Nanoparticle Interaction with Biological Membranes: Does Nanotechnology Present a Janus Face?

PASCALE R. LEROUEIL,[‡,⊥] SEUNGPYO HONG,[†,⊥] ALMUT MECKE,[§,⊥] JAMES R. BAKER, JR.,[⊥] BRADFORD G. ORR,[§,⊥] AND MARK M. BANASZAK HOLL*,[†,‡,⊥,⊗]

Program in Macromolecular Science and Engineering, Departments of Chemistry and Physics, Graham Environmental Sustainability Institute, and Michigan Nanotechnology Institute for Medicine and Biological Sciences, University of Michigan, Ann Arbor, MI 48109

Ⓦ **This paper contains enhanced objects available on the Internet at http://pubs.acs.org/journals/achre4.**

ABSTRACT

Polycationic organic nanoparticles are shown to disrupt model biological membranes and living cell membranes at nanomolar concentrations. The degree of disruption is shown to be related to nanoparticle size and charge, as well as to the phase–fluid, liquid crystalline, or gel–of the biological membrane. Disruption events on model membranes have been directly imaged using scanning probe microsopy, whereas disruption events on living cells have been analyzed using cytosolic enzyme leakage assays, dye diffusion assays, and fluorescence microscopy.

Introduction

A great deal of optimism exists regarding the potential impact of nanotechnology upon the biomedical sciences. It is hoped that nanoscale materials, defined as 1–100 nm by the National Nanotechnology Initiative, the Food and Drug Administration, and ASTM International, will interact effectively and specifically with the components of cells such as membranes, proteins (both enzymatic and structural), and nucleic acids. Note that all of these important cellular structures are themselves nanometer in scale. Noncellular biological species such as viruses and prions may also most effectively be controlled and explored using nanomaterials and nanotechnology.

The development of therapeutics and diagnostics that take advantage of well-defined nanoscale polymeric scaffolds called poly(amidoamine) (PAMAM) dendrimers is a major focus of the interdisciplinary team working at the Michigan Nanotechnology Institute for Medicine and Biological Sciences (MNiMBS).[1–3] PAMAM dendrimers can be synthesized and purified to provide excellent polydispersity values (~1.01) and, as graphically illustrated in Figure 1, provide a range of sizes relevant to the nanoscale components of biology.

A particularly successful implementation of these materials has been the development of targeted chemotherapeutic agents. A combination of synthetic and analytical chemistry, *in vitro* cell biology, and *in vivo* experiments employing xenograft KB tumors in mice has allowed the nanoengineering of a therapeutic agent on a generation 5 (G5) PAMAM platform that can effectively deliver methotrexate to the tumor with no apparent side effects to the animal (Figure 2).[1,3] In this case, the nanoscale drug delivery platform has increased the therapeutic effectiveness by at least 10-fold.

* Corresponding author. Email: mbanasza@umich.edu. Tel: (734)763-2283.
‡ Department of Chemistry
⊥ Michigan Nanotechnology Institute for Medicine and Biological Sciences.
† Program in Macromolecular Science and Engineering.
§ Department of Physics.
⊗ Graham Environmental Sustainability Institute.

James R. Baker, Jr., was born in Oak Park, IL, in 1953. He graduated from Williams College in Williamstown, MA, and did his medical education at Loyola-Strich School of Medicine in Maywood, IL. After an internship and internal medicine residency at the Walter Reed Army Medical Center in Washington, D.C., he completed an Allergy and Clinical Immunology Fellowship, also at Walter Reed and at NIAID. In 1988, Dr. Baker was appointed as Associate Professor in the Departments of Medicine and Surgery at the Uniformed Services University of the Health Sciences. He joined the faculty of the University of Michigan in 1989 as an Associate Professor, Department of Internal Medicine, Division of Allergy, and has served as Chief of the Division of Allergy in the Department of Internal Medicine from 1993 to the present. He was promoted to Professor of Medicine in 1996 and appointed to Director of the Center for Biologic Nanotechnology in 1998, which was restructured into the Michigan Nanotechnology Institute for Medicine and Biological Sciences in 2005, and Dr. Baker serves as its Director. Dr. Baker is the Ruth Dow Doan Professor of Biologic Nanotechnology.

Bradford G. Orr was born in Coronado, CA, in 1958. He graduated from the University of Minnesota in 1980 with a B.S. and 1985 with a Ph.D. degree. He spent 2 years as postdoctoral fellow at the IBM T. J. Watson research laboratory in Yorktown Heights, NY, prior to accepting a position as an assistant professor of physics at the University of Michigan, where he is currently Professor of Physics and Director of Applied Physics.

Mark M. Banaszak Holl was born in Ithaca, NY, in 1964. He graduated from the University of Chicago in 1986 and obtained his Ph.D. degree at Cornell University in 1991. He spent 1 year as postdoctoral fellow at the IBM T. J. Watson research laboratory in Yorktown Heights, NY, prior to accepting a position as an assistant professor of chemistry at Brown University (1992–1995). He accepted a position at the University of Michigan in 1995 where he is currently Professor of Chemistry and Macromolecular Science and Engineering.

Pascale R. Leroueil was born in Auburn, WA, in 1981. She obtained her B.A. from Whitman College in 2003 and is working towards her Ph.D. in Chemistry at the University of Michigan.

Seungpyo Hong was born in Chuncheon, South Korea, in 1974. He obtained B.S. (1999) and M.S. (2001) degrees in polymer engineering at Hanyang University, Seoul, Korea. After working as a researcher at Korea Institute Science and Technology for a few years, he started his Ph.D. study working with his advisors Prof. Mark Banaszak Holl and Prof. James Baker, Jr., at the University of Michigan. He graduated with his Ph.D. in Macromolecular Science and Engineering in 2006 and is currently a Postdoctoral Associate in the lab of Prof. Robert Langer at MIT.

