# In-vitro Testing of Arsenic Sulfide Nanoparticles for the Treatment of Multiple Myeloma Cells

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## ABSTRACT

In this study, α- and β-phase $As_4S_4$ nanoparticles were prepared by wet nanomilling using sodium dodecyl sulfate as a surfactant. The properties of the nanoparticles were characterized by particle size distribution and Raman scattering. A monomodal distribution, in the 150 – 200 nm range characterized the particle size of both phases after milling. Raman scattering revealed a common pattern for all studied particles. The existence of pararealgar was documented by Raman spectroscopy. The anticancer effects, of the milled species were confirmed for two multiple myeloma cell lines (RPMI-LR5 and OPM1) in-vitro.

***Keywords*:** nanomilling, arsenic sulfide, cancer, multiple myeloma, pararealgar

## INTRODUCTION

The medicinal use of arsenic and its derivates dates back to ancient times. Traditional Chinese medicines employ different forms of mineral arsenicals, such as orpiment $As_2S_3$, realgar $As_4S_4$ (REA) and arsenolite $As_2O_3$ (ATO), for the external (or even oral) treatment of psoriasis, syphilis, epilepsy, ulcers and malaria [1]. In Western pharmacological history, approximately 60 different arsenic preparations have been developed for medicinal use [2]. In 1878, the ability of Fowler's solution (1 % ATO in $K_2CO_3$) to reduce the white blood cell counts in leukemia patients was reported [3]. Currently, ATO (brand name Trisenox) is applied in the treatment of acute forms of leukemia [2, 4-5]. However, the toxicity of ATO is high, and the drug form causes severe side effects. REA is much less toxic than ATO – the acute oral toxicities are 3.2 g/kg and 32 mg/kg for REA and ATO, respectively [1]. The curative potential of REA has been reviewed recently [6]. However, its solubility (and consequently bioavailability) is low, and some sort of pretreatment is needed. Mechanochemistry, which applies several modes of high-energy milling (including nanomilling) involves the mechanical treatment of solids [7]. Nanomilling seems to be an effective pretreatment procedure that modifies the physico-chemical properties of solids [8-10]. The studies of the mechanochemistry of sulfides has also brought new stimuli into cancer research [11-12]. Here, the various forms of polymorphs and their improved bioavailability play a crucial role in the application of new drugs.

In this paper, we report a mechanochemical method to prepare nanosuspensions of α- and β-phases of arsenic sulfide. Their anticancer activity was tested, and their solid state properties were evaluated using various physico-chemical methods.

## MATERIALS AND METHODS

### Materials

The investigation was carried out with arsenic(II) sulfide (98 % $As_4S_4$ in purity, Sigma-Aldrich, USA, CAS 1303-32-8). In a separate experiment β-phase was prepared from α-phase by heating in an argon atmosphere at 270 °C during 60 min. The XRD-analysis estimated α-$As_4S_4$ (JCPDS 01-072-0686) and β-$As_4S_4$ (JCPDS 24-0078) in the samples under study.

### Nanomilling

The nanomilling of α- and β-phases was performed in a laboratory stirred mill PE-075 (Netzsch, Germany). The following milling conditions were used: 1 gram of α- or β-phase, respectively, 200 mL of 0.075 % sodium dodecylsulfate solution, material of the milling balls $Y_2O_3$ stabilized $ZrO_2$, loading of the mill 1176 g of 1.5 mm diameter balls, rotation speed of the mill shaft 1000 $min^{-1}$, milling time 30 min.

### Characterization of solids

The XRD measurements were performed by employing a X-ray diffractometer Rigaku Miniflex

(Rigaku, Japan). The following measuring conditions were applied: 2Θ range 0–40°, Co radiation, scan speed: 2 deg 2 theta/min, scan step: 0.2 theta 2 theta.

