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Hexyltrimethylammonium ion enhances the copper-chelating properties of ammonium thiomolybdate in an in vivo zebrafish model

--Manuscript Draft--

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Jian-Ping Jin & Henry Forman
Editor-in-Chief.
Archives of Biochemistry and Biophysics

December 12th, 2023.

Dear Editors J. Jian-Ping and H. Forman,

I am pleased to present an original research article entitled "HEXYLTRIMETHYLAMONIUM ION ENHANCES THE COPPER CHELATING PROPERTIES OF AMMONIUM THIOMOLYBATE IN AN *IN VIVO* ZEBRAFISH MODEL" for consideration and publication in the Archives of Biochemistry and Biophysics (ABB).

Originally intended for submission to the Journal of Inorganic Biochemistry, our manuscript was redirected to ABB by the editor, who suggested that the scope of your journal aligns more closely with the content of our research. After a thorough examination of ABB's topics, we wholeheartedly concur with this recommendation.

We previously found interesting results in exploring the chelating properties of octyltrimethylammonium thiotungstate, therefore, this manuscript was inspired by our previous study to incorporate hexyltrimethylammonium ion and evaluate its effects on the chelating properties of thiomolybdate and thiotungstate. Through comprehensive in vitro and in vivo assay techniques, coupled with cyclic voltammetry, we sought to evaluate the impact of this incorporation on the chelating properties of both thiomolybdate and thiotungstate. We firmly believe that this manuscript contributes valuable insights to the field and is well-suited for publication in ABB.

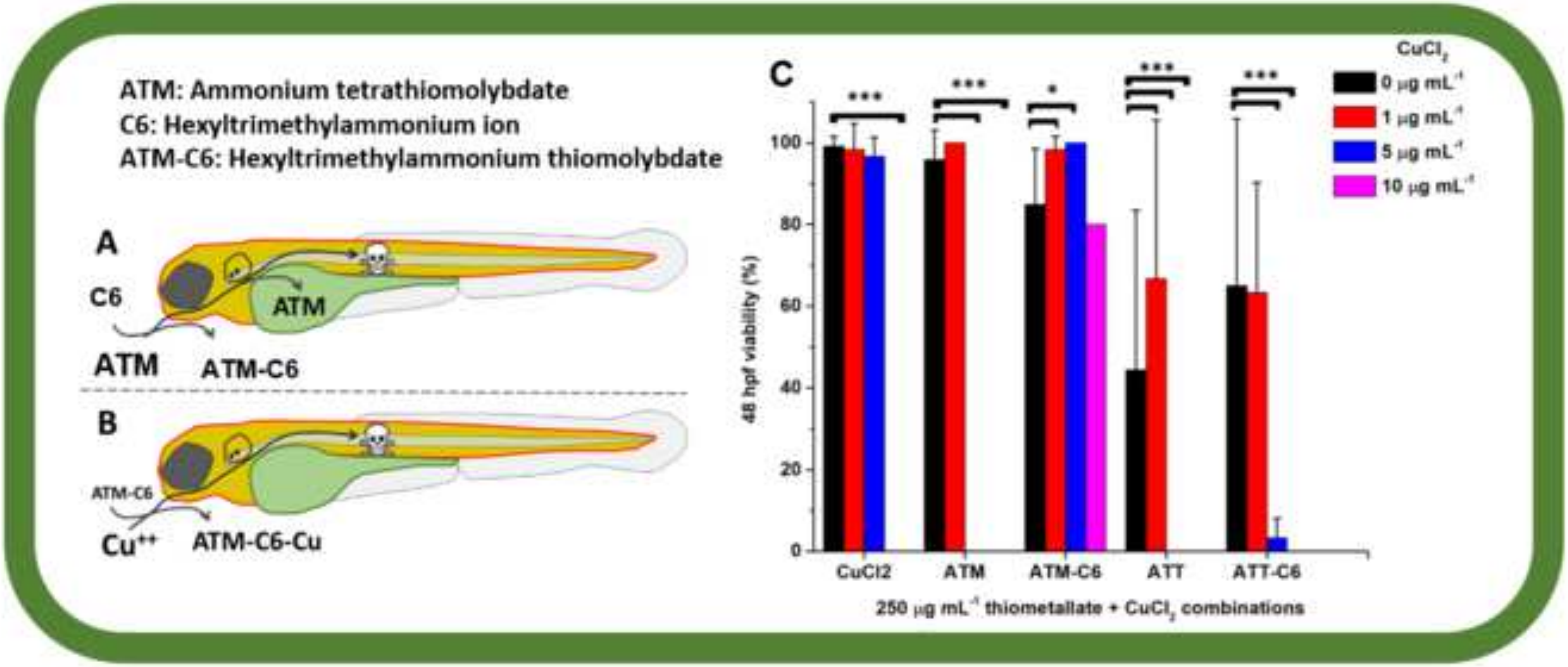
This manuscript has not been published before and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

We appreciate your time and consideration of our submission.

