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Quantum Dot-Encoded Beads

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Summary

Multicolor optical coding for biological assays has been achieved by embedding semiconductor quantum dots into mesoporous and macroporous beads at precisely controlled ratios. Owing to their novel optical properties such as size-tunable emission and simultaneous excitation, quantum dots are ideal fluorophores for wavelength-and-intensity multiplexing. Kinetics study reveals that quantum dot doping of porous silica and polystyrene beads can be completed from seconds to minutes. The use of 10 intensity levels and six colors could theoretically code 1 million nucleic acid or protein sequences. Imaging and spectroscopic measurements indicate that the quantum dot-tagged beads are highly uniform and reproducible, yielding bead identification accuracies as high as 99.99% under favorable conditions. DNA hybridization studies demonstrate that the coding and target signals can be simultaneously read at the single-bead level. This spectral coding technology is expected to open new opportunities in gene expression studies, high-throughput screening, and medical diagnostics.

Key Words

Quantum dots; nanoparticles; encoding; multiplexing; spectroscopy; gene expression; decoding.

1. Introduction

Recent advances in bioanalytical sciences and bioengineering have led to the development of DNA chips ***(1,2)***, miniaturized biosensors ***(3,4)***, and microfluidic devices (e.g., microelectromechanical systems or bioMEMS) ***(5–7)***. These enabling technologies have substantially impacted many areas in biomedical research, such as gene expression profiling, drug discovery, and clinical diagnostics. As current research in genomics and proteomics produces more sequence data, there is a strong need for new and improved technologies that can rapidly screen a large number of nucleic acids and proteins. Here we report detailed protocols for preparing quantum dot-encoded beads in the size range of 100 nm to 100 μm in diameter.

From: *Methods in Molecular Biology, vol. 303: NanoBiotechnology Protocols*
Edited by: S. J. Rosenthal and D. W. Wright © Humana Press Inc., Totowa, NJ

Key steps in preparing quantum dot-encoded beads include the synthesis of highly monodisperse beads with suitable pore structures and the formulation of solvent conditions that strongly favor the partitioning of quantum dots from the solution into the pores. In this chapter, we discuss these two aspects and provide detailed procedures for three types of encoded beads: (1) polymeric nanobeads, (2) mesoporous polystyrene microbeads, and (3) mesoporous silica microbeads.

2. Materials

1. Technical grade (90%) trioctylphosphine oxide (TOPO).
2. Trioctylphosphine (99% pure).
3. Cadmium oxide (CdO) (99.7%).
4. Selenium (more than 99%).
5. Stearic acid (98%).
6. Hexamethyldisilathiane ($[TMS]_2S$).
7. Dimethlycadmium; store and use in inert air.
8. Dimethylzinc ($ZnMe_2$) (10% wt in hexane); store and use in inert air.
9. Hexadecylamine (HDA) (98%).
10. Styrene (99%); store at –80°C after removing inhibitor.
11. Divinylbenzene (80%); store at –80°C after removing inhibitor.
12. Acrylic acid (99%); store at –80°C after removing inhibitor.
13. Inhibitor remover disposable column, polyvinylpyrrolidone (PVP).
14. 2,2′-Azobisisobutyronitrile (AIBN) (98%).
15. PVP (mol wt = 40,000).
16. Polystyrene beads.
17. Silica beads (obtained from high-performance liquid chromatography column).
18. Dibutyl phthalate (DBP).
19. Sodium dodecyl sulfate (SDS) (98%).
20. Sodium nitrite (99.5%).
21. Benzoyl peroxide (70%).
22. Poly (vinyl alcohol) (mol wt = 85,000–124,000).
23. (3-Mercaptopropyl)trimethoxysilane (more than 97%).
24. Tetraethoxysilane (more than 99%).
25. Specialized equipments (*see* text).

3. Methods

Multicolor quantum dots are incorporated into *porous* polymer beads mainly via hydrophobic interactions. In this section, we describe the methods of making and reading quantum dot-tagged microbeads: preparation of quantum dots, synthesis of microbeads, bead encoding and surface functionalization, bead characterization.

