Available online at www.sciencedirect.com
SCIENCE DIRECT®
ELSEVIER
Journal of Magnetism and Magnetic Materials 293 (2005) 655–662
Journal of magnetism and magnetic materials
www.elsevier.com/locate/jmmm

Magnetically-modulated optical nanoprobes (MagMOONs) and systems

Jeffrey N. Anker, Caleb J. Behrend, Hongmei Huang, Raoul Kopelman*

Department of Chemistry, University of Michigan, 930 North University, Ann Arbor, MI 48109-1055, USA

Available online 2 March 2005

Abstract

Magnetically-modulated optical nanoprobes (MagMOONs) are micro and nano particles that emit different fluxes of light in different orientations, and that rotate in changing magnetic fields. The particles blink as they rotate, allowing in situ background subtraction and chemical imaging with individual particles. The probes also function as torque sensors to measure the drag on the particle and local rheological properties.

Keywords: Sensors; MagMOONs; Fluorescence; Spectroscopy; Microscopy; Blinking particles; Torque sensor; Rheology

1. Introduction

Fluorescent dyes make excellent chemical probes, functioning as either indicators for chemical imaging of intracellular ions and small molecules, or as labels for detection of proteins and larger molecules in solution. The strong optical signals from fluorescent dyes can be detected using standard photodetectors and optical instruments—down to the single molecule level [1,2]. However, most biological materials exhibit strong background fluorescence that can obscure the signal from fluorescent dye sensors. Separating sensor fluorescence from background requires careful preparation of blanks, as a consequence, imperfect background removal limits sensitivity, especially when the background changes in location or time.

Magnetically-modulated nanoprobes (MagMOONs) are microscopic particles that are designed to blink in changing magnetic fields, allowing rapid in situ background subtraction. The particles orient like a compass in external magnetic fields, and rotate in response to rotating magnetic fields; they are also designed to emit different amounts of light in different orientations, and thus blink as they rotate. Demodulating the blinking MagMOON signals dramatically increases signal-to-background levels, enabling homogeneous mix and measure immunoassays with a wide range of fluorescent labels, and enhancing detection limits for chemical imaging

*Corresponding author. Tel.: +1 734 764 7541; fax: +1 734 936 2778.
E-mail address: kopelman@umich.edu (R. Kopelman).

0304-8853/$ - see front matter © 2005 Published by Elsevier B.V.
doi:10.1016/j.jmmm.2005.01.031

of ions, dissolved gases, and small molecules. The rotation rate and behavior of particles in response to changing magnetic fields also reveals information about torques acting on the particles. Fig. 1 shows a chart of applications being explored for MagMOONs and Brownian MOONs.

In this paper, we focus on metal-capped MagMOONs [3] produced as shown in Fig. 2. Aspherical magnetic particles are another type of MagMOON with similar applications but blinking at twice the frequency [4]. In addition, it is possible to form hybrid particles, such as chains of metal-capped MagMOONs, or metal-capped MagMOONs with chains tethered to the ends of the particles. The particles can be oriented (rotated) in external magnetic fields, and pulled (translated) with magnetic field gradients. Another method of modulation is to pull individual magnetic particles and swarms of magnetic particles into and out of view with magnetic field gradients, or to magnetically pull on magnetic labels attached to tension sensitive dyes. We have also produced fluorescent