Almut Mecke was born in Schwenningen am Neckar, Germany, in 1971. After graduating with a Diplom degree in Physics from the University of Tübingen in Germany in 1997, she joined the Ph.D. program of the Department of Physics at the University of Michigan. She performed her dissertation research in collaboration with the Department of Chemistry and the Center for Biologic Nanotechnology (now Michigan Nanotechnology Institute for Medicine and Biological Sciences). She obtained her Ph.D. degree in 2004 and continued to work as a postdoctoral fellow at Michigan and in Basel, Switzerland, until 2006. She is currently employed at F. Hoffmann-La Roche Ltd.
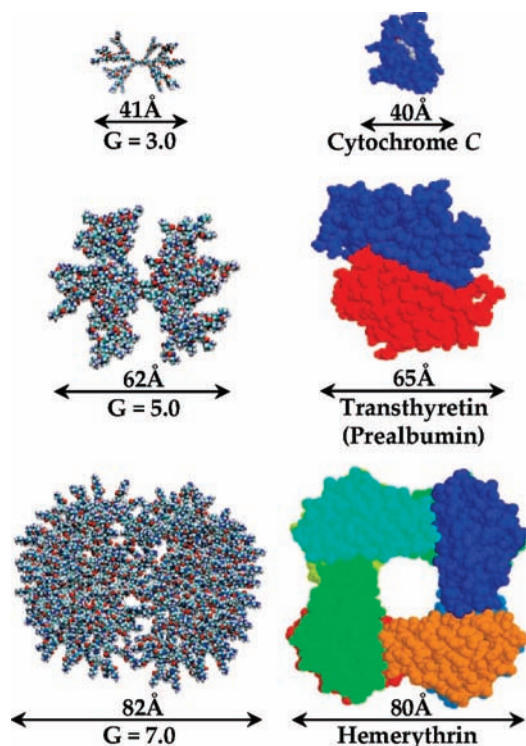
FIGURE 1. Absolute size comparison of PAMAM dendrimers to several key proteins.
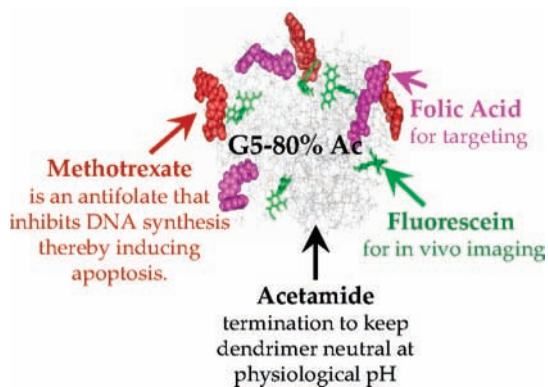

FIGURE 2. Schematic of multifunctional targeted nanodevice based on the PAMAM dendrimer scaffold.

At the dose ranges published to date, which are greater than the LD_{50} value for free methotrexate, no harmful effects have been noted in histology studies.[1]

In the course of the research program to develop the functional nanoparticle highlighted in Figure 2, we noted that many of the nanoparticles invented, both by us and by others, appeared inherently nonselective and that the nanosize scale of the devices might play a role in how the materials nonselectively passed through the plasma membrane of cells. Understanding this aspect of the nanoparticles is a key to designing the most selective, and therefore effective, chemotherapeutic platforms. Similar polymeric nanoparticle platforms are employed for cell transfection and are being developed for *in vivo* gene delivery; thus the behavior of this class of nanoparticles is of substantial general interest.[4] Another perspective regarding nonselective uptake into cells by nanoparticles relates to concerns

regarding cytotoxicity.[5–12] Does this behavior betray a tendency of nanoparticles to penetrate the membranes of cells and therefore provide clues to a potential negative impact upon human health and the environment? This was the scientific context existing in our group as we initiated a program to explore these questions as part of our interdisciplinary National Cancer Institute Unconventional Innovations grant funded in 1999.

Initial Studies—The Surface Scientist's View

Many studies already existed in the literature describing the interactions of PAMAM dendrimers, as well as other chemically similar polymers, with biological membranes. These studies, employing a wide range of techniques including dye diffusion in liposomes,[13] electron paramagnetic resonance (EPR),[14] and a variety of biological assays,[15–18] provide convincing evidence that the PAMAM dendrimers, and other amine-containing polymers, interact strongly with lipid bilayers and cell plasma membranes to induce substantial membrane permeability and, if sufficiently concentrated, cell lysis.[19–21] Indeed, these membrane disrupting properties were taken advantage of for the commercial development of cell transfection agents such as Superfect and jetPEI. Despite extensive work in this field, the vast majority of experimental studies examined the interaction using bulk techniques.

In order to obtain a nanoscale view of the interactions, we turned to scanning probe microscopy (SPM) studies of supported lipid bilayers (SLBs).[22–27] Key considerations when selecting a SLB for experiments include the head group, lipid chain length, and presence of unsaturation, which all have an impact on a crucial parameter, lipid bilayer phase. We wanted to mimic typical mammalian cell behavior so we selected dimyristoylphosphatidyl choline (DMPC), which has a zwitterionic head group and exists in the liquid-crystalline fluid phase under our typical imaging conditions. The interactions of G5 and G7 PAMAM dendrimers (~10–20 nm in diameter when spread on a surface[28]) with DMPC bilayers are illustrated in Figure 3, panel I.[24,27] The primary amine-terminated macromolecules, containing 128 and 512 positives charges, respectively, per nanoparticle at neutral pH, interact with the lipid bilayer to varying degrees. The G7 dendrimer initiates the formation of ~20 nm holes in the plateau regions of the SLB and expands the size of existing holes. The G5 dendrimer does not initiate the creation of new holes in the bilayer but does expand the size of existing holes and defects.

