

Toward Intelligent Nanosize Bioreactors: A pH-Switchable, Channel-Equipped, Functional Polymer Nanocontainer

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ABSTRACT

To develop an intelligent sensor–effector functionality on the nanoscale, a pH-switchable, controlled nanoreactor based on amphiphilic copolymer membranes was built. The nanovesicles were equipped with bacterial transmembrane ompF pore proteins and the pH-sensitive enzyme acid phosphatase, resulting in a switchable substrate processing at pH 4–6.5. Ideal pH and substrate concentrations for the reaction were determined experimentally. In future, the reactor might be used for self-regulating targeted diagnostic and therapeutic applications in medicine.

Nanotechnology is believed by many to be one of the most important scientific topics of the next decade with a huge impact on biology, pharmacology, and medicine.^{1–3} In particular, medicine with its reliance on safe, potent, user-friendly, and target-specific therapies for important diseases such as arteriosclerosis, cancer, infections, or autoimmune disorders will profit from nanometer-sized devices for diagnostic and therapeutic applications.

Specifically, injectable nanometer-sized particles, micelles, and vesicles (typical size 50–250 nm) made of advanced materials are currently under investigation for targeted delivery of toxic or water-insoluble drugs or contrast agents in biological systems.^{4,5} Necessary key elements for a secure and directed transport of such nanocarriers in the bloodstream are high chemical and mechanical stability, low protein absorbance, no immunogenicity, good biocompatibility and biodegradability, specific targeting and clearance properties, and most of all defined functionality.^{6–8}

Promising candidate structures for a targeted delivery of drugs or contrast agents in humans are polymer-based vesicles consisting of amphiphilic diblock⁹ (hydrophilic–hydrophobic) or triblock¹⁰ (hydrophilic–hydrophobic–hydrophilic) copolymer building blocks. Depending on the

chain length and end group modifications of the polymer constituents, the building blocks can form unilamellar vesicles with specific diameters in aqueous solution, allowing the encapsulation of lipid-soluble (in the case of diblock membranes) or water-soluble (in the case of triblock membranes) substances such as drugs, enzymes, nucleotides, radioisotopes, or contrast media into the self-assembled nanostructures.

By choosing biocompatible and protein-repellent polymers such as poly(ethylene glycol) (PEG),¹¹ poly[*N*-(2-hydroxypropyl)methacrylamide] (pHPMA)¹² or poly(methyl oxazoline) (PMOXA)¹³ for the hydrophilic side blocks, the nanovesicles can be used for biomedical applications. These surface polymers ensure a higher grade of protection against endogenous opsonins such as IgG antibodies and complement factors, an essential prerequisite for immunogenic stealth properties. In contrary to these long-circulating stealth vesicles with protecting polymers, unprotected nanostructures show very short circulation lifetimes due to the rapid uptake into the macrophages of the mononuclear phagocyte system (MPS, also known as reticuloendothelial system RES) in liver and spleen.¹⁴ Furthermore, the uptake of artificial polymers into macrophages can lead to the presentation of these substances on class II major histocompatibility complexes (MHC) and subsequent T cell mediated immune reactions.

To enable a targeted delivery of the carrier to specific cell lines or receptors in biological systems, the polymeric strands can be chemically end group-modified to bear targeting