The Raman micro-spectroscopy was studied using a dispersive T64000 triple monochromator dispersive system (Hariba–Jobin Yvon, USA). Radiation of 647 nm from a Kr-ion was brought to a tight focus (1 micron spot size on the samples using a 0.5 mW power to excite Raman scattering).

The specific surface area was determined by the low temperature nitrogen adsorption method in a Gemini 2360 sorption apparatus (Micromeritics, USA).

The particle size distribution was measured by the method of photon cross correlation spectroscopy on particle sizer Nanophox (Sympatec, Germany).

## Biological activity

Suspensions of the arsenic sulfide nanoparticles for testing their in-vitro biological activity were prepared by filtration of wet nanomilled samples through a 0.22- μm filter. For testing in-vitro, the human multiple myeloma cell lines OPM1 and RPMI-LR5 were cultured in RPMI 1640 medium that had been supplemented with 10 % fetal calf serum as suspension cell cultures. All cells were maintained in 6 -cm dishes in a humidified incubator at 37 °C with 5 % $CO_2$. The effect of milled α- and β-polymorphs (serial dilutions) on the survival of cells was determined by an MTT assay [13]. The number of viable cells is proportional to the content of the MTT metabolite, formazan. The absorbance of formazan crystals dissolved in DMSO was measured at 540 nm and 690 nm in a microplate reader (Dynatech Lab Inc., USA). The $IC_{50}$ values were calculated from median-effect plots [14], taking into account the fractions of affected and unaffected cells (the $IC_{50}$ represents the concentration of arsenic that is required for 50 % inhibition with respect to the control sample). After a 30-min incubation in the dark at 37 °C, the cells were analyzed using a flow cytometer. Data acquisition was performed on Coulter Epics Altra flow cytometer equipped with a 488 nm excitation laser. For each analysis, $1x10^4$ cells were acquired as previously described [15]. Viability (fluorescein diacetate/propidium iodide) and apoptosis (Annexin-V-binding) were determined by standard flow cytometric assays. The data were analyzed with FCS software (DeNovo Software).

# RESULTS AND DISCUSSION

## Nanoparticles size distributions

The comminution of arsenic sulfide α- and β-phases by nanomilling is accompanied by an increase in the number of ultrafine particles and by the generation of fresh surfaces. The size distribution of nanoparticles is given in Fig 1. The as-received α-phase (1) showed a broad polymodal distribution of particles in the range 100 – 10,000 nm. After nanomilling, the distribution changed to a monomodal pattern where the main particle population had an average hydrodynamic parameter of 150 – 200 nm (2, 3). The patterns of the α- and β-phases were similar with values $D_{90}$ = 227 nm and 233 nm for the α- and β-forms, respectively ($D_{90}$ denotes that 90 % of particles have a lower diameter D than the given value) (Table 1). Nanosuspensions with a mean particle size in the 100 – 250 nm range are generally suitable for preclinical pharmaco-kinetic evaluation [9].

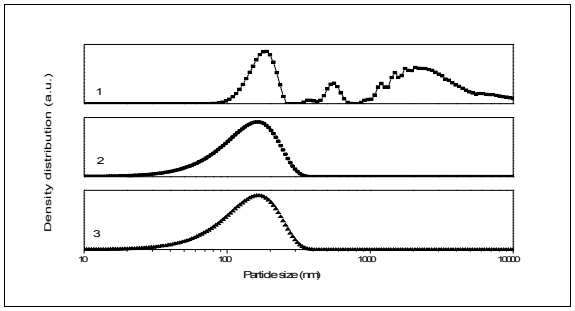


Fig. 1 Size distribution of arsenic sulfide particles: 1 – as received α-$As_4S_4$ (non-milled), 2 – α-$As_4S_4$ (milled), 3 – β-$As_4S_4$ (milled)

| Sample | $D_{10}$ | $D_{50}$ | $D_{90}$ |
|---|---|---|---|
| | [nm] | | |
| As received α-phase (non-milled) | 154 | 1458 | 4348 |
| α-phase (milled) | 63 | 138 | 227 |
| β-phase (milled) | 65 | 141 | 233 |

