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Functional Magnetic Nanoparticles for Biodefense and Biological Threat Monitoring and Surveillance

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A new methodology toward biodefense and threat surveillance by water-dispersible, bactericidal, paramagnetic nanoparticles is presented. The nanoparticles consist of magnetite clusters (~70 nm) modified by polyethyleneimine (PEI) and poly(hexamethylene biguanide) (PHMBG) and are prepared by a two-step procedure at an acceptable cost. The cationic nanoparticles are colloidally stable in water at pH ≤10, where they sequester DNA or whole microbes. The nanoparticles and bound DNA are captured by high-gradient magnetic separation. Methods of efficient extraction and quantification of the DNA by real-time PCR are developed. Broad bactericidal activity of the nanoparticles is demonstrated at concentrations far below cytotoxicity levels for mammalian cells. The levels of the DNA detection sensitivity obtained in our experiments allow us to project the applicability of the developed method for the detection of DNA molecules of various germs.

The continuing threat of illnesses resulting from bioterrorism and related infectious diseases elevates the need for effective biodefense and surveillance systems that can detect and monitor the course of an outbreak.[1,2] Biodefense success depends on parameters such as the time passing between initial recognition of a suspected threat and the efficiency of its provisional neutralization *in situ*, transportation of samples for analysis, and time needed for detection. An ideal biodefense system would allow elimination of a broad range of biohazards and enable effective surveillance, while being inexpensive and sufficiently rapid in its application. Limitations on the routine detection of biohazards are due to the sensitivity of the detection method and the quantity of material available.[3]

Nanomaterials applicable for simultaneous pathogen detection and magnet-mediated separation are beginning to emerge[4] and offer significant potential for biodefense purposes. Capture probes conjugated to paramagnetic nanoparticles for extraction of microorganisms and DNA from water and soil have been reported,[5−7] but they lack the ability to kill germs *in situ*. Magnetic beads modified with immunoaffinity agents such as antibodies[8] or nucleotide probes (molecular beacons and specific sequences)[9] possess high specificity and can be recovered magnetically, but they are expensive, relatively unstable and cannot be applied *in situ* for biodefense. Zerovalent iron is known to kill bacteria and viruses in water via reduction and adsorption;[10] however, it is not useful for monitoring and surveillance, because, upon oxidation in water, it is converted to Fe ions and hydroxides that are unstable and nonparamagnetic.

Recently, a promising technology for airborne pathogen detection based on multiplexed polymerase chain reactions (PCRs) was developed at Lawrence Livermore National Laboratory (LLNL).[11] The technology is based on nucleic acid analysis of germs dispersed in air. It is fully automated and integrates different procedures, including collection and concentration of particles, DNA extraction and amplification of germ-specific DNA. Such a system, tested with weaponizable germs such as *Bacillus anthracis* and *Yersinia pestis*, is capable of early and rapid detection of microorganisms without the costs associated with sample transportation. The system utilizes a large array of 5.6-μm polystyrene microspheres coupled to pathogen-specific nucleic acid sequences.

Although the LLNL system is effective in the detection of airborne pathogens, it is not readily applied to biothreat monitoring of potentially compromised *water* resources. As with other micrometer-size particulates modified with specific DNA sequences, the polymer beads permitting the multiplex approach[11] would not remain dispersed in water for extended periods of time due to rapid sedimentation, and the DNA attached to the bead surfaces would be susceptible to degradation by bacterial DNA-hydrolyzing enzymes present in natural aquatic habitats. The present work addresses these issues and establishes an approach

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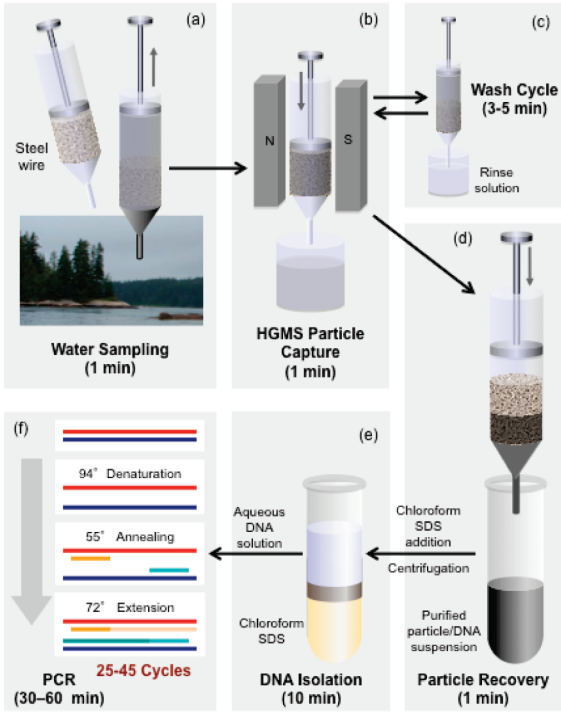

Figure 1. Schematic of proposed biothreat monitoring and surveillance: (a) sampling of water to capture nanoparticles with DNA, bacterial cells, and viruses; (b) capture and concentration of nanparticles using HGMS; (c) washing of nanoparticles to remove other suspended matter; (d) recovery of washed nanoparticles and adsorbed biological material by flushing with buffer solution with magnetic field removed; (e) extraction and separation of DNA from nanoparticles using a chloroform/SDS solution and centrifugation; and (f) real-time PCR to identify specific nucleic acids that pose a threat.

using magnetic nanoparticle clusters that merges *in situ* biodefense with a sensitive method of biohazard monitoring and surveillance.

