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Lipid Encapsulation of Arsenic Trioxide Attenuates Cytotoxicity and Allows for Controlled Anticancer Drug Release

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Arsenic trioxide (ATO, As_2O_3) is emerging as a front line agent for treatment of acute promyelocytic leukemia[1] with a complete remission rate of 83−95%. ATO also shows significant activity in relapsed/refactory multiple myeloma;[2] however, expansion of clinical utility to other cancers has been limited by toxicity at higher doses, including peripheral neuropathies and liver failure.[3] Here we describe the development of new bioavailable As(III) materials that undergo pH-triggered release of active drug and exhibit a significantly attenuated cytotoxicity. We show that cytotoxicity can be controlled via the inorganic phase transitions of the drug and suggest a mechanism for further improvement of the risk/benefit ratio of As_2O_3 for cancer treatment.

The activity of anticancer drugs can be controlled via encapsulation.[4] Liposomal doxorubicin (Doxil),[4,5] for instance, is successful in the clinic; however, a persistent challenge in the development of other encapsulated drugs has been in loading the agent to high densities within the liposome and allowing the controlled drug release in physiological conditions. Previous attempts to encapsulate ATO involved hydrating lipids in a concentrated solution of ATO followed by freeze-drying.[6] Unfortunately, the resulting liposome−arsenic vesicles lost substantial amounts of the drug within a few hours, thus limiting the application of this method. This is expected as ATO is present primarily in physiological solutions as the neutral H_3AsO_3 species ($pK_{a1} = 9.3$),[7] which readily diffuses across lipid membranes. Indeed, we find that passively encapsulated ATO leaks out of liposomes consisting of dipalmitoylphosphatidylcholine (DPPC)/dioleoylphosphatidylglycerol (DOPG)/cholesterol (Chol) = 51.4/3.6/45% (mol) with a half-life ($t_{1/2}$) of less than 50 min at 4 °C and pH 7.2 (Figure S4). By taking advantage of weak acid transport properties and the fact that arsenite forms insoluble transitional metal (e.g., Ni^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, Fe^{2+}, and Pb^{2+}) compounds (see Supporting Information),[8−11] we now describe an efficient system for loading ATO into liposomes at high arsenic density that retains the key biological activity.

First, concentrated metal salt solutions, such as 300 mM nickel(II) acetate ($Ni(OAc)_2$) (Figure 1 and Supporting Information), are passively loaded into 100 nm liposomes. Upon washing, the extraliposomal metal solution is removed, forming a gradient from the internal to the external aqueous phase of the liposomes. Addition of 10 mM As_2O_3 solution at pH 7.2 results in the rapid ($t_{1/2} \approx 5$ min at 50 °C) and active loading of arsenic(III) into the liposome, giving a final As/lipid (mol) ratio of 0.5 ± 0.1 ($n = 8$, the number of separate experiments). Acetate salts of Co^{2+}, Cu^{2+}, and Zn^{2+} also allow for rapid arsenic uptake ($t_{1/2} < 10$ min, Supporting Information), and the extent of arsenic loading increased with increasing concentrations of intraliposomal metal salt (Figure S5). In contrast, the monovalent sodium acetate gave little arsenic uptake (Supporting Information), presumably due to the high aqueous solubility of sodium arsenite. Here we specify arsenic-loaded liposomes as Lip(Ni,As), Lip(Co,As), Lip(Cu,As), or Lip(Zn,As), according to the internal metal ions. Using this method, one liposomal core (with a 100 nm size) can be loaded up to 273 ± 33 mM ($n = 8$) arsenic for Lip(Ni,As) and 261 ± 30 mM ($n = 4$) for Lip(Co,As) (Figure S2). Such accumulation of arsenic inside liposomes could be visualized by transmission electron microscopy (TEM) and verified by energy-dispersive X-ray analysis (EDX)[12] (Figures 2 and S3).

