

Selective Antimicrobial Activity Associated with Sulfur Nanoparticles

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Many sulfur compounds are known to exhibit widespread antimicrobial activity. The latter is often the result of an intricate redox biochemistry whereby reactive sulfur species, such as organic polysulfanes, interact with pivotal cellular signaling pathways. The S₈ unit in elemental sulfur resembles certain aspects of the chemistry of polysulfanes. As a consequence, water-soluble S₈-sulfur nanoparticles are active against some smaller organisms, including nematodes, yet are non-toxic against human cells. In contrast, selenium and tellurium nanoparticles are less active. Together, the ease of production of the sulfur nanoparticles, their chemical stability in aqueous dispersion, amenable physical properties and selective toxicity, turn sulfur nanoparticles into promising antimicrobial prototypes for medical as well as agricultural applications.

Keywords: Nanoparticle, Sulfur, Selective Antimicrobial Activity, Selenium, Tellurium.

1. INTRODUCTION

Numerous plants, bacteria and fungi contain organic sulfur compounds (OSCs) which often exhibit distinct biological activities.¹ The antimicrobial properties of garlic-based extracts rich in allicin and polysulfanes,^a for instance, have been known for years,² while an emerging, selective cytotoxicity against certain cancer cells is currently attracting considerable interest. Polysulfanes, in particular, show considerable promise in the context of antimicrobial and anticancer drug development as well as in the field of 'green' and eco-friendly pesticides.³

Interestingly, many of the properties associated with such polysulfanes (Fig. 1) are also found in the S₈ ring of

elemental sulfur. Both polysulfanes and S₈ contain sulfur-sulfur bonds which may react with biomolecules, such as cysteine containing proteins and enzymes. The reduced forms of polysulfanes and S₈, notably RS_xH (R ≠ H, x ≥ 2) and H₂S_x (x ≥ 2), respectively, are able to form superoxide radical anions (O₂^{•-}) under physiological conditions, bind to diverse metal ions (e.g., iron, copper, zinc) and interact readily with proteins and cellular membranes. Compared to natural long-chain organic polysulfanes, such as diallyltetra-, penta- or hexasulfide, elemental sulfur is even more stable chemically, more readily available and also less intrusive when it comes to smell. In many aspects, S₈ may therefore match or even outperform polysulfanes in the context of practical applications. There is already some evidence from recent studies which point towards a pronounced bactericidal activity of sulfur particles, for instance against gram negative *Pseudomonas aeruginosa* and gram positive *Staphylococcus aureus*.⁴

Nonetheless, while polysulfanes have recently experienced considerable interest among biological and pharmaceutical chemists, elemental sulfur, with a few notable

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^aPolysulfanes have the general formula RS_xR (R ≠ H, x ≥ 2), and differ chemically from sulfides (RSR) and disulfides (RSSR). The biological literature often refers to these agents as 'polysulfides,' which is slightly incorrect from a chemical perspective yet widely used in the naming of natural products. Here, we will refer to 'polysulfides' in the context of anionic species, such as RS_xH (R ≠ H, x ≥ 2) and H₂S_x.

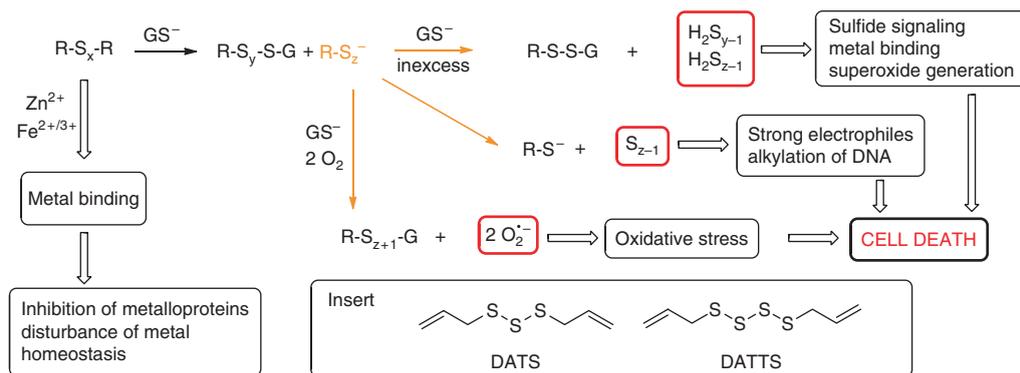


Fig. 1. Multifaceted reactivity of polysulfanes and their interactions with biomolecules. Apart from binding to metal sites and hydrophobic pockets in proteins and interacting with membranes, polysulfanes such as DATS and DATTS (see insert) react readily with cellular thiols to form mixed disulfides, thiols and perthiols. This chemistry is illustrated with glutathione (GSH), which is highly abundant in mammalian cells. The same reactions also occur, of course, with other redox sensitive thiols in peptides, proteins and enzymes. Follow-on products with suspected biological activity include H_2S , inorganic polysulfides and even superoxide radical anions. Some of these chemical species are highly damaging and trigger cellular signaling pathways, including pathways leading to apoptosis.

exceptions, has been largely ignored by this community, perhaps because it is considered insoluble and hence inaccessible for biological systems. This development is rather unfortunate, since elemental sulfur is nowadays readily available for ‘aqueous’ applications in form of sulfur nanoparticles, which can be generated by redox comproportionation of Na_2SO_3 and $Na_2S \cdot 9H_2O$.⁵ This method produces stable particles, is comparably simple and can be used for large-scale production.