The basic concepts of our approach are summarized schematically in Figure 1. Magnetic nanoparticle clusters modified by poly(hexamethylene biguanide) (PHMBG), which is a broad-range antiseptic commonly used for swimming pool sanitization,[12] are dispersed in threatened surface or groundwater bodies, where their small size and high charge density ensure their long-term colloidal stability in the aquatic environment. These polycationic magnetic particles bind polyanionic DNA nonspecifically, via electrostatic forces, while the membrane-binding PHMBG[13,14] on the surfaces of the particles ensures a high binding affinity for bacterial cells and viruses. In Figure 1a, samples of the particles with their captured germs and DNA debris are collected and then are passed through a bed of stainless steels wires placed within a strong magnetic field to trap and concentrate the loaded magnetic nanoparticles (Figure 1b), through a process known as high-gradient magnetic separation (HGMS). In this process, magnetizable wires distort the magnetic field to induce large local field gradients near the wire surfaces; the magnetic forces acting on the nanoparticles are proportional to the local field gradient, and, thus, the particles are attracted to and retained by the wires. The captured particles can be resuspended in a rinsing solution on removal of the magnetic field, and captured again as the rinse solution is discarded through the magnetic filter with the field applied once again (Figure 1c); this step can be repeated as often as needed to remove unbound materials. Following this capture and rinsing procedure, particles are released by removal of the magnetic field and flushed out of the steel wire matrix with fresh water (Figure 1d). In Figure 1e, the DNA is desorbed from the particles and extracted into a separate phase, then, it is identified by a real-time polymerase chain reaction (PCR) using primers developed for the set of germs to be surveyed (Figure 1f). Note that, in this description, we show the samples being collected using a syringe, but any sampling procedure may be used. In particular, large volumes of the water can be sampled by pumping it through an HGMS system on-site so that the magnetic nanoparticles are captured and concentrated relative to their original concentration in the contaminated water body.

In this paper, we show that (i) the dispersed particles can kill and capture a variety of bacteria, viruses, and fungi on contact, but at particle concentrations that are harmless to mammalian cells, and (ii) these particles, if appropriately sized, can be recovered readily by HGMS. We also show that PCR, which is well-recognized as the most sensitive method for the detection of nucleic acids, including viral and bacterial genomes, can be effective for both the monitoring of DNA molecules present in various environmental and bodily liquids as breakdown products of degraded bacteria, viruses, and other infectious agents, and for the analysis of DNA extracted from the germs captured by nanoparticles.

To the best of our knowledge, there have been no reports of a system that combines all the features of the concept reported herein, despite its seeming simplicity. In the present work, we experimented with model DNA and micro-organisms, as the work with potentially lethal germs would be limited to the government laboratories. Nevertheless, all of the key aspects of our approach have been demonstrated, as described below.

EXPERIMENTAL SECTION

The materials and the characterization methods used in this work are described in detail in section S1 in the Supporting Information.

Particle and Polymer Synthesis. *Polyethyleneimine-Modified Magnetite (PEI-M).* Magnetic nanoparticles were synthesized by chemical coprecipitation of iron(II) and iron(III) chlorides. Namely, 7.58 g (28 mmol) of $FeCl_3 \cdot 6H_2O$ and 2.78 g (14 mmol) of $FeCl_2 \cdot 4H_2O$ were added to 40 mL of deionized water, and the solution was deaerated by nitrogen purge in a stirred 250-mL three-necked flask and the temperature of the flask contents was brought to 80 °C. An aqueous solution of a branched, 25 kDa PEI (10 g polymer in 40 mL water, pH adjusted to 6) was added to the flask and the resulting mixture was equilibrated at 80 °C for 10 min while gently stirring under nitrogen purge. The nitrogen purge then ceased and the contents of the flask were added, at once, to 100 mL of a 28% ammonium hydroxide solution, and the mixture, which rapidly turned black, was stirred vigorously for 5−10 min. The resulting precipitate possessed strong magnetic properties and was thus separated from the liquid by decantation using a Franz isodynamic

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magnetic separator (Trenton, NJ). The precipitate was dried under vacuum until constant weight and was resuspended in deionized water by sonication for 30 s with a Branson Model 450 sonifier (Branson Ultrasonics Corp., Danbury, CT) at an output of 50%. The solids were separated from the liquid by magnetic decantation and resuspended in dialyzed water, and the suspension was further dialyzed against excess deionized water (membrane MW cutoff, 12−14 kDa) and lyophilized. Elemental analysis (%): C, 26.1; H, 5.87; Fe, 39.3; N, 13.7.

Poly(hexamethylene biguanide)- and Polyethyleneimine-Modified Magnetite (PHMBG−PEI-M). PEI-M particles (5 g) were suspended in 50 mL of 7 wt % PHMBG aqueous solution with sonication for 30 s. To this suspension, 10 mL of 25% aqueous glutaraldehyde were added and the resulting suspension was quickly stirred and then shaken at room temperature for 3 h, at which point viscosification was observed. The resulting viscous suspension was stirred and lyophilized. The solids were ground by mortar and pestle and suspended in deionized water (70 mL) using a Branson sonifier, resulting in a two-component suspension. Larger particles quickly sedimented in the gravitational field, while smaller particles remained suspended in water. The aqueous layer was separated and dialyzed against deionized water (membrane MW cutoff, 12−14 kDa). After lyophilization, the solids were weighed. Elemental analysis (%): C, 35.8; H, 7.75; Fe, 27.6; N, 18.3. The procedure resulted in ∼56 wt % yield of PHMBG−PEI-M particles dispersible in aqueous media, relative to the initial PEI-M particles.

Poly(hexamethylene biguanide)- and Polyethyleneimine-Modified Magnetite Labeled by 5(6)-Carboxyfluorescein N-Hydroxysuccinimide. A freshly prepared solution of 5(6)-carboxyfluorescein *N*-hydroxysuccinimide (9.5 mg, 20 mmol) in dimethylsulfoxide (DMSO) (1 mL) was added to 10 mL of 7.5 wt % PHMBG aqueous solution and the resulting mixture was kept in the dark while stirring for 3 h at room temperature. Then, 0.5 g of PEI-M particles were added to the solution and the particles were suspended with brief (3 s) sonication. A chilled (4 °C) solution of 30% glutaraldehyde (2 mL) was added to the suspension and the mixture was kept at room temperature in the dark for 16 h while shaking. The suspension was snap-frozen and dried under vacuum, and the resulting solids were suspended, with sonication, in 20 mL of ethanol. Solids were separated by the magnetic separator and the procedure was repeated twice more. The resulting labeled particles were then dried, resuspended in 20 mL of deionized water, and dialyzed (MWCO, 12−14 kDa) against dialyzed water, and the suspension was lyophilized. The labeled particles were strongly fluorescent in aqueous media ($\lambda_{em} = 495$ nm, $\lambda_{ex} = 525$ nm).