3.1. Preparation of Multicolor Quantum Dots

High-quality hydrophobic quantum dots are prepared according to literature procedures with modifications ***(8–12)***.

1. Dissolve 1 mmol of CdO precursor in 1 g of stearic acid with heating until a clear solution is formed.
2. Add a TOPO and HDA mixture (10 g) as reaction solvents, and then heat to 250°C under argon for 10 min.
3. Briefly raise the temperature to 360°C, and quickly inject an equal molar selenium solution into the hot solvents. The mixture will immediately change color to orange-red, indicating the formation of quantum dots.
4. Reflux the dots for 10 min.
5. Slowly add a capping solution of 20 m*M* $ZnMe_2$ and $(TMS)_2S$ to protect the CdSe core. These ZnS-capped CdSe dots have excellent chemical and photo stability.
6. Cool the dots to room temperature, and rinse repeatedly with methanol to remove free ligands.
7. For characterization, use ultraviolet (UV) adsorption, fluorescence emission spectra, and transmission electron microscopy.

The emission spectra of quantum dots can be continuously tuned by changing particle size and component materials during synthesis (*see* **Note 1**). Spanning the visible spectrum, simultaneous excitation of 10 emission colors can be achieved with a single near-UV (350-nm) lamp ***(13)***. For microbead encoding, the dots are stored in a mixture solvent containing 5% chloroform and 95% butanol.

3.2. Synthesis of Porous Microbeads

Depending on the bead size, quantum dot-encoded beads can be used for multiplexed bioassays and multiplexed biolabeling. In the first application, optically encoded microcarriers in the size range of 3–30 μm are linked to DNA probes or antibodies for recognition and detection of target molecules in a homogeneous solution ***(14–19)***. In the second application, small beads in the size range of 100–500 nm are used for spectrally encoded tagging of cells and tissue specimens ***(20–26)***. Some of the mesoporous beads suitable for these applications are available from chromatographic or latex reagent companies such as Phenomenex (Torrance, CA) or Bangs Lab (Fisher, IN). To describe the procedures of preparing microbeads with tunable size and porosity, we outline them in the following three subsections.

3.2.1. Polystyrene-Based Nanobeads

Polystyrene nanobeads are synthesized by using emulsion polymerization ***(13)***.

1. Purify styrene, divinylbenzene, and acrylic acid with an inhibitor-remover column, and store at –80°C in small aliquots.
2. Add the three monomers into an ethanol/water mixture (8:1 [v/v]) in a ratio of 98:1:1, together with PVP as a stabilizer. Briefly sonicate the whole system and purge with N_2 for 2 min.

3. Initiate polymerization by adding AIBN (1.5 mg/mL) and heating to 70°C.
4. Shake or stir the reaction for 10 h.
5. Rinse the resulting polymer beads with ethanol for three rounds and ethanol/chloroform (9:1) for another three rounds.
6. Store the isolated beads in butanol for encoding experiments.

3.2.2. Polystyrene-Based Mesoporous Microbeads

Porous polystyrene microbeads are prepared according to literature procedures ***(27,28)***. Monodispersed nonporous polystyrene beads with a diameter of 1 μm are used as a shape template.

1. Swell the beads (60 mg) with 0.2 mL of DBP in 0.25% SDS solution for 10 h, and with a styrene emulsion (porogen) and benzoyl peroxide for another 10 h.
2. Add sodium nitrite (0.3 mg/mL) and poly (vinyl alcohol) at a final concentration of 1% to the swelled beads before purging the emulsion mixture with nitrogen. Conduct the polymerization at 70°C for 24 h.
3. Add an emulsified mixture of 5 mL of styrene, divinylbenzene, DBP, and benzoyl peroxide in 0.25% SDS solution to the reaction and mix for 10 h under stirring. Again, purge the emulsion with nitrogen and polymerize at 70°C.
4. Rinse the resulting beads with water and methanol before extracting with toluene using a Soxhlet extractor.