We have therefore turned our attention to possible applications of nanosized sulfur particles, bearing in mind that the biological activity of most polysulfanes is associated with their distinct redox activity and interactions with the cellular thiolstat. A major focus of our study has been the question if such particles are generally toxic or if a certain selectivity exists. Since the biological activity of chalcogen-containing agents is often accelerated when turning from sulfur compounds to their respective selenium and tellurium analogues, we have also considered the possibility that selenium and tellurium particles may even be more reactive chemically and may exhibit an enhanced biological activity.⁶

Overall, our findings confirm that nanosized sulfur particles can be obtained with comparable ease, and that these particles exhibit considerable toxicity, especially against microbes, while human cells are virtually not affected. The antimicrobial activity associated with these sulfur nanoparticles is comparable to the one found for organic sulfanes, while the selenium and especially the tellurium particles are less active.

2. EXPERIMENTAL DETAILS

2.1. Chemicals

Chemicals were purchased from Sigma Aldrich (Darmstadt, Germany). They were of analytical grade

and used without further purification. All solutions were prepared with deionized MilliQ water ($R = 18.2 M\Omega$).

2.2. Synthesis of Nanoparticles and Nanowires

Sulfur nanoparticles were synthesized and purified according to the procedure of Lange et al. with small modifications. After synthesis, the nanoparticles were washed extensively with MilliQ water and centrifuged several times in order to remove all soluble, unreacted impurities. The sulfur nanoparticles were then filtered with a 25 mm syringe filter consisting of a cellulose acetate membrane ($w/0.2 \mu m$).⁵

Selenium nanoparticles were synthesized and purified following the procedure of Chen et al.⁷ Tellurium nanowires were produced by the method of Lin et al.⁸ Poly(D,L-lactide-co-glycolide) (PLGA) nanoparticles were prepared as described by Nafee et al.⁹ Citrate stabilized gold nanoparticles (AuNP) were prepared following the procedure of Turkevich et al.^{10,11}

2.3. Characterization of Particles

Nanoparticles and nanowires were studied with a transmission electron microscope (TEM) including X-ray diffraction analysis (XRD) (JEOL 2010 instrument from JEOL GmbH) and with a Zetasizer Nano (Malvern Instruments Ltd, Germany). The zeta potentials of the nanoparticles and nanowires were measured at 25 °C, pH 7 in triplicate with the Zetasizer.

2.4. Cultivation of *Plasmodium falciparum* and Determination of IC_{50} Values

The chloroquine-sensitive *Plasmodium falciparum* strain 3D7-Netherlands was grown in continuous culture as described by Trager and Jensen with slight modifications.¹²

Unless stated otherwise, parasites were maintained at 1 to 10% parasitemia and 3.3% haematocrit in an RPMI 1640 culture medium supplemented with A⁺ erythrocytes, 0.5% lipid-rich bovine serum albumin (Albumax), 9 mM (0.16%) glucose, 0.2 mM hypoxanthine, 2.1 mM L-glutamine, and 22 $\mu\text{g/ml}$ gentamicin. All incubations were carried out at 37 °C in 3% O₂, 3% CO₂, and 94% N₂. Synchronization of parasites in culture to ring stages as starting population was carried out by treatment with 5% (w/v) sorbitol.¹³

Isotopic drug sensitivity assays by means of the semi-automated microdilution technique¹⁴ were employed to investigate the susceptibility of *P. falciparum* to the nanoparticles. The procedure depends on the incorporation of radioactive ³H-hypoxanthine, which is taken up by the parasite as a precursor of purine deoxynucleotides for DNA synthesis, and was performed according to the modifications of Fivelman et al.¹⁵ In 96-well microtitre plates (Nunc^R), a two-fold serial dilution of the starting concentration of each pharmacologically active compound to be tested was carried out. Parasites were incubated at a parasitemia of 0.25% (>70% ring forms) and 1.25% haematocrit in hypoxanthine-free medium. After 48 h, 0.5 μCi ³H-hypoxanthine was added to each well and the plates were incubated for another 24 h. The cells of each well were then harvested on a glass fiber filter (Perkin-Elmer, Rodgau-Juegesheim, Germany), washed and dried. Their radioactivity in counts per min was considered to be proportional to the number of parasites in the well. All IC₅₀ values were determined in triplicate.

2.5. Nematode Assay

The nematode *Steinernema feltiae* was purchased as a friable soft cake product used in gardening from Sautter & Stepper company (Ammerbruch; Germany) which was stored at 4 °C until the time of use. A suitable suspension of the nematodes was prepared by mixing 1–2 g of the nematode friable soft cake with about 250 ml doubly distilled water. The suspension was left on the bench at room temperature for 15–30 min before use to allow the nematodes to revive from their preparation. The experimental samples for the nematode assay contained doubly distilled water, nanoparticle dispersion and 100 μl of prepared nematode suspension. The final concentrations of the compounds tested were as follows: 1, 5, 10, 25, 50, 75 and 100 $\mu\text{g/ml}$. For the (big and small) gold nanoparticles and also for the PLGA nanoparticles the following concentrations were used: 1, 2.5, 5, 10, 25 and 50 $\mu\text{g/ml}$. A negative control was established consisting of doubly distilled water and 100 μl of nematode suspension.

Three replicate tests for each concentration were carried out. From each replicate, four samples, 100 μl of each, were transferred into 4 wells in a 96-well sterile, flat bottom tissue cell culture plate with a lid (Greiner bio-one,

Cellstar) and examined immediately under the microscope at four-fold magnification.^b Living and dead nematodes in each sample were counted and the total number of nematodes, as well as the percentage of living nematodes were calculated. Afterwards, the plate was covered and incubated at room temperature in the dark. It was re-examined after 4 and 24 h.