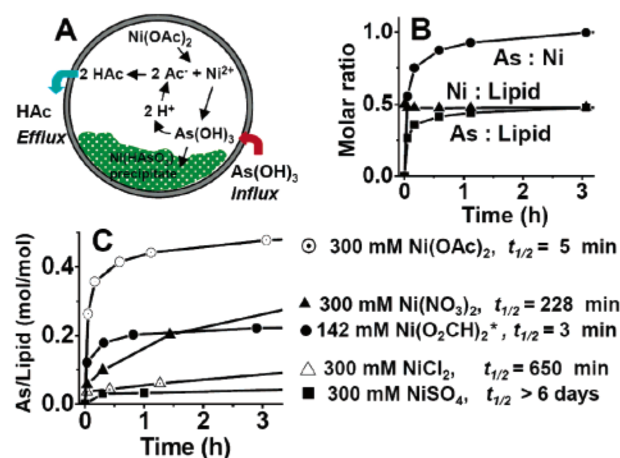

Figure 1. (A) Schematic of arsenic loading mechanism. (B) Molar ratios of As/lipid, Ni/lipid, and As/Ni as a function of incubation time during arsenic loading into liposomes at 50 °C using 300 mM $Ni(OAc)_2$ (pH 6.8) as intraliposomal medium. (C) Kinetics of arsenic loading into liposomes using various Ni(II) solutions as the intraliposomal medium at pH 6.8; ∗, the low solubility of Ni(II) formate prevented its examination at 300 mM. Lipid composition (B, C): DPPC/DOPG/Chol = 51.4/3.6/45, mol %).

During one cycle of loading arsenic into a liposome (Figure 1A), the external neutral $As(OH)_3$ diffuses across the membrane to form insoluble nickel(II) arsenite complexes internally, for instance, Ni($HAsO_3$) (Supporting Information). Protons are released and associate with the basic acetate anions. The resulting weak acid (HAc) then diffuses out of the liposome in exchange for $As(OH)_3$, leading to significant accumulation of arsenic inside liposomes. Both the formation of insoluble nickel(II) arsenite complexes and the efflux of acetic acid drive arsenic uptake. When other counterions were used in place of the acetate, the rate of arsenic loading was significantly different (Figure 1C); for instance, little uptake occurs

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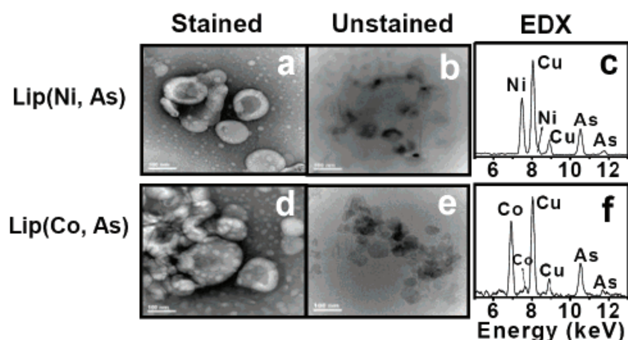

Figure 2. TEM images and EDX spectra of Lip(Ni,As) (a−c) and Lip-(Co,As) (d−f). Samples of a and d were stained with 4% uranyl acetate; b and e are unstained and show discrete electron-dense inorganic cores within liposomes; the single-particle EDX spectra c and f correspond to b and e, respectively, revealing Ni and As (c) as well as Co and As (f) cores (Cu peaks arise from EM grid; scale bar = 100 nm).

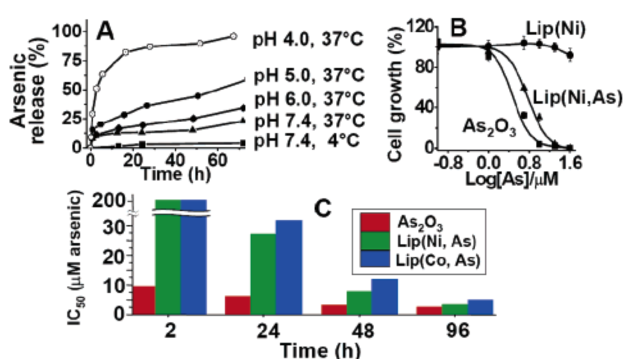

Figure 3. (A) Temperature and pH-triggered arsenic release from Lip-(Ni,As) in an external buffer of 300 mM NaCl, 20 mM HEPES. (B) Cytotoxicity effect of free As_2O_3 (■), Lip(Ni) (●, same lipid and Ni concentration as in ▲), and Lip(Ni,As) (▲) on SU-DHL-4 human lymphoma cells after 48 h incubation. (C) Cytotoxicity (IC_{50}) of free As_2O_3, Lip(Ni,As), and Lip(Co,As) after incubation for 2, 24, 48, and 96 h.