3.2.3. Silica-Based Mesoporous Microbeads

Mesoporous silica beads are synthesized by using pore-generating templates such as self-assembled surfactants or polymers (called porogens) ***(29)***. After synthesis, removal of the templates generates mesosized pores, which are either ordered or random depending on the template structures. In general, the porous silica beads can be prepared with a broad size range (nanometers to micrometers). Alternatively, mesoporous silica beads (5 μm diameter) with pore sizes of 10 or 32 nm are available from Phenomenex. The pore surfaces are coated with a monolayer of Si-$C_{18}H_{37}$ (octadecyl, an 18-carbon linear-chain hydrocarbon). The beads are repeatedly rinsed with ethanol and butanol before use.

3.3. Bead Tagging and Surface Functionalization

Single-color doping is accomplished by mixing porous beads with a controlled amount of quantum dots in an organic solvent mixture (butanol and chloroform). Quantum dot concentration is measured based on its fluorescence absorption profile ***(8)***. In parallel, the number of microbeads is counted using a hemacytometer. Owing to the completeness and evenness of quantum dot incorporation into porous beads, the number of quantum dots per bead can be calculated and can be used to generate working curves (**Fig. 1**). Based on these curves, intensity-coded beads can be prepared by adding predetermined

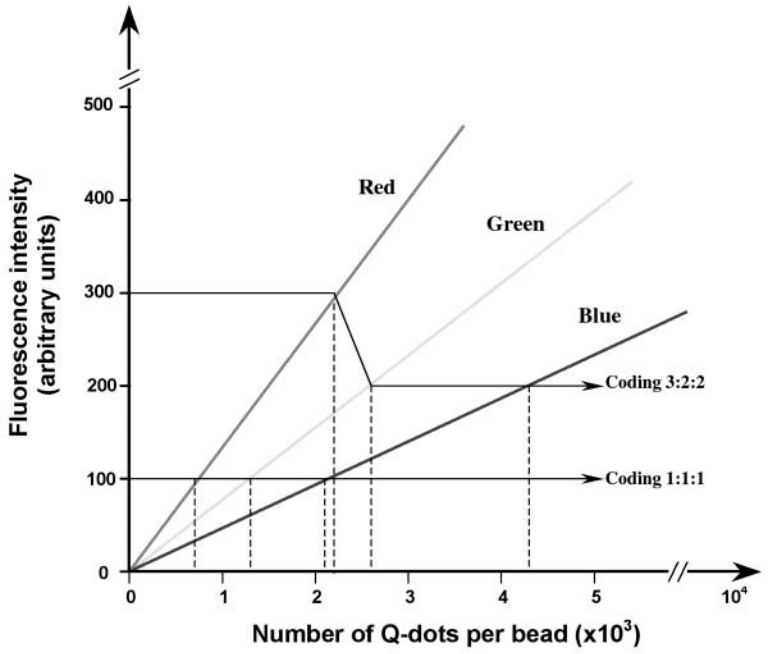

Fig. 1. Schematic drawing of working curves for preparation of multicolor quantum dot microbeads. Arrows depict two representative beads coded with intensity ratios of 1:1:1 and 3:2:2, and the number of quantum dots (or concentration) can be obtained from the *x* axis. Each single bead may contain quantum dots (Q-dots) ranging from several hundred to millions, depending on the bead size and surface area.

amounts of quantum dots in a stock solution. For beads with a pore size more than 30 nm, the doping process is complete in less than 10 min (essentially no free dots left in the supernatant). For beads with smaller pores, more extended times are used to allow quantum dot incorporation (*see* **Note 2**). For multicolor doping, different-colored quantum dots are premixed in precisely controlled ratios, determined by a fluorometer. Porous beads are then added to an aliquot of this premix solution in a similar manner to the monochromatic beads. Doped beads are isolated by centrifugation and are washed three times with ethanol.