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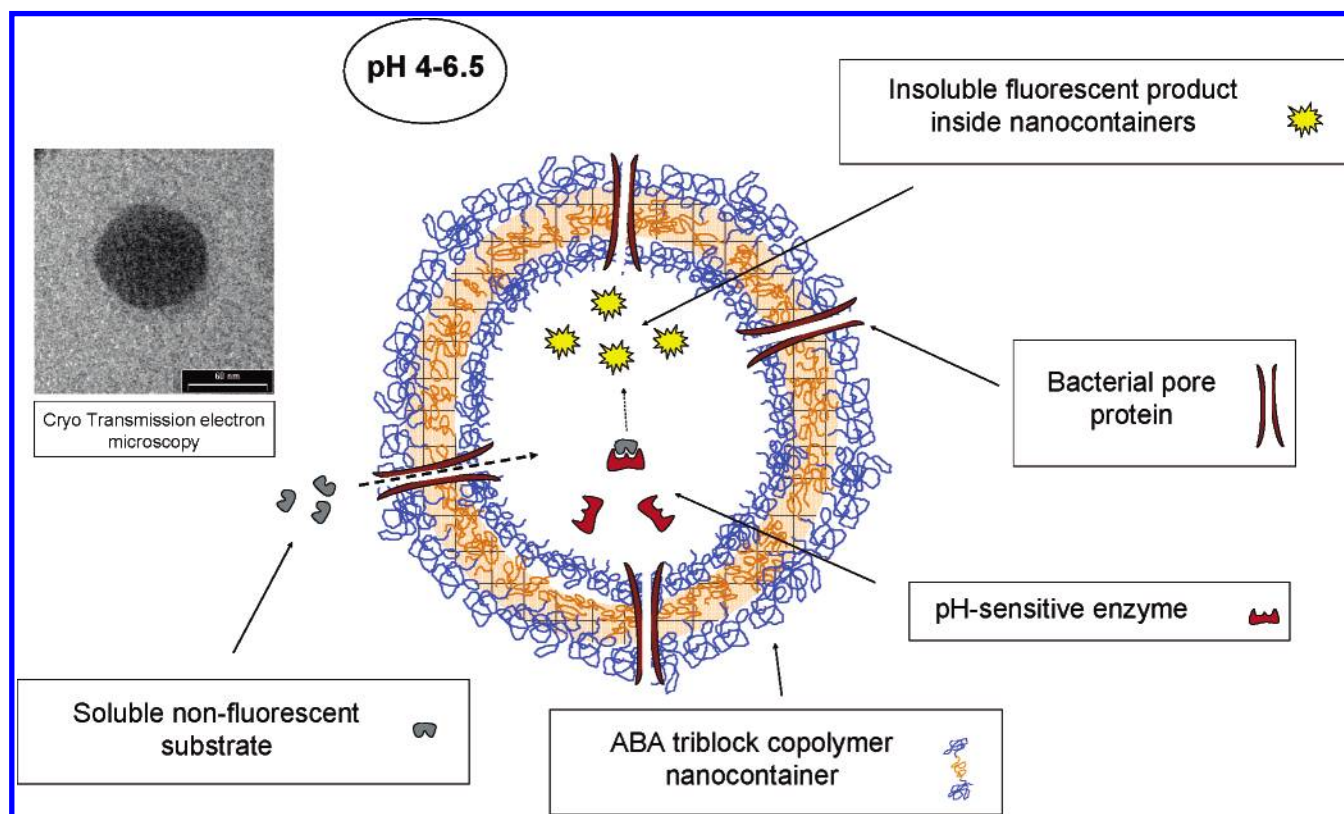


Figure 1. Two-dimensional outline and visualization of the nanoreactor system: The nanoreactors are based on a synthetic triblock copolymer membrane functionalized with bacterial ompF pore proteins that make intact, size-selective channels for passive diffusion across the membrane. Encapsulated acid phosphatase enzyme processes a nonfluorescent substrate into an insoluble, fluorescent reaction product at pH 4–6.5. Visualization of the final nanoreactor was done by cryogenic transmission electron microscopy, where a distinct core is surrounded by a fine halo, corresponding to the polymer membrane.

moieties such as antibodies,¹⁵ peptides,¹⁶ carbohydrates,¹⁷ polyamines,¹⁸ or oligonucleotides.¹⁹ The targeting moiety allows a receptor- or cell type specific transport of the long-circulating polymeric nanovesicles. Polymeric nanovesicles have been used for targeting of cancer cells, the blood–brain barrier, mononuclear phagocytes, dendritic cells, and endothelial cells.⁴

A revolutionary new concept is the combination of these target-specific nanometer-sized polymer vesicles with multifunctional biological components such as transmembrane proteins, pore proteins or enzymes. The goal is the creation of target-specific and furthermore responsive nanosystems that can reach their desired cellular or subcellular target with the help of a defined targeting moiety and that exhibit a controlled and triggered diagnostic signal or therapeutic effect upon activation. By combining sensing and effector functionality on the nanoscale, it will be possible to generate a defined response of the nanostructure that depends on environmental factors such as pH, substrate concentrations, or temperature at the target site. In contrary to this bottom-up, auto-regulated approach, currently used concepts for a triggered action of nanostructures rely on externally applicable signals such as near-infrared light,²⁰ laser irradiation,²¹ or magnetic fields.²² These signals trigger the release of bound or encapsulated substances from the nanostructures or the generation of cytotoxic molecules, leading for instance to the killing of cancer cells.