2.6. Cyclic Voltammetry

Cyclic Voltammograms were recorded on a BAS CV-50W workstation linked to a dropping mercury electrode. The mercury electrode (drop size 16) served as working electrode, together with a standard Ag/AgCl reference electrode and a platinum wire counter electrode. The reference electrode was calibrated against ferrocene. Cyclic Voltammograms were recorded between –1200 and –200 mV versus Ag/AgCl, with four full cycles i.e., eight segments per experiment, and a scan rate of 250 mV/s. A stock of the nanoparticle dispersion was prepared in MilliQ[®] water. The stock dispersion was transferred to the electrolysis cell containing 20 ml of buffer (phosphate pH 7.4) to obtain a final concentration of 100 $\mu\text{g/ml}$ of the nanoparticle dispersion. For DATS and DATTs, the stock solution was prepared with pure methanol and then transferred to the electrolysis cell containing 20 ml NaPi (pH 7.4) and methanol (33%) to yield a final concentration of 100 μM of these sparingly soluble compounds. The solution in the electrolysis cell was stirred and purged with pure nitrogen to expel the oxygen in the solution prior each experiment. All experiments were performed at room temperature and in triplicate.

3. RESULTS

3.1. Synthesis of Chalcogen-Based Nanoparticles

Sulfur nanoparticles were synthesized by redox comproportionation of Na₂SO₃ and Na₂S · 9H₂O according to the procedure by Lange et al., which was slightly modified for our purposes.⁵ After synthesis, the particles were centrifuged and washed extensively with MilliQ[®] water (*R* = 18.2 M Ω) to remove any soluble impurities (such as unreacted sulfite) and then filtered to remove any larger debris. The particles were subsequently characterized by transmission electron microscopy (TEM) including XRD analysis and by using a state-of-the art Zetasizer at 25 °C, pH 7. Figure 2(a) shows a microscopic image of the sulfur nanoparticles. These particles possess an average diameter of 150 nm and a zeta potential of approximately –30 mV at pH 7. The size distribution was determined using the *Image J* software. As Figure 2(b) illustrates, the sulfur particles obtained by this method exhibit a monodisperse

^bIn essence, each concentration of each compound was therefore examined in total 12 times.

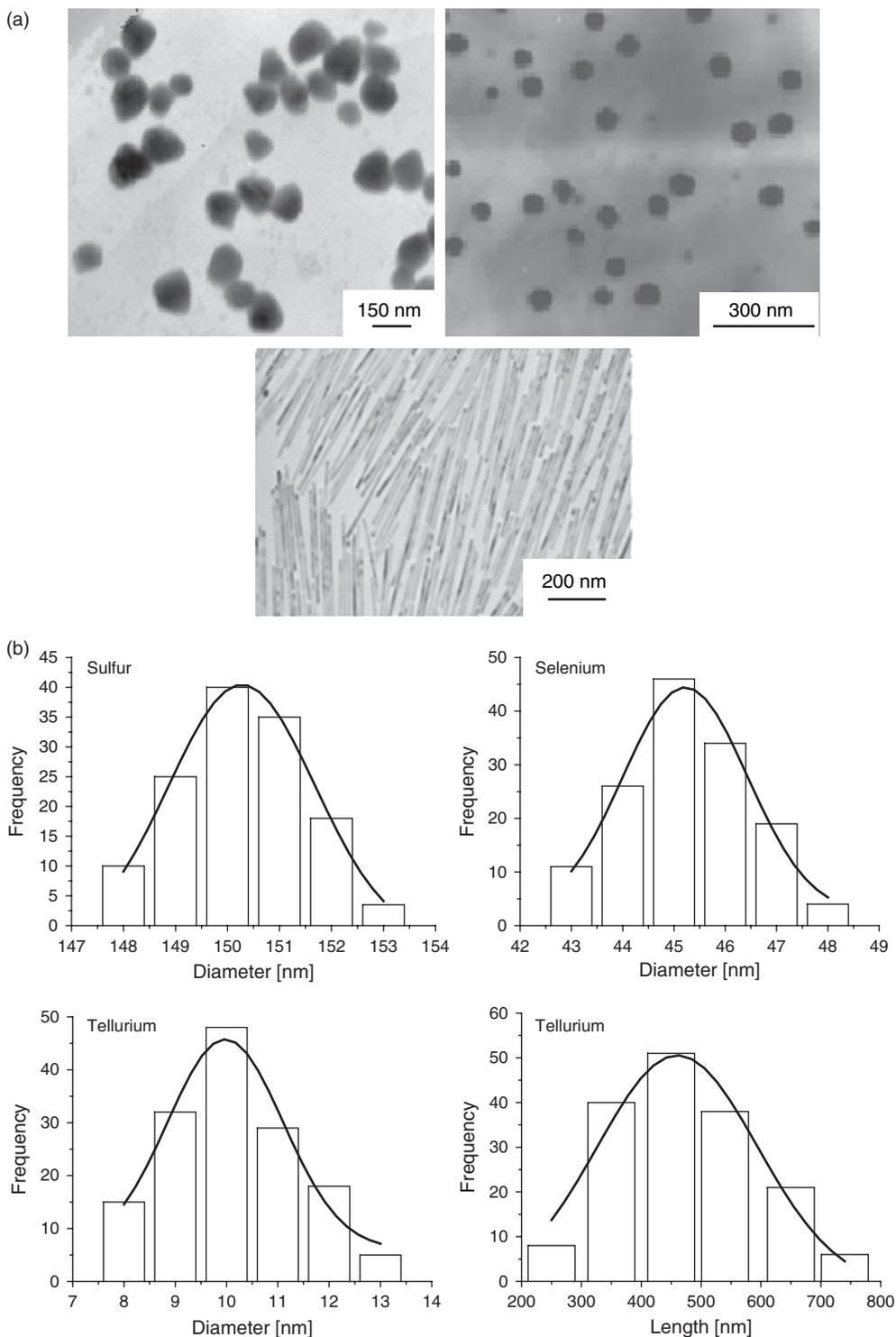


Fig. 2. Nanoparticles of sulfur, selenium and tellurium. (a) From the top left: Images of the spherical sulfur and selenium particles and the tellurium nanowires recorded using Transmission Electron Microscopy (TEM). (b) Size distribution of particles as determined by *Image J* software. Please note that the sulfur and selenium particles are characterized by their respective diameters, while the tellurium wires have a rather narrow distribution of diameter, but show a wider range of different wire length.

size distribution and are rather uniform regarding size and shape. According to the XRD analysis, the particles are highly pure, i.e., do not contain any notable impurities. This finding is supported by the TEM analysis, which also

points towards the absence of any visible contaminations, such as salt crystals.