Sincerely,
Dra. Lilian Beatriz Romero Sánchez.

Highlights

- Ammonium and hexyltrimethylammonium thiometallates show low toxicity
- Thiometallates form complexes with copper at a 2:1 Cu:thiometallate ratio
- Hexyltrimethylammonium ion reduces toxicity in extended thiometallate treatments
- Hexyltrimethylammonium ion inhibits thiomolybdate accumulation in zebrafish
- Hexyltrimethylammonium thiomolybdate exhibits excellent copper-chelating properties


ATM: Ammonium tetrathiomolybdate
C6: Hexyltrimethylammonium ion
ATM-C6: Hexyltrimethylammonium thiomolybdate
A
C6
ATM
ATM
ATM-C6
B
ATM-C6
Cu++
ATM-C6-Cu
C
CuCl2
0 μg mL-1
1 μg mL-1
5 μg mL-1
10 μg mL-1
***
***
*
***
***
48 hpf viability (%)
0
20
40
60
80
100
CuCl2
ATM
ATM-C6
ATT
ATT-C6
250 μg mL-1 thiometallate + CuCl2 combinations

Hexyltrimethylammonium ion enhances the copper-chelating properties of ammonium thiomolybdate in an *in vivo* zebrafish model

Autores

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Abstract

Ammonium and hexyltrimethylammonium thiomolybdates (ATM and ATM-C6) and thiotungstates (ATT and ATT-C6) were synthesized. Their toxicity was evaluated using both *in vitro* and *in vivo* approaches via the zebrafish embryo acute toxicity assay (ZFET), while their copper-chelating properties were studied using cyclic voltammetry, as well as *in vivo*. Cyclic voltammetry indicated that all thiometallates form complexes with Cu in a 2:1

Cu:thiometallate ratio. Both *in vitro* and *in vivo* assays demonstrated low toxicity in BALB/3T3 cells and in zebrafish embryos, with high IC_{50} and LC_{50} values. Furthermore, the hexyltrimethylammonium ion played a crucial role in enhancing viability and reducing toxicity during prolonged treatments for ATM and ATT. In particular, the ZEFT assay uncovered the accumulation of ATM in zebrafish yolk, averted by the incorporation of the hexyltrimethylammonium ion. Notably, the copper chelation assay highlighted the exceptional viability of embryos when cultured in $CuCl_2$ and ATM-C6, even at high $CuCl_2$ concentrations, emphasizing its potent copper-chelating properties. The hatching assay further confirmed that copper-chelation properties of ATM-C6 mitigates inhibitory effects induced by thiomolybdates and $CuCl_2$ when administered individually.

Keywords

Chelating activity, Anti-copper therapy, Alkyltrimethylammonium thiometallate, Zebrafish model, Cyclic voltammetry.

1. Introduction

Copper (Cu) at small concentrations is known to participate in numerous biological reactions, which are essential for human health[1]. However, it is categorized as a heavy metal, that if found at high concentrations in living organisms, may lead to poisoning and neurodegenerative diseases, such as Wilson´s, Parkinson´s and Alzheimer´s, as well as oxidating and inflammatory processes such as multiple sclerosis, arthritis, and cancer. Metal chelation therapy is a commonly used strategy for the treatment of metal intoxication, which helps to decrease metal concentrations in living organisms[2]. Chelating compounds may form complexes with metallic ions in solution through the interaction between the ligand and a central metal atom, generating a complex ring-like-structure. Once the metal has been chelated, its bioavailability decreases and it can be eliminated efficiently from the organism, preventing metabolic damage.