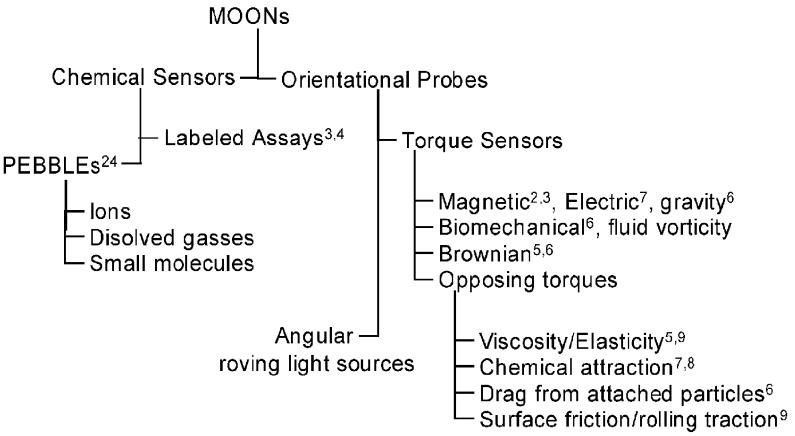

Fig. 1. Applications being explored for MagMOONs and Brownian MOONs.

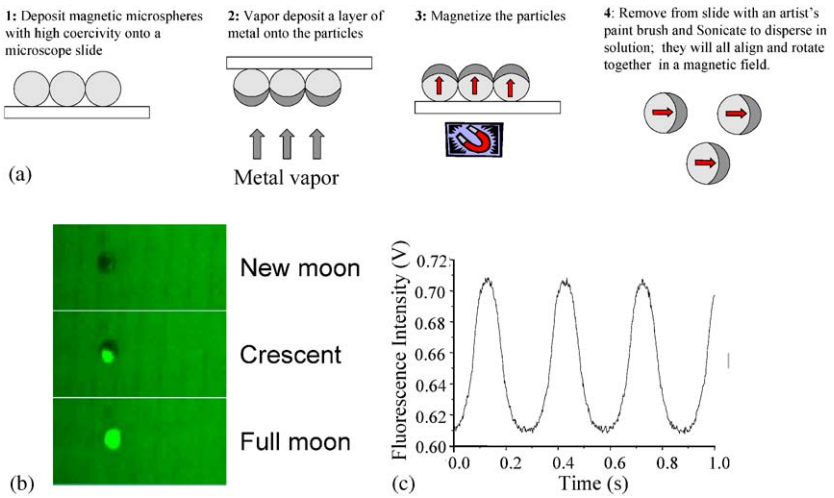

Fig. 2. Principle of MagMOONs. (a) Preparation method for MagMOONs. (b) An aluminum-capped 4.4 μm MagMOON in three different orientations. (c) Intensity time series for an ensemble of rotating aluminum-capped 4.4 μm MagMOONs.

and reflective "Brownian MOONs", where random thermal motion causes metal-capped particles to tumble and blink erratically [3,5,6].

2. Orientation and torque sensors

Most fluorescent nanospheres and microspheres emit light uniformly in all directions. Coating these particles with a metal hemisphere shell breaks this symmetry, causing the particles to emit different amounts of light in different orientations, and allowing the particle orientation to be tracked in time. Tracking particle rotation in time [3–9] reveals any torques acting on the particle from sources such as: electrical fields [7], magnetic fields [3], gravity [6], Brownian forces [5,6], biomechanical forces in macrophages [6], and chemical attraction [7,8]. The rotation also reveals fluid vorticity, translation/rotation coupling (rolling traction), and the opposing torque of the medium's viscous drag and elasticity [5,9] (Fig. 1).

Rotational viscosity and elasticity is a fundamental property of materials of interest to chemistry, biology and materials science. Macroscopic instruments determine viscosity by measuring the resistance of a fluid to mechanical movement. The same principle applies at a microscopic scale, using magnetic forces to torque MagMOONs, and observing the individual response of the MagMOONs to the driving fields [9]. Conversely, if the medium is homogeneous, then the response of individual MagMOONs can be compiled into comparative statistics giving information about the size distribution, morphology, magnetic properties, and the interactions of MagMOONs with surfaces. Similar methods have used magnetic response to determine particle orientation, but needed ensemble measurements with large numbers of magnetic particles [10]. In the absence of external magnetic fields, or for particles without magnetic material, the thermal fluctuations of Brownian MOONs can also be used to mechanically probe rotational viscosity [5,9].

