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Brief Communication

Inorganic Mercury Detection and Controlled Release of Chelating Agents from Ion-Responsive Liposomes

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SUMMARY

A liposome system that can detect and detoxify mercury in aqueous solution is demonstrated. The system consists of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine and 20% PEG-PE (PEG MW 2000 Da) that forms liposome, which encapsulates self-quenching fluorescein for detection, and chelating agent *meso*-2,3-dimercaptosuccinic acid (*meso*-DMSA) for chelating detoxification through Hg^{2+}-responsive release of fluorescein and *meso*-DMSA. This system can detect mercury levels as low as 10 nM with high selectivity. In particular, the release profile of *meso*-DMSA by the local concentration of Hg can be modulated, so that more chelators are released in regions of high concentration and less chelators are released in regions of low concentration. The design has been demonstrated both in vitro and in HeLa cells. This "budgeted" release profile is particularly useful in situations in which the local levels of Hg contamination vary, or if such contamination is time dependent.

INTRODUCTION

Mercury exposure has been linked to a number of health problems, such as damage to the central nervous system, endocrine system, brain, kidney, and fetus development (Harris et al., 2003). Because inorganic mercury is found in water, soil, food sources, and a broad range of environments, there is a substantial need for both sensitive detection and efficient detoxification of mercury—just like we do for sensing other biologically important metal ions (Nolan and Lippard, 2003; Kikuchi et al., 2004; Komatsu et al., 2005; Wang and Guo, 2006; Qian et al., 2009). To meet the need, a number of mercury sensors have been developed based on small organic molecules (Yoon et al., 1997; Guo et al., 2004; Caballero et al., 2005; Yoon et al., 2005; Yang et al., 2005; Nolan et al., 2006; Kim et al., 2006; Ha-Thi et al., 2007; Nolan and Lippard, 2007; Yoon et al., 2007), oligonucleotides (Ono and Togashi, 2004; Wang et al., 2008), conjugated polymers (Liu et al., 2007), foldamers, (Zhao and Zhong, 2006), micelles, (Pallavicini et al., 2007) genetically engineered cells (Virta et al., 1995), membranes (Chan et al., 2001), electrodes (Widmann and van den Berg, 2005), enzymes (Frasco et al., 2007), antibodies (Matsushita et al., 2005), proteins (Chen and He, 2004; Wegner et al., 2007), DNAzymes (Hollenstein et al., 2008; Liu and Lu, 2007), functionalized nanotubes (Heller et al., 2006), and nanoparticles (Lee et al., 2007; Xue et al., 2008). Despite the progress of mercury detection in organic solvents, there remains a need for sensors with high sensitivity and selectivity that can detect mercury in water (Guo et al., 2004; Caballero et al., 2005; Ha-Thi et al., 2007; Prodi et al., 2000; Szurdoki et al., 2000; Liu and Tian, 2005; Song et al., 2006; Zhao et al., 2006), and no single platform can detect and detoxify mercury at the same time. Moreover, the distribution of mercury can be location and time specific; a systemic dose of detoxifying agents can be inefficient in dealing with various concentrations of mercury, whereas a large dose can potentially lead to undesirable side effects. In this paper, we report a novel liposome-based system that can detect and respond to the presence of mercury with a concentration as low as 10 nM, the maximum contamination level defined by the US Environmental Protection Agency (EPA) for mercury in drinking water. In particular, we are able to modulate the release profile of Hg chelators by the local concentration of Hg so that more chelators are released in regions with high-concentration Hg and low or no chelators are released in regions of low concentration. This budgeted release profile will be particularly useful in situations in which the local levels of Hg contamination vary, or if such contamination is time dependent.