In this sense, ammonium thiometallates from metals like molybdenum (ATM) and tungsten (ATT) show excellent chelating properties, useful for anti-copper therapies[3]–[6] since these compounds bind strongly to copper, making it easy to eliminate it through urine. ATM forms complexes with copper and albumin to reduce copper concentrations in blood for the treatment of Wilson’s disease[7]–[11], cancer or even in animal models of fibrotic, inflammatory, and autoimmune diseases[12]–[15]. These thiometallates also possess interesting anti-inflammatory and antioxidant properties.

Unlike ATT, ATM has been widely studied and tested for many clinical applications. For example, Brewer et al. [7]–[10], [16], [17] have contributed to show the effectiveness of ATM in the treatment of Wilson´s disease and have developed experimental models that suggest that the therapy with ATM inhibits angiogenesis, tumoral necrosis and suppresses metastasis from breast, oesophagus, prostate, colorectal, cerebral and neck cancer[13]–[15]. On the other hand, the United States Food and Drug Administration (FDA) approved the phase III clinical trial for the treatment of Wilson´s disease using ATM (NCT00004339). Moreover, at least 12 registered assays include the use of ATM for the treatment of metastatic non-small cell lung (NCT01837329, phase I), oesophagus carcinoma (NCT00176800, phase II), and primary biliary cirrhosis (NCT00805805, phase III), among others.

ATM and ATT have been historically employed as precursors for MoS_2 and WS_2 hydrotreating catalysts. An important advance in the development of these types of materials was the incorporation of carbon in the precursors, which provides the catalysts with crystalline structure defects that function as active sites for the adsorption of molecules, resulting in enhanced catalytic activity[18]–[21]. In the late 90´s, Alonso et al. [22], [23] proposed a novel synthesis method where carbon was incorporated into the catalysts parting from tetraalkylammonium thiometallates in aqueous medium $(NR_4)_2MS_4$, where M= Mo or W and R = alkyl chains. More recently, it was found that ATT substituted with the octyltrimethylammonium ion (ATT-C8) presented chelating and antioxidant activity, as well as better biocompatibility when compared to its non-carbon containing analogous compound[6].

Chelation and biocompatible properties of new reagents may be preclinically tested both *in vitro* and *in vivo* in several animal models. *In vitro* cell culture and *in vivo* tests such as the chorioallantoic membrane (CAM), the mouse corneal micropocket, or the rodent model assay[24] are examples of these models. Due to its external and abundant offspring, zebrafish (*Danio rerio*) provides a powerful model of transparent embryonic development[25], which is increasingly used in cost-effective screens[26]–[30], e.g. toxicity or inflammation, angiogenesis or regeneration modulatory effects of reagents, and drug testing over models of human disease[25], [26], [31]. Furthermore, to test chelating properties of reagents *in vivo*, zebrafish embryo assays are also increasingly used [6], [32]–[34].

The toxicity of copper and biocompatibility and the chelating ability of thiotungstate salts have been previously tested in zebrafish embryos[6]. Following this experimental initiative, the aim of this work is to evaluate the chelating and biocompatibility properties of thiomolybdates and thiotungstates to which the hexyltrimethylammonium ion has been incorporated: ATM-C6 and ATT-C6. In this study, we synthesized the thiometallates and assessed their toxicity *in vitro* using BALB/3T3 murine embryonic fibroblast cells, as well as in vivo using zebrafish embryos, respectively. We evaluated copper-chelating properties of the thiometallates through both electrochemical method and *in vivo* assays using cyclic voltammetry and zebrafish embryos, respectively. These results were further analyzed to deduce the influence of the metal and the hexyltrimethylammonium ion.

2. Materials and Methods

2.1. *Thiometallates synthesis*

2.1.1. Synthesis of ammonium thiometallates:

A mixture of an aqueous ammonium sulfide solution($(NH_4)_2S$) and the corresponding ammonium salt ($(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ for ATM; $(NH_4)_6H_2W_{12}O_{40} \cdot xH_2O$ for ATT) previously dissolved in water was prepared[20], [35]. Reactions were performed in a reflux system at 60°C with constant stirring. Afterwards, solutions were placed in an ice bath to enhance crystallization. The final products, which appeared as needle-like red and yellow crystals, respectively for ATM and ATT, were vacuum filtered and stored at inert atmosphere to prevent oxidation.