after 6 days for 300 mM $NiSO_4$. Given the pK_a values[13] of acetic acid (4.7), formic acid (3.7), HNO_3 (<0), HCl (<0), HSO_4^- (2.0), and H_2SO_4 (<0), arsenic loading efficiency correlates with the base strength of the anion, as long as the resulting weak acid is a neutral compound. This allows for rapid efflux of protons and internal precipitation of the inorganic phase (Figure 1A).

The arsenic drug is stably entrapped under storage conditions (i.e., it exhibits shelf lives > 6 months at 4 °C and pH 7.4, Figures 3A and S7A), but it is not inert: arsenic release can be triggered at a lower pH and/or higher temperature (Figure 3A). After 24 h at 37 °C for Lip(Ni,As), 90% of the arsenic was released at pH 4.0, compared to 13% release at pH 7.4. Lip(Co,As) particles appear to be more acid-sensitive than those of Lip(Ni,As) (Figure S7A), where 90% of arsenic was released after only 3 h at pH 4.0. For both Lip(Ni,As) and Lip(Co,As), 30−60% of the arsenic was released after 24 h at pH 5.0, a pH similar to that found in endocytic compartments involved in cellular uptake. Possibly, cellular uptake of liposomal arsenite could directly facilitate drug release.

The cytotoxicities of Lip(Ni,As), Lip(Co,As), and controls were evaluated against SU-DHL-4 human lymphoma cells (Figures 3B, C, S7B, and S8). After a 48 h incubation, both Lip(Ni,As) (IC_{50} = 6.0 μM) and Lip(Co,As) (IC_{50} = 9.6 μM) show arsenic bioavailability but lower cytotoxicities than free As_2O_3 (IC_{50} = 3.0 μM). In contrast, the arsenic-free liposomes are nontoxic at comparable metal and lipid concentrations (Figures 3B and S7B). At long incubation times (>48 h), the cytotoxicity of arsenic-loaded liposomes increased greatly, approaching that of the free As_2O_3 (Figures 3C and S8), consistent with gradual release of the drug at 37 °C (Figures 3A and S7A). When cells were incubated with drugs for 2 h, washed twice by phosphate-buffered saline (PBS), and then incubated 22 h more in drug-free medium (Figure 3C and Supporting Information), the IC_{50} values (>200 μM) of Lip(Ni,As) and Lip(Co,As) were much greater than that of free As_2O_3 (9.7 μM). This indicates that free As_2O_3 has high permeability through the cell membrane, and that the resultant acute toxic effect has been reduced by shielding an inorganic arsenic phase with the liposome bilayer.

In summary, we have developed a novel system for encapsulating arsenic-based drugs with transition metal ions into 100-nm-scale liposomes at high density and excellent retention. The resultant therapeutic agents appear to be stable in physiological situations but release the drug cargo at low pH, as encountered in endosomes.[14] Such arsenic nanoparticles exhibit the bioavailability of As(III) with the attenuated cellular toxicity relative to As_2O_3. The possible cellular endosomal uptake and acidification could facilitate $As(OH)_3$ release from liposomes for its biological activity. Liposomes loaded with either essential or nonessential transition metal ions alone were nontoxic under these conditions, but it remains to be seen which type of element will exhibit synergistic activity or toxicity with As(III) species. When combined with cancer cell targeting strategies (such as the clinically monoclonal antibody Rituxan), these arsenic-containing liposomes open a variety of new avenues for increasing the therapeutic index of inorganic arsenic-based anticancer drugs.

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Supporting Information Available: Additional experimental details. This material is available free of charge via the Internet at http://pubs.acs.org.

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