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Nature's Hat-trick: Can we use sulfur springs as ecological source for materials with agricultural and medical applications?

Lisa Faulstich [a, e], Sharoon Griffin [a, b, c], Muhammad Jawad Nasim [a], Muhammad Irfan Masood [a, c], Wesam Ali [a], Salah Alhamound [a], Yousef Omran [a], Hyejin Kim [a], Ammar Kharma [a], Karl-Herbert Schäfer [c], Rainer Lilischkis [d], Mathias Montenarh [e], Cornelia Keck [b], Claus Jacob [a, *]

[a] Division of Bioorganic Chemistry, School of Pharmacy, Saarland University, D-66123 Saarbruecken, Germany
[b] Institute of Pharmaceutics and Biopharmaceutics, Philipps-Universität Marburg, D-35032 Marburg, Germany
[c] Department of Biotechnology, University of Applied Sciences, Kaiserslautern, 66482 Zweibruecken, Germany
[d] Department of Information Technology and Microsystem Technology, University of Applied Sciences, Kaiserslautern, 66482 Zweibruecken, Germany
[e] Medical Biochemistry and Molecular Biology, Saarland University, D-66424 Homburg, Germany

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ABSTRACT

Sulfur and its various compounds play a major role in agriculture and medicine. Natural waters rich in hydrogen sulfide may therefore be seen as a sustainable resource for biologically active sulfur species. By sampling such waters from two readily accessible mineral wells in Germany, we are able to show that such waters exhibit interesting nematicidal and antimicrobial activity which may be used in an agricultural context. Whilst applications in the field of agriculture could, in theory, result in an amalgamation of irrigation, soil enrichment and phyto-protection, therapeutic uses are more complex and complicated by the many physiological effects associated with hydrogen sulfide and its oxidized derivatives. The latter may include polysulfides (S_x^{2-}) as well as small sulfur particles. Indeed, we have recently noted significant cytotoxic properties of clean, mechanically produced sulfur nanoparticles against HCT-116 colon cancer cells. Since sulfur-rich natural waters are known to deposit elemental sulfur upon oxidation, they may therefore be used as a natural (re)source of sulfur particles, possibly obtained by direct oxidation on air, mild oxidation with sulfur dioxide or enzymatic oxidation employing *Thiobacillus*. A similar biotechnological approach involving *Staphylococcus carnosus* and selenite (SeO_3^{2-}) produces biologically active selenium nanoparticles of excellent quality and with a pronounced biological activity. Eventually, natural spa waters rich in sulfide seem to open up various interesting opportunities in medicine and eco-friendly agriculture.

1. Introduction

Sulfur and its plethora of chemically diverse organic and inorganic compounds are known to exhibit a wide and often diverse spectrum of biological activities, ranging from antioxidant action to antimicrobial and even anticancer properties. Our own studies on natural Organic Sulfur Compounds (OSCs) – in particular with allicin and diallylpolysulfanes found in *Allium* plants such as garlic and onions – on many occasions have corroborated older reports of pronounced activities against diverse pathogens, and even point towards a selective action against certain cancer cells (Allah et al., 2015; Czepukojc et al., 2013a, 2013b; Saidu et al., 2013b). A particularly fruitful field for possible applications of such natural sulfur compounds seems to be eco-friendly agriculture, and indeed, several companies, such as Ecospray Limited in the UK have developed sulfur-based preparations for eco-friendly agricultural uses (Hamilton et al., 2014).

Nonetheless, such agricultural applications are faced with several drawbacks. First of all, the materials applied have to be "safe", not only for humans and animals but also for the

* Corresponding author.
E-mail addresses: karlherbert.schaefer@hs-kl.de (K.-H. Schäfer), rainer.lilischkis@fh-kl.de (R. Lilischkis), Mathias.Montenarh@uks.eu (M. Montenarh), cornelia.keck@pharmazie.uni-marburg.de (C. Keck), c.jacob@mx.uni-saarland.de (C. Jacob).

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environment. The resulting, fairly restrictive licencing policy for new products in agriculture, which in the EU today is comparable to the one in medicine, has therefore shifted the attention towards "natural" products, such as the garlic-derived materials referred to above. Secondly, however, the material has to be available readily, economically and, in any case, in rather large amounts. To satisfy an agricultural demand, tons rather than milligrams (as in medicine) are required, and plant-derived materials, such as the ones based on garlic, are not necessarily always competitive. Indeed, many natural products based on plants are derived from a more or less expensive source which is also not cultivated everywhere and at all times - hence requiring transportation and storage. An alternative approach based on "turning (agricultural) waste into value" has therefore been advocated to circumvent some of these issues (Griffin et al., 2016).

Inspired by the idea that anything found in the bin or sewer may serve as a cheap and readily available resource, and guided by the incentive smell of hydrogen sulfide emanating from sulfur-rich waters streaming seemingly endlessly from natural wells in towns such as Aachen or Bad Nenndorf, we have decided to investigate (a) if such natural waters may serve as biologically active materials in agriculture and (b) if they could be refined to yield more advanced sulfur-based materials with possibly new or improved biological activities. For instance, one may envisage a simple oxidation or redox comproportionation involving sulfide and elemental sulfur particles according to Eq. (1), which is thermodynamically favourable (Riedel, 1988).

$$3H_2S + \frac{3}{2}O_2 \rightarrow 3S + 3H_2O \quad \Delta H^0 = -664 kJ/mol \tag{1}$$

Here we report our first results of this study, which despite the fact that they are still preliminary, substantiate the idea that such natural (re)sources could be valuable as phyto-protectants and that sulfur nanoparticles, which might be obtained from such waters, are interesting from a medical point of view.