In addition to microviscometers, particles can serve as a modulated directional light source for angular illumination (roving moonlight) as shown in Fig. 3. Local fluorescence illumination provides structural information in the vicinity of the probe, and could be combined with chemical sensing, for instance in studying stretch activated channels [11].

3. Optochemical sensors

3.1. Chemically responsive dyes

Elucidating cellular and physiological responses to pathogens, toxins, drugs, and other stimuli is a major challenge in medicine and biology. These responses are often directly or indirectly manifest in changes in the concentration of ions and small molecules. MagMOONs promise to improve measurements inside cells, where autofluorescence has limited the range of indicator dyes and the

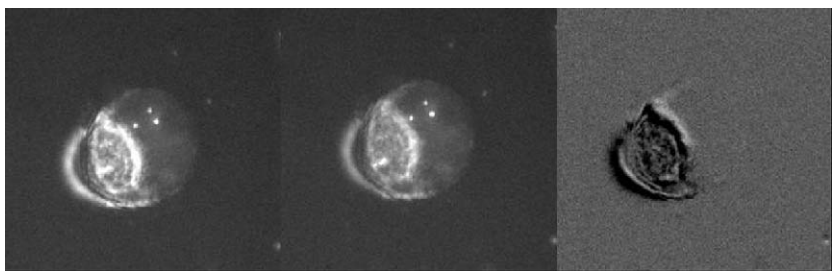

Fig. 3. Roving moonlight from an out of focus 2 μm Brownian MOON rotating above a macrophage. (a) and (b) are images separated by 1 s. Three 300 nm Brownian MOONs in different orientations (brightnesses) are also visible inside the macropahge, although they did not rotate in this short time interval. An auotofluorescent background from the macrophage is also visible. (c) Shows the difference image (a) minus image (b) resulting from the rotating Brownian MOON.

types of samples that can be detected with reasonable signal-to-noise ratios. Our recently developed Photonic Explorers for Bioanalysis with Biologically Localized Embedding (PEBBLE) [12–14] nanosensors are able to measure concentrations of ions and small molecules within a single cell, rapidly, sensitively, with high spatial resolution, and without interference from cellular proteins. Universal delivery techniques (such as liposomal delivery, pico-injection, or gene gun) may deliver all sensor types into cells regardless of the particular analytes they sense. Combining PEBBLEs with MagMOONs can increase the sensitivity to intracellular analytes using fewer probes. Magnetic modulation and background separation can increase the range of dyes that can be used, including blue and ultraviolet excited dyes as well as weakly fluorescing dyes. MagMOONs can also be used in a wider range of samples, including highly fluorescent cells, tissues, and stained cells [15,16].

Fig. 4 illustrates how MagMOON spectra can be separated from backgrounds. 4.4 μm aluminum-capped MagMOONs were prepared by vapor depositing an aluminum cap on one hemisphere of magnetized fluorescent microspheres containing chromium dioxide (Spherotech, IL) (Fig. 2). The MagMOONs were dispersed in egg white (ovine albumen), a solution of similar composition to many biological environments. A drop of the solution was placed on a microscope slide and viewed. The particles were then oriented "On", and "Off", by orienting a permanent magnet interfaced to a Pentium computer. A program written in Labview oriented the MagMOONs and acquired 32 pairs of "On" and "Off" spectra. Fig. 4(a) shows a yellow aluminum-capped MagMOON in the green autofluorescent albumin, in the "On", and "Off" orientations. Fig. 4(b) illustrates spectra from the same MagMOON in both "On" and "Off" orientations (green and black dashed line, respectively, to be read on the left-hand axis), as well as the "On" minus "Off" spectrum of the MagMOON (black line to be read on the right-hand axis). The MagMOON spectrum was separated from a mercury lamp peak background at 800 nm, and from the green fluorescence of ovine albumin. Spectral subtraction reduced the background mercury lamp peak by a factor of 5,000 (from 9 to 0.002) and rendered negligible the green albumen fluorescence. Using principal components analysis (PCA) further improves the separation of signal from background.