Table 1 $D_{10}$, $D_{50}$ and $D_{90}$ values of arsenic sulfide particles

## Bulk changes

The structure of both arsenic sulfide phases was elucidated using Raman scattering (Fig. 2). To analyze the Raman lineshape of the samples, the Raman spectrum of non-milled α-phase (1) was also recorded. In all three samples, we can observe a Raman lineshape that is, more or less, common and representative of the majority phase in the samples. The line shape is extremely rich; close to 12 lines are observed, which are all reasonably well-resolved. However, the milled samples (2, 3) have less-intensive spectra in comparison with the non-milled sample (1), which can be the result of the partial loss of crystallinity as a consequence of milling [7]. The Raman spectrum is characterized by a pair of strong peaks at 150 – 250 $cm^{-1}$

and 320 – 380 $cm^{-1}$, which resemble the As-As-S bending and As-S stretching frequencies of realgar, respectively [16]. The peak A at 271 $cm^{-1}$ appears only in the Raman spectrum of pure pararealgar [17] and does not overlap with the other arsenic sulfide Raman peaks.

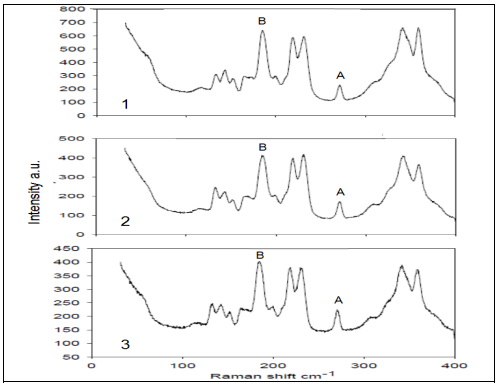


Fig. 2 Raman lineshape of arsenic sulfide particles: 1 – as received α-$As_4S_4$ (non-milled), 2 – α-$As_4S_4$ (milled), 3 – β-$As_4S_4$ (milled)

We have selected peak B at 184 $cm^{-1}$ as a reference "non-pararealgar" peak representing the samples under study. The values $I_A/I_B$ = 0.33, 0.41 and 0.43 have been calculated as a measure of the possible transformation to pararealgar for α-$As_4S_4$ (non-milled) and α-$As_4S_4$ (milled) and β-$As_4S_4$ (milled), respectively. There is practically no difference in $I_A/I_B$ values between two milled samples and only a slight enhancement in milled samples in comparison with the non-milled one.

The interpretation of the observed changes is not straightforward. The impact of milling energy could play some role. The used wet milling mode is usually applied in solids to create surface changes (particle size, surface area, etc.) and to a lesser extent for bulk changes [7]. However, transformation to pararealgar is also very sensitive to light management during sample manipulation as well as the applied excitation light during Raman experiments. Justification of the above mentioned assumptions needs further experiments.

## Biological activity

Multiple myeloma (MM) accounts for 10 % of all hematologic malignancies. In principle, the disease remains incurable [3]. For biological tests, the human MM cell lines RPMI-LR5 and OPM1 were selected. In Fig. 3 the inhibition effects of α- and β-phase arsenic sulfide on the cell line RPMI-LR5 are depicted. The data represent the proportion of dead cells in samples treated with arsenic sulfide in comparison to the control untreated sample. The $IC_{50}$ values were also calculated.