2. Experimental

2.1. Materials

All Chemicals were purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Plantacare® 2000 UP was purchased from BASF (Ludwigshafen, Germany). Biological assays were carried out using distilled water. All chemicals were used without further purification. The fungus *Candida albicans* was kindly provided by the research group of Prof. Reichrath (Department of Dermatology, UKS, Homburg, Germany). HCT-116-cells were cultivated in the group of Prof. Montenarh (Department of Medical Biochemistry, UKS, Homburg, Germany). *Steinernema feltiae* was purchased from Sautter and Stepper GmbH (Ammerbuch, Germany). *Escherichia coli* was cultured in the group of Prof. Jacob (Department of Bioorganic Chemistry, UdS, Saarbruecken, Germany).

Size reduction was carried out using the ball mill FastPrep 24 high-speed homogenizer MP Biomedicals, Solon, OH, USA, fitted with Precellys Kits from Bertin Technologies, Montigny-le-Bretonneux, France. A MICCRA D-9 Homogenizer (MICCRA GmbH, Muellheim, Germany) was used for High Speed Stirring (HSS), whilst an APV Gaulin LAB40 (APV GmbH, Mainz, Germany) was employed for High Pressure Homogenizing (HPH). For the analysis of the particles, a Mastersizer 2000 (Laser Diffraction (LD) analysis) and a Zetasizer (Nano ZS Photon Correlation Spectroscopy (PCS) analysis) from Malvern Instruments, England, were used. A ZEISS Supra 40 field emitter microscope (Carl Zeiss NTS GmbH, Oberkochen, Germany) combined with a Bruker Quantax EDX system (Bruker Nano GmbH, Berlin, Germany), was utilized for Microscopy. For MTT assays, an Elisa Reader (TECAN infinite M200PRO) was employed, whilst viability of nematodes was observed with the microscope TR 200 (VWR International, Belgium). Growth of *E. coli* and *C. albicans* was determined via optical density measurements on a Varian Cary 50 Bio UV–Visible-Spectrophotometer (Varian Australia Pty Ltd., Australia).

2.2. Production of chalcogen nanoparticles via milling and homogenization

Chalcogen nano-sized material was obtained via a sequential top-down approach. Starting off, the elemental chalcogens were reduced in size using a ball mill. After consecutive cycles, the dry-milled samples were suspended as 1% w/w suspensions in 1% Plantacare®. Distilled water was used as the solvent while Plantacare was utilized as surfactant for stabilizing the resulting suspensions. Subsequently, the suspensions were put through rotor-stator HSS at 15,000 rpm, before performing adequate homogenizing cycles. HPH was used for initial and final homogenizing procedures. Initial homogenizing included three cycles of 200, 500, 1000 bar pressure, respectively, while ten cycles of 1500 bar pressure were performed as part of final homogenizing (Estevam et al., 2016).

2.3. Characterization of mechanically generated nanoparticles and natural deposits

The production of chalcogen particles was followed by a thorough characterization of their relevant physico-chemical properties, and by involving static and dynamic light scattering measurements as described by us in recent publications (Estevam et al., 2016; Griffin et al., 2016). Moreover, classical techniques such as Scanning Electron Microscopy (SEM) for visualization and X-ray Diffraction (EDX) for *in situ* elemental analysis of microscopy samples were performed (Estevam et al., 2016). The presence of particles in natural sulfur-rich water samples was investigated primarily with the help of EDX, which enabled a rapid determination of elemental composition of the "spots" selected under the microscope (Estevam et al., 2016).

2.4. Cytotoxicity screen in HCT-116 cells

HCT116 cells (ATCC number: CCL-247) were maintained in 5% CO_2 at 37 °C and in McCoy's 5A medium with 10% fetal calf serum (FCS) (Mosmann, 1983). The suspension of 1% w/w nanoparticles in 1% Plantacare was diluted ten times with distilled water and subsequently was used as stock solution. Cells were incubated with a 0.001% *v/v* suspension of the respective chalcogen nanoparticles. A negative control and a solvent control (0.001% *v/v* Plantacare) were performed in order to avoid any false positive results. Cell viability was determined after 4 and 24 h by the colourimetric MTT (3-(4,5)-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. This assay was routinely performed according to a well-established literature method (Allah et al., 2015).

2.5. Antimicrobial and nematicidal activity against selected (micro) organisms

The water samples collected from Bad Nenndorf and Aachen were tested against three model organisms, including the Gram-negative bacterium *E. coli*, the agricultural nematode *S. feltiae* and the infectious or pathogenic fungus *C. albicans*. The assays were performed according to well-established methods described in the literature (Czepukojc et al., 2013b; Estevam et al., 2016; Hughes et al., 1987). Cell density was determined at time point 0 h and 24 h after incubation at 37 °C. A mixture of penicillin, amphotericin B and streptomycin was used as positive control for *E. coli* while ketoconazole served as positive control for *C. albicans.* A 50% dilution of medium in distilled water was used as negative control. The viability assay of *S. feltiae* was performed according to literature (Czepukojc et al., 2013b). Viability readings were taken immediately after incubation (at 0 h) to assess toxic effects and after 24 h incubation to consider a more long-term impact on this organism. Ethanol (70% *v*/*v*) was used as positive control. Experiments were generally performed in triplicate and on three independent occasions (*de facto*, each measurement was repeated nine times).