The generality of the membrane disruption behavior observed for the PAMAM dendrimers was explored by studying an additional set of polycationic organic polymers, namely, poly(L-lysine) (PLL), polyethyleneimine (PEI), and diethylaminoethyl-dextran (DEAE-dextran), as well as two neutral polymers, poly(ethylene glycol) (PEG) and poly(vinyl alcohol) (PVA).[23] PLL, PEI, and DEAE-dextran were selected because they are commonly used and commercially available materials for cell transfection as nonviral gene delivery vectors. These polymers have
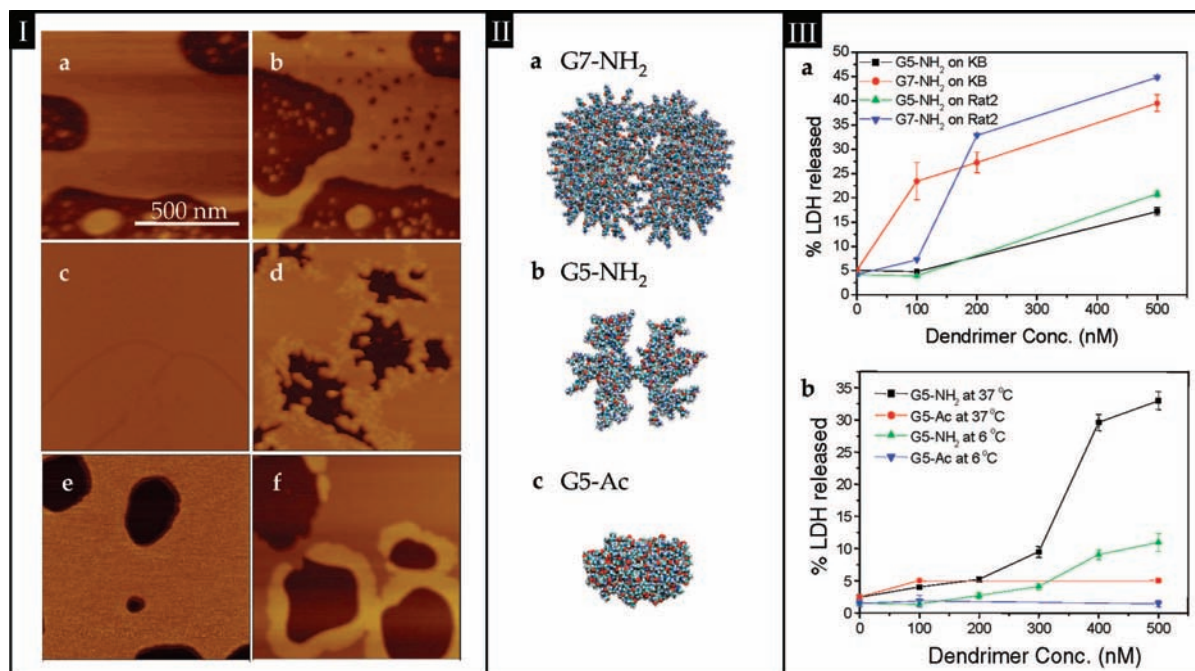
FIGURE 3. Dendrimer interactions with biological membranes. Panel I: AFM observation of DMPC-supported lipid bilayers (a,c,e) before and after incubation with (b) G7-NH$_2$, (d) G5-NH$_2$, and (f) G5-Ac PAMAM dendrimers. Panel II: Space-filling models of chemical structures of (a) G7-NH$_2$, (b) G5-NH$_2$, and (c) G5-Ac PAMAM dendrimers. Panel III: LDH leakage as a result of cell exposure to PAMAM dendrimers, showing (a) size effect of G7-NH$_2$ and G5-NH$_2$ on the LDH leakage out of KB and Rat2 cells after incubation at 37 °C for 3 h and (b) surface group dependency on the LDH leakage at different temperatures. Note that larger dendrimers (G7-NH$_2$) induce formation of new nanoscale holes in the bilayers as seen in the AFM images and cause a greater amount of LDH leakage out of live cells than G5-NH$_2$. G5-NH$_2$ dendrimers do not cause new hole formation in the lipid bilayers but instead expand pre-existing defects. In contrast, G5-Ac dendrimers do not cause hole formation, expansion of pre-existing defects, or LDH leakage out of live cells.

Ⓦ Movies in AVI format of panel Ia,b, panel Ic,d, and panel Ie,f are available.

also been employed as platforms for drug delivery applications. PEG and PVA were selected because they are commonly employed "biocompatible" polymers that are neutral. The polycationic polymers once again exhibited substantial membrane disruption behavior, including nanoscale hole formation (Figure 4, panel I). The substantial polydispersity index of the PEI (3.44) and DEAE-dextran (32.90) make detailed mechanistic comparisons of the action of the polymers difficult. For such polydisperse materials, we do not know which portion of the sample is active with the membrane. Thus, specific comparison and contrast of the physical properties of these materials, such as the role of size and charge density, is premature. The neutral polymers PEG and PVA did not induce membrane disruption over a similar concentration range.

The selection of DMPC to achieve a liquid-crystalline SLB phase turned out to be of critical importance. Experiments carried out using a cooled DMPC membrane containing both the gel and liquid-crystalline phases showed that only the liquid-crystalline phase was disrupted (Figure 5).[26]

These experimental results were intriguing for a number of reasons: (1) nanoscale hole formation in lipid bilayer membranes was directly imaged; (2) the trend of dendrimer molecular weight with hole formation efficiency was consistent with previously published transfection, dye diffusion, and cytotoxicity data;[22] (3) the observations were shown to