Fig. 5 shows 300 nm aluminum-capped Brownian MOONs, fluorescent nanospheres capped with aluminum, in different orientations within rat macrophages, illustrating that MOON shaped particles can survive the harsh conditions within endosomes for more than 24 h. The change in particle orientation in time can be used to observe biomechanical torques on the endosomes [6], and enables the separation of particles emission spectra from the background.

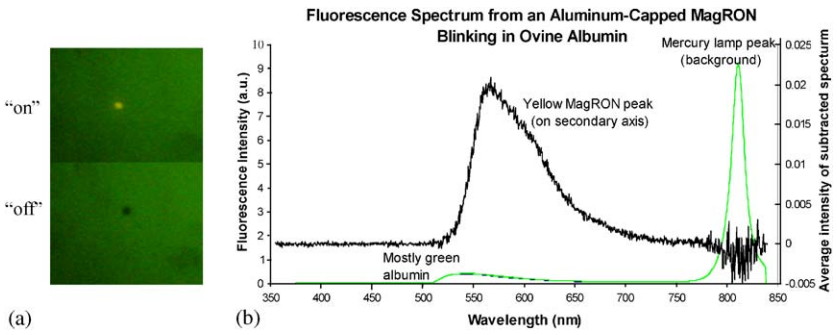

Fig. 4. An aluminum-capped MagMOON blinking in ovine albumen. (a) Images of a MagMOON oriented "On" and "Off". (b) Spectra of the MagMOON oriented "On" (green line) and "Off" (black line just below the green). Spectral subtraction reduced background at 800 nm from mercury-lamp peak by a factor of 5,000 and rendered negligible the green albumin fluorescence. The "On minus Off" spectrum (black line peaking at 565 nm) is plotted on the secondary axis on the right of the graph.

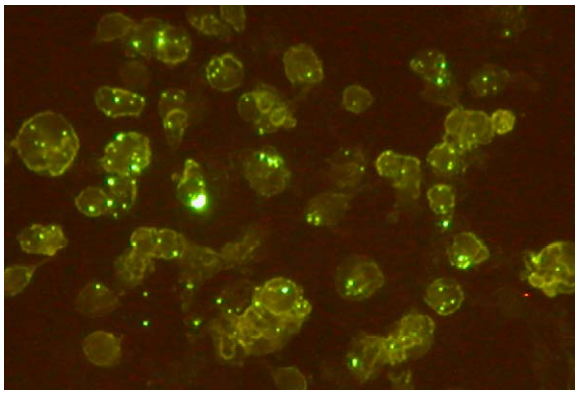

Fig. 5. 300 nm Brownian MOONs in endosomes within macrophages.

3.2. Assays

MagMOONs can be made to detect and quantify proteins using immunoassay techniques. Functionalizing either hemisphere of a MagMOON with antibodies or molecular recognition elements allows it to bind to and capture specific analytes from solution. Subsequently labeling the captured antigen with a fluorescent tag, in a sandwich assay, increases the brightness of the MagMOON. Measuring this increase in brightness allows quantification of the antigen in solution. Alternatively, in a competitive assay, fluorescently labeled analyte competes with native analyte for free binding sites on the MagMOON, thus a high MagMOON fluorescence indicates many available binding sites, and a low native analyte concentration.

Fig. 6 shows two schematics for MagMOON assays. Fig. 6(a) shows an assay, reported earlier [3,4], measuring the relative concentration of biotin labeled with two different fluorescent dyes, Oregon green and Phycoerythrin. The experiment verified that magnetic modulation could be used to separate the MagMOON spectrum from electronic background signals, autofluorescence, and excess label not attached to the particles. Fig. 6(b) shows a competitive assay using chain-shaped aspherical MagMOONs. A solution of biotin functionalized fluorescent microsphere tags, 2 μm in diameter, was added to a solution of superavidin coated magnetic microspheres or nanospheres in a 96 well