For different applications, the encoded beads can be either used directly or sealed before conjugation to biomolecules. In one example, 3-mercaptopropyltrimethoxysilane is adsorbed onto the embedded quantum dots in ethanol, and the free compound is removed by centrifugation. The beads are then resuspended in ethanol with the addition of tetraethoxysilane (sealant). The silane compounds are polymerized inside the beads on addition of water and,

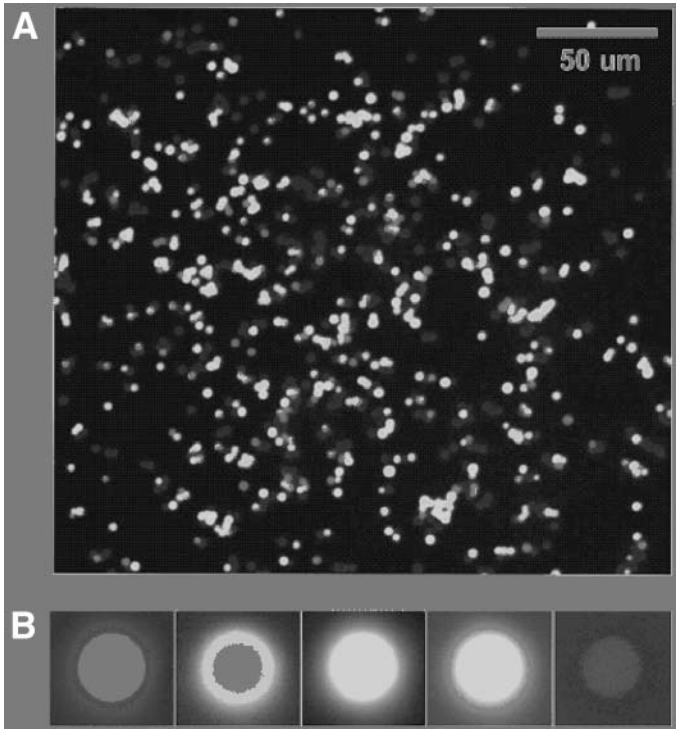

Fig. 2. Fluorescence images of mesoporous silica beads (5-µm diameter, 32-nm pore size) doped with single-color quantum dots emitting light at 488 nm, 520 nm, 550 nm, 580 nm, or 610 nm. **(A)** Wide-field view of large population of doped beads, prepared in batches and then mixed; **(B)** enlarged views of single monochromatic beads. Each bead contains up to 2 million dots of the same color.

therefore, seal the pores. A variety of sealing procedures can be used for different types of beads and applications (*see* **Note 3**).

The sealed beads can then be linked to biomolecules by covalent binding or adsorption and used for bioassays, sharing the same detection principles as the organic dye-labeled microspheres. Owing to this similarity, detailed procedures can be found in the references ***(14–26,30)*** and are thus not discussed here.

3.4. Optical Imaging and Spectroscopy

True-color fluorescence imaging is achieved with an inverted Olympus microscope (IX-70) equipped with a digital color camera (Nikon D1), a broadband UV (330- to 385-nm) light source (100-W mercury lamp), and a long-pass interference filter (DM 400; Chroma Tech, Brattleboro, VT). **Figure 2**

shows true-color fluorescence images of quantum dot–doped silica beads (5.0 ± 0.3 μm in diameter) with an average pore size of 32 ± 4 nm. These "monochromatic" beads (coded with single-color quantum dots) are mixed and spread on a glass surface for fluorescence imaging. Although only a single light source (a near-UV lamp) is used for excitation, all the doped beads are observed and are clearly distinguishable. Note that simultaneous excitation of multiple emission colors is a unique property of quantum dots and is not possible with organic dyes or lanthanide compounds. The quantum dot-doped beads are remarkably bright and can be recorded by using a digital color camera and a mercury lamp.

Wavelength-resolved spectra are obtained by using the inverted microscope coupled with a single-stage spectrometer (SpectraPro 150; Roper, Trenton, NJ). A schematic diagram of the instrument is shown in **Fig. 3**. Multicolor quantum dots are excited with a broadband excitation in the UV or blue region, and the Stoke-shifted fluorescence is passed through a long-pass filter. A pinhole is placed at the objective image plane between the spectrograph and microscope to reject the out-of-focus lights. The beads are manually positioned, and spectra are recorded with a thermoelectrically cooled charge-coupled device (CCD). The quantitative results have shown that the intensity ratios of multicolor quantum dots are remarkably uniform, leading to the development of advanced devices and algorithms to read the doped beads at high accuracies and speeds (*see* **Note 4**).