In this work, we present the successful equipping of a polymer vesicle platform (called nanocontainers) with proven cell targeting properties¹⁹ with a switchable enzyme functionality responsive to external conditions by integrating bacterial pore proteins into the triblock copolymer membrane.^{23–25} This system demonstrates the feasibility of a nanometer-sized bioreactor with size-selective substance diffusion and a defined trigger mechanism that allows external control of a defined functionality inside the enzyme-protecting, pore containing synthetic polymer nanocontainer.

The plant-derived enzyme acid phosphatase was chosen for encapsulation inside the nanocontainers due to the broad spectrum of substrates²⁶ and the well-established pH-dependent activity. The enzyme shows fast dephosphorylation of phosphate substrates between pH 4–7, whereas at highly acidic, neutral, or basic pH the enzyme is inactive.

To enable a controlled substance transport across the otherwise highly impermeable polymer membrane,²⁷ the bacterial outer membrane protein F (ompF)²⁸ was integrated into the synthetic bilayer. The pH-stable²⁹ ompF belongs to the porins, a group of proteins that build hydrophilic pores in the outer membranes of Gram-negative bacteria.³⁰ The 340-amino-acid-residue ompF was first detected in *Escherichia coli* bacteria and consists of three similar subunits. The resulting pores in the amphiphilic polymer membranes are known to remain fully functional and allow the passive

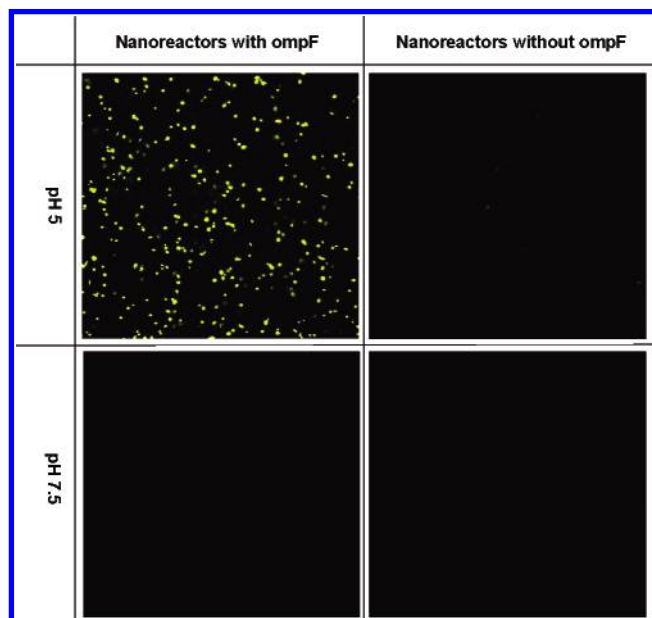


Figure 2. Channel- and pH-dependent nanoreactor activity: Active nanoreactors with ompF pores in the upper left panel show strong fluorescent activity at pH 5, whereas controls at pH 7.5 and without the ompF pores show no significant fluorescent activity in confocal microscopy after 3 h. Substrate concentration in this experiment was 75 μ M.

diffusion of polar molecules smaller than 600 Da through the channel of this transmembrane protein.^{25,28}

Nanoreactor properties were controlled by light scattering and transmission electron microscopy, the functionality of the complete nanosystem was evaluated at variable pH and substrate concentrations. The pH-switchable functionality of the complete system was demonstrated using enzymatic

hydrolysis of a water-soluble, nonfluorescent phosphatase substrate, leading to the production and precipitation of the water-insoluble fluorescent molecule ELF97 within the nanoreactor. Analysis was performed with fluorescence microscopy and confocal laser scanning microscopy (see Supporting Information for details).

The resulting nanoreactors showed a mean diameter of 93 nm and a homogeneous size distribution with a standard deviation of 15 nm in transmission electron microscopy. Enzyme-equipped nanocontainers showed a distinct core (mean diameter 60 nm, standard deviation 9.5 nm) in cryogenic transmission electron microscopy (see Figure 1), whereas empty nanocontainers exhibited no increase in contrast. The high-density core is covered by a distinguishable halo (mean thickness 16.5 nm, standard deviation 3 nm), representing the polymer membrane.