RESULTS AND DISCUSSION

Liposomes are assemblies of lipid molecules in the form of closed spherical bilayers. They have been used extensively for drug delivery applications. Because the lipid bilayer separates the encapsulated content from the external environment, efficient encapsulation of a broad range of cargo is possible (Samad et al., 2007; Dass, 2008). It has been shown that inorganic mercury binds to phosphatidylethanolamine (PE) lipid

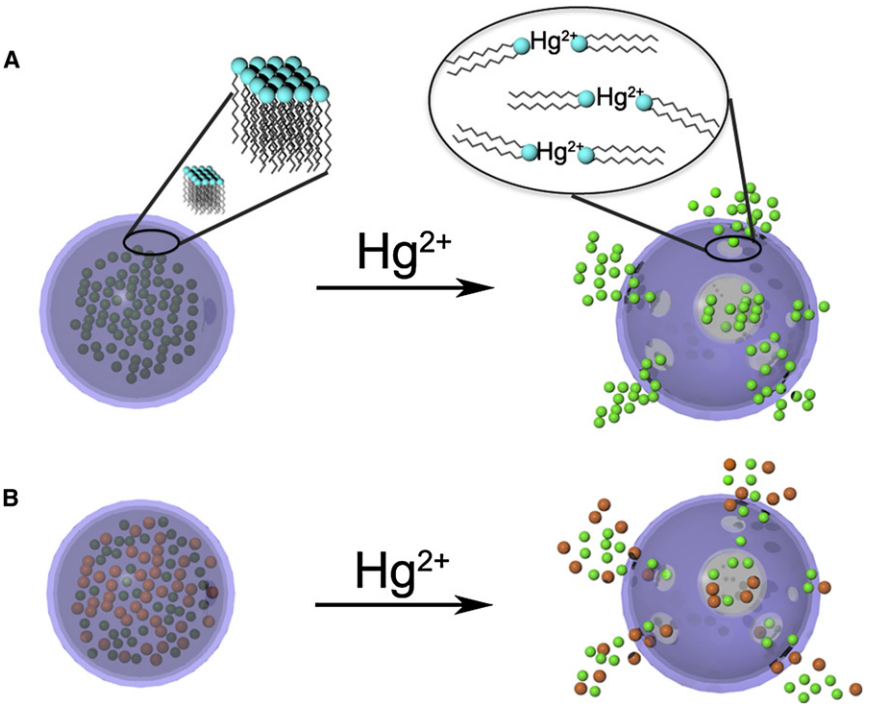

Figure 1. Schematic Illustration of Hg^{2+}-Responsive PEG-Liposomes for Selective Detection and Controlled Release of Chelating Agents

(A) Interaction of Hg^{2+} with fluorescein-encapsulated DOPE liposomes resulted in release of fluorescein and selective Hg^{2+} sensing.

(B) Interaction of Hg^{2+} with *meso*-DMSA and fluorescein-encapsulated DOPE liposomes resulted in release of both *meso*-DMSA and fluorescein and thus Hg^{2+} detection and detoxification.

Green and orange circles represent fluorescein and *meso*-DMSA, respectively. Enlarged areas show lipid molecules on the liposome surface and their interaction with Hg^{2+} that destabilizes liposomes.

headgroups strongly and can reorient them (Figure 1A inset) (Girault et al., 1996). We hypothesized that we might be able to exploit this effect to make a mercury sensing and controlled release platform using the PE-based PEG-liposomes. To test this hypothesis, we developed PEG-liposomes encapsulating both fluorescent molecules and mercury-specific chelators. These liposomes are designed to rupture in the presence of mercury, thus releasing the encapsulated fluorophores and the chelators. Because the self-quenching of the fluorophores inside of the liposome is reversed upon liposome rupture, the fluorescent signal increases, thereby providing a readout of the release profile (Figure 1A). Importantly, as the chelators are released to reduce the concentration of free mercury (Figure 1B), the release of chelators is downregulated, so that the PEG-liposomes can be maximally available to interact with free mercury elsewhere.

