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Fluorescent Nanogel of Arsenic Sulfide Nanoclusters**

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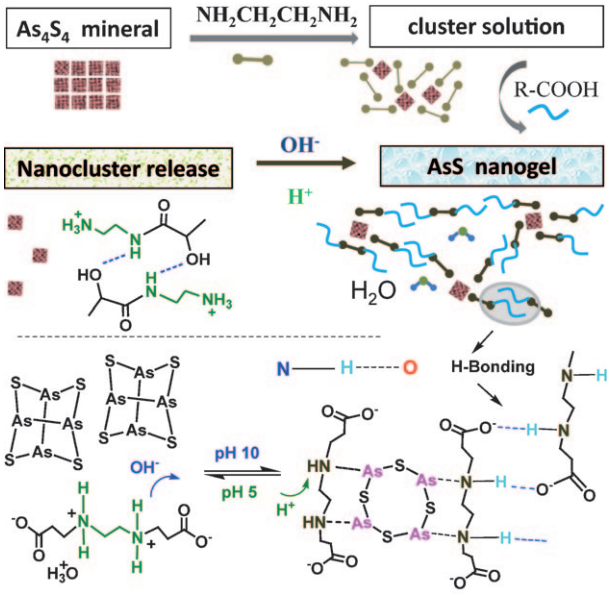
Hydrogels are smart and environmentally sensitive materials that can be chemically tailored to exhibit responses to various stimuli. The three dimensional networks of hydrophilic polymers imbibing large amounts of water make hydrogels soft and elastic and offer excellent properties for many applications.[1] Stimuli-sensitive hydrogels responsive to pH,[1a] temperature,[1b] light,[1b] biomoleclules,[1c] and drugs[1d] hold great promise as intelligent materials for application in the biomedical fields. Currently, the primary focus for hydrogel research is on their biodegradability and drug-delivery kinetics.[1e,2] The hydrogel network can be held together by covalent, ionic, and noncovalent forces, and this variety provides the flexibility for stimuli-responsive assembly and disassembly, as well as easy biodegradation.[3] The idea of integrating semiconductor nanoclusters within the three-dimensional hydrogel network to form functional hybrids has not been considered. Such hybrid material could potentially display stimuli-responsive optical and electronic properties. Herein, we describe a route to synthesize nanosized hydrogel (nanogel) containing arsenic sulfide nanoclusters as an integral part of its bonding framework. The design is motivated by the good chelation properties of arsenic atoms with the amine groups in small organic molecules and amino acids; the latter undergoes nanogel formation with hydrogen bonding. As a result, highly hydrophilic nanogel containing arsenic sulfide nanoclusters can be produced. Interestingly, such a composite nanogel was found to retain the quantum-confined optical properties of the arsenic sulfide nanoclusters, whereby the specific optical properties can be controlled by adjusting the pH value of the material. Moreover, the release of arsenic sulfide nanocluster from the nanogel can be influenced by changes in pH. This feature is promising for pharmaceutical applications because the bulk form of arsenic(II) sulfide, realgar, has been traditionally used as a drug for its antipyretic, antiulcer, anticonvulsive, and anti-schistosomiasis actions as well as a treatment for promyelocytic leukemia.[4] Recently it had been demonstrated that the nanosized form of realgar shows greater cytotoxic effects for cancer cells.[5,6] The release of arsenic sulfide nanocluster from the nanogel thus affords a vehicle for releasing a "molecular drug." Indeed, we show herein that such composite nanogels combine the useful properties of fluorescent labeling and good in vitro cytotoxicity towards cancer cells.

