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Synthesis of hemicyanine dyes for 'click' bioconjugation

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Abstract—A new type of 2-propynyl substituted hemicyanine dyes have been synthesized in a facile route, which have showed superior solubility in water and good optical properties. The terminal alkynyl groups were employed for bioconjugation through a 1,3-dipolar cycloaddition with azides under mild conditions.

1. Introduction

Huisgen 1,3-dipolar cycloaddition of azides and alkynes to afford 1,2,3-triazole ring has been widely used in organic synthesis.[1] The high reaction yield, simple reaction and purification conditions, and the tolerance of other functionalities of this reaction make it an ideal prototype to demonstrate the concept of '*Click Chemistry*' developed by Sharpless and co-workers.[2,3] The new Fokin–Sharpless–Meldal modification, in which Cu(I) is used as the catalyst, affords superior regioselectivity and almost quantitative transformation under extremely mild conditions.[4,5] It accelerates the application of this 'click process' in medicinal chemistry and biomedical research.[6] Alkyne and azide groups are very small in size, are highly energetic with particularly narrow distribution of reactivity, can be conveniently introduced into organic compounds, and are quite indifferent to solvent and pH. Therefore, the Cu(I) catalyzed cycloaddition reaction can be used to link biomolecules in an aqueous environment under mild physiological conditions with high efficiency and chemoselectivity.[7–10]

In our study of the interfacial self-assembly of bionanoparticles (BNPs), we hope to investigate how the surface charges of BNP will influence the self-assembly process. Cyanine and hemicyanine dyes are ideal regents to introduce positive charges on the exterior surface of BNP since they usually have good solubility in water, which is crucial for the bioconjugation reaction. Cy3, Cy5, and other cyanine based fluorescent analogs are among the most commonly used bio-labeling fluorescent reagents.[11–15] Since only traditional methods for the attachment of cyanine dyes to biomolecules have been exploited so far, such as the reaction of *N*-succinimidyl ester for amino groups,[16–18] or iodoacetamide for thiol groups,[19,20] the synthesis of bio-labeling cyanine dyes in general was quite challenging. Herein, we describe the synthesis of a novel type of water soluble alkyne substituted hemicyanine dyes **I**–**V** (Scheme 1), which could be successfully conjugated to biomolecules via the 'click' bioconjugation reaction.

2. Results and discussion

Similar to our previous work in the synthesis of laser-used hemicyanine dyes,[21–23] the synthesis of compounds **I**–**V** was very straightforward (Scheme 2). Since the terminal alkyne group is inert to all reaction conditions adopted in our synthesis, no additional protection and deprotection steps were necessary. The synthesis of **I**–**III** was started from 2-methylbenzothiazole **X**, which was converted to **XI** by refluxing with propargyl bromide. Condensation of **XI** with 4-*N*,*N*-dimethylaminobenzaldehyde or 4-*N*,*N*-di-(2-carboxyethyl)aminobenzal-dehyde or 4-*N*,*N*-dimethylaminocinnamaldehyde resulted in dyes **I**–**III**. The dyes **IV** and **V** were synthesized analogously starting from 1,1,2-trimethyl-1 *H*-benzoindole. All the dyes show superior solubility in