Experiments performed with the pH-controlled nanoreactors showed a strong fluorescent signal, when nanocontainers with the integrated ompF were tested near the enzyme pH optimum (pH = 5), while a series of control experiments documented the expected behavior of the different bioreactor subsystems (see Figure 2): Nanocontainers without the ompF porins showed no signal due to missing access of the substrate to the enzyme. Experiments with pH > 6.5 or pH < 4 (see Figure 3) showed no detectable increase in fluorescent signal intensity because of the switch functionality of the enzyme. Long-term experiments showed a time-dependent concentration increase of fluorescent product inside the nanocontainers over 3 h (see Figure 4), whereas equimolar concentrations of free acid phosphatase processed the substrate within 10 min. Substrate dependency of the nanoreactor system was tested with increasing phosphatase substrate concentrations, the ideal working concentration in

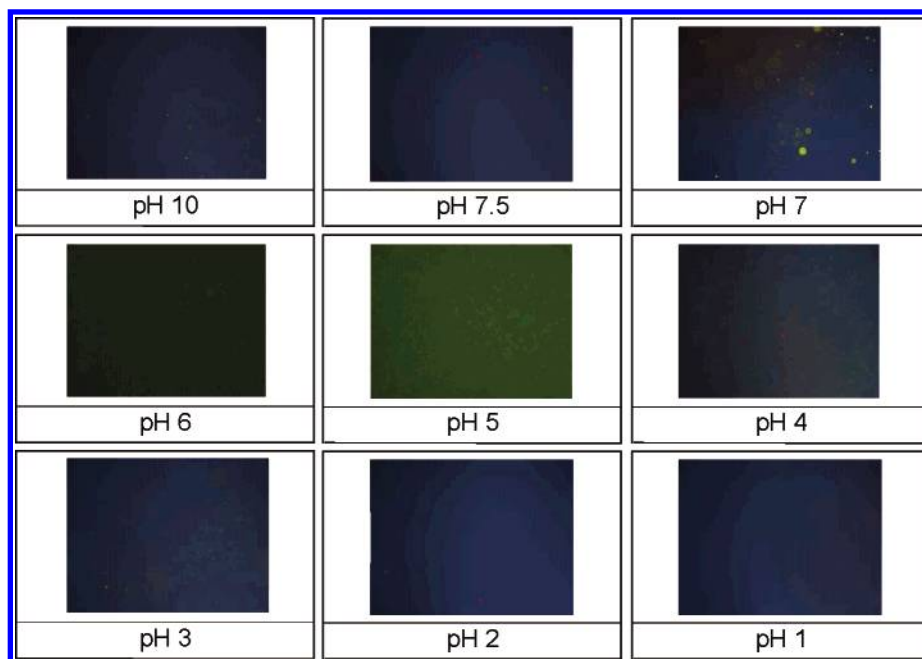


Figure 3. Nanoreactor activity in acidic, neutral and basic environment: Active nanoreactors with ompF pores were incubated with 75 μ M phosphatase substrate at changing pH conditions. The resulting fluorescent activity was measured and visualized after 3 h reaction time by fluorescence microscopy. Strong green-yellowish fluorescence was observed at pH 5, weaker fluorescence could be observed in the pH range between 6.5 and 4. At highly acidic, neutral and basic conditions, the nanoreactor showed no enzyme activity.

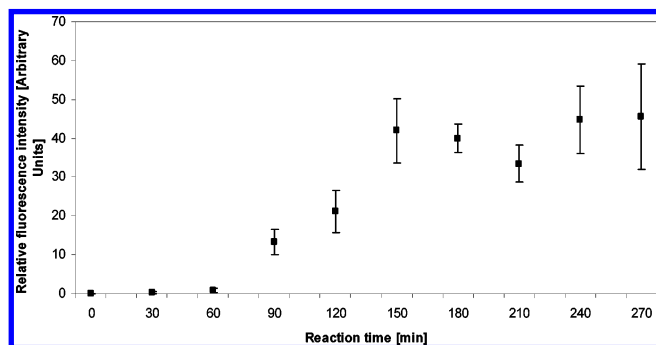


Figure 4. Time-dependent nanoreactor activity: Active nanoreactors with ompF pores were incubated with 75 μM phosphatase substrate at pH 5. Repetitive measurements over more than 4 h indicate slow increase of fluorescence intensity with a maximal value after 3 h (the error bars represent the standard deviation over a series of experiments).

