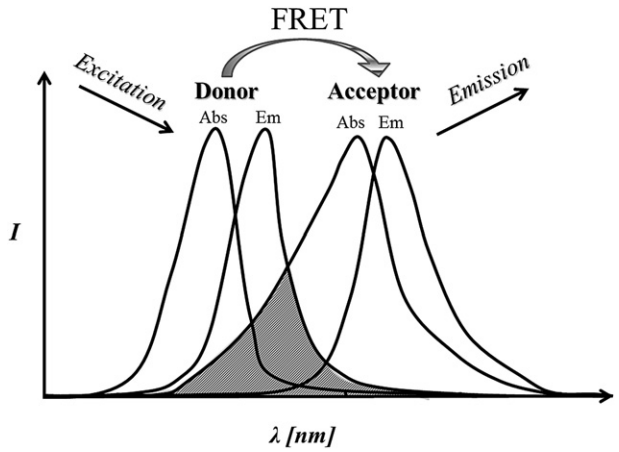

Fig. 2. The principle of FRET. The shadowed area represents the overlapping between the emission spectrum of the donor and the absorption spectrum of the acceptor.

$Ti(OPr)_3$-acac solution with 0.8 mL of water/n-propanol solution (1:9 v/v). The coating solution was then spread on a clean silicon substrate, spun at 3000 rpm for 1 min and calcined at 400 °C for 1 h. A well-adhered film having a thickness of approximately 90 nm, and made of sintered nanocystallites was obtained.

A photolithography process, performed at a clean room facility, was used to pattern the TiO_2 films into equal-width, alternating stripes of non-coated and phorphoric acid-coated titania (Fig. 3). The procedure included cleaning in acetone, methanol, isopropanol and DI Water, drying by spinning, and baking to complete dryness. For patterning, a S1818 G2 (Shipley Ltd.) positive photoresist was used. Here, the photoresist was spun at 5000 rpm for 1 min, pre-baked at 110 °C for 1 min, exposed through an appropriate mask for 4.5 s on a MA-6 mask-aligner, developed in Microposit MF-319 developer for 35 s, and post baked for 3 min at 125 °C. The samples were then dipped into a 1 M H_3PO_4 solution for 30 s and washed with flowing DI water for 1 min. The last step in the preparation was the stripping of the photoresist by soaking in acetone for 1 min, following by washing in water. Three different types of samples were prepared having line-widths of 10 μm, 5 μm and 2 μm. All samples had the same shape and size (10 × 10 mm).

The adsorption of the phosphoric acid on exposed TiO_2 films was verified by FTIR using an IFS-55 (Bruker) spectrophotometer. Likewise, the striped samples were characterized by surface probe microscopy using a Pico+ (Molecular Imaging) SPM machine.

2.2. Adsorption of dyes and FRET measurements

Choosing the right pair of chromophores is a very important for the success of the reported method. In this study, Fluorescein 5(6)-

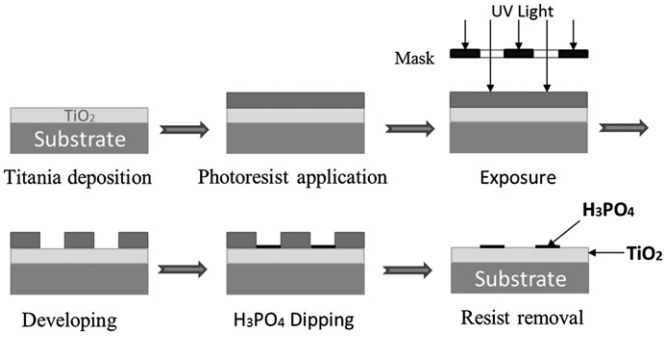

Fig. 3. A schematic view of the model systems preparation procedure.

isothiocyanate (FITC, Sigma–Aldrich) was used as a donor, whereas Tetramethylrodamine B isothiocyanate (TRITC, Sigma–Aldrich) was used as an acceptor. These chromophores were chosen based on their known qualities as commercial FRET pair and following preliminary screening measurements of their selective adsorption. The samples were first immersed in the TRITC solution for 45 min under dark conditions, cleaned with methanol and dried under an air-stream. Then, the samples were immersed into the FITC solution for 2 min and dried. Non-patterned samples (both treated and non-treated with phosphoric acid) served as controls. Different concentrations were used in various solvents as described below.