Copolymer of Poly(hexamethylene biguanide) and Polyethyleneimine (PHMBG−PEI). To an aqueous solution (25 mL) of branched, 25 kDa PEI (2.5 g) and PHMBG (1.75 g), 5 mL of 25% aqueous glutaraldehyde were added with stirring and viscosification of the solution was immediately observed. The mixture was kept at room temperature for 16 h, and then the paste-like suspension was sonicated briefly, dialyzed against deionized water (membrane MW cutoff, 12−14 kDa), snap-frozen, and lyophilized. ^{1}H NMR (400 MHz, D_2O): δ 3.0 (m, $-CH_2$ located α,β to $-N$; β,α to $-N(C)C$ imino), 2.52 (m, $-CH_2$ located α to $-N$; β to $-C$), 2.47 (m, $-NH_2$), 1.37 ($-CH_2$ located β to $-N$, β to $-C$), 1.16 ppm ($-CH_2$ located β to $-C$).

Magnetic Particle Capture by HGMS. Dispersions of nonfluorescent PEI-M and PHMBG−PEI-M particles (0.05−0.1 mg/mL) in fresh water or 5 mM Tris buffer (pH 7.4) were passed, by means of a hand-driven piston, through a polypropylene syringe with an internal diameter of 5 mm and a length of 7 cm tightly packed (packing length, 30 mm) with 0.21 g of Type 430 fine-grade stainless steel wool (40−66 mm diameter) supplied by S.G. Frantz Co., Inc. (Trenton, NJ) placed inside a Model L-1CN separator (S.G. Frantz Co.). The maximum flux density generated inside the magnet separator was 1.3 T. Alternatively, HGMS was performed using a portable quadrupole magnet system comprised of four nickel-plated neodymium iron boron 40 MGOe permanent magnets (each 18 cm × 1.8 cm × 1.8 cm in size; Dura Magnetics, Inc., Sylvania, OH). The flux density generated inside the packed column was ca. 0.73 T. The particle dispersion was passed through a stainless steel wire column (height, 22 mm; internal diameter, 13.5 mm; weight, 1.78 g) tightly packed inside a 10-mL syringe (Beckton Dickinson) placed inside of the quadrupole. The concentrations of iron in the initial suspension (C_0) and separated suspensions (C_{HGMS}) were measured by inductively coupled plasma emission spectrometry (ICP) and the efficiency of separation was estimated as

$$\text{efficiency (\%)} = 100 \times \frac{1 - C_{HGMS}}{C_0}$$

Dispersions of the PHMBG−PEI-M particles labeled by 5(6)-carboxyfluorescein *N*-hydroxysuccinimide in 5 mM Tris buffer (pH 7.4) were separated by the same method, but the particle concentration measurement was accomplished using the fluorescence emission intensity ($\lambda_{ex} = 495$ nm, $\lambda_{em}^{max} = 525$ nm). Emission intensity versus concentration calibration curves were developed and the separation efficiency was expressed as previously shown. Experiments with the fluorescent particles were conducted in triplicate.

DNA Capture and Purification. HGMS capture and purification of DNA samples was performed as follows. A DNA sample (0.3 mL) was contacted with 1.0 mL of a 0.1 mg/mL suspension of PHMBG−PEI-magnetite particles in 10 mM Tris buffer containing 10 mM EDTA (pH 7.4). The resulting suspension was vortexed and kept at room temperature while shaking for 1 h. The suspension was then passed, by means of a hand-driven 1-mL piston, through a polypropylene syringe packed with stainless steel wool as described previously. The syringe was placed inside a Model L-1CN separator. The wool was purified in boiling water prior to loading onto the column. The collected liquid was then diluted by 1 mL of the Tris buffer, loaded, and passed through the column; the procedure was repeated three times. The magnet was then turned off and 1.6 mL of 10 mM Tris buffer containing 10 mM EDTA was passed through the column to collect and resuspend the washed particles. To separate the DNA from the particles, the suspension was mixed with 1.5 mL of 10 mg/mL solution of sodium dodecylsulfate (SDS) in chloroform. The resulting mixture was vortexed, yielding an opaque, lightly brownish-colored emulsion. The emulsion was centrifuged at 15 000 *g* for 5 min and the upper, transparent aqueous layer was carefully separated and stored at −80 °C. The efficiency of DNA

capture and recovery was measured using a fluorescent DNA fragment labeled by succinimidyl ester of 6-carboxyfluorescein (6-FAM). Calibration curves of fluorescence intensity of DNA solutions ($\lambda_{ex} = 495$ nm, $\lambda_{em}^{max} = 517$ nm) in 5 mM Tris buffer versus concentration were developed in the concentration range of 6.67−66.7 ng/mL. Using the initial DNA concentration before binding to the particles (C_0) and after particle binding and HGMS separation, followed by the DNA extraction from the particles (C_r), the efficiency of the DNA recovery was calculated as

$$\text{recovery (\%)} = 100 \times \frac{1 - C_r}{C_0}$$

Analogous experiments were conducted with DNA from salmon testes, which were used to develop the procedure of DNA extraction off the particles. The concentration of this DNA in 5 mM Tris buffer was measured spectrophotometrically ($\lambda_{max} = 260$ nm, extinction coefficient 6600 M^{-1} cm^{-1}). The presence of the particles (electronic absorbance up to 570 nm) in DNA solutions interfered strongly with such measurements, but no interference was observed in DNA solutions separated from the particles by the SDS/chloroform extraction and centrifugation, as previously noted. Such solutions were colorless and transparent. dsDNA of pUC18 plasmid and ssDNA of M13 bacteriophage were used to develop a monitoring system by quantitative real-time PCR. The DNA was captured and extracted from the particles as described previously, followed by an additional purification using a QIAquick nucleotide removal kit (Quiagen, Germantown, MD) according to the manufacturer's protocol. Finally, DNA was dissolved in nuclease-free water and aliquots of serial dilutions were used for the real-time PCR reaction.