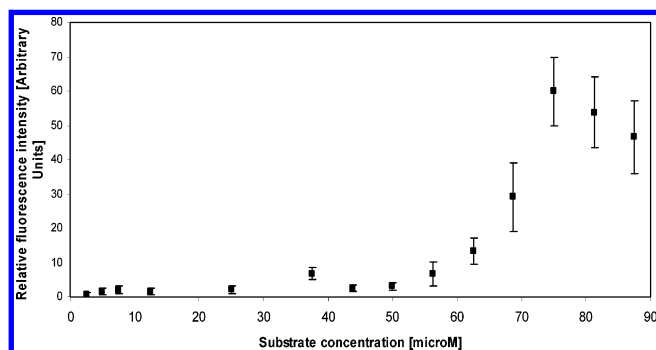


Figure 5. Substrate-dependent nanoreactor activity: Active nanoreactors with ompF pores were incubated with changing concentrations of phosphatase substrate at pH 5. After 3 h, the resulting fluorescence intensity was measured. The results indicate a hyperbolic curve in the area between 55 and 90 μM substrate concentration (the error bars represent the standard deviation over a series of experiments).

the solution lies between 55 and 90 μM . The resulting enzyme activity curve as defined by increase of relative fluorescence intensity over time shows an approximation to a theoretically expected hyperbolic curve, corresponding to Michaelis–Menten kinetics (see Figure 5).

As conclusion, we have presented the successful self-assembled nanofabrication of an enzyme nanoreactor with environment-controlled and triggerable sensor–effector functionality by combining synthetic triblock copolymer membranes with complex biological components. By variation of the pH of the surrounding solution, the nanoreactor was able to change its state of activity as demonstrated by producing a water-insoluble fluorescent dye inside the polymeric vesicle. The bacterial pores that were integrated into the polymer membrane remained functional (even though the polymer membrane is 3–4 times thicker than a biological, lipid-based membrane) and allowed the passive diffusion of both protons for activity control of the encapsulated acid phosphatase, as well as the diffusion of the nonfluorescent substrate into the nanoreactor.

Even though the nanoreactor showed a substrate concentration dependent enzyme kinetic, the bacterial pore proteins seem to be the key limiting element of the nanosystem. As

mentioned above, free nonencapsulated acid phosphatase was able to process equimolar substrate concentrations within minutes (no increase of fluorescence intensity after 10 min) at pH 5. The complete nanoreactor system showed a steady increase in fluorescence intensity over a time period of 3 h, where the system reaches steady-state. Regarding the size limitation of the ompF pores of 600 Da and the molecular weight of the phosphatase substrate of 431 Da, it seems to be evident that the pore number per nanocontainer influences the nanoreactor kinetics and allows an adjustment of the processing velocity according to the need of the application.

This proof of concept of externally triggerable nanoreactor is a step forward in equipping artificial nanosystems with an increasingly complex range of biological functionalities from plant and bacterial origin and has the potential for technical as well as biomedical application.

In the field of targeted delivery of diagnostic or therapeutic agents in medicine, the switchable bioreactor functionality enlarges the possible applications of such polymer-based systems massively. Not only might it be possible to release an encapsulated prodrug upon activation at the desired target structure (e.g., site of inflammation, site of infection, lysosome), the nanoreactors can also be considered as artificial automated nanosystems with complex functionality inside or outside of cells. The flexibility, stability, biocompatibility, and multifunctionality of our triblock copolymer nanocontainer platform make it a promising candidate system for an easily producible, injectable, target-specific, and stable nanometer-sized carrier with complex and automated diagnostic signal and/or therapeutic effect.