To demonstrate the rupture of liposomes in the presence of Hg^{2+}, liposomes were prepared using 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) and PEG-PE (PEG MW 2000 Da) as the building blocks. We found that by incorporating 20% w/w of PEG we were able to achieve stable liposomes. However, when the liposomes consist of 100% DOPE, they were not stable due to the intrinsic negative curvature. Fluorescein was encapsulated in the liposomes with a concentration of 50 mM, which is sufficiently high to ensure fluorescence self-quenching and detection. The interaction of mercury ions with DOPE headgroups, and the resultant induced reorientation of the headgroups, can in principle lead to rupture of the vesicles, thereby releasing and unquenching the fluorescein. When 1 μM $Hg(ClO_4)_2$ was added to the liposomes, a significant increase in the fluorescence was indeed observed (Figure 2A). The fluorescein release at this concentration of mercury is determined to be the maximum by comparing it with the fluorescein release by Triton-X (a detergent), which should rupture all lipid vesicles in the solution. To further quantify this effect, different amounts of Hg^{2+} were added into the liposome solution and the change in fluorescence spectra was monitored over a 15 min period. The fluorescence increase with 10, 25, 50, 100 nM and 1 μM Hg^{2+} was determined as 20.66 ± 6.88%, 24.66 ± 7.54%, 59.8 ± 19.74%, 74.28 ± 5.85%, and 97.83 ± 3.05%, respectively. As shown in Figure 2B, fluorescence increased with increasing concentration of Hg^{2+}, which is consistent with the hypothesis that mercury is the inducing agent for liposome rupture and fluorescein release. At 10 nM Hg^{2+}, the maximum contamination level defined by the US EPA, the system can still display signal above the background (Figure 3).

To test whether Hg^{2+}-lipid interaction can be used to make ion-responsive liposomes for the budgeted release of chelating agents, *meso*-2,3-dimercaptosuccinic acid (*meso*-DMSA), a molecule routinely used in clinic for the treatment of mercury exposure, was encapsulated in the liposomes in order to chelate mercury. *Meso*-DMSA is one of the most commonly used chelators because it is water-soluble, odorless, stable, and has low toxicity (Blanusa et al., 2005). It binds to the inorganic mercury and forms nontoxic metal-chelator complex (Maiorino et al., 1993; Fang and Fernando, 1994; Fang et al., 1996; Garza-Ocanas et al., 1997). To test the efficiency of *meso*-DMSA chelation, we incubated Hg^{2+} with *meso*-DMSA for 5 min and then added it to the fluorescein-encapsulated liposomes for a final concentration of 1 μM Hg^{2+}. In contrast to high fluorescence intensity (97.83 ± 3.05%) at the same concentration of Hg^{2+} in the absence of *meso*-DMSA, the fluorescence intensity remained unchanged in the presence of *meso*-DMSA (Figure 2B, 1μM + *meso*-DMSA). This experiment indicates that the mercury ions that bind to *meso*-DMSA can no longer interact with new lipid headgroups and therefore do not break up liposomes that encapsulate additional chelators.

In order to quantify the detection and budgeted chelation of Hg^{2+} simultaneously, we encapsulated 50 mM fluorescein with 0, 100, 1000, or 2000 μM *meso*-DMSA in the liposomes. The liposomes were then incubated with 1 μM Hg^{2+} for 15 min. As shown in the inset of Figure 3, the concentration of *meso*-DMSA is