Steady-state fluorescence measurements under ambient air conditions were taken each time the samples were immersed in the dye solutions by a Wallac 1420 VICTOR^2 spectrophotometer. The measuring area was constant in all measurements. For samples immersed in FITC a F485 excitation filter and F535 emission filter were used, whereas samples immersed only in TRITC were measured with a F535 excitation filter and F618 emission filter.

In this manuscript, analysis of the data is often presented in terms of Fluorescence Efficiency (FE), defined as the ratio between the measured fluorescence intensity in the presence of both donor and acceptor and the sum of the measured fluorescence of the donor and the acceptor alone, at the same wavelength. For all cases, i.e. in solution or on dried substrates, care was taken to make the comparison under the same conditions, namely the same solvent and the same concentration of the liquid. For surfaces, the fluorescence selectivity between different surfaces is evaluated by calculating the fluorescence selectivity(FS) values, defined as the measured fluorescence intensity from TiO_2 following immersion in the fluorophore divided by the measured fluorescence intensity from H_3PO_4-coated TiO_2, after immersion in the same fluorophore.

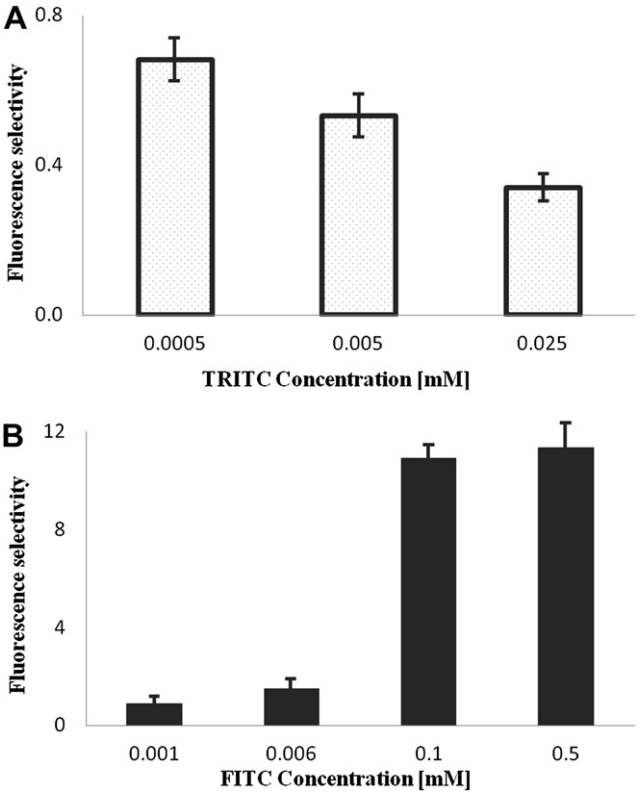

Fig. 4. The Fluorescence Selectivity (FS) measured upon immersing TiO_2 and H_3PO_4-coated TiO_2 substrates in solutions of TRITC (A) and FITC (B) for 10 minutes. Results are given for various concentrations of fluorophores.

3. Results and discussion

It is known that fluorescence efficiency values may strongly depend on both the excitation wavelength and the wavelength at which the emission is measured. As an example, one may consider the FE values obtained upon excitation of TRITC (5 μM in methanol), 5-FITC (10 μM in methanol) and a mixture prepared by mixing the two solutions (1:1 by vol.). In this example excitation at 485 nm yielded a FE value of 42 when measured at an emission wavelength of 535 nm, but only a value of 14 when measured at an emission wavelength of 618 nm. For the specific case presented in this manuscript the data in the liquid phase might be less relevant than the tendency for selective adsorption, leading to selectivity in the fluorescence. The fluorescence selectivity is of large importance in choosing the optimal conditions for measuring the contour line length, as the method is based on adsorption matching between substrates and fluorophores. In other words, there is a need that the FS value of one fluorophore will be well above unity, while the FS value of the second flurophore should be below unity and as small as possible. Fig. 4 presents the fluorescence selectivity measured upon immersing the substrates in alcoholic solutions of TRITC (A) and FITC (B) for 10 min. Results are given for several concentrations of various fluorophores, evidently demonstrating that the concentration of the fluorophores may have a significant effect on the selectivity between the two substrates. That way, the FS values for TRITC were reduced by a factor of 2 upon increasing the concentration from 0.5 μM to 25 μM. In the same manner, the FS values for FITC were increased by a factor as large as 12 upon increasing the concentration of FITC from 1 μM to 500 μM. Hence, it can be concluded that FITC tends to adsorb more selectively on these surfaces than TRITC.