Bulk arsenic sulfide is soluble in liquid ammonia,[7a] anhydrous amines,[7b] and ethylenediamine,[7c] owing to the chemical bonding of arsenic with amine groups. For example, the two nitrogen atoms[7c] in ethylenediamine can donate their lone pairs to the empty d orbitals of arsenic to form chelate bonds. A wide range of amines with different pK_a values can chelate to the arsenic atoms, thus allowing the tuning of the zeta potential of the complex. Scheme 1 illustrates the two stages in the formation of the assemblies: first molecular complex or nanocluster formation and then gel formation. Depending on the molar ratio of ethylenediamine to arsenic sulfide, arsenic sulfide chelates of the amine solvent with different degree of complexation can be formed.[5,7c] Upon the addition of carboxylic acid, ethylenediamine is converted into an amide (Figure S1 in the Supporting Information) or a secondary amine (Scheme S1 in the Supporting Information),



Scheme 1. Formation of nanogel assemblies: Stage 1: Formation of arsenic sulfide (AsS) nanocluster/ethylenediamine solution from bulk As_4S_4 mineral. Stage 2: Formation of a AsS nanogel after the addition of carboxylic acid. The AsS nanocluster can be released from the nanogel at low pH, and the AsS nanocluster is coordinated by ethylenediamine to regenerate the nanogel at high pH.

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which acts as a stabilizer of the arsenic sulfide complex in the aqueous environment. These ethylenediamine derivatives have rich hydrogen-bonding properties (Figure S2 in the Supporting Information) and form a three-dimensional (3D) hydrogen-bonded cross-linked hydrogel network with arsenic sulfide as the hydrophobic core and the ethylenediamine derivative as the hydrophilic linker; this constitutes the second level of supramolecular bonding and gives rise to the hydrogel formation.

The ethylendiamine derivatives in the hydrogel can convert between neutral or anionic state (high pH) and cationic stage (low pH) by deprotonation and protonation. Changing the pH value of the hydrogel converts it from a jelly-like semisolid state at high pH into a semiliquid state at low pH (Figure 1 a). The chelation between nitrogen and arsenic is readily disrupted by protonation of the nitrogen (amine) atom in acidic environment, thus causing the arsenic sulfide nanocluster to be released from the hydrogel. The addition of base deprotonates the nitrogen (amine) atom and causes ethylenediamine to coordinate to the arsenic sulfide nanocluster and revert to the semisolid hydrogel state. In humid environments, the hydrogel adsorbs water and swells (TEM images in Figure S3 in the Supporting Information).

The jelly-like 3D network of the hydrogel readily dissolves and forms colloidal dispersion of nanogel in water.

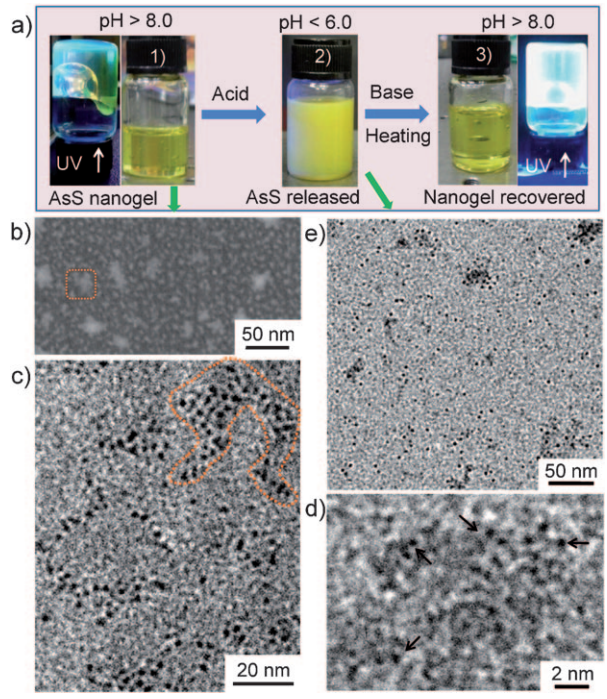

Figure 1. a) Reversible semisolid to semiliquid transition of the AsS nanogel in response to high or low pH conditions, respectively. 1: The semisolid state is transparent and strongly fluorescent under UV illumination; 2: fluorescence is quenched in the semiliquid state with the release of AsS nanoclusters; 3: recovery of transparent and fluorescent semisolid state at pH > 8.0. b) Scanning transmission electron microscopic image of the nanogel. c) TEM image showing the assembled nanolcusters inside the nanogel; the orange line traces the outline of one nanogel particle. d) High-resolution TEM image of the nanoclusters. e) TEM image showing AsS nanoclusters that were released from the nanogel at pH 5, after re-dispersion in ethanol.