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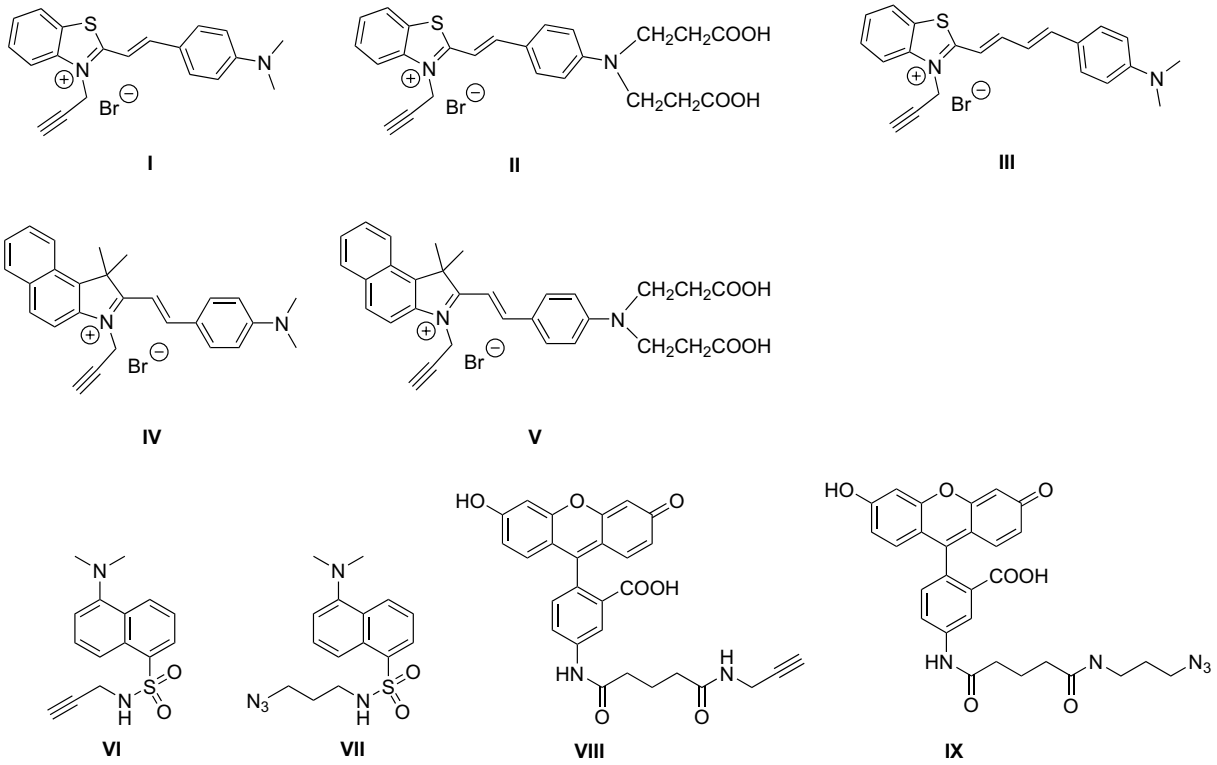
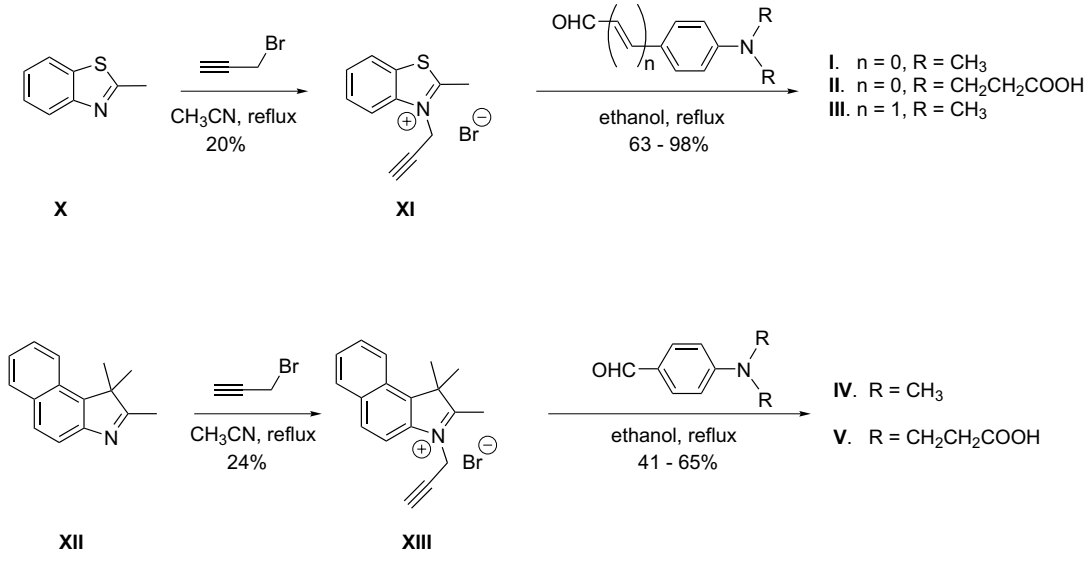


Scheme 1. Structures of fluorescent dyes used in 'click' bioconjugation.