It is known that, in terms of FRET efficiency, the optimal concentration of donor and acceptor depends not only on the specific fluorophores but also on the type of solvent. For FITC and TRITC, DMSO is regarded as the preferred solvent and is used in biological systems [14,15]. Indeed, a study on the effect of the TRITC/FITC concentration ratio on the fluorescence efficiency, measured in the liquid phase, revealed to us that the optimal ratio in DMSO was 0.5, yielding a FE value as large as 42. In our case, the adsorption of DMSO-dissolved fluorophores on the substrates under-study was negligible. Hence, DMSO was considered inadequate despite its high liquid-phase FE.

As mentioned above, a set of substrates containing alternating stripes of H_3PO_4- treated and non-treated TiO_2 were prepared by photolithography. Fig. 5 presents an AFM phase-contrast image, obtained in the non-contact mode. The H_3PO_4-treated and the non-treated TiO_2 stripes, both 5 μm in width, are clearly observed, reflecting a noticeable difference between the stiff inorganic TiO_2 surface, and the softer phosphoric acid over-coating. Topography images of the stripes were found to be less clear, and indicated that

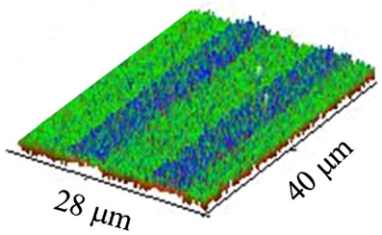

Fig. 5. Phase-contrast AFM image of a substrate made of alternating stripes of TiO_2 and H_3PO_4-coated TiO_2.

the thickness of the overcoating phosphoric acid was 1–2 nm, which may correspond to a few layers of molecules. Images of substrates that had been immersed in FITC showed very poor contrast in both topography and phase modes, in correlation with selective adsorption on bare TiO_2 but not on the acid-overcoated stripes. Along this line, images of alternating-stripe substrates that were first immersed in TRITC solution, washed and then immersed in a FITC solution, were characterized by a reasonable contrast between the two types of domains.

Fig. 6 presents the fluorescence intensity measured at 535 nm following excitation at 485 nm. The data was obtained by measuring the fluorescence intensity emitted from alternating-stripe substrates (TiO_2 and H_3PO_4/TiO_2) that were immersed successively in the two dyes, and washed with ethanol after each immersion. Care was made to optimize the concentration of the dye solutions according to the results presented in Fig. 4, i.e. a concentration of 0.1 mM in methanol for the FITC solution and a concentration of 0.025 mM in methanol for the TRITC solution. Such an optimization is important to assure minimization of contributing signals from areas that are not along the contour lines between the two types of domains. Two sets of data are presented: a set of data where the samples were first soaked in FITC, washed in ethanol, soaked again in TRITC and washed again and a second set of data where samples were first soaked in TRITC, washed in ethanol, soaked again in FITC and washed again. As presented in the figure, in both cases the larger the contour length was the higher the fluorescence intensity was. Moreover, the correlation was found to be linear (R^2>0.99 for both cases), as could be expected from a theoretical point of view. The observation of a linear correlation between the contour line length and the intensity of the FRET fluorescence suggests that FRET fluorescence can be used as a means to estimate the length of contour lines, provided that a calibration curve is prepared in advance. Repeated measurements with more sets of samples made by the "first TRITC, then FITC" procedure confirmed the high linearity and large slope presented in Fig. 6, thus strengthening the statistical value of this analysis of the data.