generalize to other polycationic polymer species such as PLL, PEI, and DEAE-dextran;[23] (4) membrane phase was shown to be crucial,[26] suggesting that interpretation of cell biology experiments exploring uptake of materials at low temperature must be carefully considered; (5) a mechanism for hole formation in lipid bilayer membranes and cell plasma membranes was proposed. Although the results obtained using SLBs were both surprising and intriguing, there were many possible caveats in applying any of the lessons learned to understanding the interactions of the dendrimers and polycationic polymers and nanoparticles more generally with biological membranes. In particular, the DMPC SLBs employed lack many of the key components of a cell plasma membrane including the variety of head groups, tail lengths, and degrees of saturation, glycolipids, protein (~50% of a mammalian cell membrane), and cholesterol.[29] Furthermore, the biological environment also includes a variety of cations and anions. The interaction of polycationic materials with biological membranes has been shown to be strongly counterion dependent in a series of papers by Sakai and Matile.[30–32] Although it is possible to create much more sophisticated SLB model systems taking into account a wide variety of additional parameters noted above, they still remain quite crude approximations of a living cell's plasma membrane. Thus, rather than performing experiments increasing the complexity of the SLB model, we decided it would be best to assess whether the lessons learned from
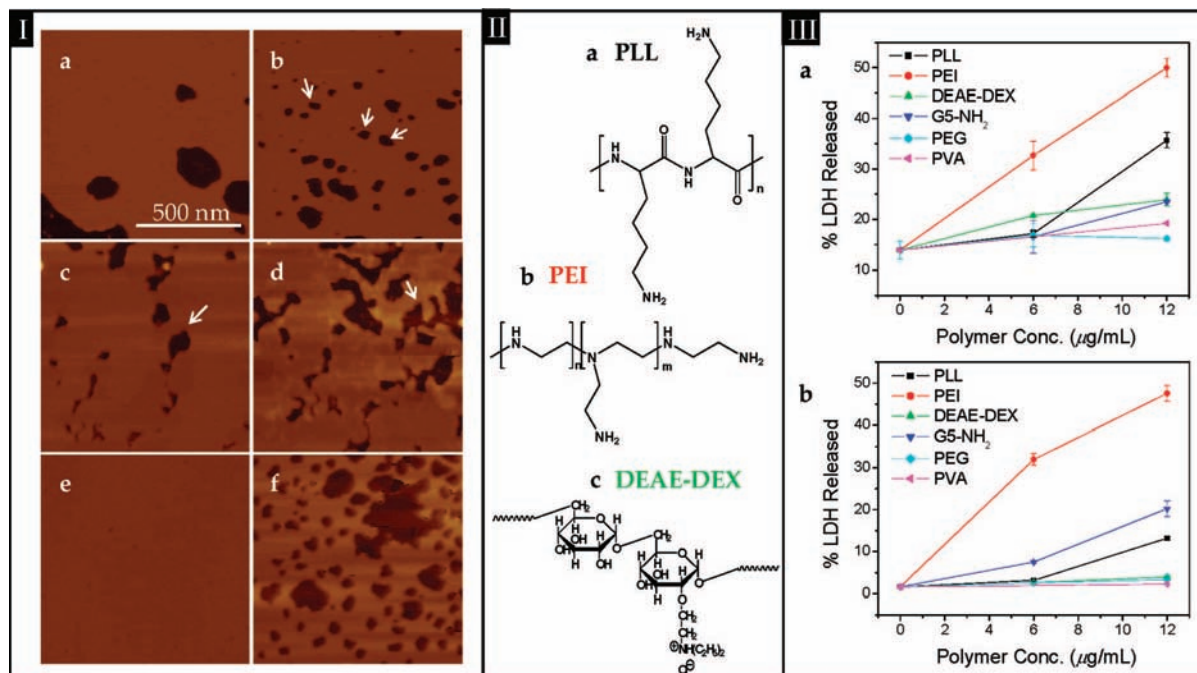
FIGURE 4. Interactions of other polymeric nanoparticles with biological membranes. Panel I: AFM observation of DMPC-supported lipid bilayers (a,c,e) before and after incubation with (b) PLL, (d) PEI, and (f) DEAE-DEX, respectively. Panel II: Chemical structures of (a) PLL, (b) PEI, and (c) DEAE-DEX. Panel III: LDH leakage out of (a) KB and (b) Rat2 cells as a result of exposure to the various polymeric nanoparticles at 37 °C for 3 h. Note that polycationic polymers induce the enzyme leakage whereas charge neutral polymers such as PEG and PVA do not.
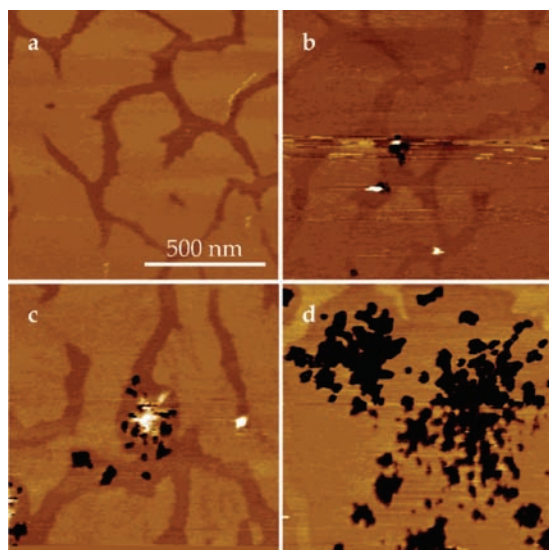
Ⓦ A movie in AVI format of panel Ic,d is available.

FIGURE 5. Interaction of G7-NH$_2$ with a supported DMPC bilayer consisting of both gel (Lβ* phase, lighter shade) and liquid-crystalline phase (L$_\alpha$ phase, darker shade). The G7-NH$_2$ preferentially forms holes in the liquid-crystalline phase of the bilayer.

the SLBs had any relevance to experimental observations on plasma membranes for living cells in culture.

A Polymer Scientist Visits the Biology Laboratory

Numerous studies in the literature indicated that polycationic organic polymers such as PAMAM dendrimers, PLL, PEI, and DEAE-dextran permeabilize cell plasma membranes.[20,33] In order to obtain the best comparison with

the SPM data that we had obtained (Figures 3–45),[24,26,27] we re-examined this line of experimentation.

If nanoscale holes are being introduced into the living cell plasma membranes, cytosolic enzymes such as lactate dehydrogenase (LDH) or luciferase (Luc) may be released into the cell media. In addition, if the membrane is challenged with charged small molecule dyes, for example, cationic propidium iodide (PI) or anionic fluorescein, the normally excluded dyes should diffuse across the membrane barrier if nanoscale holes or pores are present.