DNA Concentration Using HGMS. The method of concentrating DNA solutions by means of DNA capture by magnetic particles, followed by DNA recovery, was demonstrated as follows. A 1 μg/mL solution of FAM-labeled fragment (71 bp) of the *Drosophila* diphtericin gene (0.3 mL) was dissolved in 990 mL of 10 mM Tris buffer containing 10 mM EDTA (pH 7.4). To the solution, 10 mL of 5 mg/mL suspension of PHMBG−PEI-M particles in the Tris buffer was added and the resulting dilute suspension was gently shaken at room temperature for 8 h. The suspension was then passed, using gravitation, through a polypropylene column (height, 27 cm; internal diameter, 10 mm) connected to a polypropylene syringe tightly packed with 0.21 g of type 430 fine-grade stainless steel wool (40−66 μm diameter) placed inside a Model L-1CN separator with the maximum flux density of 1.3 T. The wool was purified in boiling water prior to loading onto the column. The magnet was then turned off and 9.0 mL of 10 mM Tris buffer containing 10 mM EDTA was passed through the syringe to collect and resuspend the washed particles. To separate the DNA from the particles, the suspension was mixed with 9 mL of 10 mg/mL solution of SDS in chloroform. The resulting mixture was vortexed, yielding an opaque, lightly brownish-colored emulsion. The emulsion was separated into six 3-mL samples, each of which was centrifuged at 15 000 *g* for 5 min and the upper, transparent aqueous layer was carefully separated. Three 3-mL aqueous samples were generated and subjected to the fluorescence measurement. Calibration curve of fluorescence intensity of DNA solutions ($\lambda_{ex} = 495$ nm, $\lambda_{em}^{max} = 517$ nm) in 10 mM Tris buffer versus concentration was developed (see section S2 in the Supporting Information). Using the initial DNA concentration before the addition of the particles ($C_0 = 0.3$ ng/mL) and after particle binding, concentration and HGMS separation followed by the DNA extraction from the particles (C_c), the degree of the DNA concentration was calculated as

$$\text{degree of the DNA concentration} = \frac{C_c}{C_0}$$

Polymerase Chain Reaction (PCR). Real-time quantitative PCR (qPCR) was conducted in a 25-μL reaction mixture that contains 5 μL of DNA aliquots, 10 pmol of each primer, 12.5 μL of 2× PCR master mix (Promega), and 1.5 μL of 10,000-fold diluted sample of SYBR Green fluorescent dye (Molecular Probes). Primers used for the detection of pUC18 plasmid or M13 phagemid were M13 Universal Forward (−20) (5′-GTAAAACGACGGCCAGT-3′) and M13 Reverse (−24) (5′-GGAAACAGCTATGACCATG-3′), both obtained from Invitrogen Corp. These primers are designed for the detection of the M13 phage sequences and a variety of vectors, including the pUC18 plasmid containing fragments of bacteriophage M13. For identification of the DNA isolated from *E. coli* cells captured by nanoparticles we amplified the DNA region encoded by pPro-EX plasmid. Primers were 5′-GAGCGGATAACAATTTCACA-3′ (pProEx forward) and 5′-AAATCTTCTCTCATCCGCCA-3′ (pProEx reverse). Primers for identification of diptericin gene were 5′-CCACCGCAGTACCCACTCAAT-3′ (diptericin forward) and 5′-CGATGACTGCAAAGCCAAAACCA-3′ (diptericin reverse).

The PCR reaction cycling parameters were as follows: denaturation for 2 min at 94 °C, 35−40 cycles of denaturation, annealing and extension for 30 s each at 94, 55, and 72 °C, respectively. The reaction specificity was confirmed by measuring melting temperature (T_m) of the amplification products after the last reaction cycle. All reactions were performed in triplicate. Quantitative real-time PCR analysis was performed using a Corbett Research Rotor-Gene Model RG-3000 thermal cycler (Corbett Robotics, San Francisco, CA) and software following the manufacturer's manual. Calibration curves were obtained with DNA standards in the range of 10 fg to 10 ng (see sections S-3−S-5 in the Supporting Information). DNA concentrations in samples were calculated based on calibration curves obtained with standards. Typical results are shown in section S3 in the Supporting Information.

Analysis of the captured bacteria DNA, detailed descriptions of the methods of determination of the minimum inhibitory concentration, cytotoxicity assays with mammalian cell line, and *E. coli* reduction factor test are all described in sections S6−S8 in the Supporting Information.

RESULTS AND DISCUSSION

Particle Design and Properties. Magnetite particles were synthesized with branched polyethyleneimine (PEI, 25 kDa) as the stabilizing compound by the coprecipitation of iron(II) and iron(III) chlorides by ammonia and characterized by a variety of methods (see sections S9−S13 in the Supporting Information). A Fe^{2+}/Fe^{3+} molar stoichiometry of 1:2 was chosen, such that,

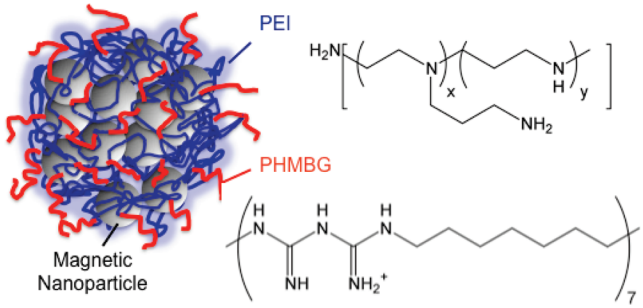

Figure 2. Magnetic nanoparticle clusters (~50−100 nm) functionalized with PEI and PHMBG; these particles are dispersed stably in the threatened aquatic environment.