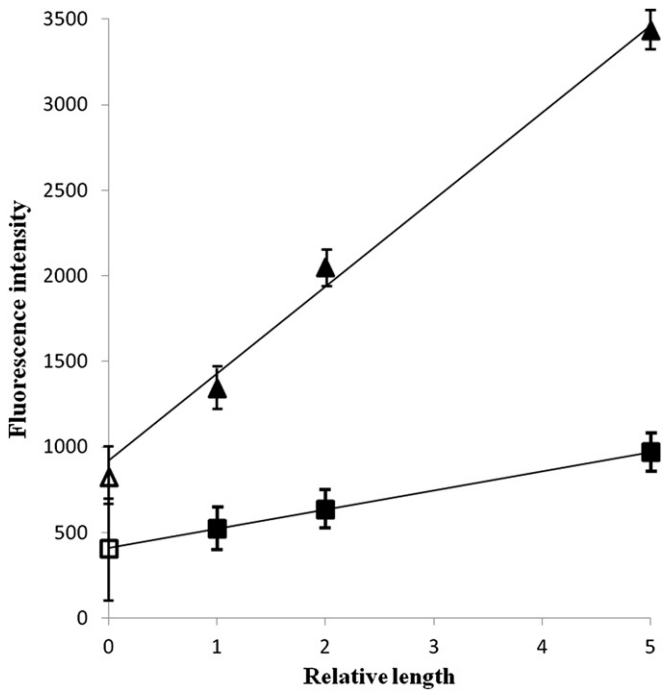

Fig. 6. The intensity of FRET fluorescence versus the relative length of contact line between TiO_2 domains and H_3PO_4 domains. Results are given for a "first TRITC, then FITC" process (triangles) and for a "first FITC then TRITC" process (squares). Empty symbols represent averaged measurements taken with non-patterned surfaces. The excitation/emission wavelengths were 485/535 nm, respectively.

Extrapolation of the "first TRITC, then FITC" curve to a zero contour line gives a fluorescence intensity value of 920, whereas extrapolation of the "first FITC, then TRITC" curve gives a value of 410. These values can be compared with measurements made with non-patterned substrates (both TiO_2 and H_3PO_4-coated TiO_2). Here, the intensity (averaged over the two types of substrates) for a case where the substrates were first immersed in TRITC and then in FITC was found to be 820, whereas the intensity (averaged over the two substrates) for a case where the substrates were first immersed in FITC and then in TRITC was found to be 405. The excellent matching between the extrapolated values and the measured "background" results may serve to strengthen the validity of the linear correlation presented in the figure.

A few words need to be said about the difference in the background values between "first FITC, then TRITC" and "first TRITC, then FITC". These values reflect a contribution of fluorescence from the whole covered area. Such contribution might stem either from less than perfect adsorption selectivity (causing some adsorption of a certain dye on an undesirable substrate) or from adsorption of one dye on its pre-adsorbed partner. The latter case may affect the fluorescence intensity either negatively or positively, depending on absorption coefficient, thickness and wavelength. The higher "background" intensity for the "first TRITC, then FITC" in comparison with the intensity of the "background" of "first FITC, then TRITC" can be explained by the lower adsorption selectivity of TRITC compared with FITC (Fig. 4). Here, the lower adsorption selectivity of TRITC is manifested, when introduced first to the system, by some adsorption on the TiO_2 surface. Once FITC is added, this undesired adsorption creates contact points between the two dyes that are manifested by an increase in the fluorescence. Indeed, the fluorescence measured on a TiO_2 substrate following immersion in TRITC increased by a factor of two upon a second immersion in FITC. At the same time, the fluorescence measured on H_3PO_4-coated TiO_2 substrate following immersion in FITC was not changed upon immersing in TRITC.

The conclusion of this analysis is that in order to reduce the undesirable contribution from the area of the domains, it is preferable to adsorb the dye with the higher selectivity before adsorbing the second dye. For our case, from the point of view of reducing background, it is preferable to adsorb FITC before adsorbing TRITC.

As explained in the introduction section, the intensity of FRET fluorescence depends, for a given set of donor and acceptor pairs and for a given impinging photon flux, on both the excitation wavelength and the measured emission wavelength. For this reason, it was important to study the effect of the excitation and emission wavelengths on the correlation between fluorescence intensity and the length of the contour lines. Fig. 7 presents fluorescence measurements taken with the same system described above (i.e. alternating stripes of TiO_2 and H_3PO_4/TiO_2 onto which FITC and TRITC were adsorbed, respectively) under three sets of excitation/emission wavelengths: 485 nm/535 nm, 485 nm/618 nm and 535 nm/618 nm. From the data, it is evident that positive correlation between the length of the contour lines and the fluorescence intensity exists in all three cases. The correlation is linear upon exciting with 485 nm, but deviates when the excitation was performed with 535 nm photons. A comparison between the 485 nm/535 nm graph and the 485 nm/618 nm graph reveals a constant ratio in the fluorescence at a given length (1.95). This constant ratio, which appears also in the extrapolation to zero contour length, suggests that almost all the measured fluorescence at 535 nm and at 618 nm originate from FRET, regardless of the relative contribution from the bulk. This is not the case upon excitation with 535 nm light that is mostly absorbed by the acceptor