Scheme 2. Synthetic routes of the hemicyanine dyes.

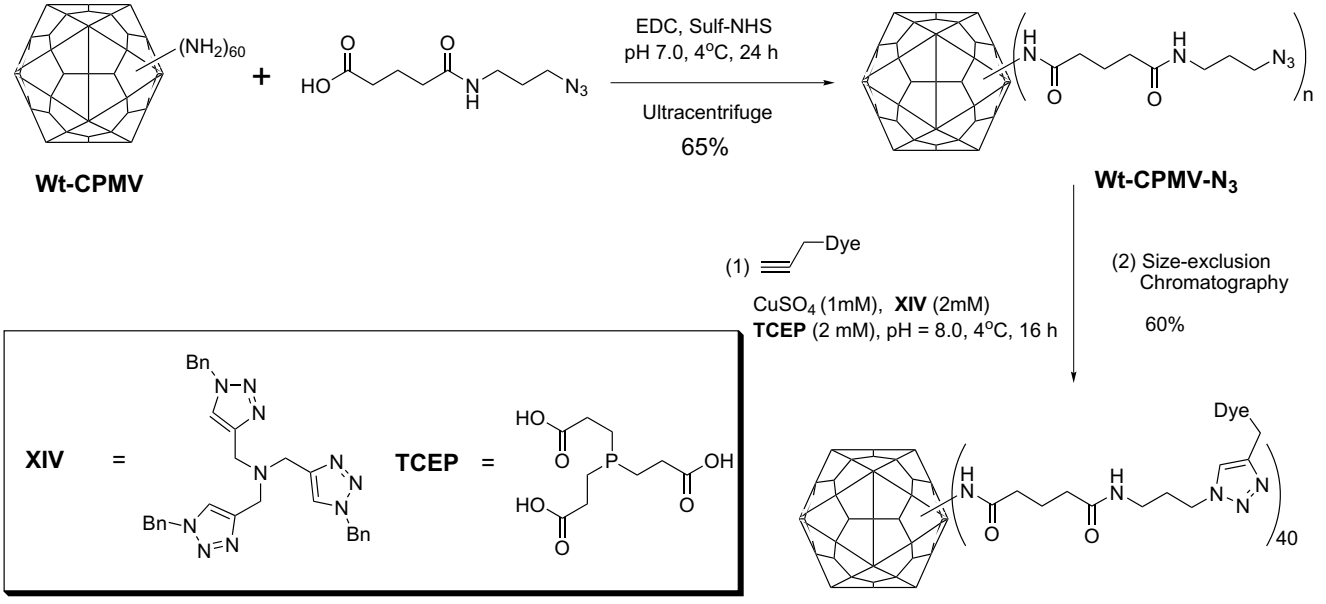
water, and have a strong absorbance as well as a large Strokes shift of the fluorescence emission maxima (Table 1). The spectral properties of **I** and **II**, as well as **IV** and **V**, are very similar to each other due to the structural similarity.

To test the reactivity in bioconjugation, icosahedral cowpea mosaic virus (CPMV) was applied as a biomolecular prototype. CPMV has been demonstrated as

Table 1. Absorption and fluorescent emission properties in 0.1 M potassium phosphate buffer (pH 7.0) for dyes **I**–**V**