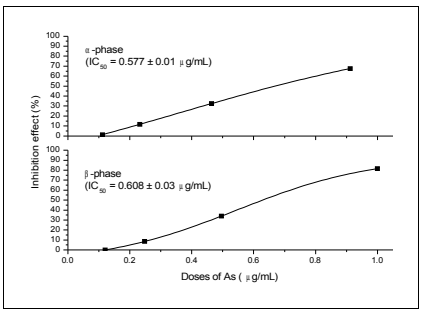


Fig. 3 Inhibition effect vs. dose of As for RPMI-LR5 cells treated with α-$As_4S_4$ and β-$As_4S_4$ (both milled)

Plots for the inhibition effect were similar for both arsenic sulfide phases. The general trend for the reduction of viability with increasing arsenic content is well documented. The values of $IC_{50}$ were 0.577 ± 0.01 μg/mL and 0.608 ± 0.03 μg/mL for α-phase and β-phase, respectively. The resemblance of the plots for both phases, as well as the similarity of the $IC_{50}$ values, demonstrates the equivalence of the applied arsenic sulfide forms, despite the different pretreatment processes used (nanomilling and/or thermal treatment + nanomilling). It is possible to interpret this phenomenon from a physical perspective: it is known that upon exposure to visible light, both α- and β-phases can transform from red solids into bright yellow pararealgar, at least partly [16-17]. This phenomena may play decisive role in this study. The Raman study presented in the previous paragraph supports this interpretation. On the other hand, equivalency of both polymorphs in their biological activity overcome the frequently registered fact in pharmacy that only one from several polymorphs are effective as drug [18].

Because of similarity of both phases, the further study on biological activity was concentrated only on α-$As_4S_4$ nanoparticles (milled) induce an increase of G2M phase of multiple myeloma OPM1 cells (Fig. 4), decrease of viable cells and an increase of proportion of affected cells (Fig. 5). Apoptotic (Annexin-V+PI-) and late apoptotic (Annexin-V+PI+) cells were observed. The calculated $IC_{50}$ = 0.21 ± 0.09 μg/mL is lower than the $IC_{50}$ values for RPMI-LR5 cells. The OPM1 cells are less resistant and more sensitive to the arsenic treatment.

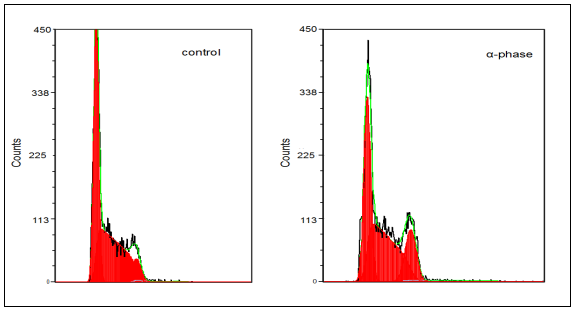


Fig. 4 Increase of G2M phase for OPM1 cells

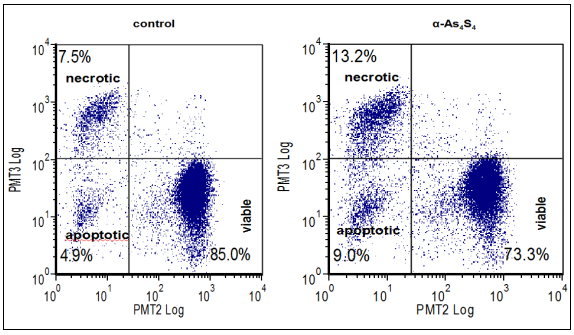


Fig. 5 Viability of OPM1 cells

## CONCLUSIONS

A shift in the particle size distribution from the micro- to nano-sized region of the arsenic sulfide α- and β-phase $As_4S_4$ nanoparticles was detected as a consequence of nanomilling. Raman scattering revealed common pattern of all particles under study and existence of pararealgar. The treatment of the human multiple myeloma cell lines RPMI-LR5 and OPM1 with the α- and β-phases produced by nanomilling resulted in similar biological activity, with a promising potential to substantially reduce the viability of the cancer cells. The obtained results show that arsenic sulfide nanoparticles can be considered a potential drug for cancer treatment.

## ACKNOWLEDGEMENTS

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