3. Results

Overall, our studies indicate that the two individual samples of sulfur-rich water collected at two different sites in Germany ("Neue Landgrafenquelle" in Bad Nenndorf and "Elisenbrunnen" in Aachen) possess some activity against smaller organisms, including the model agricultural nematode *S. feltiae* and the human pathogen fungus *C. albicans*. This activity is likely due to the presence of upto millimolar amounts of hydrogen sulfide (as H_2S and HS^-), yet other factors such as the presence of certain minerals (*e.g.* $CaSO_4$) cannot be ruled out. At closer inspection, these probes also contain particulate matter, which may be, in part, based on inorganic elemental sulfur. Employing well-established methods from the arsenal of nanotechnology, we have therefore produced such inorganic sulfur particles in a chemically pure form and noticed a significant activity of this material against cultured HCT-116 colon cancer cells. Whilst tellurium particles produced alongside are more active than sulfur particles, and H_2S seems to exhibit various activities on its own, we have also instigated first attempts to use the natural waters as a source for the manufacture of natural sulfur particles. These results will now be presented in more detail.

3.1. Nematicidal and antimicrobial activities associated with natural waters from mineral wells in Aachen and Bad Nenndorf

There are various mineral wells in Germany which are particularly rich in sulfide. Based on the especially high content of sulfur, and comparably low(er) amounts of other redox active substances, such as iodides, we have selected the "Elisenbrunnen" in Aachen and the "Neue Landgrafenquelle" in Bad Nenndorf for our initial studies, with the option to venture to other wells later on. Fig. 1 indicates the location of both wells. Whilst the "Elisenbrunnen" is a natural spring in the west of Germany, the "Neue Landgrafenquelle" in the North is an artificial well accessed via an approximately 300 m deep borehole. In any case, both wells provide a cheap, reliable and sustainable source for sulfide-rich water for years to come.

In order to estimate if these waters exhibit any noticeable biological activity, we have initially employed a nematode screen involving the model nematode *S. feltiae*. This screen has been used by us several times before (Schneider et al., 2011). It is not only fairly simple and robust - it does not require sterile conditions, staining or any major instrumental analysis - but also provides initial information about a possible effect on a multicellular organism. *S. feltiae* is a good choice of nematode for such studies as it is a harmless model of more aggressive nematodes relevant in an agricultural context.

Fig. 2 shows the results obtained in this screen. It is immediately apparent that both samples, form both wells, exhibit a significant nematicidal activity which is pronounced after 24 h. This activity is concentration dependent and also seems to differ between the two samples. The sample from Bad Nenndorf, which is known to contain around 2.4 mM of sulfide, is somewhat more active, in line with its higher content of sulfide – approximately 67 μM in the water from the "Elisenbrunnen". It is notable that the water from Bad Nenndorf is still statistically significantly active at dilutions of 1:10, implying that it may serve as a nematicidal "stock solution" which may be watered down considerably for possible nematicidal applications. Indeed, when employed at full strength, the water of Bad Nenndorf killed almost all *S. feltiae* present in the sample, with just 3% viable nematodes remaining. Compared to many other suspected nematicidal compounds and materials tested by us in the past, this activity is truly astonishing, bearing in mind that we are not dealing with any eloquent new chemical compound here, or with extensively processed materials derived from medical plants etc.

Encouraged by these results, we have then turned our attention to a wider screen involving a (Gram-negative) bacterium, *E. coli*, and a single cell fungus, *C. albicans*. Both organisms are potential human pathogens and are used by us routinely to probe a possible medical application. Whilst the water from Bad Nenndorf showed slight activity against *C. albicans* (around 10% inhibition of growth when used in a 1:1 dilution in distilled water), both waters did not show any significant activity against *E. coli* (data not shown). Generally, the water sampled at Bad Nenndorf, which contains a higher concentration of H_2S compared to the water sampled at Aachen, has also shown a higher activity against the organisms we have studied when compared to the water from Aachen.

Whilst some of our findings, such as an apparent lack of activity against *E. coli*, at first sight may be disappointing, they nonetheless confirm a certain selectivity against particular organisms - and therefore count against a general toxicity, which may be less desirable. Indeed, sulfides are known to trigger various cellular responses, and some organisms, including humans, enjoy a complex and complicated network of hydrogen sulfide signalling. Eventually, practical applications of (hydrogen) sulfide are therefore context-dependent, and we will discuss this issue later on.

3.2. Particulars about the water

Since our previous studies with - chemically generated - sulfur nanoparticles have shown that such materials may be active, yet less toxic than sulfides, and since there are notable precipitations of elemental sulfur in and at many sulfide-rich wells, we have wondered if such - biologically active - sulfur particles could also be present in and eventually obtained from the waters flowing freely at Aachen or Bad Nenndorf. Besides visual inspection (Fig. 3 a), we have therefore employed a combination of microscopy and EDX analysis.

Fig. 3 b and c provide representative microscopic analyses of a sample from Aachen and Bad Nenndorf, respectively, with a representative EDX analysis of the sample from Bad Nenndorf in Fig. 3 c (table). From these images, it is apparent that the samples obtained from the wells contain some particles with diameters at the hundreds of nanometer scale, and that these particles also contain significant amounts of sulfur (around 10%) (Fig. 3 c). EDX is unable, however, to distinguish between different forms of sulfur, and the considerable presence of calcium as well as oxygen may also point to the presence of $CaSO_4$ in those particles. The latter is known to contribute to the hardness of water, and whilst there may be some elemental sulfur in or at those particles as well, the situation is "messy" and the material is poorly defined, physically as well as chemically.