The results of the enzymatic leakage assays are presented in panel III of Figures 3 and 4.[22] Both G5 and G7 PAMAM dendrimers cause the leakage of LDH. The magnitude of LDH leakage is greatest for G7, consistent with the observation that the G7 dendrimer is also the most active in nanoscale hole formation for SLBs. Similar results were also observed for the leakage of Luc from KB and Rat2 cells transfected to express this enzyme in their cytosols. The G5 PAMAM dendrimer that has had the surface primary amine groups acetylated, G5-Ac, no longer protonates in aqueous solution at pH 7.[34] In the same concentration ranges, G5-Ac does not initiate nanoscale hole formation in SLBs (Figure 3, panel I) nor does it cause LDH or Luc leakage (Figure 3, panel III). Following this same trend, the cationic polymers PEI, PLL, and DEAE-dextran also cause LDH leakage, whereas the neutral polymers PEG and PVA do not (Figure 4, panel III).[23] Note that concentrations of the polymers in Figure 4 were expressed by weight (μg/mL) instead of using nanomolar concentrations since PDIs of those polymers were signifi-
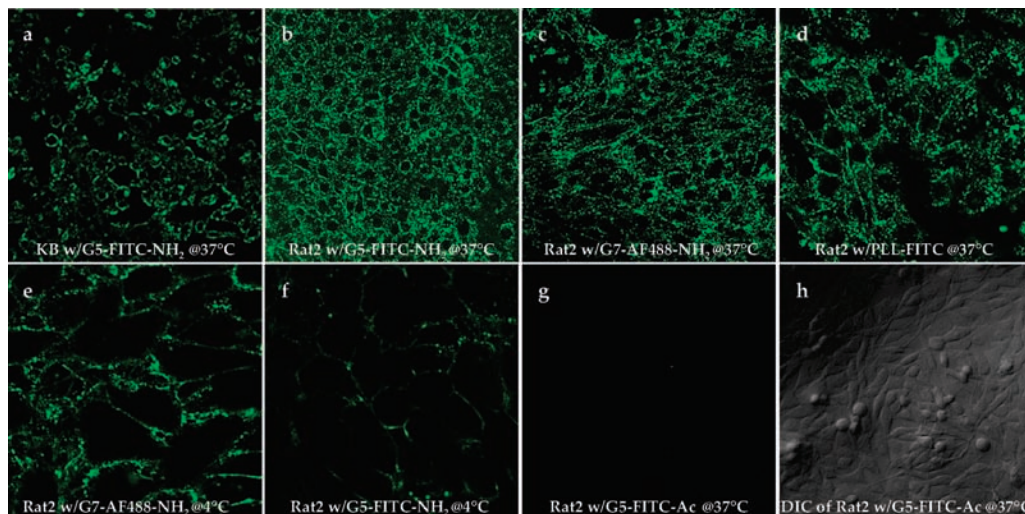
FIGURE 6. Internalization of polycationic polymers into cells observed by confocal laser scanning microscopy: (a) KB cells and (b) Rat2 cells incubated with 200 nM G5-NH$_2$–FITC at 37 °C for 1 h; Rat2 cells incubated with (c) 200 nM G7-NH$_2$–AF488 and (d) PLL–FITC at 37 °C for 1 h. Images e and f show Rat2 cells incubated with (e) G7-NH$_2$–AF488 and (f) G5-NH$_2$–FITC at 4 °C for 1 h. Note that G7-NH$_2$–AF488 dendrimers exhibit some degree of internalization at the low temperature as compared with G5-NH$_2$–FITC. Panel g shows Rat2 cells incubated with G5-Ac–FITC, used as a negative control, at 37 °C for 1 h. Panel h shows a differential interference contrast (DIC) image of confocal image e. Green fluorescence indicates polymeric nanoparticles conjugated with FITC or AF488.

cantly higher than those of dendrimers and the molarity of these solutions is not well defined.[23]

Do nanoscale polymers crossing the cell plasma membrane necessarily cause LDH leakage? We explored this question by measuring the degree of LDH leakage for G5-NH$_2$ and comparing it with the amount of LDH leakage for the uptake of G5-Ac–FA (FA = folic acid).[22] The uptake of G5-Ac–FA follows a receptor-mediated endocytosis pathway that is wholly blocked by the presence of an excess of FA indicating that receptor-mediated endocytosis is the only pathway by which this material enters the cell. The G5-Ac–FA particle enters the cell with no LDH leakage demonstrating that cytosolic enzyme leakage is not an intrinsic part of a ~5 nm particle entering a cell.

The effect of surface charge and morphology on polymer uptake into KB and Rat2 cells was tested by using the fluorescently labeled nanoparticles G5-NH$_2$–FITC, G5-Ac–FITC, G7-NH$_2$–AF488, and PLL–FITC (Figure 6) (FITC = fluoroscein isothiocyanate; AF488 = AlexaFluor 488). All of the polycationic materials, G5-NH$_2$–FITC, G7-NH$_2$–AF488, and PLL–FITC, were observed to internalize when incubated with the cells for 1 h at 37 °C. By way of contrast, the neutral G5-Ac–FITC did not internalize or even bind to the cell membrane. Recall that G5-Ac does not cause cytosolic enzyme leakage or cause holes to form in SLBs (Figure 3). The observation that G5-NH$_2$ did not cause cytosolic enzyme leakage at 6 °C, whereas G7-NH$_2$ still did,[22] prompted experiments with cells at low temperature to see whether the lack of cytosolic enzyme leakage was accompanied by a change in the degree of polymer internalization. The experiments were also prompted by the observation that G7-NH$_2$ only appears to disrupt fluid liquid-crystalline phase regions of SLBs

and does not cause holes in gel phase regions (Figure 5). Consistent with these observations, it was noted that G7-NH$_2$–AF488, which causes cytosolic enzyme leakage even at 6 °C, still internalized into Rat2 cells at 4 °C (Figure 6e), albeit to a lesser degree than at 37 °C (Figure 6c). By way of contrast, G5-NH$_2$–FITC, which does not cause cytosolic leakage at 6 °C, did not internalize into Rat2 cells but did bind to the cell plasma membrane (Figure 6f).

What Is a "Hole" or "Pore" in a Cell Plasma Membrane?

Our experiments point to the formation of a "hole" or "pore" in the living cell membranes as a possible mechanistic hypothesis. The meaning of the term "hole" or "pore" with respect to a living cell's membrane requires clarification. The limiting case is the complete loss of a region of the plasma membrane in direct analogy to the holes observed experimentally for the SLBs (see Figures 3–45).[24,26,27] In this case, a literal hole in the bilayer membrane exists (Figure 7b). Questions to be answered regarding such holes include the distribution of sizes, density in the membrane, and lifetime. In principle, the distribution of sizes could be tested as a bulk experiment employing differently sized probes. The density of holes or pores and the lifetime require direct measurements in single cells and are thus more challenging.