4. Notes

1. The preparation of multicolor quantum dots, especially CdSe/ZnS quantum dots, has been reported by many groups. The emission wavelengths can be tuned by several factors, such as chemical composition and particle size. For example, the use of semiconductor materials with higher band gap or preparation of dots with bigger particle size results in longer emission wavelength, and vice versa. Detailed procedures are discussed in **refs. *8–12***.
2. The key to making quantum dot-encoded microspheres is the preparation of porous microstructures, because quantum dots are much bigger than traditional organic fluorophores. It has been shown that completion of quantum dot doping can be as fast as minutes if microbeads with a pore size bigger than 30 nm are used, whereas the same process could take hours for beads with 10-nm pores ***(31)***. Beads with small pores need to be swelled before quantum dot doping. A typical solvent for this purpose is chloroform, which not only enlarges the polystyrene microbeads, but also increases the solubility of quantum dots. For hydrocarbon-capped silica beads, the addition of chloroform also improves the doping kinetics, by increasing the flexibility of hydrocarbons on the bead wall and quantum dot surface ***(13,31)***.
3. A variety of pore-sealing or surface-coating methods could be used for different applications. To seal the pores completely, polymerization can be conducted

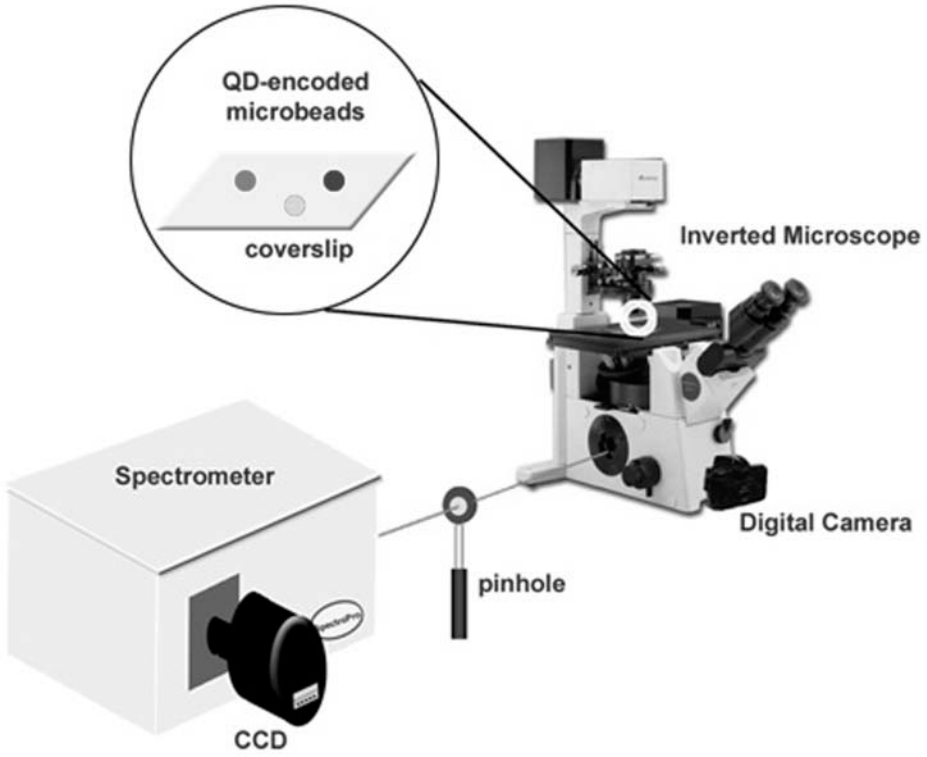

Fig. 3. Schematic diagram of integrated fluorescence imaging and spectroscopy system. The coding signals are read out by a CCD camera and a single-stage spectrometer attached to the side port of an inverted microscope.

within the pores using silanes, styrene and derivatives, and so forth. This approach completely isolates the embedded dots with the environment and protects them. However, the area for biomolecule conjugation is limited to the beads' outer surface. Another approach that can be used to protect the dots is to overcoat the pores and the embedded dots with a layer of polymers, so that bioconjugation can occur on both the interior and the outside walls of the beads.