Table 1. Composition and Physical Properties of PEI-M and PHMBG−PEI-M Particles

parameter	PEI-M	PHMBG−PEI-M
Fe_3O_4 content (%)	54.3	38.2
polymer content (%)	45.6	61.8
PEI	45.6	52.4
PHMBG	0	9.7
hydrodynamic diameter (nm)[a]	56.3	63.2
average ζ-potential (mV)[b]	31.4	39.8
saturation magnetization[c] (emu/g)	62.2	43.8

[a] Mean number-average, measured at pH 7.4. [b] Measured at pH 7.4. [c] Measured at 300 K.

under nonoxidizing conditions, magnetite (Fe_3O_4) was formed.[15] Prior to the precipitation by ammonia, the Fe ions in the aqueous mixture were complexed with PEI under slightly acidic conditions (pH 6). Complexation of PEI with Fe ions is well-known.[16]

The PEI-M particles were further conjugated with PHMBG via linking of the amino groups of PEI with the amino/imino groups of PHMBG by glutaraldehyde, resulting in the PHMBG chains being exposed to the exterior of the nanoparticle clusters (see Figure 2). Such a Schiff-base conjugation is analogous to enzyme immobilization onto PEI-coated magnetite particles using glutaraldehyde.[17,18]

The presence of both PEI and PHMBG on the particles was shown by the Fourier transform infrared (FTIR) spectroscopy (see section S9 in the Supporting Information). Transmission electron microscopy (TEM) images of the PHMBG−PEI-M particles showed ~7-nm primary magnetite particles flocculated into ~60-nm clusters surrounded by the polymer layers (see section S10 in the Supporting Information). The number-average particle diameters measured by dynamic light scattering in the pH range of 2.5−10 (see Table 1) were consistent with the TEM observations. High surface charge densities, with ζ-potential values above 40 and 30 mV for PHMBG−PEI-M and PEI-M particles, respectively (see section S10 in the Supporting Information) ensured the colloidal stability of the particles in an aqueous milieu, as evidenced by the absence of visible sedimentation in dilute (0.01−0.5 wt %) suspensions of either PEI-M or PHMBG−PEI-M particles for weeks in fresh and ocean water at room temperature. At pH >10, exceeding the pK_a of PEI and PHMB (reported to be 9.7 and 11, respectively),[19,20] the particles became uncharged and, hence, aggregated.

Both PEI-M and PHMBG−PEI-M particles exhibited a lack of hysteresis in the magnetization-field coordinates at 300 K, indicating that they were superparamagnetic (see section S12 in the Supporting Information). Their saturation magnetizations, calculated per gram of magnetite in the particles, are shown in Table 1. The smaller magnetizations, relative to the bulk value of 92 emu/g for magnetite, are due to the significant volume fraction of the polymers and the existence of a well-developed surface layer with reduced magnetization on the individual nanoparticles. The contents of magnetite and polymers in the different particles were determined by thermogravimetric analysis (see section S13 in the Supporting Information) and by elemental analysis and are collected in Table 1.

Particle Capture by HGMS. The most common and simplest type of magnetic separation is magnetocollection, where magnetic materials are passed over a magnet and collected while nonmagnetic materials pass through. Magnetic trapping becomes increasingly difficult as the particle sizes decrease, or as magnetic susceptibility decreases. Simple magnetocollection is not effective for collecting particles smaller than ~75 μm in diameter. Alternatively, high gradient magnetic separation (HGMS) has been developed to trap smaller particulates. An HGMS column consists of magnetically susceptible, typically stainless steel wires ~50 μm in diameter, packed into a column that is placed between the poles of an electromagnet. Because the wires are magnetizable, but the open space between them is not, high magnetic field gradients are formed near the wires. Magnetic nanoparticles that pass through the column are attracted to the wires according to formula[21]

$$\mathbf{F_m} = \mu_0 V_p \mathbf{M_p} \cdot \nabla \mathbf{H} \quad (1)$$

where μ_0 is the permeability of free space, V_p the volume of the particle, $\mathbf{M_p}$ the magnetization of the particle, and $\mathbf{H}$ the magnetic field at the location of the particle.

For magnetic capture to occur, the force of magnetism must be strong enough to overcome fluid drag forces and diffusional resistances to particle buildup. Capture becomes more difficult as the size of the particles decreases.[21]

Flocculation of the small primary nanoparticles of magnetite in our process of coprecipitation of the iron(II) and iron(III) chlorides chelated with PEI into larger (50−70 nm) clusters afforded not only sufficient colloidal stability, but also the possibility of removal of such clustered particles by HGMS using syringes packed with stainless steel wool. The HGMS efficiency for the separation of particles smaller than 50 nm is impacted negatively by the dominant thermal diffusion effects and therefore calls for HGMS columns of increased length for sufficient particle

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capture.[21,22] In accordance with the foregoing concept, we aimed at portable and simple devices for the particle collection; therefore, the mean particle size of >50 nm that resulted from the applied synthetic procedure was quite opportune. As described in the Experimental Section, we explored two modes of HGMS: (i) collection of the samples into small, 1-mL syringes prepacked with stainless steel wool, which affords sampling and transportation of the collected samples into a laboratory setting for HGMS, sample concentration, and DNA recovery using an electromagnet; and (ii) *in situ* particle separation by placing a prepacked syringe into a portable permanent magnet, followed by transportation of the thus-concentrated sample into the laboratory. The overall process of the water sampling using a prepacked syringe followed by particle (and adsorbed DNA) capture by HGMS, particle recovery, DNA extraction from the captured particles and detection using real-time PCR is depicted schematically in Figure 1. To remove the captured particles from the steel wire, the syringe is removed from a permanent magnet or the current in an electromagnet is turned off and the particles that are no longer attracted to the wire by magnetic force are washed away by an aqueous buffer passing through the wire column.