Membrane permeability could also arise from a reduction in density of the plasma membrane. In this case, a hole or pore corresponds to a region of reduced material (lipid, protein, cholesterol, etc.) (Figure 7c). Another possibility for the nature of the hole or pore involves a change in plasma membrane content. For example, the formation of dendrimer/lipid vesicles could create a localized region that was lipid-poor and protein- and
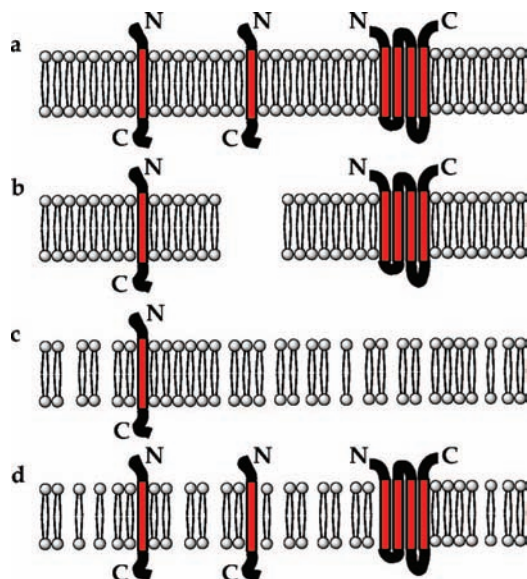
FIGURE 7. Variants of "holes" in cell plasma membranes. (a) intact membrane containing lipid and protein; (b) membrane with hole; (c) membrane containing low-density regions in which the amount of lipid and protein is reduced; d) membrane containing lipid-poor region.

cholesterol-rich (Figure 7d). For all cases, the distribution of sizes, the density, and the lifetime of such lipid-poor regions remain to be quantified.

The term hole or pore can refer to a wide range of structural changes that could lead to enhanced permeability ranging from the formation of an actual hole in the membrane to more subtle changes in content of the membrane leading to enhanced diffusion. Understanding the details of these changes that lead to nanoparticle-induced membrane permeability is a major challenge for the field.

Connections to the Literature: Mechanisms of Nanoparticle Internalization into Cells

The interaction of polymer nanoparticles and biological membranes is a complex process made difficult to understand in detail by the heterogeneity of both the nanoparticles and the cell membranes. Despite these obstacles, great interest exists in uncovering general principles that govern the interactions as well as details specific to a particular polymer or type of cell. This is due to the promise of polymeric nanoparticles for drug and gene delivery applications. The experiments from our laboratory are specifically focused on understanding the nanoparticle interaction with the cell plasma membrane and the mechanism of transport—active, passive, or both—into cells. The transfection process is commonly used as a primary assay to study polymer transport into cells.[16,35] We have consciously avoided this approach for these studies because of the number and complexity of the steps required for successful transfection after the polymer has breached the plasma membrane. In addition, the polymer/DNA complexes, ranging from ~10 to 2000 nm in size and typically referred to as "polyplexes", are substantially larger than the polymers themselves, and this may lead to mecha-

nistic changes.[36–39] Three primary hypotheses for the uptake of polycationic nanoparticles into cells have been postulated in the literature: (1) energy-dependent endocytosis;[16,35] (2) energy-dependent formation of nanoscale membrane holes;[22,23] (3) energy-independent membrane translocation.[40–42] The relationship of the three mechanisms to the data presented in this Account will now be discussed.

A variety of energy-dependent endocytosis processes have been proposed including the recent suggestions of fluid-phase phagocytosis by Behr et al.[35] and lipid raft mediated endocytosis by George et al.[16] Should these endocytosis processes be intrinsically leaky allowing cytosolic enzymes to escape? Our data indicates that neutral PAMAM dendrimers terminated with acetamide groups, which do not normally internalize into the cell or interact with cell membranes, will endocytose when conjugated to an appropriate targeting ligand such as folic acid and that cytosolic enzyme leakage is not inherently a part of the process.

If nanoparticles do follow endocytosis pathways, as has been proposed, is the endocytosis process itself leaky or is another mechanism present that causes the cytosolic enzyme leakage? In order to address this question, we turned to low-temperature studies. The inhibition of polymer uptake into cells at low temperatures (~4–6 °C) has generally been considered to be evidence for the inhibition of an ATP-driven endocytosis process. However, we have recently pointed out that cooling lipid membranes resulting in a change from fluid phase to gel phase also inhibits hole formation in SLBs.[26] Employing G5-NH$_2$, we noted that uptake into cells ceased at low temperature as did LDH leakage. Employing G7-NH$_2$, we observed that both LDH leakage and polymer uptake decreased but still clearly occurred. Since the only parameter changed in these experiments was the size and charge density of the polymer, this suggested that membrane disruption, in the form of hole or pore formation, was responsible for the continued LDH leakage and uptake at low temperature. The G7-NH$_2$ polymer had previously been shown to be more active towards hole formation in SLBs and to give a greater magnitude of LDH leakage.

In order to maintain the position that endocytosis is responsible for the continued uptake and LDH leakage of G7-NH$_2$ at ~4–6 °C, it has to be posited that the G7-NH$_2$ polymer's greater size or charge density or both results in a lower energetic barrier for the ATP-driven endocytosis process. On the face of it, a lower barrier appears counterintuitive since the G7 particle has a substantially larger volume (~3.4× larger) and surface area (~2.2× larger).[43] However the larger size also allows the particle to interact with a larger number of heparan sulfate proteoglycans (HSPGs), which have been hypothesized to trigger the endocytosis process.[35] Work recently published by Rothen et al. is quite interesting in this regard. They studied the uptake of polystyrene and TiO$_2$ particles in red blood cells and pulmonary macrophages.[44,45] These studies were particularly interesting because red blood cells lack the typical cellular machinery for endoctyosis or phagocytosis, yet the particles still penetrated the cell. The authors concluded that

particles enter the cells via an adhesive or diffusive mechanism and not the typically invoked endocytosis or phagocytosis mechanisms. This mechanistic proposal is roughly consistent with the mechanistic hypotheses developed from the SLB studies (Figures 3–45) and from our own cell culture studies.[5,22,27] Clearly, more studies are needed to fully understand the process by which nanoparticles cross the cell plasma membrane.

Summary

The mechanism by which nanoparticles cross the cell plasma membranes is not well understood. Gaining a better understanding of this mechanism has important implications for design of drug delivery, cell transfection, and gene therapy agents. Controlling the balance between effectively crossing the cell plasma membrane and inducing toxic effects is one of the key challenges for these fields. Concerns regarding cell plasma membrane disruption and resulting toxicity are paramount in the minds of nanoparticle designers focused on these applications. However, the results of our studies indicate that the key features of the nanoparticles related to membrane disruption, size and charge, are common to nanoparticles used in a wide variety of applications. Indeed, amine termination of nanoparticles is a common strategy employed to make materials water soluble and chemically reactive. The unique properties that make synthetic nanoparticles so fascinating for a wide variety of applications are a double-edged sword. In answer to the question posed by the title, we believe nanoparticles as a class will serve as powerful new therapeutics and, when present at sufficient doses, have the potential to act as dangerous toxins. This behavior is not surprising. The natural nanoparticles, oligonucleotides and proteins, as well as more complex functional nanoparticles such as viruses, have always presented humanity with a similar Janus face.