The nanogel is made up of several units of AsS nanocluster coordinated to ethylenediamine derivatives. The size of the nanogel is related to the concentration of AsS in water. When the concentration of AsS and ethylenediamine increases, the small colloidal units cross-link together (aggregate) into 3D hydrogel through hydrogen-bonding among ethylenediamine derivatives. As seen in the TEM image in Figure 1, AsS nanogels consist of 3–4 nm arsenic sulfide nanocluster colloidal units (highlighted in Figure 1 c). These colloidal units appear to be interlinked to form shaped features with constant interparticle distance. In contrast, Figure 1 e shows that after the release of nanoclusters from the nanogel in acidic conditions, the arrangement of the nanoclusters becomes more random. Elemental analysis of released nanoclusters confirmed the presence of arsenic and sulfur in a ratio close to 1 (Figure S4 in the Supporting Information). In the nanogel-encapsulated state, the solubility of arsenic sulfide nanocluster can be improved to several $mg\,mL^{-1}$ of arsenic sulfide in water (bulk realgar is generally thought to be insoluble in water at pH 7). We make a distinction between arsenic sulfide nanoclusters and nanocrystals in this study. The high-resolution TEM image in Figure 1 d shows that these nanolcusters resemble molecular with sizes less than 1 nm. Neither electron diffraction pattern (EDX) nor X-ray diffraction (XRD) analysis reveal evidence of crystallinity in the nanogel. In fact, it can be demonstrated that these arsenic sulfide–ethylenediamine cluster solutions are the precursor state to the AsS nanocrystal, and the transformation can be brought about by thermal treatment (Scheme S2 in the Supporting Information).

Taking a cue from the nanogel formation between ethylenediamine, arsenic sulfide, and acrylic acid, we hypothesize that the use of ethylenediamine-*N*,*N*′-bis(3-propionic acid) dihydrate (EPH; $^{-}OOCC_2H_4NH_2^{+}C_2H_4NH_2^{+}C_2H_4COO^{-}\cdot 2\,H_2O$)[8] as the chelating agent with As_4S_4 should provide a one-step route to hydrogel formation, as shown in Scheme 1. Indeed, a transparent gel with similar properties can be obtained by treating EPH with As_4S_4. Alternatively, when two molar equivalents of $CH_2{=}CH{-}COOH$ and As_4S_4–$NH_2C_2H_4NH_2$ cluster solution (ca. $5\,mg\,mL^{-1}$) were mixed, phase separation of As_4S_4 (confirmed by EDX and XPS) and highly crystalline EPH occurred (see the Supporting Information). The two phases of As_4S_4 and EPH could be combined again to form a half-transparent gel when the pH value of the mixture was increased to 10 with mild heating (70 °C). The resulting gel was similar to that prepared by the one-step process under basic conditions.

Interestingly, the AsS nanogel was observed to be highly fluorescent (quantum yield greater than 5 % in water) in the visible region. The fluorescence can be switched on or off by adjusting the pH value (Figure 2). The fluorescence of the nanogel decreased significantly upon release of AsS nanoclusters at acidic conditions and transformation into a semiliquid. As discussed above, under acidic conditions, the coordination of ethylenediamine derivatives to AsS is disrupted following protonation of the amine. As a result, unpassivated AsS clusters are released. The fluorescence could be recovered when base is added and ethylenediamine again coordinates to AsS (semisolid state). The need for