2.1.2. Synthesis of hexyltrimethylammonium thiometallates:

The incorporation of hexyltrimethylammonium ion in the thiometallates was performed in aqueous medium, following reports by Alonso et al. [22]. The corresponding thiometallate (ATM; ATT) was mixed with hexyltrimethylammonium bromide ($C_{19}H_{42}BrN$) at a 1:2 molar ratio. Mixtures were continuously stirred for three hours at room temperature and furtherly vacuum filtered, resulting in red and yellow fine powders for ATM-C6 and ATT-C6, respectively, which were stored at inert atmosphere.

2.2. Characterization methods

Optic spectroscopy assays were performed to analyze the chemical structure of the synthesized thiometallates. Ultraviolet-Visible (UV-Vis) analysis was performed in a VWR UV-1600PC spectrometer at a wavelength range of 200-500 nm using water as solvent for the sample preparation. Fourier-Transform infrared spectroscopy was performed at transmission mode in a Perkin-Elmer Spectrum GX spectrometer at a 4000-400 cm^{-1} wavenumber range. The composition analysis of light elements (C, S, N and H) was assessed in LECO TruSpec(R) CHN equipment suited with IR and TCD detectors.

2.3. Electrochemical studies

The copper-chelating properties of the thiometallates was studied using cyclic voltammetry in a standard three-electrode cell, with a Metrohm Autolab PGSTAT 302N potentiostat/galvanostat. The working electrode consisted in a 3 mm diameter glassy carbon electrode, which was polished with powdered alumina prior to every experiment, the reference electrode was a KCl saturated Ag/AgCl (BASI-RE5) electrode, while a platinum plate was used as counter electrode. Cyclic voltammograms were performed at a 50 mV/s scan rate, starting from the OCP. The working electrode surface was activated via cyclic voltammetry prior to the presented voltammograms.

2.4. In vitro assays

2.4.1. Cell culture

BALB/3T3 (ATCC CCL-163) murine embryonic fibroblasts cell line was cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. Culture was performed in a humidified atmosphere with 5% CO_2 at 37 °C.

2.4.2. Cytotoxicity assay

The assay consists of the culture of 5000 cells in culture medium for 24 h using a 96-well microplate. Furtherly, 100 μL of previously resuspended solutions of the studied thiometallates at different concentrations (0.1-1000 μg mL^{-1}) were added. Cells were incubated for 72 h at 37 °C in a humidified atmosphere with 5% CO_2. After the exposure time, compound cytotoxicity was determined with the aid of a MTT kit (Roche, Switzerland), where 0.5 mg mL^{-1} of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) were added to each well and incubated for an additional four hours at the same conditions. Afterwards, 100 μL of solubilizing solution were added, and cells were incubated overnight. Lastly, absorbance was measured using a microplate reader in a Multiskan FC, Thermo Scientific (Waltham, MA, EE. UU.) photometer. Inhibitory concentration at 50% (IC_{50}) was calculated considering untreated control cells to have 100% viability.

2.5. In vivo assays

2.5.1. Zebrafish embryo collection

Offspring embryos of AB wild type and/or *Tg(fli-1:EGFP)y1*[36] adults were used. Adults zebrafish were provided by the Centre of Experimentation and Animal Behaviour at the University of Malaga and at IBIMA-platform Bionand. After fertilization, eggs were collected and incubated in E3 embryo medium at 28 ± 0.1ºC. Fish were handled under National Laws and approval by the University of Malaga Bioethics commission.

2.5.2. Zebrafish embryo toxicity assay (ZFET)

Ten embryos per well at four hours-post-fertilization (hpf) were incubated in 3 mL of embryo medium[37] in 6-well plate. Stock solutions of thiometallates were prepared under inert atmosphere and stored at 4 ºC. Working solutions were prepared just prior to use. Various solutions of thiomolybdates and thiotungstates were tested, and the exposures lasted four days at 28 ºC. At 120 hpf, zebrafish were anesthetized and observed on the plate under a magnifying microscope. The survival/mortality, hatching rates, and morphological changes of zebrafish embryos and larvae in each treatment group were assessed daily determined. The lethal concentration 50 (LC_{50}, $\mu g\ mL^{-1}$) was determined by analyzing cumulative mortality at 48 and 72 hpf and and represents the concentration causing the death of 50% of the zebrafish.

Additionally, in this assay, chorion hardening was studied via X-Ray Diffraction (XRD) of chorions obtained from dechorionated embryos previously incubated with thiomolybdates. Tungsten accumulation in embryo yolks was studied using X-Ray Photoelectron Spectroscopy (XPS) of embryos treated with thiotungstates. Prior to this procedure, embryos were freeze-dried, and their yolk opened with tweezers.