Selenium nanoparticles were obtained by the method of Chen et al., which involves the reduction of H_2SeO_3 with

L-cysteine.⁷ These particles are also highly pure. They are rather uniform regarding their respective diameter and are smaller than the sulfur particles (Fig. 2(a)). They have a diameter of around 45 nm and a zeta potential of approximately +10 mV at pH 7 (Fig. 2(b)).

The tellurium nanowires were synthesized according to the procedure by Lin et al., i.e., by reduction of TeO₂ with hydrazine hydrate.⁸ These wires are free of any noticeable contaminations. They are around 10 nm in diameter but vary with regard to their length (Figs. 2(a and b)). On average, these wires are 454 nm long. They exhibit a zeta potential of approximately +8 mV at pH 7.

The key parameters of the different chalcogen-particles are summarized in Table I. It should be noted from the outset that the sulfur, selenium and tellurium particles generated by the simple chemical methods employed here are truly elemental, i.e., they are chemically highly pure and do not contain any additional coating or other material for surface stabilisation. This aspect is important when it comes to chemical reactivity and biological interactions associated with the particle surface. Furthermore, one should bear in mind that the chalcogen particles used in this study not only differ in their elemental composition, but also in size, shape and surface charge.^c

3.2. Electrochemical Properties of Nanoparticles

One aspect frequently associated with the biological activity of polysulfanes such as diallyltrisulfide (DATS) and diallyltetrasulfide (DATTS) is their distinct redox behaviour.¹⁶ The electrochemical properties of the chalcogen particles were therefore briefly surveyed using Cyclic Voltammetry in combination with a dropping mercury working electrode. The electrochemical results, obtained in a 0.1 M sodium phosphate (NaPi) buffer solution are summarized in Table I.^d Albeit of a preliminary nature, they confirm that the sulfur nanoparticles are redox active, with a reduction potential E_{pc} of around -635 mV and an oxidation potential E_{pa} of around -573 mV versus the Ag/AgCl electrode in 0.1 M phosphate buffer at pH 8.0. The $E_{1/2}$ value characteristic for the sulfur particles is -604 mV, almost identical with the value measured for

^cWhile more elaborate synthetic methods may well be developed to generate similarly sized and shaped particles across the chalcogen spectrum, our focus in this study has been primarily on readily accessible particles for possible practical applications.

^dOne should note that the electrochemical potentials of these sulfur species depend heavily on the kind of solvent and pH employed. Here, we report and compare uncorrected values which have been obtained under identical experimental conditions and on the same day as described in the experimental section. It should further be emphasized that such electrochemical measurements are prone to adsorption phenomena on the mercury electrode surface. The values presented here should therefore be considered as an estimation only. Nonetheless, they confirm certain trends, such as a similarity in the redox behaviour of sulfur nanoparticles and sulfides, and a considerably more reducing redox behaviour of the tellurium nanowires when compared to the sulfur and selenium particles.

Na₂S ($E_{1/2} = -603$ mV) under the same conditions and significantly more negative than the value determined for the thiol/disulfide cysteine redox couple of glutathione ($E_{1/2} = -377$ mV). The electrochemical properties of the sulfur nanoparticles are therefore not too different from the ones of inorganic sulfide, but differ considerably from the ones of glutathione.

Interestingly, the redox behaviour of the sulfur particles is also similar to the one of biologically active polysulfanes. DATS and DATTS have equally negative redox potentials, with $E_{1/2} = -613$ mV for DATS and $E_{1/2} = -627$ mV for DATTS. The $E_{1/2}$ value of selenium nanoparticles ($E_{1/2} = -583$ mV) is close to the one of the sulfur particles, while the tellurium nanowires are considerably less oxidizing (and in their reduced forms hence more reducing), with an $E_{1/2}$ value of -984 mV.

3.3. Selective Toxicity of Nanoparticles

The electrochemical measurements have confirmed two major aspects of the particles used in this study: Firstly, sulfur, selenium and tellurium nanoparticles are redox active in aqueous dispersion. Secondly, the $E_{1/2}$ value of the sulfur nanoparticles is rather negative and close to the one of DATS and DATTS, pointing towards a possible biological (redox) activity similar to the one observed for these polysulfanes. We have therefore studied the toxicity of the particles in four independent, representative and biologically relevant assays, i.e., against *Plasmodium falciparum*, the nematode *Steinernema feltiae*, cultured human epithelial A431 carcinoma cells and human histiocytic U937 lymphoma cells.

Plasmodium falciparum was selected for two reasons. Firstly, this particular plasmodium is an important medical target: it is the cause of malaria, a serious human disease affecting millions of people in Africa every year.¹⁷ Secondly, plasmodia lack the usual thiol-based antioxidant defence and hence are rather sensitive to redox changes, such as the ones caused by reactive sulfur species.¹⁸ In our study, the sulfur nanoparticles inhibit the growth of *Plasmodium falciparum* with an IC₅₀ value of 9.76 μg/ml in solution as determined by a semi-automated microdilution technique. This finding is in line with recent studies which have evaluated similar sulfur nanoparticles—albeit of a smaller size of around 10 nm in diameter—in concentrations of 30 μg/ml and 150 μg/ml as potential bactericidal agents.⁴ Yet little is known about the toxicity of sulfur particles against more complex small organisms, such as nematodes—which are of particular interest in medicine and agriculture, and about the cytotoxicity against human cells.

In the context of nematocidal activity, the nematode *Steinernema feltiae* provides an excellent, reproducible and robust toxicity assay which has been employed by us

Table I. Overview of selected physical and chemical properties of the nanoparticles and nanowires synthesized as part of this study. See text for further details.