References

(1) (a) Kukowska-Latallo, J. F.; Candido, K. A.; Cao, Z. Y.; Nigavekar, S. S.; Majoros, I. J.; Thomas, T. P.; Balogh, L. P.; Khan, M. K.; Baker, J. R. Nanoparticle targeting of anticancer drug improves therapeutic response in animal model of human epithelial cancer. *Cancer Res.* **2005**, *65*, 5317–5324. (b) Thomas, T. P.; Majoros, I. J.; Kotlyar, A.; Kukowska-Latallo, J. F.; Bielinska, A.; Myc, A.; Baker, J. R. Targeting and inhibition of cell growth by an engineered dendritic nanodevice. *J. Med. Chem.* **2005**, *48*, 3729–3735.

(2) Majoros, I. J.; Thomas, T. P.; Mehta, C. B.; Baker, J. R. Poly(amidoamine) dendrimer-based multifunctional engineered nanodevice for cancer therapy. *J. Med. Chem.* **2005**, *48*, 5892–5899.

(3) Hong, S.; Leroueli, P. R.; Majoros, I.; Orr, B. G.; Baker, J. R.; Banaszak Holl, M. M. The binding avidity of a nanoparticle-base multivalent targeted drug delivery platform. *Chemistry and Biology* **2007**, *14*, 107–115.

(4) Reineke, T. M.; Grinstaff, M. W. Designer materials for nucleic acid delivery. *MRS Bull.* **2005**, *30*, 635–639.

(5) Hong, S.; Hessler, J. A.; Banaszak Holl, M. M.; Leroueil, P. R.; Mecke, A.; Orr, B. G. Physical interactions of nanoparticles with biological membranes: The observation of nanoscale hole formation. *Chem. Health Saf.* **2006**, *13*, 16–20.

(6) Borm, P. J. A.; Kreyling, W. Toxicological hazards of inhaled nanoparticles - Potential implications for drug delivery. *J. Nanosci. Nanotechnol.* **2004**, *4*, 521–531.

(7) Donaldson, K.; Stone, V.; Tran, C. L.; Kreyling, W.; Borm, P. J. A. Nanotoxicology. *Occup. Environ. Med.* **2004**, *61*, 727–728.

(8) Duncan, R.; Izzo, L. Dendrimer biocompatibility and toxicity. *Adv. Drug Delivery Rev.* **2005**, *57*, 2215–2237.

(9) Oberdorster, G.; Oberdorster, E.; Oberdorster, J. Nanotoxicology: An emerging discipline evolving from studies of ultrafine particles. *Environ. Health Perspect.* **2005**, *113*, 823–839.

(10) Nel, A.; Xia, T.; Madler, L.; Li, N. Toxic potential of materials at the nanolevel. *Science* **2006**, *311*, 622–627.

(11) Hoet, P. H. M.; Nemmar, A.; Nemery, B. Health impact of nano-materials. *Nat. Biotechnol.* **2004**, *22*, 19.

(12) Hoet, P. H. M.; Bruske-Hohlfeld, I.; Salata, O. V. Nanoparticles - known and unknown health risks. *J. Nanobiotechnol.* **2004**, *2*, 12.

(13) Zhang, Z.-Y.; Smith, B. D. High-generation polycationic dendrimers are unusually effective at disrupting anionic vesicles: Membrane bending model. *Bioconjugate Chem.* **2000**, *11*, 805–814.

(14) Ottaviani, M. F.; Matteini, P.; Brustolon, M.; Turro, N. J.; Jockusch, S.; Tomalia, D. A. Characterization of starburst dendrimers and vesicle solutions and their interactions by CW- and pulsed-EPR, TEM, and dynamic light scattering. *J. Phys. Chem. B* **1998**, *102*, 6029–6039.

(15) Kukowska-Latallo, J. F.; Bielinska, A. U.; Johnson, J.; Spindler, R.; Tomalia, D. A.; Baker, J. R. Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 4897–4902.

(16) Manunta, M.; Tan, P. H.; Sagoo, P.; Kashefi, K.; George, A. J. T. Gene delivery by dendrimers operates via a cholesterol dependent pathway. *Nucleic Acids Res.* **2004**, *32*, 2730–2739.

(17) Haensler, J.; Szoka, F. C. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjugate Chem.* **1993**, *4*, 372–379.

(18) Sonawane, N. D.; Szoka, F. C.; Verkman, A. S. Chloride accumulation and swelling in endosomes enhances DNA transfer by polyamine-DNA polyplexes. *J. Biol. Chem.* **2003**, *278*, 44826–44831.

(19) Malik, N.; Wiwattanapatapee, R.; Klopsch, R.; Lorenz, K.; Frey, H.; Weener, J. W.; Meijer, E. W.; Paulus, W.; Duncan, R. Dendrimers: Relationship between structure and biocompatibility in vitro, and preliminary studies on the biodistribution of I-125-labelled polyamidoamine dendrimers in vivo. *J. Controlled Release* **2000**, *65*, 133–148.

(20) Fischer, D.; Li, Y. X.; Ahlemeyer, B.; Krieglstein, J.; Kissel, T. In vitro cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* **2003**, *24*, 1121–1131.

(21) Tajarobi, F.; El-Sayed, M.; Rege, B. D.; Polli, J. E.; Ghandehari, H. Transport of poly amidoamine dendrimers across Madin-Darby canine kidney cells. *Int. J. Pharm.* **2001**, *215*, 263–267.

(22) Hong, S.; Bielinska, A. U.; Mecke, A.; Keszler, B.; Beals, J. L.; Shi, X.; Balogh, L.; Orr, B. G.; Baker, J. R.; Banaszak Holl, M. M. The interaction of polyamidoamine (PAMAM) dendrimers with supported lipid bilayers and cells: hole formation and the relation to transport. *Bioconjugate Chem.* **2004**, *15*, 774–782.