3.3. Mechanically generated chalcogen particles

We have therefore decided to continue our investigations with

Fig. 1. Geographical location of the two mineral wells sampled as part of this study. The "Neue Landgrafenquelle" in Bad Nenndorf is to some extent man-made, whilst the "Elisenbrunnen" in Aachen is a surface-water spring. Map generated via *Google Maps*.

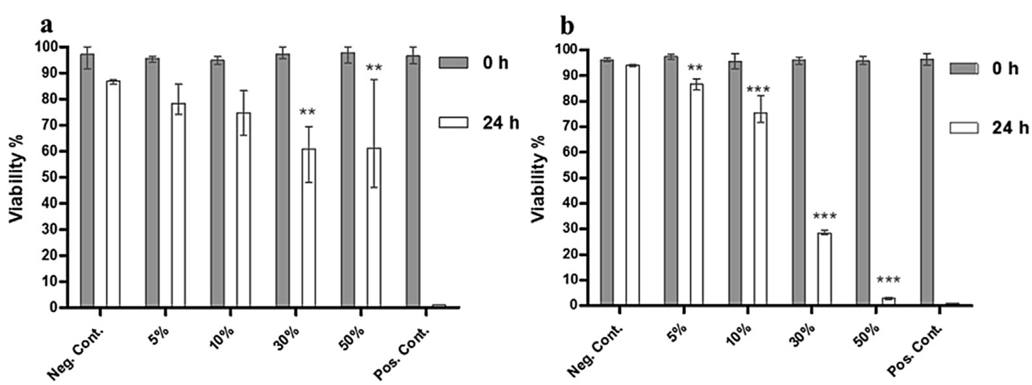


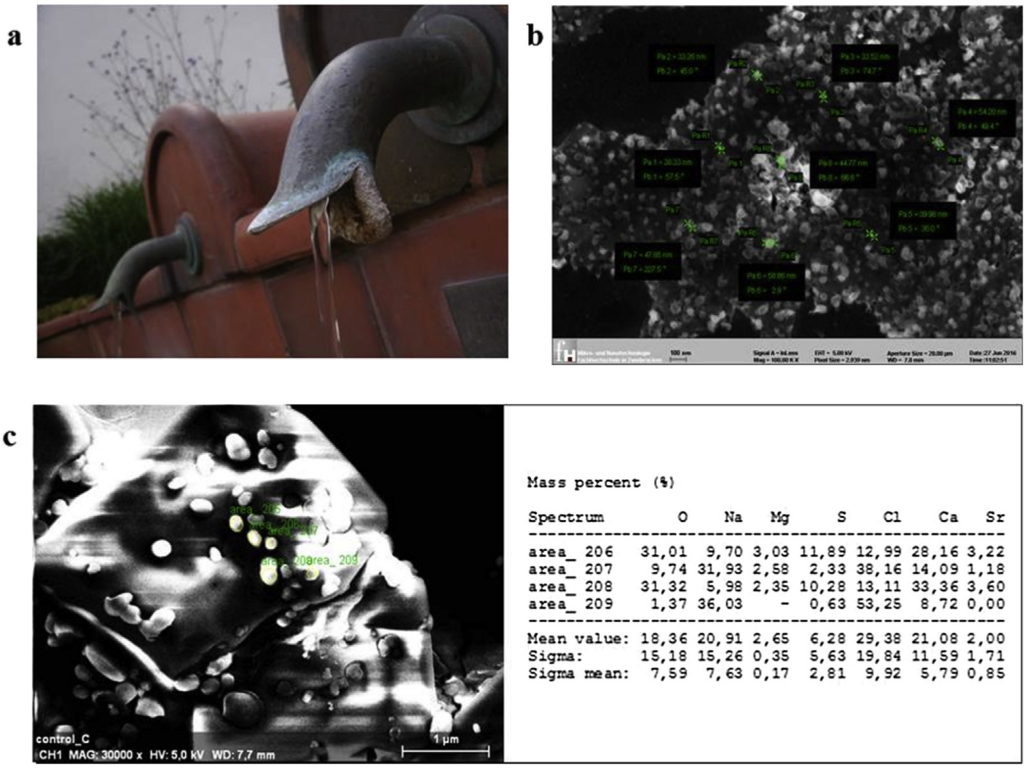
Fig. 2. Activity of different dilutions of sulfur-rich water obtained from the "Elisenbrunnen" in Aachen (**a**) and the "Neue Landgrafenquelle" in Bad Nenndorf (**b**) against the model nematode *S. feltiae*. Statistical analysis was performed by using two-way ANOVA (GraphPad Prism 5.03). $^{**}p < 0.01$, $^{***}p < 0.001$. See text for further details.

"clean" sulfur particles generated by a combination of milling and HPH. This method for particle production has been employed by us previously to produce good-quality particles of elemental selenium, and as Fig. 4 shows, can also be used readily for the production of elemental particles of the other relevant chalcogens, *i.e.* sulfur and tellurium.