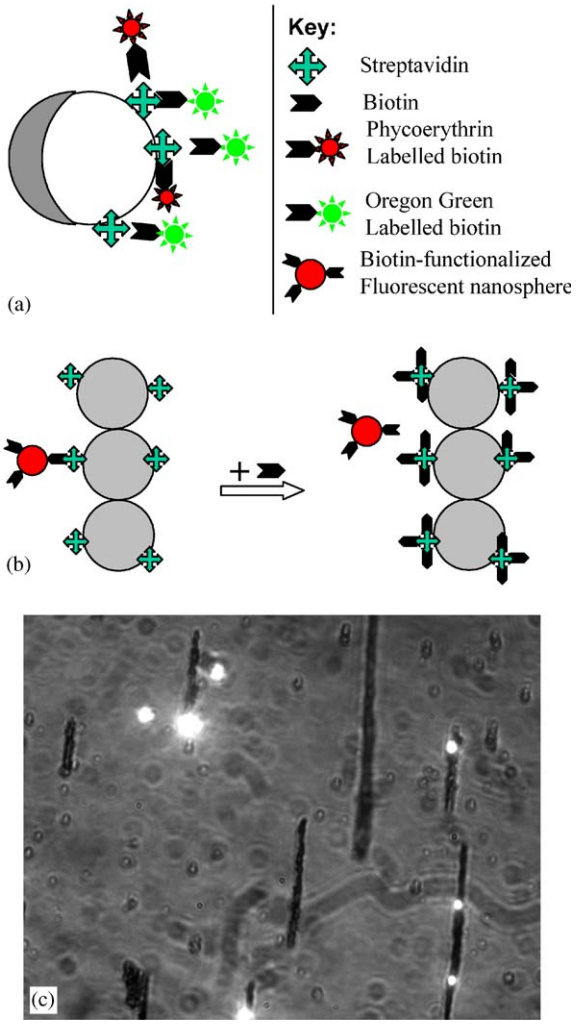

Fig. 6. Schematics for MagMOON assays. (a) A schematic to measure relative concentrations of biotin molecules labeled with two different fluorescent dyes. (b) A schematic for a biotin assay based on unlabeled biotin competing with fluorescently labeled biotin nanospheres for binding sites on a chain-shaped magMOON. (c) An image of a 2 μm fluorescent biotin labeled microspheres (Bangs labs) attaching to chains (aspherical magMOONs) of 0.86 μm superavidin functionalized magnetic microspheres (Spherotech). Rotating the MOONs modulated the fluorescence analogous to Venetian blinds. Adding biotin reduces binding between the microsphere labels and the chain-shaped MagMOONs.

microtiter plate. Ten minutes later, a solution of unlabelled biotin (the analyte) was added to a final concentration of 10^{-12} to 10^{-16} M biotin. The

solution was allowed to sit for 24 h. Then a permanent magnet was held a few centimeters from the solution so that the magnetic particles formed chains (an aspherical MagMOON) and the magnet was rotated in order to rotate the chains. Rotating the chain modulates the fluorescence from the tags attached to the chain, allowing signal separation [4]. Addition of unlabelled biotin reduced binding between the fluorescent tags and the MagMOONs resulting in a weaker modulated signal. Fluorescent nanoparticles may contain millions of fluorescent dye molecules, dramatically amplifying the signal from every binding event. A second dye that fluoresces at a different wavelength from the nanoparticles may be added, or reflection from the particle may be used, as an internal reference standard to account for changes in lamp intensity, sample geometry, and number of MagMOONs in view.

Unlike standard fluorescence immunoassays, where the fluorescent tags attach to the fixed surface of a microplate well, the capture surface in a MagMOON assay is on particles that are dispersed in solution and rotate. The MagMOON fluorescence can be separated from unbound dye fluorescence, solution autofluorescence, and electronic backgrounds by modulating the MagMOON and subtracting background. This signal separation enhances sensitivity in assays with high background variation and enables MagMOONs to be used for no-wash homogenous mix-and-measure assays. Homogeneous assays allow simpler procedures, as well as measurements of binding in situ, in real time, and with weakly binding proteins where washing would cause loss of analyte. Unlike most homogeneous assays, such as fluorescence polarization and fluorescence resonance energy transfer (FRET) assays, MagMOONs assays are not strongly effected by fluorescent backgrounds. Lifetime discrimination in time-resolved FRET (TR–FRET) largely overcomes this background problem, but requires specialized fluorescent tags and specialized instruments. MagMOONs can be interfaced to standard optical equipment, with addition of an external magnet or electromagnet.