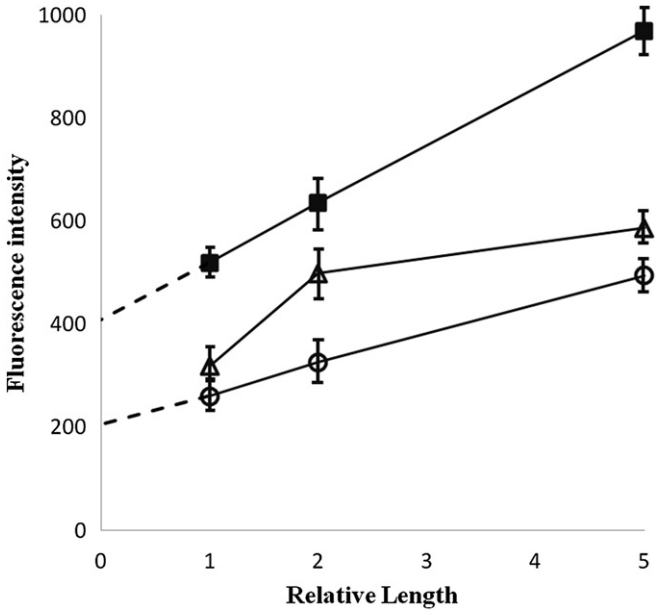

Fig. 7. The intensity of FRET fluorescence versus the relative length of contact line between TiO_2 domains and H_3PO_4-coated domains. Results are given for "first FITC then TRITC" process. The excitation / emission wavelengths were 485 nm/535 nm (filled squares), 485 nm/618 nm (circles), and 535 nm/618 nm (triangles).

(TRITC). Hence, the results point out the fact that in order to measure the effect reported hereby it is not necessarily required to have the exact set of filters, so far as there is no overlap between the excitation wavelength and the absorption spectrum of the acceptor. Although in this case the ratio between the signal and the background is constant (suggesting that the wavelength at which emission is measured has no importance in this range), it is still recommended, for reasons that have to do with equipment sensitivity, to use the wavelength at which the intensity of the emission is higher.

4. Conclusion

The feasibility of using the FRET phenomenon in order to gain information on the length of contour lines between neighboring domains was demonstrated. The technique is based on selective adsorption of a donor and an acceptor on the two types of domains, giving rise to a fluorescence signal that is linearly proportional to the length of the contour line. As a first example of its kind, we have used FITC as a donor and TRITC as an acceptor that adsorb selectively on TiO_2 and on H_3PO_4-overcoated TiO_2.

Based on the results obtained with this specific system, it can be concluded that a strong adsorption selectivity of at least one of the dyes is required. In addition, the sequence of adsorption seems to be important. Here, it is recommended to adsorb first the more selective dye. At any case, one has to take into account some contribution to the FRET signal from the bulk. This contribution is not, however, prohibitive to the application of the method, as it can be deducted during the preparation of the calibration curve. The results show that although the wavelengths have an effect on the signal to noise ratio, linear correlation between length and intensity can be obtained within a wide range of wavelengths.

The large number of donor–acceptor pairs that are either commercially available or can be easily synthesized may facilitate the locating of adequate pairs for a large number of substrates. This makes the phenomenon demonstrated in this manuscript a technique that can be implemented commercially, as a method for characterization of contour lines between domains. This method is potentially not only cheaper than electron microscopy or AFM, but also benefits from the fact that the manpower requirement is less stringent in terms of required expertise. A simple variation of the above-mentioned scheme may provide a way to measure local adhesion between polymer sheets that are joined together, similar to the method proposed before to study polymer chain interpenetration kinetics [16] and the coalescence of polymer particles [17]. Another possible use of this technique in materials science is for verifying the quality of overcoating of particles or surfaces by thin films, for example polymer-metal core-shell particles [3], and polymer–polymer core-shell particles [18]. Here, the measuring of FRET fluorescence might indicate incomplete coverage by the overcoating film. An example of an area that may benefit from such a technique is microencapsulation.