The particles obtained via this combination of mechanical methods were more or less spherical in shape and, after successive homogenization, showed diameters in the range of 800 nm. It should be mentioned that the same method was also applied to produce particles of elemental (red) selenium and (amorphous) tellurium. In the case of selenium, the particles obtained were more or less spherical with diameters around 500 to 1000 nm. As for tellurium, the particles were round to lozenge-shaped, with (the longer) diameters around or slightly above 1000 nm. This is interesting, as chemically or enzymatically generated tellurium particles usually appear as rod-like objects and are not really well-suited for most biological applications.

These mechanically generated particles of known chemical composition, shape and size (distribution) were subsequently studied for their activity against cultured HCT-116 colon cancer cells. This particular cell line was chosen as it represents a "surface" tumour (in the colon) which can be approached by nanoparticles (a systemic delivery would surely be considerable more complex). Furthermore, we have used this cell line extensively in the past and hence are able to compare activities of the different compounds against diverse "benchmarks" (Busch et al., 2010; Saidu et al., 2013a).

Fig. 5 illustrates the activity of the different chalcogen particles against HCT-116 cells. It seems that at the concentration selected for this first screen (0.001% chalcogen content, corresponding roughly to 313 μM sulfur, 130 μM selenium and 78 μM tellurium, based on the total amount of chalcogen, not on the number of particles, see below), the sulfur particles are quite active, reducing the viability of



Spectrum	O	Na	Mg	S	Cl	Ca	Sr
area_ 206	31,01	9,70	3,03	11,89	12,99	28,16	3,22
area_ 207	9,74	31,93	2,58	2,33	38,16	14,09	1,18
area_ 208	31,32	5,98	2,35	10,28	13,11	33,36	3,60
area_ 209	1,37	36,03	-	0,63	53,25	8,72	0,00
Mean value:	18,36	20,91	2,65	6,28	29,38	21,08	2,00
Sigma:	15,18	15,26	0,35	5,63	19,84	11,59	1,71
Sigma mean:	7,59	7,63	0,17	2,81	9,92	5,79	0,85

Fig. 3. The “Marktbrunnen” in the suburb Burtscheid of Aachen, clearly illustrating the considerable deposits of solid matter which precipitate from the Aachen mineral water (**a**). A microscopic image of particles contained within water from the “Elisenbrunnen” at 10,000-fold magnification (**b**). EDX analysis of the water of Bad Nenndorf confirms the presence of sulfur and a range of other elements, such as calcium and oxygen (**c**). Please note that in contrast to the “Marktbrunnen”, the outlet of the “Elisenbrunnen” in Aachen (see Fig. 1) is cleaned regularly from such deposits as it is a mineral well certified for human consumption.

cells to around 50%. This effect becomes most pronounced after the 24 h interval, hence an inhibition of growth rather than direct kill seems to be the most likely explanation for this observation. A similar, yet lower activity is seen for the selenium particles. In contrast, the tellurium particles are particularly active, reducing cell viability to around 60% after 24 h. Interestingly, these particles also cause an almost immediate loss of viability (to about 70% compared to the control), pointing towards a more aggressive, possibly cytotoxic impact.

When discussing these activities in the context of molar concentrations, however, one must bear in mind that these preparations represent chalcogens (suspended) in a solution, not as or in solution. Here, the reactive surface area, which is determined by the shape and size of the different particles, by large determines the “true” activity of available and reactive chalcogen atoms. By simplifying these suspensions to simple solutions, one may therefore compare apples with horse apples.

4. Discussion

The results obtained as part of this study have revealed various interesting aspects of sulfur in the context of the environment, agricultural applications and medicine which require some comment. Returning briefly to our initial idea that mineral springs may represent a better source of biologically active sulfur when compared to, for instance, garlic and onions, we note that this hypothesis is supported by some of our findings, yet also needs some refinement. The ultimate scheme of such a process, which may embrace environmental (re-)sources and conversions in bioreactors, is shown in Fig. 6. Whilst this scheme is more or less a speculative “game plan” at this time, certain aspects can be discussed based on the results obtained already.

4.1. Taking the waters?

It is apparent from our studies that the mineral waters we have sampled exhibit some biological activity, even when diluted. Admittedly, the dilution factors, two-, three- or four-fold for the more active sample from Bad Nenndorf, are modest compared to biologically active compounds, such as garlic oil, which is active in ten-to hundred-fold dilution. Furthermore, the activity we have observed is also limited to some organisms, such as *S. feltiae* and *C. albicans*, whilst other organisms are less affected.

Still, one needs to bear in mind that the mineral waters employed, unlike concentrated oils from plants, are in any case highly diluted with respect to any matter dissolved in them. In the water from Bad Nenndorf, we basically start with a 2.4 mM solution of sulfide, the water from Aachen is even more “diluted” when it comes to sulfide and other potentially biologically active ingredients. It should also be noted that the mineral waters contain inorganic forms of sulfur, implying that the chemical diversity and activity is somewhat restricted when compared to OSCs. Later on, we will therefore turn to the idea of processing. Another aspect worth mentioning is that the mineral waters, as unprocessed natural products, are obviously not “pure” in a chemical sense. The presence of minerals other than sulfide, such as phosphate, borate and various cations, clearly complicates the picture and also renders an unambiguous causal relationship between a constituent - such as sulfide - and a particular biological activity very difficult.