4. The coding uniformity is mainly determined by the intrinsic variation in bead size. It has been reported previously that ratiometric measurements are considerably more reliable than absolute intensities because the ratio values are often not affected by simultaneous drifts or fluctuations in the individual signals ***(32)***. Therefore, the use of ratios for bead decoding could help in the development of advanced devices and algorithms that can read the doped beads at high accuracies and speeds.

Acknowledgments

We are grateful to Dr. Mingyong Han (Department of Materials Science, National University of Singapore) for help with the microbead synthesis and for fruitful discussions. This work was supported in part by the National Institutes of Health, the Department of Energy, and the Coulter Translational Research Program at Georgia Tech and Emory University.

References

1. Fodor, S. P. A., Read, J. L., Pirrung, M. C., Stryer, L., Lu, A. T., and Solas, D. (1991) Light-directed, spatially addressable parallel chemical synthesis. *Science* **251,** 767–773.
2. Schena, M., Shalon, D., Davis, R. W., and Brown, P. O. (1995) Quantitative monitoring of gene-expression patterns with a complementary-DNA microarray. *Science* **270,** 467–470.
3. Dickinson, T. A., Michael, K. L., Kauer, J. S., and Walt, D. R. (1999) Convergent, self-encoded bead sensor arrays in the design of an artificial nose. *Anal. Chem.* **71,** 2192–2198.
4. Clark, H. A., Hoyer, M., Philbert, M. A., and Kopelman, R. (1999) Optical nanosensors for chemical analysis inside single living cells: fabrication, characterization, and methods for intracellular delivery of PEBBLE sensors. *Anal. Chem.* **71,** 4831–4836.
5. Harrison, D. J., Fluri, K., Seiler, K., Fan, Z., Effenhauser, C. S., and Manz, A. (1993) Micromachining a miniaturized capillary electrophoresis-based chemical analysis system on a chip. *Science* **261,** 895–897.
6. Ramsey, J. M., Jacobson, S. C., and Knapp, M. R. (1995) Microfabricated chemical measurement systems. *Nat. Med.* **1,** 1093–1096.
7. Woolley, A. T. and Mathies, R. A. (1994) Ultra-high-speed DNA fragment separations using microfabricated capillary array electrophoresis chips. *Proc. Natl. Acad. Sci. USA* **91,** 11,348–11,352.
8. Murray, C. B., Norris, D. J., and Bawendi, M. G. (1993) Synthesis and characterization of nearly monodisperse CdE (E = S, Se, Te) semiconductor nanocrystallites. *J. Am. Chem. Soc.* **115,** 8706–8715.
9. Hines, M. A. and Guyot-Sionnest, P. (1996) Synthesis of strongly luminescing ZnS-capped CdSe nanocrystals. *J. Phys. Chem. B* **100,** 468–471.
10. Peng, X. G., Schlamp, M. C., Kadavanich, A. V., and Alivisatos, A. P. (1997) Epitaxial growth of highly luminescent CdSe/CdS core/shell nanocrystals with photostability and electronic accessibility. *J. Am. Chem. Soc.* **119,** 7019–7029.
11. Peng, Z. A. and Peng, X. G. (2001) Formation of high-quality CdTe, CdSe, and CdS nanocrystals using CdO as precursor. *J. Am. Chem. Soc.* **123,** 183, 184.
12. Qu, L., Peng, Z. A., and Peng, X. (2001) Alternative routes toward high quality CdSe nanocrystals. *Nano Lett.* **1,** 333–337.