Dye	Absorption λ_{max}/nm	ε/M^{-1} cm^{-1}	Emission λ_{max}/nm
I	533	90,460	588
II	530	61,370	593
III	553	69,330	680
IV	578	55,000	604
V	571	54,700	605

a robust platform for many organic reactions.[24] A number of functional groups and biomolecules have been attached to the native reactive lysines and genetically inserted cysteines of CPMV.[25–28] CPMV can be made inexpensively on the gram scale and it is very easy to separate the chemical modified viral particles from unreacted small reagents. Therefore, it is an ideal model system to test new bioconjugation reactions. In previous reports, fluorescent compounds (**VI–IX**) were used to study the influences of pH value, copper source, reducing reagent, copper concentration, and ligand structure in copper(I) catalyzed 1,3-dipolar cycloaddition of azides and alkynes.[7,29] A standard 'Click' protocol for bioconjugation applications has been established using a catalytic system including $CuSO_4$ as the copper source, either a copper wire or tris(carboxyethyl)phosphine (TCEP) as the reducing agent, and tris(triazolylamine) as the ligand.[7] Using a similar protocol (Scheme 3), we first prepared the azide modified CPMV (**Wt-CPMV-N_3**),[7] which was then conjugated with **I–V** catalyzed by $CuSO_4$ (1 mM), TCEP (2 mM) and tris(triazolyl)amine **XIV** (2 mM). After 15 h incubation at 4 °C and purification over gel filtration column, the products were analyzed with UV–vis spectrometer and transmission electron microscopes. While dyes **I**, **III**, and **IV** degraded all the viral particles, dyes **II** and **V** afford high recovery yield of intact viral particles (more than 60%) and high conjugation efficiencies (>80%). Figure 1



Scheme 3. Bioconjugation of alkyne substituted fluorescent dyes to CPMV by Cu(I) catalyzed 1,3-dipolar cycloaddition reaction.

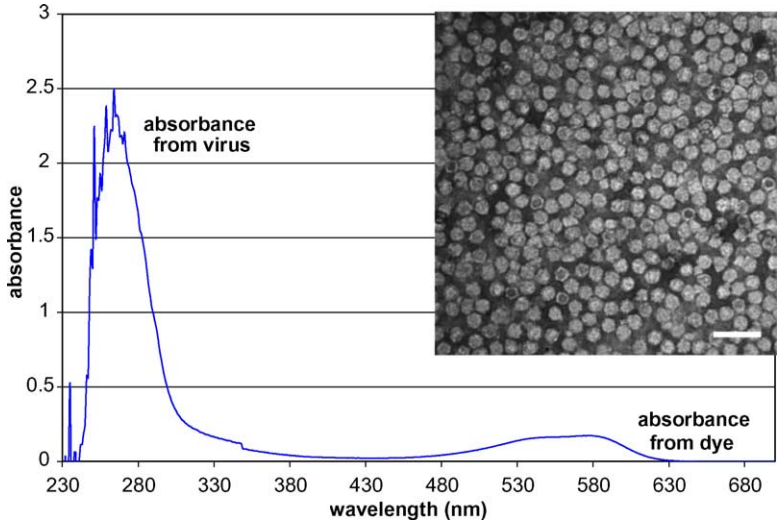


Figure 1. UV–vis absorbance spectrum of CPMV modified with **V**. The peak of 260 nm is contributed by virus and the peak around 570 nm is contributed by attached dye. (inset) The transmission electron microscope picture of CPMV-**V** conjugates after negative staining. The scale bar is 100 nm.

shows the UV–vis absorbance spectrum and negative staining transmission electron microscopy pictures of **V** modified CPMV. The only structural difference between **I** and **II** (or **IV** and **V**) is the terminal carboxyl groups. When CPMV (2 mg/mL) was incubated with **I**–**V** (1.0 mM) alone, non-specific binding between dyes and CPMV was not observed and intact viral particles could be collected in high recover yield in all situations. Therefore, the degradation of the viral particles when click with **I**, **III**, and **IV** was caused by the reaction. Since the reaction system was much more complicated than a simple catalytic cycloaddition,[7] many factors could interfere the stability of the viral particles, which will be of great interest for future investigation.

In conclusion, water soluble hemicyanine dyes **I**–**V** can be prepared in a facile manner and could be used for bioconjugation reactions under mild 'click' reaction conditions. These reagents may also be used in labeling other biologically important molecules. Compared to common functionalities for bioconjugation, such as *N*-succinimide ester, isothiocyanate, or maleimides, the terminal alkyne group used in this study is more synthetically friendly. Further modification and improvement of the hemicyanine dyes structures in order to afford better spectral and physical properties are under exploitation.