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Supporting Information Available: Text giving material and methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- (1) Emerich, D. F. *Expert Opin. Biol. Ther.* **2005**, *5*, 1–5.
- (2) Wickline, S. A.; Lanza, G. M. *Circulation* **2003**, *107*, 1092–1095.
- (3) Seaton, A.; Donaldson, K. *Lancet* **2005**, *365*, 923–924.
- (4) Moghimi, S. M.; Hunter, A. C.; Murray, J. C. *FASEB J.* **2005**, *19*, 311–330.
- (5) Duncan, R. *Nat. Rev. Drug Discovery* **2003**, *2*, 347–360.
- (6) Discher, D. E.; Eisenberg, A. *Science* **2002**, *297*, 967–973.
- (7) Photos, P. J.; Bacakova, L.; Discher, B.; Bates, F. S.; Discher, D. E. *J. Controlled Release* **2003**, *90*, 323–334.
- (8) Anderson, J. M.; Shive, M. S. *Adv. Drug Delivery Rev.* **1997**, *28*, 5–24.
- (9) Kwon, G. S. *Crit. Rev. Ther. Drug Carrier Syst.* **1998**, *15*, 481–512.
- (10) Nardin, C.; Hirt, T.; Leukel, J.; Meier, W. *Langmuir* **2000**, *16*, 1035–1041.
- (11) Owens, D. E., III; Peppas, N. A. *Int. J. Pharm.* **2006**, *307*, 93–102.
- (12) Kamei, S.; Kopeček, J. *Pharm. Res.* **1995**, *12*, 663–668.
- (13) Woodle, M. C.; Engbers, C. M.; Zalipsky, S. *Bioconjugate Chem.* **1994**, *5*, 493–496.

- (14) Moghimi, S. M.; Szebeni, J. *Prog. Lipid Res.* **2003**, *42*, 463–478.
- (15) Olivier, J.-C.; Huertas, R.; Lee, H. J.; Calon, F.; Pardridge, W. M. *Pharm. Res.* **2002**, *19*, 1137–1143.
- (16) Dufes, C.; Muller, J.-M.; Couet, W.; Olivier, J.-C.; Uchegbu, I.; Schätzlein, A. *Pharm. Res.* **2004**, *21*, 101–107.
- (17) Dufes, C.; Schätzlein, A. G.; Tetley, L.; Gray, A. I.; Watson, D. G.; Olivier, J.-C.; Couet, W.; Uchegbu, I. F. *Pharm. Res.* **2000**, *17*, 1250–1258.
- (18) Brown, M. D.; Gray, A. I.; Tetley, L.; Santovena, A.; Rene, J.; Schätzlein, A. G.; Uchegbu, I. F. *J. Controlled Release* **2003**, *93*, 193–211.
- (19) Broz, P.; Benito, S. M.; Saw, C.; Burger, P.; Heider, H.; Pfisterer, M.; Marsch, S.; Meier, W.; Hunziker, P. *J. Control Release* **2005**, *102*, 475–488.
- (20) Hirsch, L. R.; Stafford, R. J.; Bankson, J. A.; Sershen, S. R.; Rivera, B.; Price, R. E.; Hazle, J. D.; Halas, N. J.; West, J. L. *PNAS* **2003**, *100*, 13549–13554.
- (21) Lapotko, D.; Lukianova, E.; Potapnev, M.; Aleinikova, O.; Oraevsky, A. *Cancer Lett.* **2006**, *239*, 36–45.
- (22) Maier-Hauff, K.; Rothe, R.; Scholz, R.; Gneveckow, U.; Wust, P.; Thiesen, B.; Feussner, A.; von Deimling, A.; Waldoefner, N.; Felix, R.; Jordan, A. *J. Neuro-Oncology* **2006**, 1–8.
- (23) Nardin, C.; Meier, W. *J. Biotechnol.* **2002**, *90*, 17–26.
- (24) Meier, W.; Nardin, C.; Winterhalter, M. *Angew. Chem., Int. Ed.* **2000**, *39*, 4599–4602.
- (25) Ranquin, A.; Versees, W.; Meier, W.; Steyaert, J.; Van Gelder, P. *Nano Lett.* **2005**, *5*, 2220–2224.
- (26) Gellatly, K. S.; Moorhead, G.; Duff, S.; Lefebvre, D. D.; Plaxton, W. C. *Plant Physiol.* **1994**, *106*, 223–232.
- (27) Graff, A.; Sauer, M.; Van Gelder, P.; Meier, W. *PNAS* **2002**, *99*, 5064–5068.
- (28) Koebnik, R.; Locher, K. P.; Van Gelder, P. *Mol. Microbiol.* **2000**, *37*, 239–253.
- (29) Nestorovich, E. M.; Rostovtseva, T. K.; Bezrukov, S. M. *Biophys. J.* **2003**, *85*, 3718–3729.
- (30) Chen, R.; Kramer, C.; Schmidmayr, W.; Henning, U. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 5014–5017.
- (31) Prilipov, A.; Phale, P. S.; Van Gelder, P.; Rosenbusch, J. P.; Koebnik, R. *FEMS Microbiol. Lett.* **1998**, *163*, 65–72.

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