(23) Hong, S.; Leroueil, P. R.; Janus, E. K.; Peters, J. L.; Kober, M. M.; Islam, M. T.; Orr, B. G.; Baker, J. R.; Holl, M. M. B. Interaction of polycationic polymers with supported lipid bilayers and cells: Nanoscale hole formation and enhanced membrane permeability. *Bioconjugate Chem.* **2006**, *17*, 728–734.

(24) Mecke, A.; Uppuluri, S.; Sassanella, T. J.; Lee, D. K.; Ramamoorthy, A.; Baker, J. R.; Orr, B. G.; Banaszak Holl, M. M. Direct observation of lipid bilayer disruption by poly(amidoamine) dendrimers. *Chem. Phys. Lipids* **2004**, *132*, 3–14.

(25) Mecke, A.; Lee, D. K.; Ramamoorthy, A.; Orr, B. G.; Banaszak Holl, M. M. Membrane thinning due to antimicrobial peptide binding - An AFM study of MSI-78 in DMPC bilayers. *Biophys. J.* **2005**, *89*, 4043–4050.

(26) Mecke, A.; Lee, D.-K.; Ramamoorthy, A.; Orr, B. G.; Banaszak Holl, M. M. Synthetic and natural polycationic polymer nanoparticles interact selectively with fluid-phase domains of DMPC bilayers. *Langmuir* **2005**, *21*, 8588–8590.

(27) Mecke, A.; Majoros, I.; Patri, A. K.; Baker, J. R.; Banaszak Holl, M. M.; Orr, B. G. Lipid bilayer disruption by polycationic polymers: The roles of size and chemical functional group. *Langmuir* **2005**, *21*, 10348–10354.

(28) Mecke, A.; Lee, I.; Baker, J. R.; Banaszak Holl, M. M.; Orr, B. G. Deformability of poly(amidoamine) dendrimers. *Eur. Phys. J. E* **2004**, *14*, 7–16.

(29) Alberts, B.; Johnson, A.; Lewis, J.; Raff, M.; Roberts, K.; Walter, P. *Molecular Biology of the Cell*, 4th ed.; Garland Science: New York, 2002.

(30) Nishihara, M.; Perret, F.; Takeuchi, T.; Futaki, S.; Lazar, A. N.; Coleman, A. W.; Sakai, N.; Matile, S. Arginine magic with new counterions up the sleeve. *Org. Biomol. Chem.* **2005**, *3*, 1659–1669.

(31) Sakai, N.; Matile, S. Anion-mediated transfer of polyarginine across liquid and bilayer membranes. *J. Am. Chem. Soc.* **2003**, *125*, 14348–14356.

(32) Sakai, N.; Takeuchi, T.; Futaki, S.; Matile, S. Direct observation of anion-mediated translocation of fluorescent oligoarginine carriers into and across bulk liquid and anionic bilayer membranes. *ChemBioChem* **2005**, *6*, 114–122.

(33) Benns, J. M.; Mahato, R. I.; Kim, S. W. Optimization of factors influencing the transfection efficiency of folate-PEG-folate-graft-polyethylenimine. *J. Controlled Release* **2002**, *79*, 255–269.

(34) Majoros, I. J.; Keszler, B.; Woehler, S.; Bull, T.; Baker, J. R. Acetylation of poly(amidoamine) dendrimers. *Macromolecules* **2003**, *36*, 5526–5529.

(35) Kopatz, I.; Remy, J. S.; Behr, J. P. A model for non-viral gene delivery: through syndecan adhesion molecules and powered by actin. *J. Gene Med.* **2004**, *6*, 769–776.

(36) Boeckle, S.; Fahrmeir, J.; Roedl, W.; Ogris, M.; Wagner, E. Melittin analogs with high lytic activity at endosomal pH enhance transfection with purified targeted PEI polyplexes. *J. Controlled Release* **2006**, *112*, 240–248.

(37) Glodde, M.; Sirsi, S. R.; Lutz, G. J. Physiochemical properties of low and high molecular weight poly(ethylene glycol)-grafted poly(ethylene imine) copolymers and their complexes with oligo-nucleotides. *Biomacromolecules* **2006**, *7*, 347–356.

(38) Lai, E.; van Zanten, J. H. Monitoring DNA/poly-L-lysine polyplex formation with time-resolved multiangle laser light scattering. *Biophys. J.* **2001**, *80*, 864–873.

(39) Tang, M. X.; Szoka, F. C. The influence of polymer structure on the interactions of cationic polymers with DNA and morphology of the resulting complexes. *Gene Ther.* **1997**, *4*, 823–832.

(40) Rhee, M.; Davis, P. Mechanism of uptake of C105Y, a novel cell-penetrating peptide. *J. Biol. Chem.* **2006**, *281*, 1233–1240.

(41) Christiaens, B.; Grooten, J.; Reusens, M.; Joliot, A.; Goethals, M.; Vandekerckhove, J.; Prochiantz, A.; Rosseneu, M. Membrane interaction and cellular internalization of penetratin peptides. *Eur. J. Biochem.* **2004**, *271*, 1187–1197.

(42) Fischer, R.; Kohler, K.; Fotin-Mleczek, M.; Brock, R. A stepwise dissection of the intracellular fate of cationic cell-penetrating peptides. *J. Biol. Chem.* **2004**, *279*, 12625–12635.

(43) Li, J.; Peihler, L. T.; Qin, D.; Baker, J. R.; Tomalia, D.; Meier, D. Visualization and characterization of poly(amidoamine) dendrimers by atomic force microscopy. *Langmuir* **2000**, *16*, 5613–5616.

(44) Geiser, M.; Rothen-Rutishauser, B.; Kapp, N.; Schurch, S.; Kreyling, W.; Schulz, H.; Semmler, M.; Hof, V. I.; Heyder, J.; Gehr, P. Ultrafine particles cross cellular membranes by nonphagocytic mechanisms in lungs and in cultured cells. *Environ. Health Perspect.* **2005**, *113*, 1555–1560.

(45) Rothen-Rutishauser, B. M.; Schurch, S.; Haenni, B.; Kapp, N.; Gehr, P. Interaction of fine particles and nanoparticles with red blood cells visualized with advanced microscopic techniques. *Environ. Sci. Technol.* **2006**, *40*, 4353–4359.

AR600012Y