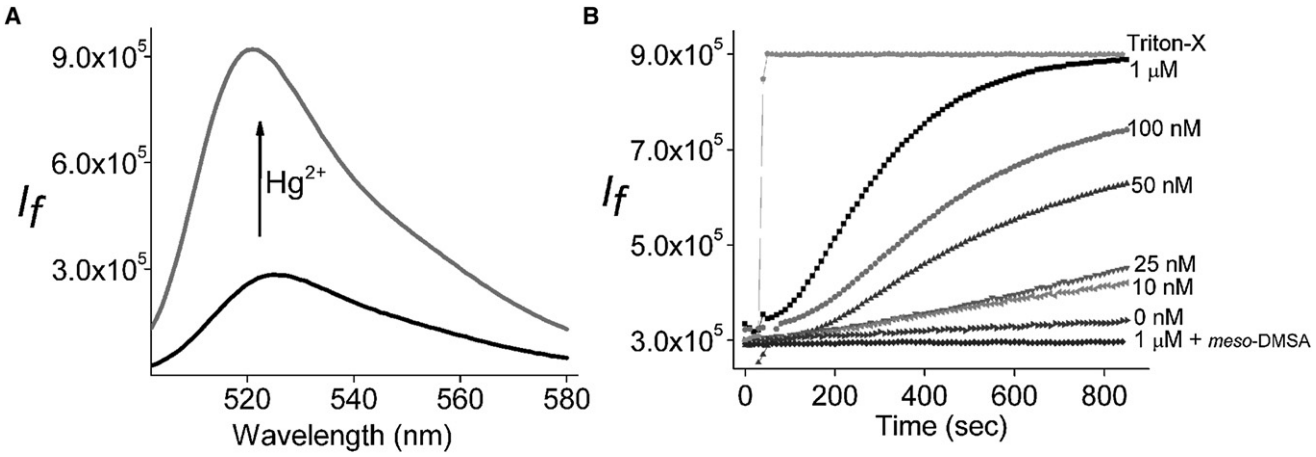

Figure 2. Fluorescence Enhancement in Fluorescein-Encapsulated Liposomes in the Presence of Hg^{2+}

(A) Fluorescence spectra of fluorescein-encapsulated liposomes before (black) and after (gray) addition of 1 μM Hg^{2+}, λ_{ex} = 495 nm.

(B) Time-dependent fluorescence change after addition of 10, 25, 50, 100, 1000 nM Hg^{2+} or 1 μM Hg^{2+} chelated with *meso*-DMSA (1 μM + *meso*-DMSA, as a negative control), or Triton-X (as a positive control).

inversely correlated with the fluorescence intensity inside of the liposomes. This result indicates that when the liposomes interact with the mercury ions, some liposomes rupture and release the encapsulated dye and *meso*-DMSA. The released dye results in the increase in fluorescence intensity while the released *meso*-DMSA binds to the mercury ions; the resulting complex is unable to rupture the liposomes. As the concentration of *meso*-DMSA increases, fewer liposomes are ruptured, resulting in smaller increase in fluorescence. This result further demonstrates that the liposomes comprise a single delivery platform that can be used for both Hg^{2+} detection and efficient Hg^{2+} chelation via the budgeted release of *meso*-DMSA from ion-responsive liposomes. Clearly, the rate of liposome rupture depends on the Hg^{2+} concentration. This important property allows for tuning the response of these liposomes by encapsulating different doses of the chelators. Because only a fraction of the liposomes are ruptured in this controlled chelation scheme, only as much chelators are released as necessary, leaving the rest intact for further detection and chelation of mercury contamination.

It is known that different ions can also interact with lipids, and potentially lead to adventitious interactions with liposomes. To test selectivity of the present system for mercury, we added 10 μM of different divalent metal ions and 1 mM Mg^{2+} and Ca^{2+} to the system. The increase in the fluorescence upon addition of 10 μM of essentially all other divalent metal ions and 1 mM Ca^{2+} or Mg^{2+} is drastically lower compared with the increase on addition of only 10 nM Hg^{2+} (see Figure 3). This result suggests that the system in not only sensitive but also selective to Hg^{2+}.

To find out if such selectivity is maintained not only when individual metal ion is tested, as shown in Figure 3, but also when mixtures of metal ions are used, we compared the increase in fluorescence intensity between the liposomes encapsulating fluorescein alone and the liposomes encapsulating both fluorescein and *meso*-DMSA in the presence of 1 μM Hg^{2+} and 10 μM Mn^{2+}, Cd^{2+}, Ba^{2+}, Co^{2+}, or Cu^{2+}. As shown in Figure 4, the liposomes