Nonetheless, the sulfide-rich waters have a range of benefits which, in our opinion, outweigh the possible drawbacks. First of all, these waters are of a natural origin and are available readily, cost-

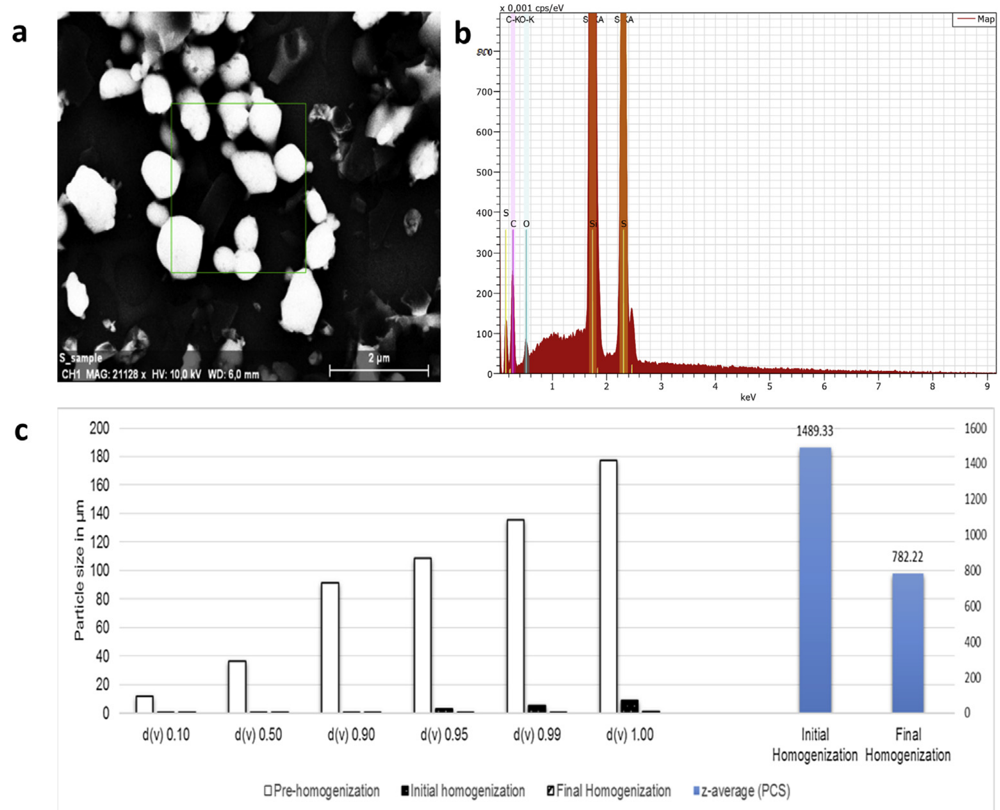


Fig. 4. Sulfur nanoparticles produced by a combination of dry milling and HPH. Microscopic image of particles at 21,000-fold magnification (**a**). EDX analysis to confirm the presence of sulfur (**b**). Laser diffraction analysis confirms the absence of large particles after HPH, while PCS analysis indicates that most particles have diameters in the range of 1500 nm after initial homogenization cycles and around 780 nm after further homogenization cycles (**c**).

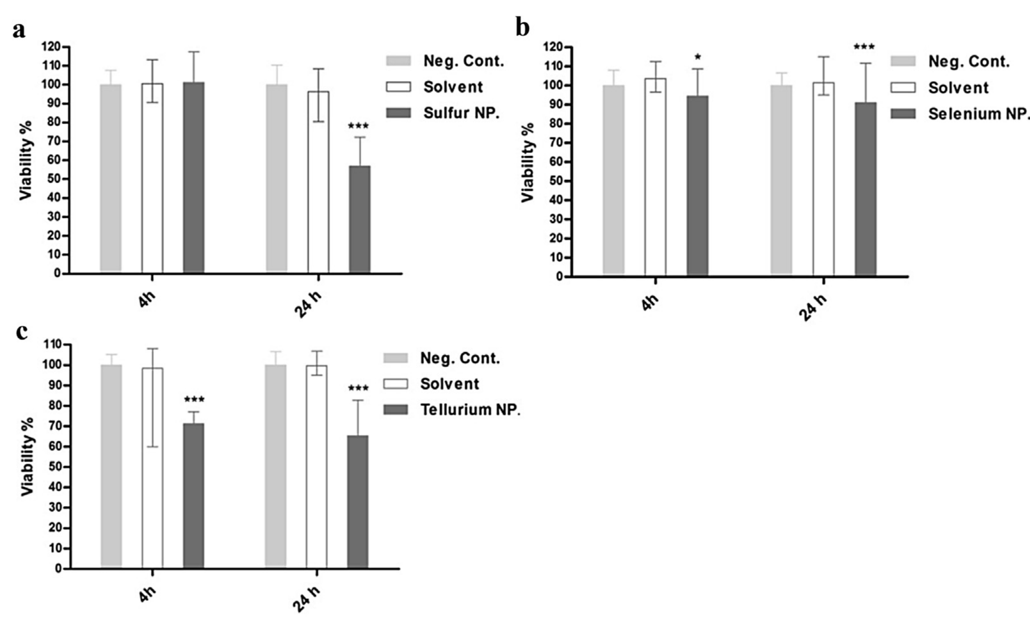


Fig. 5. Reduction of viability of cultured HCT-116 cells in the presence of mechanically generated sulfur (**a**), selenium (**b**) and tellurium (**c**) particles. Statistical significances were calculated using two-way ANOVA (GraphPad Prism 5.03). $^{*}p < 0.05$, $^{***}p < 0.001$. See text for further details.