Nanoparticle/nanowire	Starting material	Diameter/length [nm]	Zeta potential [mV]	E_{pc} [mV]	E_{pa} [mV]	$E_{1/2}$ [mV]	ΔE [mV]
Sulfur	Na ₂ SO ₃ Na ₂ S · 9H ₂ O H ₂ SO _{4conc}	150.4 ± 2.5	-30.2 ± 1.8	-635	-573	-604	31
Selenium	H ₂ SeO ₃ L-cysteine	45.3 ± 2.1	+10.4 ± 1.2	-604	-562	-583	21
Tellurium	TeO ₂ N ₂ H ₄ · H ₂ O	10.2 ± 1.8 454.2 ± 108.3	+8.1 ± 1.0	-1071	-897	-984	87

extensively in the past.^e The results obtained in this assay are summarized in Figure 3. They support the notion that sulfur-based nanoparticles exhibit considerable toxicity, not only against bacteria and plasmodia, but also against small nematodes. The LC₅₀ dose with an almost immediate effect on *Steinernema* is around 57.58 μg/ml, confirming the rather high toxicity of these particles (Fig. 3(a)). While the sulfur particles exhibit *acute* toxicity against *Steinernema*, the toxic effects become even more pronounced with time: LC₅₀ values in the nematode assay drop to below 1 μg/ml for incubations of 4 h or longer (0.71 μg/ml after 4 h, 0.61 μg/ml after 24 h). Importantly, these LC₅₀ values are considerably lower than the LC₅₀ values determined by us for highly active diallylsulfides, such as DATS or DATTS, which generally turn toxic after four or more hours of incubation and at concentrations of 200 μM or more. Interestingly, the small (19.7 ± 1.1 nm) as well as the big (35.7 ± 7 nm) metallic gold nanoparticles (Figs. 3(d) and (e), respectively) and also the polymeric poly(lactic-co-glycolic acid)-(PLGA) nanoparticles (215.2 ± 10 nm) (Fig. 3(f)), which were tested as benchmark controls, show no significant toxicity at a concentration of 50 μg/ml.

The selenium nanoparticles and tellurium nanowires exhibit a rather different biological activity in the *Steinernema* assay: The selenium particles are not particularly active initially, yet turn toxic after prolonged incubation of four (or more) hours (Fig. 3(b)). The tellurium nanowires, in contrast, show a surprisingly low toxicity: Most nematodes survive in the presence of these particles, even at particle concentrations of 75 μg/ml (Fig. 3(c)).

The toxicological data so far available for the sulfur nanoparticles points towards a widespread antimicrobial activity which may also translate into cytotoxicity against human cells. We have therefore studied the activity of these particles against cultured human epithelial A431 carcinoma cells and human histiocytic U937 lymphoma cells. These cells were chosen since they reflect distinctively

different types of human cells, are easy to culture and study and, last but not least, provide two prominent targets for anti-cancer drug development. Interestingly, the sulfur nanoparticles were entirely non-toxic in these assays up to a concentration of 145 μg/ml (data not shown). While the lack of toxicity observed may be in part due to the choice of (cancer) cell line and culture conditions (which differ from the ones used in the Plasmodium and nematode assays), these findings nevertheless point towards a rather selective toxicity of sulfur nanoparticles against lower organisms.^f

4. DISCUSSION

Our studies show that the preparation of a series of chalcogen (sulfur-, selenium- and tellurium) nanoparticles by reduction of sulfite (sulfur oxidation state +4), selenite (selenium +4) and tellurium dioxide (tellurium +4) is possible and rather straight forward. Nonetheless, there is no general method available so far which applies to all three chalcogens. Hence individually tailored reaction pathways, conditions and reducing agents have to be employed for each element, which ultimately also results in differently sized and shaped nanoparticles.

It is likely that a number of parameters control particle size and morphology, including the concentration of the reagents used, the temperature at which the redox reactions are carried out and, last but not least, the velocity of the reactions. Future studies need to investigate the synthesis of such particles in more detail, with a particular focus on controlling their sizes and shapes. Based on our knowledge of similar processes, such as precipitations and crystal growth, it may, for instance, be possible to generate smaller particles (at higher concentrations) by increasing the speed of particle formation.

From the perspective of biological activity, these studies will be of considerable interest: In addition to the elemental composition (and elemental modification), which appears to be of paramount importance, size, shape and

^eIt should be emphasized that *S. feltiae* is not a direct target for pesticide development. It is employed here due to the fact that it exhibits many biological similarities with agricultural pests, yet can be handled without the strict safety measures required nowadays for keeping ecologically damaging nematodes in the laboratory.

^fThe selenium and tellurium particles were not tested, since these elements are known to be toxic to humans. Such particles are of subordinate relevance in the context of anti-malaria drug development or possible agricultural uses.