Dilute particle dispersions collected in the field can be kept in syringes until subjected to the HGMS procedure. The efficiency of both PEI-M and PHMBG−PEI-M particle capture by HGMS conducted with 1-mL syringes was dependent on the particle size polydispersity, The particles in the initial dispersion prior to HGMS were quite polydisperse, with a significant fraction in the range of 25−50 nm in size (see section S14 in the Supporting Information). These smaller particles were not captured and were thus washed out of the column and discarded such that the capture efficiency for the as-synthesized nanoparticle dispersion was only 33% ± 7%, for dispersions in both fresh water and in 5 mM Tris buffer, in experiments with both fluorescently labeled and unlabeled particles. The nanoparticles that were captured, recovered by flushing with fresh water in the absence of the magnetic field, and reapplied to the HGMS column with the field present, were captured with a significantly improved efficiency of 87% ± 9%. After three such passes through the HGMS column, the capture efficiency of the remaining particles was in the range of 95%−97%. The <100% efficiency in these experiments is indicative of the dynamic equilibrium established between particles in the static buildup volume between and the wires and the bulk solution. Very similar results have been reported previously with small volumes of graft copolymer-based magnetic fluids passed through the HGMS columns.[21] These results indicate the importance of nanoparticle size in determining the efficiency of the process, and the need to fractionate the as-synthesized nanoparticles unless better control on their sizes is exercised during their synthesis.

DNA Extraction from the Particles and Concentration Using HGMS. One of the important parameters of the concept proposed in this work is the nanoparticle ability to capture various biological materials for their further identification. Initial experiments performed with salmon sperm DNA for evaluating DNA binding and elution showed that over 98% of the DNA from salmon testes captured by the PHMBG−PEI-M particles was recovered by extraction of the DNA from the particles with 10 mg/mL SDS solution in chloroform, followed by centrifugation. The efficiency of this process stems from the fact that the binding between DNA and strongly cationic particles is controlled by electrostatic forces. Displacement of the DNA from the DNA−cationic particle complex by an excess of anionic SDS unimers and micelles followed by centrifugation leads to an ~14-fold more-effective separation than an analogous procedure (i.e., chloroform/particle dispersion followed by centrifugation), but without SDS. We also evaluated the efficiency of the capture/elution of small size DNA (71 bp) present at low concentrations. The extracellular (dissolved) DNA present in aquatic environments at typical concentrations on the order of 10−20 μg/L[23] is at various stages of degradation (i.e., exists as smaller fragments), so that the conditions of our experiments corresponded to the most likely scenario encountered in nature. To facilitate the assessment and to make it more specific, DNA labeled with fluorescein (FAM) and incubated with PHMBG−PEI-M particles was extracted and measured fluorimetrically. In the case of DNA capture at the condition below the particle total ion-exchange capacity, a 0.71 nM solution of FAM-labeled, 71-bp DNA in Tris buffer (pH 7.4) was blended with the PHMBG−PEI-M particles (100 μg/L) and the suspension was equilibrated, the particles separated by HGMS and then the DNA was extracted off the particles as described in the Experimental Section. The ratio between the concentration of hexamethylene biguanide monomers (expressed in units of mol/L of the biguanide-containing monomer, MW 228) to the concentration of DNA (expressed in units of mol/L of basepairs) was >60 in these experiments. Alternatively, a series of experiments was conducted at the ratio of the particle biguanide group concentration close to the DNA basepair concentration. That is, a 75-nM solution of FAM-labeled 71-bp DNA in Tris-EDTA buffer (7.4) was equilibrated with 175 μg/L PHMBG−PEI-M particle suspension containing ca. 77 nM biguanide groups. In both cases, the overall recovery of the particles that had not undergone HGMS prior to the DNA capture was measured to be ~29%. However, the PHMBG−PEI-M particles that were separated from their population that was <50 nm in size by HGMS (see section S14 in the Supporting Information) showed >85% recovery under otherwise identical conditions. These experiments demonstrated that the particle capture efficiency (and not the DNA extraction off the particles) is the defining step in the overall DNA recovery by the particles, either below or close to the saturation of the particle ion-exchange capacity.

The ability of the cationic magnetic PHMBG−PEI-M particles to bind DNA was shown to be quite useful in *concentrating DNA*, which significantly reduces the limits of DNA detection. In experiments with the fluorescent fragment of *Drosophila* diphtericin gene dispersed in 1 L of aqueous buffer solution, the DNA was captured by polydisperse PHMBG−PEI-M nanoparticles, which were concentrated by HGMS into a 9-mL aqueous dispersion. The latter was then subjected to extraction with a 10 mg/mL SDS solution in chloroform, followed by centrifugation, as described previously. Using initial DNA concentration before addition of the particles (C_0 = 0.3 ng/mL) and after particle

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binding, concentration and HGMS separation followed by the DNA extraction from the particles (C_c), the degree of the DNA concentration was calculated as C_c/C_0 (see section S2 in the Supporting Information). The C_c value was measured to be 8.1 ± 0.05 ng/mL, which is 24.3% of the theoretically possible recovery. The degree of the DNA concentration was thus measured to be ~27-fold. Again, we observed that reducing the particle polydispersity by removal of the smaller particle fractions using HGMS enhanced the recovery of the particles to 72%−80%. Importantly, however, even without a specific particle workup, the recovery of DNA by HGMS through as-made PHMBG−PEI-M nanoparticles was sufficient in enabling the DNA detection by PCR, as described below.

Capture of DNA and Whole Microorganisms for Analysis by PCR. We also evaluated DNA capture/extraction and applicability of DNA preparations for further analysis by PCR. In this case, plasmid double-stranded or bacteriophage single-stranded DNA preparations were used to develop a monitoring system by quantitative real-time PCR. Both eluted DNA and lysates from the captured bacteria were further purified and subjected to molecular analysis using the PCR technique. Because of the strong inhibitory effect of even trace concentrations of the residual particles and polycations on PCR observed here and reported previously,[24] the DNA captured by and extracted from the PHMBG−PEI-M particles was further purified by passing the extracted DNA solution through columns packed with silica-gel membranes (QIAquick columns designed for selective adsorption of DNA molecules). This methodology enabled us to obtain high-quality DNA amounts sufficient for detection by PCR. Thus, we were able to reliably detect as little as 1 pg of bacteriophage DNA, which corresponds to ~1:30 000 v/v dilution of the surveyed aqueous sample (see section S3 in the Supporting Information). The recovery of DNA after incubation, capture, and extraction constituted ~10% of the initial DNA amount taken for the analysis (300 ng/mL in 1-mL samples).