We have also observed that the intrinsic fluorescence of streptavidin can be separated from background signals using magnetic modulation of streptavidin coated metal-capped MagMOONs. Streptavidin coated 4–5 μm ferromagnetic particles (Spherotech) were capped with aluminum, suspended in a solution of deionized water, and modulated by orienting them "on" and "off" in magnetic fields. A fluorescent spectrum from the weakly fluorescing streptavidin directly linked to the particles was detected. This weak signal contrasted with no detectable signal from a control (similar particles without streptavidin), and strong fluorescent signals from streptavin that had been fluorescently labeled with biotin linked fluorophores (Fig. 7).

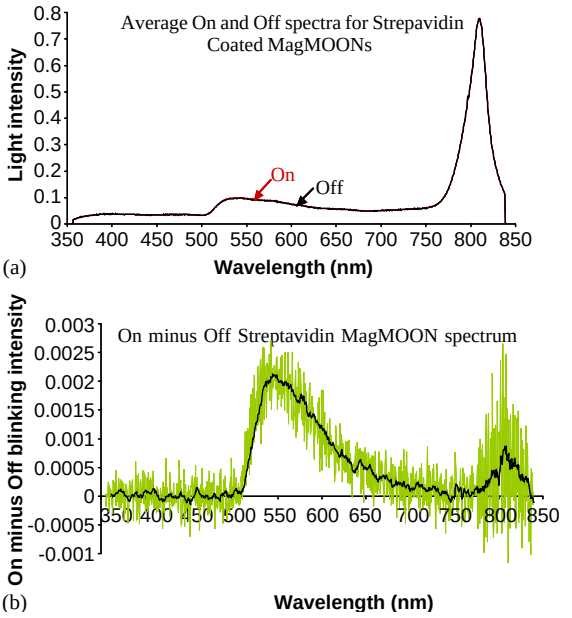

Fig. 7. (a) Average On (red) and Off (black) spectrum from a Streptavidin coated MagMOON. (b) On minus Off MagMOON spectrum reduces 800 nm mercury lamp background by a factor of a thousand. The green is the subtracted spectrum, the black line is a smoothed spectrum.

4. Noise/limitation

Modulation and demodulation of MagMOON signals allows in situ background subtraction, reducing interference from constant backgrounds and background noise that varies at different

frequencies from the MagMOONs. Background signals at other frequencies need not be discarded, as they can reveal information about the system. However, there are some types of noise such as shot noise, and read noise that occur at all frequencies and limit the signal-to-noise ratio for MagMOONs. In addition, fluctuations in excitation lamp intensity can interfere with measurements, unless one simultaneously measures and corrects these fluctuations.

Fluorescence, absorption, and reflection generally produce strong optical signals [1,2]. Even single fluorophores emit 10^4 to 10^5 photons per molecule before photobleaching, and more with oxygen depleted samples. Reflection from metal-capped particles is stronger yet. With good optical setups, using high numerical aperture lenses and high quantum efficiency photodetectors $>10\%$ of these photons can be captured and converted into electrical signal.

Careful optical techniques rely on preparing a similar blank and subtracting background signals. Uncertainty in these background levels often limits measurement sensitivity. This background variation becomes significant compared to random noise for background signals, and/or long acquisitions and averaging over many pixels. In many biological samples and "dirty" samples, background may vary between 1% and 100% from sample to sample, or at different locations and times within a sample.