13. Han, M. Y., Gao, X. H, Su, J. Z., and Nie, S. M. (2001) Quantum-dot-tagged microbeads for multiplexed optical coding of biomolecules. *Nat. Biotechnol.* **19,** 631–635.
14. Chen, J., Iannone, M. A., Li, M. S., Taylor, J. D., Rivers, P., Nelsen, A. J., Slentz-Kesler, K. A., Roses, A., and Weiner, M. P. (2000) A microsphere-based assay for multiplexed single nucleotide polymorphism analysis using single base chain extension. *Genome Res.* **10,** 549–557.
15. Fulton, R. J., McDade, R. L., Smith, P. L., Kienker, L. J., and Kettman, J. R. Jr. (1997) Advanced multiplexed analysis with the FlowMetrix™ system. *Clin. Chem.* **43,** 1749–1756.
16. Steemers, F. J., Ferguson, J. A., and Walt, D. R. (2000) Screening unlabeled DNA targets with randomly ordered fiber-optic gene arrays. *Nat. Biotechnol.* **18,** 91–94.
17. Ferguson, J. A., Boles, T. C., Adams, C. P., and Walt, D. R. (1996) A fiber-optic DNA biosensor microarray for the analysis of gene expression. *Nat. Biotechnol.* **14,** 1681–1684.
18. Ferguson, J. A., Steemers, F. J., and Walt, D. R. (2000) High-density fiber-optic DNA random microsphere arrays. *Anal. Chem.* **72,** 5618–5624.
19. Van Oosterhout, M. F., Prinzen, F. W., Sakurada, S., Glenny, R. W., and Hales, J. R., (1998) Fluorescent microspheres are superior to radioactive microspheres in chronic blood flow measurements. *Am. J. Physiol.* **275,** H110–H115.
20. Bhalgat, M. K., Haugland, R. P., Pollack, J. S., Swan, S., and Haugland, R. P. (1998) Green- and red-fluorescent nanospheres for the detection of cell surface receptors by flow cytometry. *J. Immunol. Methods* **219,** 57–68.
21. Hall, M., Kazakova, I., and Yao, Y. M. (1999) High sensitivity immunoassays using particulate fluorescent labels. *Anal. Biochem.* **272,** 165–170.
22. Zijlmans, H. J. M. A. A., Bonnet, J., Burton, J., Kardos, K., Vail, T., Niedbala, R. S., and Tanke, H. J. (1999) Detection of cell and tissue surface antigens using up-converting phosphors: a new reporter technology. *Anal. Biochem.* **267,** 30–36.
23. Kurner, J. M., Klimant, I., Krause, C., Preu, H., Kunz, W., and Wolfbeis, O. S. (2001) Inert phosphorescent nanospheres as markers for optical assays. *Bioconj. Chem.* **12,** 883–889.
24. Sondi, I., Siiman, O., Koester, S., and Matijevic, E. (2000) Preparation of aminodextran-CdS nanoparticle complexes and biologically active antibody-aminodextran-CdS nanoparticle conjugates. *Langmuir* **16,** 3107–3118.
25. Chen, Y. F., Tianhao, J., and Rosenzweig, Z. (2003) Synthesis of glyconanospheres containing luminescent CdSe-ZnS quantum dots. *Nano Lett.* **3,** 581–584.
26. Schultz, S., Smith, D. R., Mock, J. J., and Schultz, D. A. (2000) Single target molecule detection with non-bleaching multicolor optical immunolabels. *Proc. Natl. Acad. Sci. USA* **97,** 996–1001.
27. Wang, Q. C., Svec, F., and Frechet, J. M. J. (1994) Fine control of the porous structure and chromatographic properties of monodisperse macroporous poly (styrene-codivinylbenzene) beads prepared using polymer porogens. *J. Polym. Sci. Pol. Chem.* **32,13,** 2577–2588.

28. Cheng, C. M., Micale, F. J., Vanderhoff, J. W., and El-Aasser, M. S. (1992) Synthesis and characterization of monodisperse porous polymer particles. *J. Polym. Sci. Pol. Chem.* **30,** 235–244.
29. Iler, R. K. and Kirkland, J. J. (1978) Process for manufacture of macroporous microspheroids. US patent no. 4,105,426.
30. Hermanson, G. T. (1996) *Bioconjugate Techniques*, Academic, New York.
31. Gao, X. H. and Nie, S. M. (2003) Doping mesoporous materials with multicolor quantum dots. *J. Phys. Chem. B*, **107,** 11,575–11,578.
32. Deniz, A. A., Laurence, T. A., Dahan, M., Chemla, D. S., Schultz, P. G., and Weiss, S. (2001) Ratiometric single-molecule studies of freely diffusing biomolecules. *Annu. Rev. Phys. Chem.* **52,** 233–253.