effectively, in considerable amounts and without any processing. They compare well not only to sulfur-rich waste waters from farming, sewage treatment plants or Industry - which clearly cannot be applied and sprayed on fields - but also to other natural sources of sulfur. In contrast to botanical sources of sulfur, such as garlic, there is no need for plants to be grown, harvested, transported and processed. Sulfur-rich waters therefore represent a sustainable and ecological source of biologically active sulfur which

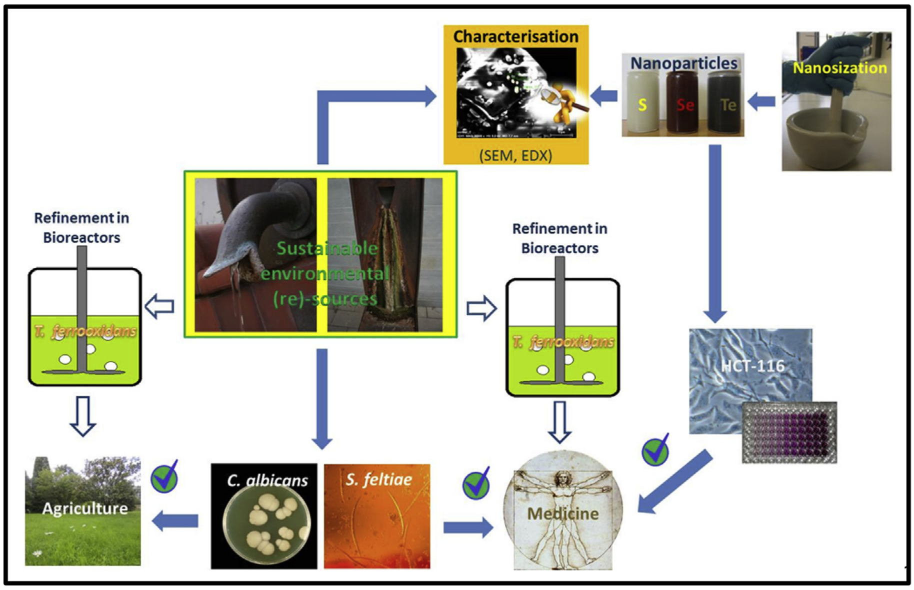


Fig. 6. Overall scheme of the possible uses of sulfur-rich waters as a sustainable natural resource. The idea of using an environmental product for subsequent environmental applications, possibly with an intermediary refinement process, is illustrated.

have been used by humans for various purposes since ancient times - in the case of the "Elisenbrunnen" since Roman Antiquity.

4.2. Potential applications of sulfur-rich water

Whilst it is correct that sulfur in these waters is "diluted" and that other minerals are also present, this possible critique can also be seen as benefit. If confirmed that such waters can act as mild phyto-protectants, for instance, they may be employed simultaneously for (a) the irrigation of fields, (b) a mineral enrichment and fertilization and (c) the protection of crops from pathogenic organisms. If such a "hat-trick" is possible will obviously depend on the precise composition of the water (some minerals may not be desired on fields), its spectrum of activity and, last but not least, the mode of application and ultimate fate of sulfide, hopefully in the soil - and not as smelly H_2S in the air. Indeed, a possible agricultural "hat-trick" will need to consider the impact of such water on the various plants and organisms in the field, on possible side effects caused by other minerals, on the enrichment of particular components in the soil, plants, and animal and human food chain, and on the ultimate fate of sulfur, which could be a matter of taste or even smell. The fact that each and every hour, hundreds of litres of these waters go into the sewer at each well, necessitates a closer look at the potential uses of such an environmental treasure.

Whilst direct medical applications of the waters in question are legend, a prolonged exposure of humans to H_2S-waters, let alone a regular oral uptake, is less attractive. Processed samples, containing, for instance, elemental sulfur or polysulfides (S_x^{2-}), may provide an attractive alternative, yet their production, biological activity, efficiency, selectivity and possible side effects need to be studied first in earnest. Here, issues related to the chemical reactivity and biological mode(s) of action will unavoidably have to accompany any activity studies in order to understand why and how such comparably simple molecules or materials are active.

4.3. Particulars, particulates and particularities

The formation of nanoparticles is another particular matter that may turn out to be complicated. Inspired by the presence of large deposits of elemental sulfur, which is frequently observed in and around such sulfur-rich mineral wells (see Fig. 3), we have therefore investigated the possibility that such waters may also contain small particles of sulfur. After all, simple oxidation of sulfide on air is sufficient to generate elemental sulfur, and during this process, the latter surely initially begins to precipitate as small(er) particles? Coincidentally, we have recently studied chemically generated sulfur nanoparticles (via the redox comproportionation of sulfide and sulfite) and have already found some interesting biological activities associated with these materials (Schneider et al., 2011).

Our present studies reveal that such waters are indeed rich in particles, yet the latter most likely are composed of various substances, from $CaSO_4$ known to be present in hard water and some other insoluble minerals to, possibly, more or less pure micro- and nanoscopic particles of elemental sulfur. It therefore appears that the matter of particulate matter in these waters is a particularly murky one, not only from the perspective of chemistry but also when discussing biological actions which may be associated with such particles. Whilst this is not entirely unexpected, we have learned the hard (water) way that we should not necessarily consider fresh, untreated mineral water of such wells as an immediate source of good-quality nanomaterials.