When sensitive measurements are needed, there are a number of methods to reduce the effects of background interference: (1) Washing background away when possible. (2) Selecting small regions for spectroscopy or imaging using confocal microscopy or total internal reflection microscopy. (3) Using dyes that are excited by red light where autofluorescence is weaker, when available. (4) Using lifetime discrimination and dyes with long lifetimes, when dyes and instruments are available. However, these techniques either require specialized instrumentation or procedures, or rely on a subset of available dyes that limit the choice of dye and number that can be observed simultaneously.

Uncertainty comes from a variety of sources including read noise, photon shot noise, dark current noise (electron shot noise), $1/f$ noise, and uncertainty in background. Eq. (1) shows the average signal-to-noise level for a CCD detector model:

$$\frac{\text{Signal}}{\text{Noise}} = \frac{St}{\sqrt{R^2 + (B + D + S)t + (\alpha B t)^2}}, \tag{1}$$

where S is the intensity of the signal of interest (in electrons per second read by the CCD), t is exposure time, R is read noise, B is the optical background intensity, D is dark current (electronic background intensity), and α is the percentage variation in background from sample to sample. The signal-to-noise ratio is reduced by a further factor of $1/\sqrt{2}$ if a blank with the same uncertainty is subtracted. The intensity of the signal $(S \cdot t)$ and background $(B \cdot t)$ depend on the exposure time, illumination intensity, optical setup, the sample properties, and the detector specifications. For a given setup, and illumination, $(S \cdot t)$ and $(B \cdot t)$ depend only on exposure time and signal-to-background level, although increasing excitation intensity, or changing setup can generally substitute for increasing exposure time. Averaging the

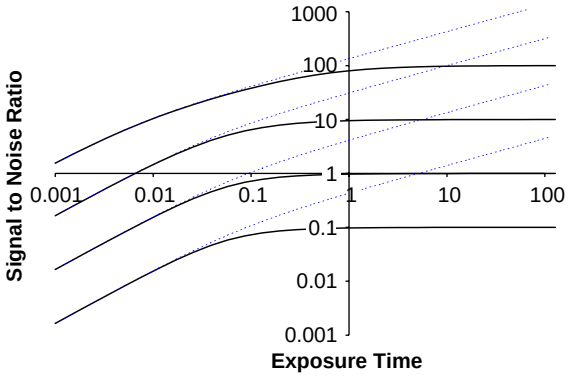

Fig. 8. Signal-to-noise ratio as a function of exposure time, for constant background but various signal-to-background levels. Calculations are for a case with $B = 2000$ electrons, $R = 12$, $D = 4$, and $\alpha = 10\%$ uncertainty in background level from sample to sample or in position and time within a sample (solid line), and 0% with MagMOONs (dotted line). Curves are plotted for $S/B = 10$, 1, 0.1, and 0.01. The advantage of in situ background subtraction becomes more pronounced when variation is large and $S \cdot t$ is large. For reference, our Roper Coolsnap ES imaging CCD has a saturation of ~12,000 electrons/pixel (or twice that for a gain of 0.5), upto 1.4 megapixels, and a read noise of ~6 electrons/pixel.

values from many pixels, or averaging over many images or spectra may also substitute for increasing exposure time, provided the signal is sufficiently intense that read noise is not significant.

Fig. 8 shows the calculated signal-to-noise ratios as a function of exposure time for a variety of signal-to-background levels, and for background uncertainties of 10%, and 0%. At low exposure times, read noise, R, is the main source of uncertainty, and the signal-to-noise ratio increases linearly with t. At higher exposure times, binning over more pixels, or for higher intensity signals, photon and electron shot noise is the most significant source of uncertainty, and the signal-to-noise ratio increases with $t^{1/2}$. For strong signals, the signal-to-noise ratio is limited to the uncertainty caused by variation in background, α. Using MagMOON modulation for in situ background subtraction removes this uncertainty, making measurements more reliable in samples with small signal-to-background ratios or large variations in background.