3. Typical experimental procedures

3.1. Synthesis of the dye I–V

3.1.1. 2-[2-(4-Dimethylamino-phenyl)-vinyl]-3-prop-2-ynyl-benzothiazol-3-ium bromide (I). 2-Methylbenzothiazole (12.0 g, 0.1 mol) was added into a solution of propargyl bromide (5.0 g, 34 mmol) in acetonitrile (20 mL) under vigorously stirring. Then the reaction mixture was refluxed for 24 h. After cooling to room temperature, the precipitation was collected to afford the product **XI** as a gray solid (5.6 g, 20% yield); mp 224–226 °C. ^{1}H NMR (DMSO-d_6) δ 8.5 (d, J = 8.1 Hz, 1H), 8.38 (d, J = 8.5 Hz, 1H), 7.95 (t, J = 8 Hz, 1H), 7.83 (t, J = 7.8 Hz, 1H), 5.78 (d, J = 2.5 Hz, 2H), 3.85 (t, J = 2.5 Hz, 1H), 3.27 (s, 3H). The solution of 4-*N*,*N*-dimethylaminobenzaldhyde (0.9 g, 6 mmol) and **XI** (1.6 g, 6 mmol) in ethanol (45 mL) was refluxed for 2 h. Then the solvent was removed and the residue was purified by column chromatography over silica gel using dichloromethane/methanol (9:1, v/v) as eluents. The product was collected as a dark purple solid (2.0 g, 81% of yield); mp 180–181 °C. ^{1}H NMR (DMSO-d_6) δ 8.30 (d, J = 7.8 Hz, 1H), 8.15 (m, 2H), 7.92 (d, J = 8.8 Hz, 2H), 7.80 (t, J = 8.0 Hz, 1H), 7.67 (m, 2H), 6.85 (d, J = 9.0 Hz, 2H), 5.75 (d, J = 2.2 Hz, 2H), 3.71 (t, J = 2.3 Hz, 1H), 3.1 (s, 6H); ^{13}C NMR (DMSO-d_6) δ 172.1, 154.6, 152.4, 141.1, 134.1, 129.7, 128.2, 127.4, 124.8, 122.2, 116.3, 112.8, 105.9, 78.9, 76.8, 38.3; HR-MS for $C_{20}H_{19}N_2S^+$ (the cation): calculated 319.1269, found 319.1269.