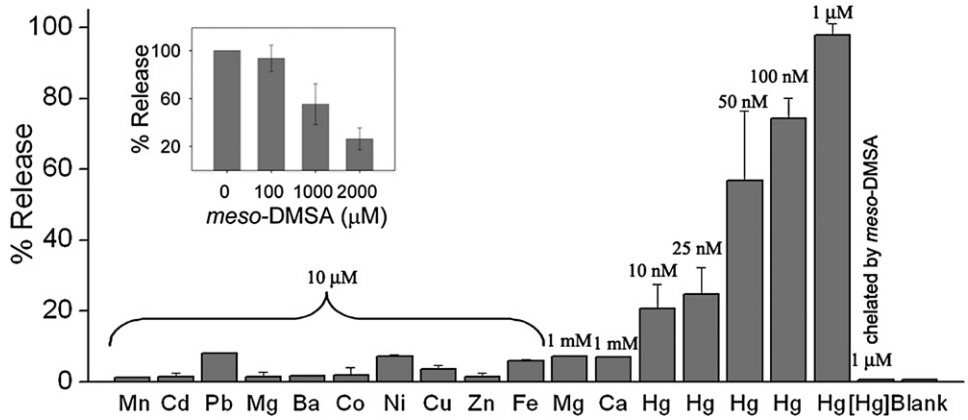

Figure 3. Percentage of Fluorescein Release in the Presence of Different Divalent Metal Ions

Inset: Percentage of fluorescein release in the presence of different concentrations of *meso*-DMSA-encapsulated DOPE liposomes treated with 1 μM Hg^{2+}. The [Hg] denotes mercury chelated with *meso*-DMSA. The error bars were determined from standard deviation of the data in triplicate.

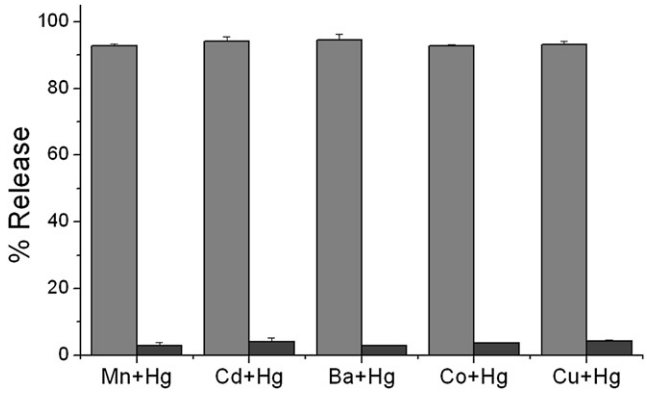

Figure 4. Percentage of Fluorescein Release in the Presence of a Mixture of Hg^{2+} and Other Divalent Metal Ions

Percentage of fluorescein release in 50 mM fluorescein-encapsulated (light gray) or 50 mM fluorescein and 3 mM *meso*-DMSA-encapsulated liposomes in the presence of 10 μM Mn^{2+}+ 1 μM Hg^{2+}, 10 μM Cd^{2+}+ 1 μM Hg^{2+}, 10 μM Ba^{2+}+ 1 μM Hg^{2+}, 10 μM Co^{2+}+ 1 μM Hg^{2+}, or 10 μM Co^{2+}+ 1 μM Hg^{2+}. The error bars were determined from standard deviation of the data in triplicate.

containing fluorescein and *meso*-DMSA have significantly less fluorescent increase than the liposomes containing only fluorescein, suggesting that Hg^{2+} effect are maintained in the presence of excess of other metal ions.

After demonstrating the controlled and budgeted release of a Hg^{2+} chelator in vitro, we assessed whether this approach can be used in a biological system. First, we utilized the HeLa cells in this study and checked the viability of the HeLa cells in the presence of Hg^{2+} at a concentration of 0, 0.2, 0.4, 0.6, 0.8, and 1.0 μM. As shown in the Figure 5A, the cell viability decreased dramatically to 35.7% in the presence of 0.2 μM Hg^{2+} and further decreased to 24.0% in the presence of 0.4 μM Hg^{2+}, which confirmed the toxicity of Hg^{2+} to the cells. Incubating the HeLa cells with liposomes without any chelator resulted in almost identical toxicity profiles as the cells in the absence of liposomes, indicating that the liposomes cannot interact with Hg^{2+} to reduce its toxicity. In contrast, when 2 μM *meso*-DMSA-containing liposomes were incubated with the HeLa cells, substantially improved viabilities were observed. The cell viabilities improved 56.8% and 43.1% when 2 μM *meso*-DMSA was added to the cell culture media containing 0.2 and 0.4 μM Hg^{2+}, respectively, than those without *meso*-DMSA (Figure 5A). The cell viability did not improve beyond 0.6 μM Hg^{2+}, probably due to the limitation of amount of 2 μM *meso*-DMSA that can be encapsulated into liposomes and their incomplete release. To provide further evidence for dosage-dependence protection, we carried out the same experiment in the presence of a constant concentration of 0.4 μM Hg^{2+} and used HeLa cells that were pretreated with increasing concentrations of either liposomes alone (LP + 0.4 μM Hg) or liposomes with *meso*-DMSA (LP-meso-DMSA + 0.4 μM Hg). As shown in Figure 5B, different concentrations of liposome-treated cells without chelator inside have little effect on cell viability, whereas increasing concentrations of liposome encapsulated *meso*-DMSA resulted in higher cell viability (from 24.0% to 49.4%), indicating the effects of controlled and budgeted release of a Hg^{2+} chelator in cellular environment.