XRD data were collected on a PANanalytical EMPYREAN automated diffractometer. Powder diffractograms were recorded in Bragg-Brentano reflection configuration by using the PIXcel 3D detector. The XPS analysis was performed in a Physical Electronics PHI VERSAPROBE II spectrometer equipped with a multichannel hemispherical electron analyzer. The Al k_α line (1486.6 eV, 15kV) was used as a monochromatic radiation excitation source. The energy scale was calibrated using Cu $2p_{3/2}$, Ag $3d_{5/2}$ y Au $4f_{7/2}$ lines at the binding energy (BE) of 932.7, 368.2 and 83.95 eV, respectively. Atomic concentrations (%) of the characteristic elements were determined from the spectra after the subtraction of a Shirley-type background and considering the relative sensitivity factors of each corresponding photoelectronic line.

2.5.3. Zebrafish embryo chelation assay

To test chelation, 15 embryos at 24 hpf were exposed to 12 experimental conditions, three for each thiometallate (1 μg mL^{-1} $CuCl_2$ + 250 μg mL^{-1} thiometallate, 5 μg mL^{-1} $CuCl_2$ + 250 μg mL^{-1} thiometallate, 10 μg mL^{-1} $CuCl_2$ + 250 μg mL^{-1} thiometallate), to which their survival-hatching rates and morphologies were daily studied. Both assays were run in triplicate and more than 60 embryos per concentration were tested. After all experiments, zebrafish embryos and larvae were euthanized and processed as organic waste[38].

2.6. Phenotypic, statistical analysis

Besides embryo viability, hatching and morphological defects, the developmental stage was also estimated each experimental day. Observations were done under a magnifying microscope (Nikon SMZ-445 model) or from digital images obtained from a Sensor Sony IMX290C-HDMI – 1 digital camera. The LC_{50} was calculated from the mortality curves using MS Excel (Microsoft Office, Windows 11) following log-linear regression models[39]. Growth delay was estimated by comparison to anatomical description previously published[40].

Statistical significance of frequencies was calculated from 2x2 contingency tables using the Chi-Square (X^2) or the Yates´ Chi-Square tests in Statgraphics (Statgraphics Technologies, Inc.) or programmed in an Excel table (Microsoft). When any expected value was below 3, Fisher´s exact test was calculated in a Past program (Oyvind Hammer). Significance was expressed with asterisks - $p < .05$ (*), $p < .01$ (**), and $p < .001$ (***) -.

3. Results and discussion

3.1. Thiometallates characterization

The obtained thiometallates were characterized using spectroscopic techniques and elemental analysis of light elements (C, S, N and H). Table 1 summarizes the obtained UV-Vis and FT-IR results, spectra are shown in Figure S1. Three bands were identified with UV-Vis spectroscopy, associated with the $(MoS_4)^{-2}$ and $(WS_4)^{-2}$ thiomolybdate and thiotungstate tetrahedral groups, respectively[22], [41]. The absorption signals for thyomolybdates are observed at 467, 316 and 242 nm for, whereas for thiotungstates, they are shown at lower wavelengths, namely 393, 278 and 216 nm. Figure S1 shows that the absorption spectra of the hexyltrimethylammonium ion-containing thiometallates is the same as the analogous materials without them.

Table 1. Spectroscopic analysis results.

Sample	**UV-Vis λ (nm)**	**FTIR ʋ(cm^{-1})**
ATM	467, 316, 242	464 ν(Mo-S); 3128 ν(N–H), 1395 δ(N-H)
ATM-C6	467, 316, 242	464 ν(Mo-S); 3005, 2925, 2852 ν(C-H); 1468 δ(C-H)
ATT	393, 278, 216	464 ν(W-S); 3128 ν(N–H), 1395 δ(N-H)
ATT-C6	393, 278, 216	464 ν(W-S); 3005, 2925, 2852 ν(C-H); 1468 δ(C-H)

In FT-IR, it is seen that all the thiometallates display an intense absorption peak at a wavenumber of 464 cm^{-1}, which corresponds to the stretching vibration of the Metal-S bond. In the spectra for the ATM and ATT samples, two peaks were obtained, at 3128 and 1395 cm^{-1}, which correspond to the stretching and bending of the N-H bonds in the NH^{+4} group. These latter signals were considerably attenuated in the spectra for the hexyltrimethylammonium tiometallates, as expected, while new signals appeared at 3005, 2925, 2852 and 1468 cm^{-1}, attributed to the stretching and bending of C-H in the methyl and methylene groups, respectively[22], [41], [42].