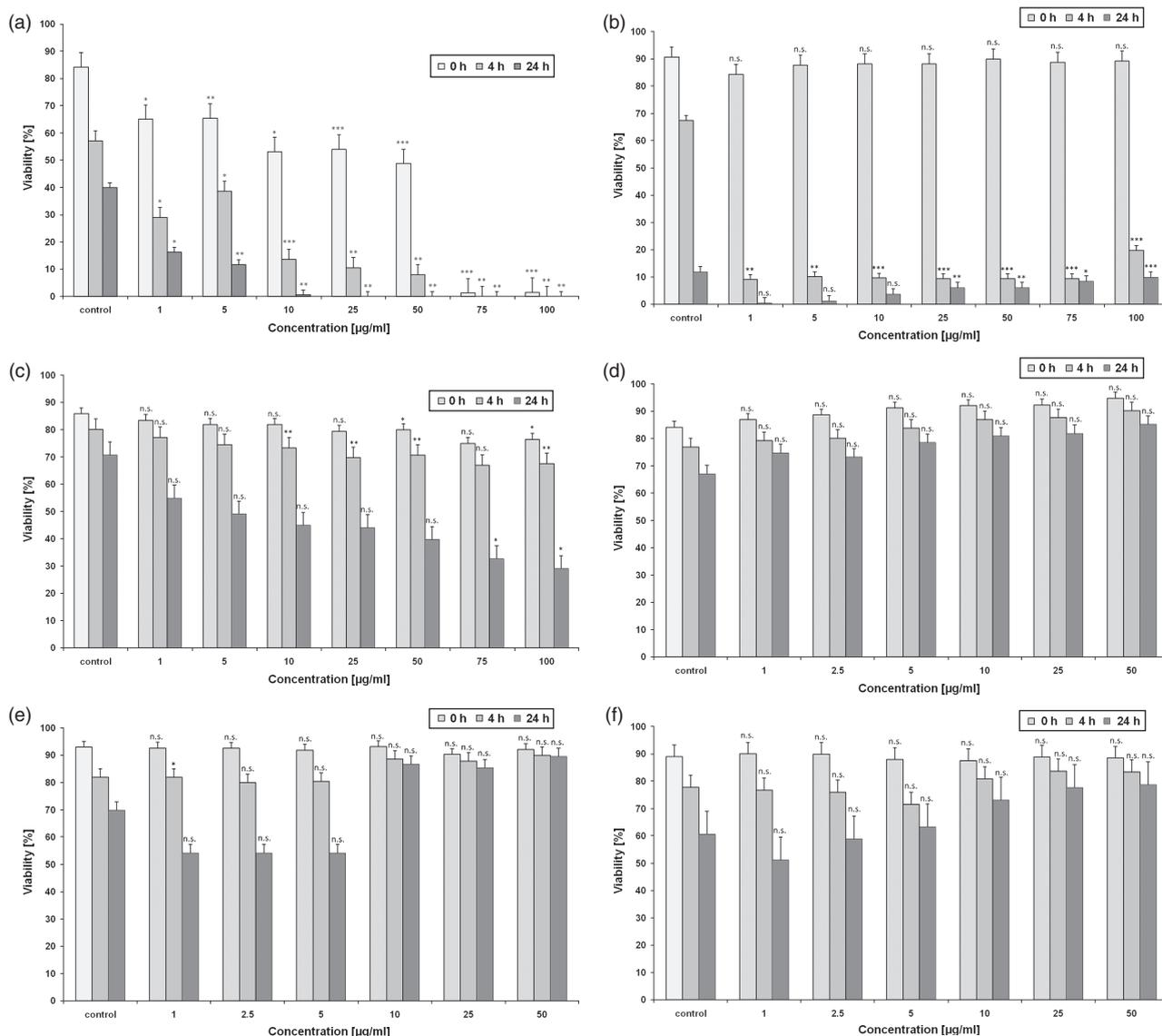


Fig. 3. Nematocidal activity of chalcogen particles. The toxicity of particles against the nematode *S. feliae* was measured after 0, 4 and 24 hours: Sulfur particles (a), selenium particles (b), tellurium wires (c), small gold particles (d), large gold particles (e) and PLGA particles (f). The high and rather immediate toxicity of the sulfur particles is clearly evident. Selenium particles are also toxic, yet only after several hours of incubation.

surface zeta potential of the particles may also be important parameters determining their chemistry and biological activity. It may, for instance, be possible to generate a range of differently sized and shaped sulfur particles, each with its own, distinct biological activity. In any case, such studies should consider the demand for easily accessible, low cost particles otherwise devoid of any toxic side products or impurities. For this reason, the redox comproportionation between Na_2SO_3 and $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ is still one of the most promising synthetic avenues.

Apart from size and morphology, the sulfur, selenium and tellurium particles also differ in their respective zeta potentials. These differences are surprising at first, since all three particle types are truly elemental, according to XRD and TEM free of any noticeable impurities, and also

devoid of any surface coatings. One possible explanation is based on differences in (redox) chemistry which distinguish the sulfur particles from the selenium particles and tellurium wires and also form the basis for a specific, sulfur-based biological activity (see below). Here, the sulfur particles are likely to form partially reduced and—at pH 7—partially deprotonated sulfide species, such as inorganic sulfide (S^{2-}) and polysulfide (S_x^{2-}) anions, on their surface, which would result in a negative surface potential. In contrast, the selenium particles and tellurium wires are unlikely to undergo this kind of redox process and their surfaces therefore do not contain any negatively charged selenium or tellurium species. These two elements are prone to oxidation, and one may speculate that the slightly positive zeta potential of the selenium

particles and tellurium wires may be the result of minor oxidation of selenium and tellurium on the surface. Alternatively, these structures may attract positively charged cations, such as sodium ions, which may result in a weakly positively charged surface. In any case, matters surrounding the chemistry of the various particle surfaces, including the formation of charged species on the surface and the attraction of ions, are of considerable interest and need to be investigated in more detail in the future.

The rather reactive S_8 unit present in elemental sulfur, once accessible in aqueous media, gives rise to an highly exciting biological activity. The antimicrobial activity against *Plasmodium falciparum* and *Steinernema feltiae* is particularly interesting in the context of medical and agricultural applications, where sulfur compounds and sulfur itself—for instance as sulfur powder or colloidal sulfur—have a long tradition of being used as antimicrobial agents. Our initial data even points towards a selective activity against lower organisms, which may in part be explained by the differences in antioxidant defence, i.e., reduced glutathione (GSH) content, which exists between microbes, nematodes and human cells.