4.4. Turning waste into value

Nonetheless, the fact that some of these waters contain millimolar concentrations of sulfide, implies that they may be used as sustainable natural "raw materials" in processes which subsequently may indeed generate good-quality particles. After all, simple oxidation of such sulfide-rich waters on air seems to occur over the years at and near those wells, and mild oxidation under controlled conditions could be envisaged to produce colloidal or even nano-sulfur (see above). Interestingly, the so-called Claus process for the manufacture of elemental sulfur (Eq. (2)) relies on the redox comproportionation of sulfide (S^{2-}) and sulfite (SO_3^{2-}), and as mentioned already, we have recently employed this particular redox chemistry to generate sulfur nanoparticles of excellent quality in the laboratory using commercial Na_2S as source of sulfide (Schneider et al., 2011; Riedel, 1988).

$$SO_2 + 2H_2S \rightarrow 3S + 2H_2O \quad \Delta H^0 = -146kJ/mol \tag{2}$$

Eventually, one may speculate that sulfide-rich waters may even be used to detoxify fumes rich in SO_2, relying on this specific redox comproportionation reaction and leading to another, environmental "hat-trick". Such an approach would be particularly elegant chemically and beneficial environmentally, as it would turn two natural sulfur materials, one freely available, the other an environmental toxin, into a valuable nano-material which may be used for a number of purposes, for instance in the context of medicine (one example will be discussed below). We have initiated experiments with oxidizing agents such as H_2O_2, oxidized glutathione (GSSG), selenite and SO_2 already, yet the results obtained so far (not shown), whilst generally promising, are complicated by issues such as pH and concentrations required for a conclusive analysis. Clearly, the most suitable conditions for any "green chemistry" approach first need to be worked out in considerably more detail, with the likelihood that biologically active - yet "impure" - particles may be generated in the end.

4.5. Bio meets nano — towards a more versatile technology

At the same time, we are also considering a more biotechnological approach for the production of sulfur particles, for instance by employing bacteria such as *Thiobacillus ferrooxidans* for the oxidation of sulfide (Janssen et al., 1996). The formation of such elemental chalcogen particles by bacteria has been reported before. Recently, we have also successfully employed this kind of biotechnology to manufacture special, coated selenium particles of excellent quality and with interesting biological activity (Estevam et al., 2016). This particulate microbial chalcogen matter obviously necessitates considerably more in depth studies in the future.

The scheme shown in Fig. 6 provides a glimpse of the possible uses of bioprocesses and bioreactors for the conversion of sulfide-rich natural waters into refined products. In those reactors, selected bacteria may be able to use sulfide as nutrient to generate sulfur nanoparticles and possibly also other, more adventurous materials. These materials may include partially oxidized polysulfides S_x^{2-}, which are known already to possess a pronounced biological activity, but also mixed chalcogen compounds or particles, such as diverse sulfur-selenium species. Indeed, such mixed chalcogens are used already widely in anti-dandruff preparations. Eventually, the natural conversion of a natural water with the help of harmless bacteria may represent the ultimate goal in the large-scale production of innovative molecules for agricultural and medical applications.

4.6. Possible applications

As such possible applications need to be defined at some stage, we have therefore decided to take a short-cut and to continue our investigations at this stage with more defined sulfur particles obtained from chemically pure elemental sulfur via a combination of dry milling and HPH. For research purposes, this method still seems to be superior to chemically or enzymatically generated particles, as we can control the shape and size of the particles. Hence reliable causal relationships in subsequent biological activity studies become possible and we can also compare the (activity of) sulfur particles with the corresponding selenium and tellurium particles manufactured by the same method.

The results obtained so far in the HCT-116 cells are promising and point towards a possible anti-proliferative activity of the mechanically generated sulfur particles. If this activity is selective, for instance for certain cancer cells, and how this activity unfolds on a more molecular level and by which mode(s) of action, still needs to be investigated as part of a considerably larger study. It is also hardly surprising that the tellurium particles show activity against the HCT-116 cells as many tellurium compounds are biologically active against a spectrum of potential targets. Still, the use of "just" tellurium, *i.e*. particles of the elemental form of the element rather than complicated Te-compounds, is intriguing.

Eventually, sulfur is probably the most "amenable" of the three chalcogens under investigation when considered in the context of possible future applications in the fields of agriculture or medicine. Sulfur is not particularly toxic and occurs in the human body in large quantities and as part of many biomolecules. At the same time, sulfur is used in traditional pharmaceutical ointments and as part of more or less "green" pesticides. Neither elemental sulfur, selenium nor tellurium are soluble in water. Therefore the idea of using nanotechnology, and within this context either chemical, mechanical or biotechnological approaches, to unlock the "chalcogen potential", deserves further attention.

5. Conclusions

Eventually, our studies have considered several avenues to channel the free flow of sulfur-rich mineral waters into practical applications for the benefit of the environment and sustainable manufacture. We hope that we have been able to demonstrate the numerous benefits associated with such a waste-turned-value approach, and that our few selected studies conducted so far have been able to underline the potential for direct applications in the field and for refinement by biotechnology manufacture of equally or even more potent and valuable materials. There is obviously the need to investigate such speculative ideas further in the future. Still, our recent experiences with similarly simple sulfur species from the realm of organic chemistry, such as allicin and diallyltetrasulfide (DATTS), and selenium particles generated naturally with the assistance of *S. carnosus*, encourage such an approach. From the perspective of the environment, as well as from the point of view of readily available natural products, such red and golden chalcogen particles may provide many innovative leads for a greener future. Eventually, you may not have to be a chameleon to realize that "loving would be easy if your colors were like my dreams, red, gold and green".

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