Both the FT-IR and UV-vis results suggest that the presence of carbon does not significantly modify the structure, bond energy and electronegativity of the Metal-S bond. Similar results have been previously reported for alkyl-containing thiometallates by other groups[22], [41], [43].

Elemental analysis of light elements was also performed, where C, S, N and H were analyzed. Results are displayed as mass percentage (wt. %) in Table 2. It was found that the C/S atomic ratio increased from 0.008 to ≈ 4.6, when ATM-C6 and ATT-C6 are compared with their analogous thiometallates without hexyltrimethylammonium ion. This significant increase in carbon content confirms the incorporation of the hexyltrimethylammonium ion in the thiomolybdates and thiotungstates, which was also supported by the UV-Vis and FT-IR results.

Table 2. Results of the quantitative analysis of light elements.

Sample	C (wt%)	S (wt%)	N (wt%)	H (wt%)	C/S atomic ratio
ATM	0.12	38.24	11.13	3.16	0.008
ATM-C6	38.93	23.18	5.45	8.01	4.481
ATT	0.09	32.88	8.10	2.31	0.007
ATT-C6	34.46	19.34	4.39	6.75	4.755

3.2. Electrochemical studies

Cyclic voltammetry was performed to explore the copper-chelating properties of the studied thiometallates. Firstly, cyclic voltammograms of copper ions from $CuCl_2$ were obtained to identify the oxidation and reduction peak positions of the species, as well as the achieved faradic currents of these peaks when $CuCl_2$ is measured in PBS solution (shown in Figure S2.). Cyclic voltammograms for $CuCl_2$ were obtained in PBS solution (pH~7.4) with 0.1 M and 0.01 M $CuCl_2$ solutions. Scanning started at the open circuit potential (OCP) (0.268 V (Ag/AgCl)) and proceeded towards positive potentials up to 0.5 V vs. Ag/AgCl, to then proceed in a negative sense for the cathodic scan up to -0.5 V vs. Ag/AgCl.

Since at the beginning of the scan, copper is only expected as Cu^{2+} ions in the solution, no further oxidations occur within the potential limit. Two peaks are observed in the following cathodic scan, signaled in Figure S2 as c_1, and c_2, in order of appearance. These peaks are associated with the reduction of Cu^{2+} to Cu^{1+} and Cu^{1+} to Cu^0 , respectively for c_1 and c_2[44], [45]. With the further anodic scan, an anodic peak a_1 corresponds to the oxidation of Cu^0 to Cu^{1+} while a_2 corresponds to the oxidation of Cu^{1+} to $Cu^{2+,}$ as expected.

To assess the ability to chelate copper ions of the studied thiometallates, cyclic voltammetry scans in the same conditions were performed, with the addition of these compounds to achieve Cu:thiometallate molar ratios of 10:1, 5:1, 2.5:1 and 2:1. The effects of these additions in the voltammograms are shown in Figure 1.