Nonetheless, it is difficult to compare the ‘true’ biological activity of nanoparticles with the activity of soluble chemical compounds, such as polysulfanes or existing drugs able to fully dissolve or dissociate in aqueous media. The studies using *Plasmodium falciparum* underline this distinct difference. In general, only agents with IC_{50} values of below $1 \mu\text{M}$ are deemed as sufficiently active against *Plasmodium falciparum* from a potential therapeutic perspective. The sulfur particles used, in contrast, were only active at higher concentrations. Here, one must bear in mind that in sulfur nanoparticles, most of the chemically reactive—and hence biologically active— S_8 -rings are buried *within* the particle and therefore cannot exert any biological activity. In order to develop the full activity associated with the IC_{50} value, the particle needs to disintegrate, for instance by continuous chemical reaction at and subsequent removal of the particle surface (see Fig. 4). An IC_{50} value of $9.76 \mu\text{g/ml}$, which corresponds to a nominal total sulfur concentration of $305 \mu\text{M}$ and a nominal total concentration of $38 \mu\text{M}$ S_8 -rings, is therefore still rather promising, bearing in mind that the concentration of accessible sulfur (or S_8) on the particle surface will be considerably smaller at a given time. In addition, the toxicity of these particles against human cells is very low, which may allow the administration of such particles in rather high concentrations. Similar considerations apply to the biological activity of sulfur nanoparticles in *S. feltiae*.[§]

[§]At this point, one must also mention the possibility that the sulfur particles used may contain inclusions of residual reactants, such as sulfite or sulfide. Although this possibility is remote because the particles were washed extensively, found to be pure using XRD and TEM, and previous reports as well as our own data with human cells point against the presence of significant amounts of such impurities, one should always be cautious when dealing with ‘precipitates’ and *in situ* generated particles.

Compared to the sulfur nanoparticles, the metallic (small and big) gold particles and the polymeric PLGA particles appear to be considerably less toxic. Together with the results obtained for selenium nanoparticles and tellurium nanowires, these findings imply that toxicity does not ‘simply’ result from particular size, shape or zeta potential, but is critically linked to particle composition, in this case to sulfur.

Based on the toxicological data and the electrochemical results available so far, we can therefore postulate a preliminary model for the biological chemistry of S_8 -containing particles (Fig. 4). In this model, S_8 on the surface of the particles would act as an oxidant, i.e., randomly S-thiolating essential cysteine residues in proteins and enzymes. Widespread S-thiolation of proteins and enzymes, which is often associated with reduced function and activity of these biomolecules, would ultimately trigger major cellular processes which may result in cell death. At the same time, the reduced form(s) of S_8 , most likely inorganic S^{2-} and S_x^{2-} anions, would be generated as part of this reaction. As already mentioned, one may speculate that the negative surface potential measured for the sulfur particles, but not for the selenium and tellurium particles, may point towards the presence of these S_x^{2-} species on the particle surface.

Sooner or later, these anions would be released from the surface of the particle. This process would ‘regenerate’ the particle surface for further interactions and, at the same time, represent a kind of redox controlled release of the biologically active polysulfides. Since polysulfides are redox active, they could develop a biological redox chemistry on their own. H_2S_x species may, for instance, act as oxidants or reduce dioxygen to $O_2^{\bullet-}$ and hence trigger a complicated cycle of radical generation and oxidative stress.¹⁹ In addition, such S_x^{2-} species are able to interact adventitiously with key metal sites in metalloproteins and enzymes, with subsequent loss of function and activity (Fig. 4). Longer chain polysulfides are also amphiphilic and may unfold proteins or disrupt cellular membranes.

The biological activity (or lack thereof), which has been observed for selenium nanoparticles and tellurium nanowires in the nematode assay, is more difficult to explain. Although speculative at this time, it is likely that the selenium particles—in contrast to the sulfur particles—are not reduced spontaneously in the presence of cellular thiols. As a consequence, these particles remain non-toxic, at least initially. Nonetheless, after a prolonged period of incubation, the selenium particles may become part of the cellular selenium metabolism, which is highly reductive and involves species such as selenodiglutathione (GSSeSG), hydrogen selenide (H_2Se), selenocysteine, dimethylselenide ($(CH_3)_2Se$), and trimethylselenide ($(CH_3)_3Se^+$).²⁰ Some of these selenium metabolites are toxic at elevated concentrations. Their presence after an initial lag phase required for metabolic activation may explain the toxicity observed for the selenium particles.