Capture of the whole microorganism by PMBG−PEI-M nanoparticles was also detected by PCR (see section S4 in the Supporting Information). The particles were dispersed at 0.1 mg/mL concentration in either overnight *E. coli* cultures or *E. coli* cultures resuspended in PBS and incubated for 3 h, and then recaptured using HGMS. The DNA was extracted by the previously described SDS/chloroform procedure with or without microwave irradiation, which facilitates lysis of bacteria and release of DNA molecules (see section S4 in the Supporting Information). The DNA was additionally purified from the supernatant using silica-gel columns and subjected to real-time PCR analysis using a set of specific primers. The sensitivity of the reaction was comparable to that obtained for the captured naked DNA (i.e., within the range of 1−10 pg). Microwave radiation afforded on the order of 5%−10% more-efficient recovery of DNA from the cells and can be of particular utility when an accelerated release of the DNA from spore-forming bacteria such as *Bacillus anthracis* is desired.[25] Based on the sensitivity of the DNA detection by PCR, we expect that it will be possible to detect intact germs captured by our nanoparticles via binding of the cationic particles to the negatively charged molecules of the cell envelope. It would appear that the efficient extraction and high sensitivity reported here address two major drawbacks in testing environmental samples by PCR: (i) the presence of enzyme inhibitors and (ii) the limited volume of the sample to be processed.[7,26]

From the standpoint of the generality of the technique described herein, the sensitivity of the PCR assays toward the target DNA of interest for biodefense may be limited by the potential presence of large excesses of nontarget DNA such as that from phytoplankton and other organisms, because the quantity of total DNA per reaction tube represents a limiting factor.[6] Different approaches were developed to enrich samples with a germ-specific DNA and thus improve sensitivity and specificity of germ identification by PCR. In some cases, prior to PCR analysis, DNA was extracted from soil or water samples using particles with attached DNA-capture fragments complementary to the target DNA.[7,11,27−30] However, the high specificity of such DNA-specific particles toward a single pathogen is a significant limitation for the surveillance of multiple germs, which calls for large arrays of the DNA sequences specific to organisms to be surveyed. In addition, particles modified with DNA capture probes are not stable, because of fast DNA degradation in the environment. Other approaches combine PCR analysis with hybridization procedure using particles modified with germ-specific DNA probes.[11] The present work involves nonspecific particles that can capture a variety of targeted as well as nontargeted microorganisms and DNA debris. With excessive nonspecific background, the likelihood of obtaining false-positive PCR results is very high. Furthermore, the concentration of germ-specific DNA in a total sample could be below detectable limits. To address these issues, we modified the procedure for identification of minute amounts of DNA in the presence of large excess of nontarget DNA. We introduced a second PCR run with nested primers to increase both the sensitivity and specificity of the analysis. The details of such nested PCR are described in section S5 in the Supporting Information. The nested real-time PCR assay has been previously reported to be several orders of magnitude more sensitive than regular real-time PCR and enabled successful detection of viruses in air, which regular real-time PCR failed to detect.[31]

Samples to be captured were prepared by mixing a pPro-EX plasmid that contained a fragment of *Drosophila* diptericin gene and nontarget DNA such as salmon sperm DNA or total DNA extracted from *E. coli* non-transformed cells. Two consecutive PCR reactions were performed, using pairs of outer and nested primers (see section S5 in the Supporting Information). Such an approach allowed us to amplify and detect a fragment of diptericin gene in the presence of an ~10^6-fold excess of nonspecific nucleic acids. Thus, both sensitivity and specificity of the analysis were significantly improved, which allowed us to project the usability

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Table 2. Minimum Inhibition Concentrations (MIC) for Aqueous Poly(hexamethylene biguanide) (PHMBG) Solutions and Dispersions of PHMBG−PEI-M Nanoparticles

	Minimum Inhibition Concentration, MIC (μg/mL)	
microorganism	PHMBG	PHMBG−PEI-M
S. aureus	25	35
L. lactis	25	25
C. albicans	265	275
E. coli	15	15
S. enterica	160	150
P. aeruginosa	35	40

of the developed analysis for identification of various germs, which is feasible through the use of sets of primers for amplification of germ-specific fragments. The latter approach has proved to be successful in identification of hepatitis A, B, and C viruses captured by PEI-modified magnetic beads with diameters in the 800-nm range.[32] Our particles, 60−70 nm in size, are more colloidally stable in water and possess other unique features that are useful for biodefense, as described below.

Nanoparticles as Disinfectants. Because our particles were designed for *in situ* protection, we examined the performance of the PHMBG-PEI-M particles as disinfectants (see section S6 in the Supporting Information), using aqueous solutions of PHMBG as a control (see Table 2). Minimum inhibitory concentrations of PHMBG in killing Gram-positive bacteria *S. aureus* and *L. lactis*, yeast *C. albicans* and Gram-negative bacteria *E. coli*, *S. enterica*, and *P. aeruginosa* corresponded well with the published data.[33,34] When tested on *E. coli*, PHMBG is bacteriostatic at concentrations $\leq$10 mg/L; however, at $\geq$10 mg/L, it is bactericidal.[34]

Importantly, MIC values obtained with the PHMBG−PEI-M particles matched those observed with the PHMBG solutions in most cases, despite the fact that the content of PHMBG in the particles did not exceed 10 wt %. This interesting result evidently reflects on the ability of the PHMBG grafted on the nanoparticle surface to bind acidic membrane lipids and DNA of the microorganism; such ability is preserved, even after the covalent bonding of PHMBG to the PEI layers complexed with magnetite. The binding of the PHMBG to the cytoplasmic membrane causing nonspecific alterations to the membrane integrity is an accepted molecular mechanism for the bactericidal action of PHMBG.[13,34] The susceptibility of pathogenic micro-organisms to the PHMBG−PEI-M nanoparticles shown here substantiates the concept of dispersed magnetic nanoparticle utilization for biodefense.