5. Conclusions

Controlling the optical symmetry of particles produces new tools for exploration of optical, chemical, and materials properties of surrounding environments. They can be used as nanoinstruments such as: nanoviscometers [17–22], nanothermometers [23], nanobarometers, and nanochemical sensors [15,24]. Modulation of Brownian MOONs and MagMOONs also allows the local optical, chemical, and material properties to be distinguished from the bulk properties for single particles and ensembles. These novel tools hold promise for sensitive intracellular chemical imaging with a wide range of dyes, high sensitivity immunoassays with simple procedures, as well as measurement of viscosities, biomagnetic torques, vorticity, and friction or rolling traction. The particles can be prepared with a wide range of size scales, from 50 nm–5 μm to date, and may use fluorescence or reflection. An exciting application for these probes is the simultaneous imaging of physical and chemical changes within systems such as living cells.

Acknowledgments

We gratefully acknowledge conversations with Brandon McNaughton and Gail Roberts as well as support from DARPA F49620-03-1-0297.

References

[1] M.A. Mason, K. Ray, G. Pohlers, et al., J. Phys. Chem. B 107 (2003) 14219.
[2] R.J. Ober, S. Ram, E.S. Ward, Biophys. J. 86 (2004) 1185.
[3] J.N. Anker, R. Kopelman, Appl. Phys. Lett. 82 (2003) 1102.
[4] J.N. Anker, C.J. Behrend, R. Kopelman, J. Appl. Phys. 93 (2003) 6698.
[5] C.J. Behrend, J.N. Anker, R. Kopelman. Appl. Phys. Lett. 84 (2004) 154.
[6] C.J. Behrend, J.N. Anker, B.H. McNaughton, et al., J. Phys. Chem. 108 (2004) 10408.
[7] H. Takei, N. Shimizu, Langmuir 13 (1997) 1865.
[8] J. Choi, Y.H. Zhao, D.Y. Zhang, et al., Nano Lett. 3 (2003) 995.
[9] C.J. Behrend, J.N. Anker, B.H. McNaughton, et al., J. Magn. Magn. Mater., in press.
[10] P.A. Valberg, J.P. Butler, Biophys. J. 52 (1987) 537.
[11] M. Glogauer, J. Ferrier, C.A.G. Mcculloch, Am. J. Physiol.-Cell Ph. 38 (1995) C1093.
[12] H. Xu, F. Yan, E.E. Monson, et al., J. Biomed. Mater. Res. A 66A (2003) 870.
[13] H. Xu, J.W. Aylott, R. Kopelman, et al., Anal. Chem. 73 (2001) 4124.
[14] E.J. Park, M. Brasuel, C. Behrend, et al., Anal. Chem. 75 (2003) 3784.
[15] T.G. Roberts, J.N. Anker, R. Kopelman, J. Magn. Magn. Mater., in press.
[16] B.H. McNaughton, J.N. Anker, R. Kopelman, J. Magn. Magn. Mater., in press.
[17] J.N. Anker, C.J. Behrend, B.H. McNaughton, et al., Mat. Res. Soc. Symp. Proc. 790 (2004) 4.4.1–12.
[18] V. Santhanam, R.P. Andres, Nano Lett. 4 (2004) 41.
[19] F.C. Mackintosh, C.F. Schmidt, Curr. Opin. Colloid In. 4 (1999) 300.
[20] Y. Nicolas, M. Paques, A. Knaebel, et al., Rev. Sci. Instrum. 74 (2003) 3838.
[21] V. Breedveld, D.J. Pine, J. Mater. Sci. 38 (2003) 4461.
[22] K.J. Van Vliet, G. Bao, S. Suresh, Acta Mater. 51 (2003) 5881.
[23] O. Zohar, M. Ikeda, H. Shinagawa, et al., Biophys. J. 74 (1998) 82.
[24] E. Monson, M. Brasuel, M.A. Philbert, R. Kopelman, in: T. Vo-Dinh (Ed.), Biomedical Photonics Handbook, CRC Press, Baco Raton, FL, 2003.