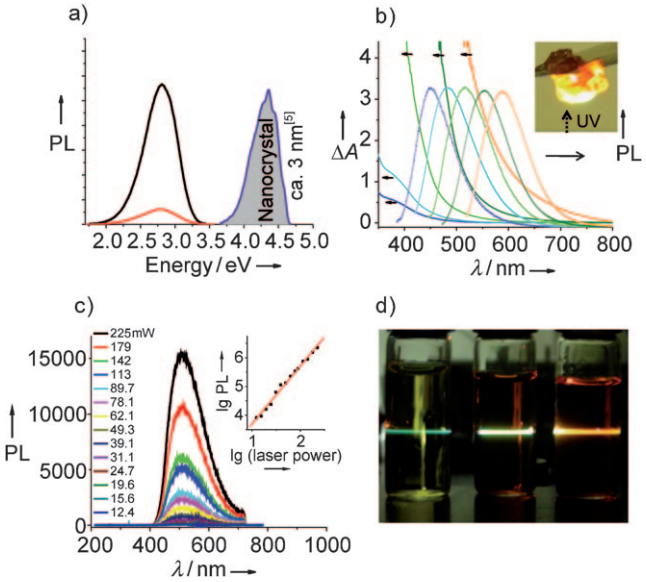

Figure 2. Optical properties of the AsS nanogel. a) The photoluminescence (PL) spectra of the nanogels before (black line) and after (red line) the release of AsS nanoclusters. The emission peak from a ca. 3 nm As_4S_4 nanocrystal[5] (gray peak) is plotted for comparison. b) Concentration-dependent absorption and PL spectra of the AsS nanogel in water (inset: photograph of the nanogel under UV illumination). There is a red shift of the spectra as the concentration of the AsS nanogel is increased from 40 to 200 mg mL^{-1}. c) Two-photon fluorescence spectra of the nanogel excited by a laser at 800 nm. Inset: quadratic dependence of fluorescence intensity with power of the laser (slope: 2.02). d) Two-photon fluorescence from AsS nanogels at three different concentrations in water.

surface passivation for these cluster materials to exhibit fluorescence suggests that its origin may be related to surface energy traps that become emissive upon stabilization. These traps could be related to the charge transfer from the N atoms in the chelated ethylenediamine ligands to the empty d orbitals of arsenic, or the recombination of carriers from conduction band and hole surface states.[9]

The emission window (450–600 nm) of the AsS nanogel is red-shifted significantly from that of arsenic(II) sulfide nanocrystal (287-450 nm). As shown in Figure 2a, the nanogel has an emission band centered at 2.8 eV, whereas ca. 3 nm arsenic(II) sulfide nanocrystal has an emission band centered at 4.3 eV at the same excitation wavelength.[5] Thus, the optical and electronic properties of the nanocluster is distinct from that of the nanocrystal. A shift in the absorbance and emission bands of the AsS nanogel with excitation wavelength was observed, which reflects not only effects from nanogel or nanoclusters of different sizes, but also a distribution of different emissive sites on each nanocluster surface.[10] Another interesting effect is the concentration dependence of the emission, which may be due to partial overlapping of the absorption edge and the emission band. Furthermore, the size heterogeneity of the nanocluster in the nanogel may allow energy transfer to proceed from smaller clusters to larger ones, leading to enhancement of the red-shifted emission. Figure 2b show that both the absorption edge and emission peak are red-shifted with increasing concentration of the nanogel in water. Resonant energy transfer through an optical near-field interaction between excited states of quantum dots has been reported.[11] Such energy transfer can occur when the excitation energy transfer length is in the nanometer range, which agrees with the physical dimensions of the nanogel matrix and the trend in concentration dependence.

Remarkably, upconversion fluorescence was also observed from the AsS nanogel (Figure 2c). The upconverted emission peak appears at 510 nm when the nanogel is excited at 800 nm by a laser. Similar to the one-photon fluorescence, the upconverted fluorescence spectra are also red-shifted with increasing concentration of the nanogel. This can be seen in the photograph of the fluorescence lines from different concentrations of nanogel in water (Figure 2d). The quadratic power dependence of the intensity of the fluorescence suggests that this is a two-photon absorption process, which may originate from intermediate surface states.[12]

The versatility of the AsS nanogel framework is such that small amino acids can be added to tune its biocompatibility. For example, instead of ethylenediamine, arginine or arginine/lactic complex can be used as the chelating agent. The distal end of arginine is capped by a complex guanidinium group with a high pK_a value of 12, thus ensuring that the group is positively charged in neutral, acidic, and even basic environments; the positive charge is favorable for cellular uptake. Because of the conjugation between the double bond and the nitrogen lone pairs, the positive charge is delocalized, enabling the formation of multiple hydrogen bonds in the nanogel. In this case, we designed a AsS nanogel based on the lactide–ethylenediamine–arginine system, in which arginine provides the first stage complexation with AsS. We observed that the nanogel is stable in the Hanks' balanced salt culture medium, and little aggregation was observed in the cytotoxicity testing. In vitro stability testing of the nanogels towards metal ions found no sign of aggregation even in aqueous solution containing 0.1 mg mL^{-1} Ca^{2+} and 50 mg mL^{-1} Fe^{3+} or Zn^{2+}.