Particle Biocompatibility. Deployment of nanoparticles in the environment (which is necessary in the present work) and their subsequent fate may, however, cause unknown environmental effects.[35] U.S. federal agencies identified research areas where

Table 3. Concentrations of Additives Allowing for 50% Survival of Mouse Fibroblast Cells (IC_{50}), Mean Concentrations of Additives Corresponding to Three-$\log_{10}$ Reduction of *E. coli* (C_{3rf}), and Biocompatibility Index (BI) of Polyethyleneimine (PEI), Poly(hexamethylene biguanide) (PHMBG), and Magnetite Particles Modified by PEI (PEI-M) and PHMBG−PEI Conjugates (PHMBG−PEI-M)

species	IC_{50} (μg/mL)	C_{3rf} (μg/mL)	biocompatibility index, BI[a]
PEI	26 ± 5	250	0.1
PEI-M	200 ± 18	330	0.6
PHMBG	158 ± 12	20	7.9
PHMBG−PEI-M	360 ± 43	20	18

[a] $BI = IC_{50}/C_{3rf}$.

risk assessments related to nanoparticles must be developed, including ecological and human health effects and life-cycle analysis.[36] Magnetic nanoparticles coated with and stabilized by tetramethylammonium hydroxide have been shown to accumulate in plant tissues.[37] Components of our particles, iron oxide (Fe_3O_4) and its nanoparticles were shown to be noncytotoxic toward mammalian cell lines,[37−39] but PEI exhibited significant cytotoxicity[40,41] and PHMBG had an acceptable cytotoxicity profile in human cells *in vitro*.[42] To address a question as to whether bactericidal concentrations of our nanoparticles would exceed their cytotoxic levels, we conducted MTT tests with the L929 mouse fibroblasts, which are a standard for cytotoxicity evaluation of various materials (see section S7 in the Supporting Information).[38,43−45] These tests reveal an IC_{50} value that is defined as the concentration allowing 50% survival of cells (see section S7 in the Supporting Information). The IC_{50} values were compared with the results of *E. coli* quantitative suspension tests, which are routinely used for testing disinfectants and antiseptics[46] and yield a three-$\log_{10}$ (99.9%) reduction of the bacterium concentration (C_{3rf}), the minimum acceptable efficacy in the presence of organic matter (see section S8 in the Supporting Information).[45] The value of the "biocompatibility index" ($BI = IC_{50}/C_{3rf}$) has been recently forwarded as an adequate parameter of the suitability of an antiseptic agent.[45] The results of these tests are collected in Table 3.

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As expected, the PHMBG polymer possessed a dramatically higher biocompatibility index (BI) than did PEI, because of its much higher bactericidal activity and lower cytotoxicity. Complexation of PEI with magnetite improved its biocompatibility, giving a greater IC_{50} value of the particles, compared to that of the PEI on its own. Because PHMBG is a widely accepted medicinal antiseptic, its cytotoxicity level may be considered to be acceptable and used as a standard to evaluate the cytotoxicity of the PHMBG-modified particles. Of importance, the IC_{50} value for the PEI−PHMBG-M particles was found to be ~2.3-fold higher than the same cytotoxicity parameter for the PHMBG solution. This, along with equal bactericidal activities of the PHMBG and the PHMBG−PEI-M particles, resulted in the high BI value for the particles. A particle concentration that is ~18-fold higher than is sufficient for bacteria killing is required for the particles to become cytotoxic to mammalian cells. Although more research is required to address all the complex issues of nanoparticle effects on the environment, this finding can help to allay concerns about the toxicity of our nanoparticles dispersed in water.

CONCLUSIONS

A novel approach toward biodefense and threat surveillance by water-dispersible, bactericidal, paramagnetic nanoparticles is presented. The nanoparticles are prepared from commercially available materials by a two-step procedure at an acceptable cost. It seems to be possible to suspend our cationic nanoparticles in water, where they remain dispersed at pH ≤10, capture the particles together with sequestered DNA or whole microbes, and then extract and quantify the DNA by real-time polymerase chain reaction (PCR). Bactericidal activity of the nanoparticles is evident at concentrations below cytotoxicity levels for mammalian cells. Based on the MIC levels, it would take ~185 tons of particles to disinfect 5 billion gallons of water, which is an annual allocation of water in a large aquifer, or over 500 years worth of a water treatment facility output with an influent flow of 25 000 gallons per day. Given the low costs of magnetite and the antiseptic used, we estimate that our approach toward *in situ* biodefense is feasible. Our next step would be to test the nanoparticles for capturing, killing, and detecting viruses and spores. The levels of the DNA detection sensitivity obtained in our model experiments allow us to project the applicability of the developed method for the detection of DNA molecules of various germs. Using sets of primers that amplify specific conserved regions of genomes of different viruses, bacteria, or other infectious agents of interest, it will be possible to monitor germs in a variety of aqueous samples. The biological material captured by and extracted from the nanoparticles can be analyzed for the presence of several germs simultaneously. Such an approach not only permits the detection of a specific germ but also allows for differentiating between genotypes to compare DNA isolates for epidemiological investigations.

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SUPPORTING INFORMATION AVAILABLE

Materials, description of particle characterization methods, description of PCR results, analysis of the captured bacterial DNA, analysis of DNA mixtures by nested PCR, detailed descriptions of the methods of determination of the minimum inhibitory concentration, cytotoxicity assays with mammalian cell line, and *E. coli* reduction factor test selected FTIR spectra, TEM images, data on particle diameter and ζ-potential, SQUID, DLS, and TGA results. This material is available free of charge via the Internet at http://pubs.acs.org.

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