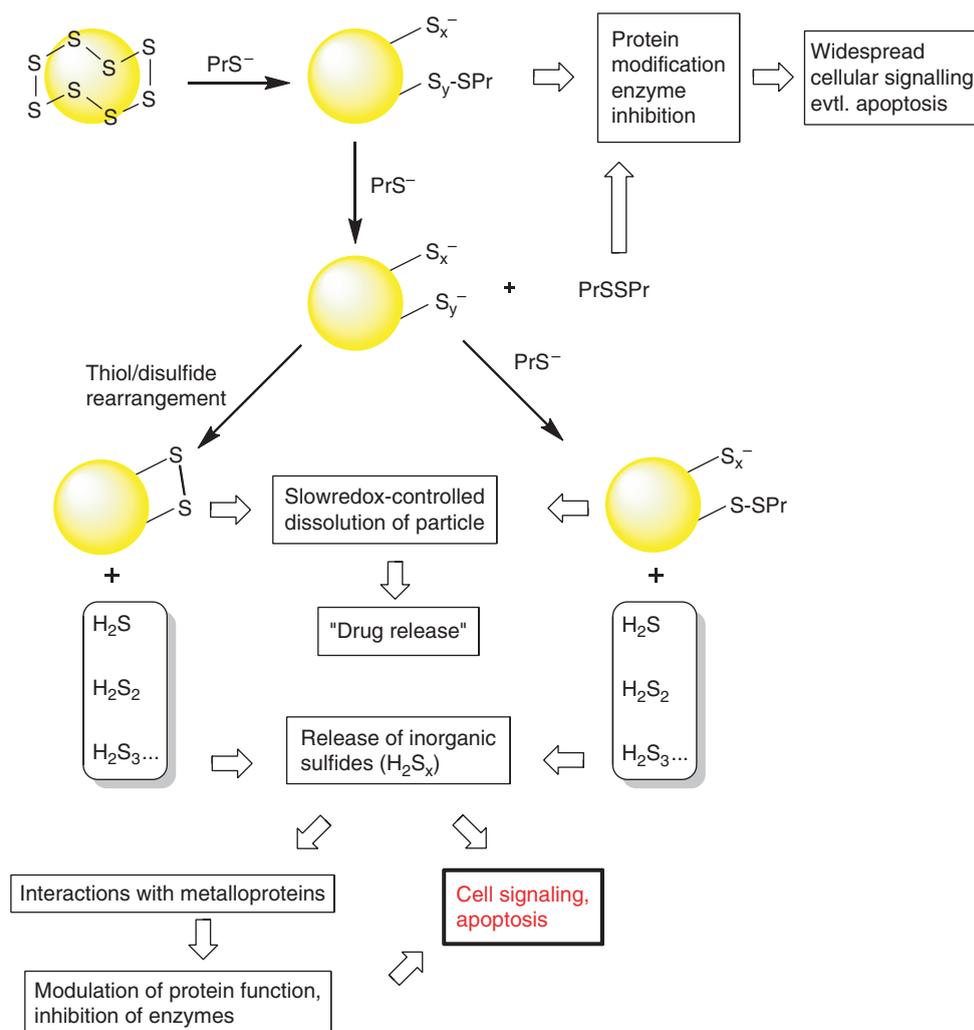


Fig. 4. A schematic overview of possible interactions of sulfur nanoparticles with biomolecules. Similar to the chemistry depicted in Figure 1 for organic polysulfanes, these particles may react with cellular thiols to form disulfides and a range of reduced inorganic sulfur species. Ultimately, this kind of redox chemistry may slowly 'dissolve' the particle, hence regenerating the reactive particle surface and releasing various reactive, biologically active species, such as H₂S and polysulfides. It should be emphasized that most of these processes are still speculative at this time, yet there is mounting evidence for widespread biochemical interactions of such reactive sulfur species with cellular components, including redox sensitive proteins, metalloproteins and hydrophobic entities, including cellular membranes.

A similar explanation may also apply in the case of the tellurium nanowires, which exhibit a surprisingly low toxicity even after several hours of exposure and at concentrations as high as 75 $\mu\text{g}/\text{ml}$. Based on their electrochemical profile, it is unlikely that these particles react spontaneously with cellular thiols. Since there is also no specific tellurium metabolism known in higher organisms, spontaneous as well as metabolic activation of these particles with subsequent development of toxicity may largely be ruled out.²¹

Chemical reactivity of the chalcogen particles, combined with certain metabolic transformations and cellular signaling pathways, may be the most obvious explanation for the biological activities observed as part of this and related studies. Nonetheless, it is possible that there are

also other explanations for the apparent toxicity of some of the chalcogen particles. Differences in the zeta potential of the particles may, for instance, play a role.²² While the sulfur particles possess a negatively charged surface, the other particles exhibit a positive zeta potential. The negatively charged particles may therefore interact more strongly with certain biological target molecules, such as proteins or enzymes. Furthermore, particle sizes and even shapes may matter. The sulfur particles are considerably larger when compared to the selenium particles and also differ significantly in size and shape from the tellurium wires.

Additional and more detailed studies are clearly required in the future to evaluate the underlying chemical and biochemical processes responsible for the selective toxicity

of sulfur nanoparticles. At the same time, a possible use of the slower acting selenium particles should not be ruled out entirely. Once the underlying chemical and biochemical mechanisms are better understood, future applications of such particles against certain microorganisms in Medicine and Agriculture may become feasible.

5. CONCLUSIONS

Our studies have shown that sulfur nanoparticles enable us to ‘unlock’ the powerful chemistry of S_8 -rings, which has traditionally been inaccessible in aqueous media due to an intrinsically low solubility of this element in water. Once formed, the sulfur nanoparticles exhibit a redox behaviour close to the one of S^{2-} and organic polysulfanes. The redox activity of the S_8 -ring may form the basis for a widespread, yet selective biological activity, which includes toxicity against various bacteria, redox sensitive plasmodia and certain nematodes. Within this context, sulfur nanoparticles join the fold of other toxic nanoparticles, whose toxicity seems to be connected to their size, including silver nanoparticles,^{23–25} titanium dioxide nanoparticles²⁶ and lanthanum calcium manganate nanoparticles.²⁷ Since nanosulfur particles do not appear to be particularly toxic against human cells, they may provide the basis for the development of future antimicrobial formulations in Medicine and Agriculture. Here, the ease and low cost of the synthesis, which is paired with high chemical stability, lack of any nasty side effects (such as smell) and an apparent low toxicity against human cells implies a considerable interest especially for agricultural applications. A more extensive screen of possible agricultural uses of sulfur (and other chalcogen-based) nanoparticles is therefore warranted.

In contrast, the selenium nanoparticles and tellurium nanowires, which have also been obtained and investigated as part of this study, exhibit a surprisingly low toxicity profile compared to their sulfur analogues. Similarly, the commonly used gold particles and polymer-based PLGA particles were also non-toxic at the concentrations used, highlighting once more the special status of the sulfur particles.

Additional studies are now required to explore the full range of biological activities associated with these particles. At the same time, the underlying chemical and biochemical mechanisms responsible for these activities need to be studied in more detail. Within this context, one particular focus will be on particle size and surface charge: It is likely that the biological activity of the particles studied not only depends on the composition, but also on the size, shape and zeta potential. If this were the case, one single element, such as sulfur, may provide us with a range of different particles, each with its own custom-made chemical properties and distinct biological activity.

Abbreviations

GSH, reduced glutathione; OS, oxidative stress; ROS, reactive oxygen species.

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