3.1.2. Dye II. Mp 143–145 °C; ^{1}H NMR (DMSO-d_6) δ 8.30 (d, J = 8.1 Hz, 2H), 8.15 (m, 2H), 7.91 (d, J = 8.9 Hz, 2H), 7.80 (t, J = 8.0 Hz, 1H), 7.7 (m, 2H); 6.90 (d, J = 9.0 Hz, 2H), 5.80 (s, 2H), 3.75 (m, 5H), 2.55 (m, 4H); HR-MS for $C_{24}H_{23}N_2O_4S^+$ (the cation): calculated 435.1379, found 435.1370. Dye **III**: mp 174 °C; ^{1}H NMR (DMSO-d_6) δ 8.33 (d, J = 7.8 Hz, 1 H), 8.16 (d, J = 7.6 Hz, 1H), 8.07 (m, 1H), 7.81 (t, J = 7.6 Hz, 1H), 7.70 (t, J = 7.6 Hz, 1H), 7.58 (d, J = 8.9 Hz, 2H), 7.51 (d, J = 14.9 Hz, 1H), 7.34 (d, J = 14.4 Hz, 1H), 7.21 (m, 1H), 6.80 (d, J = 9.0 Hz, 2 H), 5.63 (d, J = 2.2 Hz, 2H), 3.75 (t, J = 2.4 Hz, 1H), 3.05 (s, 6H). Dye **IV**: mp 175–177 °C; ^{1}H NMR (DMSO-d_6) δ 8.50 (d, J = 15.4 Hz, 1H), 8.35 (d, J = 8.9 Hz, 1H), 8.22 (d, J = 8.9 Hz, 1H), 8.13 (m, 3H), 7.96 (d, J = 8.9 Hz, 1H), 7.75 (t, J = 7.3 Hz, 1H), 7.63 (t, J = 7.5 Hz, 1H), 7.35 (d, J = 15.4 Hz, 1H), 6.92 (d, J = 9.0 Hz, 2H), 5.62 (d, J = 1.7 Hz, 2H), 3.65 (s, 1H), 3.20 (s, 6H), 1.98 (s, 6H); HR-MS for $C_{27}H_{27}N_2^+$ (the cation): calculated 379.2175, found 379.2174. Dye **V**: ^{1}H NMR (DMSO-d_6) δ 8.55 (d, J = 16.4 Hz, 1H), 8.34 (d, J = 9.8 Hz, 1H), 8.25 (d, J = 9.4 Hz, 1H), 8.14 (t, J = 8.2 Hz, 3H), 8.01 (d, J = 8.8 Hz, 1H), 7.76 (t, J = 8.2 Hz, 1H), 7.65 (t, J = 7.5 Hz, 1H), 7.43 (d, J = 15.7 Hz, 1H), 6.98 (d, J = 8.9 Hz, 2 H), 5.62 (s, 2H), 3.80 (s, 2H), 3.67 (s, 2H), 3.33 (t, J = 2.38, 1H), 2.66 (t, J = 7.2 Hz, 1H), 2.59 (t, J = 6.5 Hz, 2H), 2.07 (s, 3H), 2.00 (s, 3H), 1.18 (t, J = 7.1 Hz, 2H). HR-MS for $C_{31}H_{31}N_2O_4$ (the cation): calculated 495.2297, found 495.2284.

3.2. General procedure of 'click reactions' on derivatized viruses

Virus conjugates **WT-CPMV-N$_3$**[7] (200 μg) and dye-alkyne (0.25 mmol) were added to a solution of tris(triazolyl)amine **XIV** (2 mM) and **TCEP** (2 mM) in a mixture of 0.1 M potassium phosphate buffer (pH 8.0, 80 μL) and DMF (20 μL) at 4 °C. $CuSO_4$ (0.5 M, 0.2 μL) was then added. Following incubation at 4 °C for 16 h, the mixture was purified by passage through a P-100® (Bio-Rad) size exclusion column (centrifugation at 800*g* for 6 min). This filtration was repeated with fresh columns until all the excess reagents were removed (typically three times). The recovery of derivatized viruses was usually about 90%. All such samples were composed of >95% intact particles (determined by sucrose gradient ultra-sedimentation and TEM). Virus concentrations were determined by measuring the absorbance at 260 nm; virus at 0.1 mg/mL gives a standard absorbance of 0.8. The average molecular weight of the CPMV virion is 5.6×10^6 Da. The dye concentrations were obtained by measurement of absorbance at the maximum absorption wavelength with the new calibrated molar absorptivity.