SIGNIFICANCE

Mercury is a highly toxic and widespread pollutant in the environment. Therefore, the strategy or the system that allows for both sensitive detection and efficient detoxification of mercury is in great demand. For this reason a number of mercury sensor-related papers have been published recently. However, no single platform can detect and detoxify mercury at the same time. More importantly, because the distribution of mercury can be location and time specific, a single dose of mercury detoxification agent might be inefficient in dealing with various concentrations of Hg, and too high a concentration of the agent can potentially lead to side effects. We have demonstrated a multiple-use, liposome-based system that can simultaneously detect and detoxify inorganic mercury, both in vitro and in HeLa cells, by controlled and budgeted release of both a fluorophore for sensing and a chelating agent for detoxification. This system

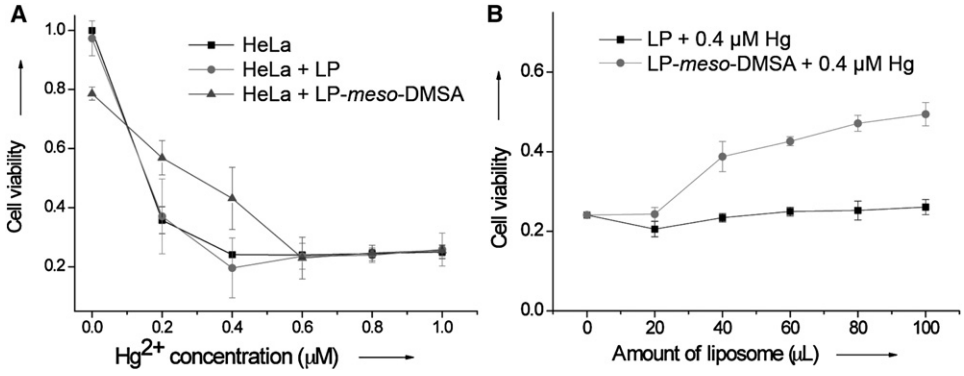

Figure 5. HeLa Cell Viability Assays with *Meso*-DMSA-Encapsulated Liposomes and Hg^{2+}

(A) Hg^{2+} concentration-dependent viability of HeLa cells alone (HeLa), HeLa cells initially treated with liposomes (HeLa + LP), or HeLa cells initially treated with liposomes containing 2 μM *meso*-DMSA (HeLa + LP-*meso*-DMSA).

(B) Liposome dosage-dependent HeLa cell viability in the presence of liposome alone treated 0.4 μM Hg^{2+} (LP + 0.4 μM Hg^{2+}) or 2 μM *meso*-DMSA-encapsulated liposomes treated with 0.4 μM Hg^{2+} (LP-*meso*-DMSA + 0.4 μM Hg^{2+}). Cell viability was measured at day 2 of liposome/cell incubation by MTT cytotoxicity assays. The error bars were determined from standard deviation of the data in triplicate.

is highly selective for mercury, and is able to detect mercury as low as 10 nM, the maximum contamination level defined by the US Environmental Protection Agency. This budgeted release profile will be particularly useful in situations where the local levels of Hg contamination vary in a location- and time-dependent manner.

EXPERIMENTAL PROCEDURES

Experimental details of liposome preparation, fluorescence measurements, and cell experiments are described in Supplemental Experimental Procedures (available online).

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures and can be found with the article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00281-6.

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