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

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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

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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).

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

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