In vitro cytotoxicity of these AsS nanogels towards certain human cancer cells was found. Plots of cell survival versus concentration of As (Figure 3a) show that for selected human ovarian (OVCAR-3) and cervical (HeLa) cancer cells, the nanogels show improved cytotoxicity.[5,6] For example, the IC_{50} value, the concentration of a drug that is required for 50% inhibition in vitro, could be less than 1 μM As for OVCAR-3 cancer cells after a 72 hour treatment period, which is much lower than that reported for nanosized realgar,[5,6] and comparable to that of arsenic trioxide.[13] In contrast, the nanogels show much less cytotoxicity towards normal human lung fibroblast cell (MRC-5) and colonic fibroblast cell (CCD-18Co) and two other cancer cells (MCF-7 and A549). The in vitro therapeutic efficacy can be combined with the optical properties of the AsS nanogel; Figure 3b shows that fluorescence labeling of the cancer cells could be obtained using the AsS nanogel. Fluorescence labeling experiments show that cells that were killed exhibited positive fluorescence, corresponding to the uptake of AsS (Figure S5 in the Supporting Information). Therefore, the

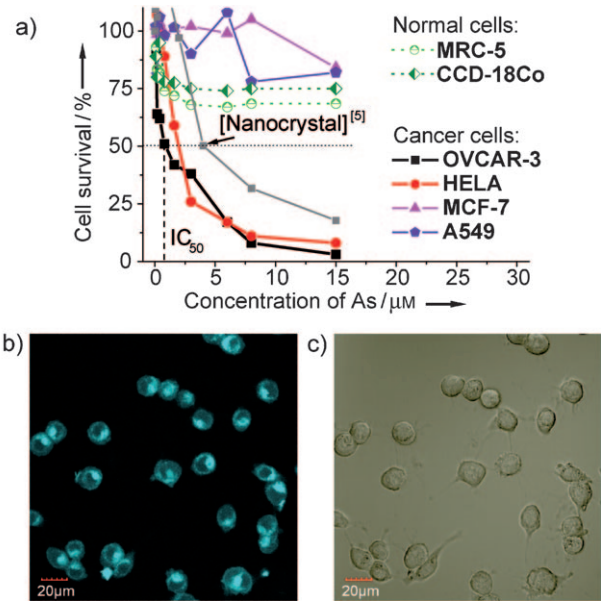

Figure 3. a) In vitro cytotoxicity of AsS nanogel assembled from lactic acid, arginine, and arsenic sulfide/ethyelenediamine solution (treated for 72 h; the gray curve shows the IC_{50} value of the nanocrystal[5] for comparison). b) Fluorescence image and c) optical image of HeLa cancer cells treated with AsS nanogel (5.0 ppm As) for 2 h.

cytotoxicity response is correlated to the uptake of the AsS nanogel.

In conclusion, we have demonstrated a new type of hybrid colloidal nanogel system that incorporates a hydrophobic inorganic nanocluster and hydrophilic ethylenediamine derivative linker. Colloidal gelation represents a simple way to assemble arsenic sulfide nanoclusters in a pH-responsive hydrogen-bonding framework. Besides imparting water solubility on the otherwise insoluble arsenic sulfide, encapsulation of the nanocluster within the nanogel framework appreciably enhances its fluorescence. In principle, this method of nanocluster colloidal gelation is very effective in solubilizing bulk arsenic sulfide and allows the design of biocompatible and molecular-level drugs based on realgar and orpiment. Furthermore, this method of nanogel assembly could be generic to all metal ions that can be chelated by amine groups. Therefore, potentially a wide range of inorganic cluster/small biomolecule hybrid nanogel systems could be assembled from its components. This work provides the first exploration of this premise and demonstrates that AsS nanoclusters embedded in nanogel combine the remarkable properties of bioimaging and therapeutics.

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