The method described in this manuscript is quite generic, and can be easily modified to ease its implementation. One of these modifications is the use of mediators in order to obtain the selective adsorption of the dyes. For example, the use of self-assembled monolayers (SAMs), tailored in a manner that enables them to chemisorb selectively to specific substrates should be considered. Here, the outer part of the SAMs should be functionalized (either before or after adsorption) with functional groups that have large selective affinity to specific dyes. Work along this line is in progress.

Acknowledgments

The authors are grateful to The Russell Berrie Nanotechnology Institute for its support and to E. Holt for his critical reading.

References

[1] Davis ME, Davis RJ, Robert J. Fundamentals of chemical reaction engineering [Chapter 5]. New York: McGraw-Hill Higher Education; 2003.
[2] Xie Z, Liu Z, Wang Y, Yang Q, Xu L, Ding W. An overview of recent development in composite catalysts from porous materials for various reactions and processes. Int J Mol Sci 2010;11:2152–87.
[3] Wang PH, Wu YZ, Zhu QR. Polymer metal composite particles: polymer core and metal shell. J Mater Sci Lett 2002;21:1825–8.
[4] Wu P, Brand L. Resonance energy transfer: methods and applications. Anal Biochem 1994;218:1–13.
[5] Lakowicz JR. Principles of fluorescence spectroscopy. 2nd ed. New York: Kluwer Academic; 1999.
[6] Stryer L. Fluorescence energy transfer as a spectroscopic ruler. Annu Rev Biochem 1978;47:819–46.
[7] Geddes CD. Reviews in fluorescence 2008, vol. 5. New York: Springer; 2010. pp. 321–488.
[8] Miller JN. Fluorescence energy transfer methods in bioanalysis. Analyst 2005; 130:265–70.
[9] Cotlet M, Gronheid R, Habuchi S, Stefan A, Barbafina A, Mullen K, et al. Intramolecular directional forster resonance energy transfer at the single-molecule level in a dendritic system. J Am Chem Soc 2003;125:13609–17.
[10] Sapsford KE, Berti L, Medintz IL. Materials for fluorescence resonance energy transfer analysis: beyond traditional donor-acceptor combinations. Angew Chem Int Ed Engl 2006;45:4562–89.
[11] Sturmey RG, O'Toole PJ, Leese HJ. Fluorescence resonance energy transfer analysis of mitochondrial: lipid association in the porcine oocyte. Reproduction 2006;132:829–37.
[12] Tsien RY, Bacskai BJ, Adams SR. Fret for studying intracellular signalling. Trends Cell Biol 1993;3:242–5.
[13] Paz Y, Luo Z, Rabenberg L, Heller A. Photooxidative self-cleaning transparent titanium dioxide film on glass. J Mater Res 1995;10:2842–8.
[14] Bergquist NR, Nilsson P. The conjugation of immunoglobulins with tetramethylrhodamine isothiocyanate by utilization of dimethylsulfoxide (dmso) as a solvent. J Immunol Methods 1974;5:189–98.
[15] Grant SA, Xu J, Mroz J, Bergeron EJ. Development of dual receptor biosensors: an analysis of fret pairs. Biosensors Bioelectron 2001;16:231–7.
[16] Morawetz H. Fluorescence study of polymer chain interpenetration and of the rate of phase separation in incompatible polymer blends. Polymer Eng Sci 1983;23:689–92.
[17] Turshatov A, Adams J, Johannsmann D. Interparticle contact in drying polymer dispersions probed by time resolved fluorescence. Macromolecules 2008;41: 5365–72.
[18] Taniguchi T, Ogawa T, Kamata Y, Kobaru S, Takeuchi N, Kohri M, et al. Preparation of core-shell coagulates by hydrophobic heterocoagulation of micron-sized poly(methyl methacrylate) and submicron-sized poly(styrene) particles. Colloids Surf A Physicom Eng Aspects 2010;356:169–75.