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References and notes

1. Huisgen, R. In *1,3-Dipolar Cycloaddition Chemistry*; Padwa, A., Ed.; John Wiley & Sons: New York, 1984; pp 1–176.
2. Lewis, W. G.; Green, L. G.; Grynszpan, F.; Radic, Z.; Carlier, P. R.; Taylor, P.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 1053–1057.
3. Kolb, H. C.; Finn, M. G.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2001**, *40*, 2004–2021.
4. Rostovtsev, V. V.; Green, L. G.; Folkin, V. V.; Sharpless, K. B. *Angew. Chem., Int. Ed.* **2002**, *41*, 2596–2599.
5. Tornoe, C. W.; Christensen, C.; Meldal, M. *J. Org. Chem.* **2002**, *67*, 3057–3062.
6. Kolb, H. C.; Sharpless, K. B. *Drug Discovery Today* **2003**, *8*, 1128–1137.
7. Wang, Q.; Chan, T.; Hilgraf, R.; Fokin, V. V.; Sharpless, K. B.; Finn, M. G. *J. Am. Chem. Soc.* **2003**, *125*, 3192–3193.
8. Speers, A. E.; Adam, G. C.; Cravatt, B. F. *J. Am. Chem. Soc.* **2003**, *125*, 4686–4687.
9. Seo, T. S.; Li, Z.; Ruparel, H.; Ju, J. *J. Org. Chem.* **2003**, *68*, 609–612.
10. Lee, L. V.; Mitchell, M. L.; Huang, S. J.; Fokin, V. V.; Sharpless, K. B.; Wong, C.-H. *J. Am. Chem. Soc.* **2003**, *125*, 9588–9589.
11. Mishra, A.; Behera, R. K.; Behera, P. K.; Mishra, B. K.; Behera, G. B. *Chem. Rev.* **2000**, *100*, 1973–2011.
12. Lipowska, M.; Patonay, G.; Strekowski, L. *Synth. Commun.* **1993**, *23*, 3087–3094.
13. Lin, Y. H.; Weissleder, R.; Tung, C. H. *Bioconjugate Chem.* **2002**, *13*, 605–610.
14. Tung, C. H.; Lin, Y.; Moon, W. K.; Weissleder, R. *Chembiochem* **2002**, *3*, 784–786.
15. Tung, C. H.; Gerszten, R. E.; Jaffer, F. A.; Weissleder, R. *Chembiochem* **2002**, *3*, 207–211.
16. Flanagan, J. H., Jr.; Khan, S. H.; Menchen, S.; Soper, S. A.; Hammer, R. P. *Bioconjugate Chem.* **1997**, *8*, 751–756.
17. Mujumdar, S. R.; Mujumdar, R. B.; Grant, C. M.; Waggoner, A. S. *Bioconjugate Chem.* **1996**, *7*, 356–362.
18. Mujumdar, R. B.; Ernst, L. A.; Mujumdar, S. R.; Lewis, C. J.; Waggoner, A. S. *Bioconjugate Chem.* **1993**, *4*, 105–111.
19. Toutchkine, A.; Nalbant, P.; Hahn, K. M. *Bioconjugate Chem.* **2002**, *13*, 387–391.
20. Gruber, H. J.; Kada, G.; Pragl, B.; Riener, C.; Hahn, C. D.; Harms, G. S.; Ahrer, W.; Dax, T. G.; Hohenthanner, K.; Knaus, H. G. *Bioconjugate Chem.* **2000**, *11*, 161–166.
21. Meng, F. S.; Yao, Q. H.; Shen, J. G.; Li, F. L.; Huang, C. H.; Chen, K. C.; Tian, H. *Synth. Met.* **2003**, *137*, 1543–1544.
22. Yao, Q. H.; Meng, F. S.; Li, F. L.; Tian, H.; Huang, C. H. *J. Mater. Chem.* **2003**, *13*, 1048–1053.
23. Meng, F. S.; Chen, K. C.; Tian, H.; Zuppiroli, L.; Nuesch, F. *Appl. Phys. Lett.* **2003**, *82*, 3788–3790.
24. Raja, K. S.; Wang, Q. Bionanoparticles and Nanotechnology. In *Encyclopedia of Nanoscience and Nanotechnology*; Marcel Dekker: New York, 2004; Vol. B, pp 321–330.
25. Wang, Q.; Lin, T.; Johnson, J. E.; Finn, M. G. *Chem. Biol.* **2002**, *9*, 813–819.
26. Wang, Q.; Kaltgrad, E.; Lin, T.; Johnson, J. E.; Finn, M. G. *Chem. Biol.* **2002**, *9*, 805–812.
27. Wang, Q.; Lin, T.; Tang, L.; Johnson, J. E.; Finn, M. G. *Angew. Chem., Int. Ed.* **2002**, *41*, 459–462.
28. Wang, Q.; Raja, K. S.; Janda, K. D.; Lin, T.; Finn, M. G. *Bioconjugate Chem.* **2003**, *14*, 38–43.
29. Lewis, W. G.; Magallon, F. G.; Fokin, V. V.; Finn, M. G. *J. Am. Chem. Soc.* **2004**, *126*, 9152–9153.