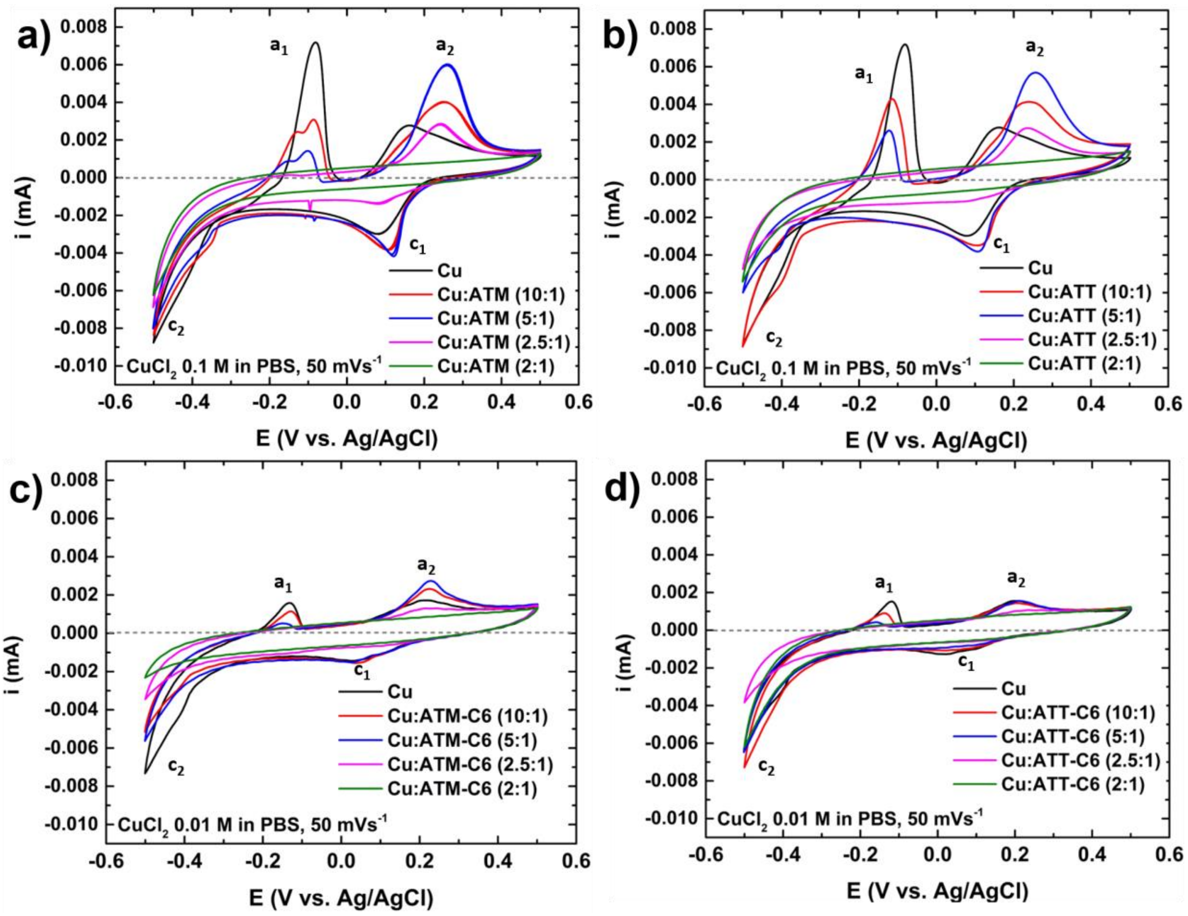

Figure 1. Cyclic voltammograms $CuCl_2$ in PBS with the correspondent additions of the thiometallates. a) ATM, b) ATT, c) ATM-C6, d) ATT-C6.

The experiments showed the appearance of two reduction peaks, along with two corresponding oxidation peaks, comparable to those in Figure S2 for $CuCl_2$ and are also signaled as c_1, c_2, a_1 and a_2. In all cases, the reduction peaks between additions, correspond in position to the reductions showed for $CuCl_2$, however, there are slight differences in the position of the oxidation peaks, which could be due to the addition of the thiometallates or to the oxidation of other species along with Cu, such as formed complexes. A special case is presented for ATM, showing two oxidation peaks in the a_1 region, possibly due to the oxidation of the formed complex. Another difference between the voltammograms of $CuCl_2$ and those with thiometallate additions is the double layer capacitance region, increasing in some cases for the latter ones due to the addition of thiometallates.

Both ammonium thiometallates (ATM, ATT), as well as ATM-C6, at the 10:1 and 5:1 ratios show a current increase for c_1 and a_2 peaks, when compared to $CuCl_2$ without thiometallate addition, which was confirmed with the integration of the mentioned peaks taking the double layer capacitance region as the baseline, and obtaining the correspondent charge at the scan rate. Results are shown in Figure 2. This, in turn confirms that, since no additional copper was added, the higher current and charge after the integration of the peaks, are due to the simultaneous reduction and oxidation of Cu-thiometallate complexes along with non-complexed Cu at these regions, which could be also the reason of the shifted position of the peaks. This phenomenon increases with the concentration of ATM, ATT and ATM-C6 up to an extent since more Cu-thiometallate complexation is expected, however, at the 2.5:1 Cu:thiometallate ratio, the current and charge start to decrease, until the peaks are not visible at the 2:1 ratio, which would mean that non-complexed Cu is not available in the solution. In ATM and ATT, this point coincides with the appearance of a precipitate in the cell, and for ATM-C6 and ATT-C6, turbidity is observed in the electrolyte, both phenomena are possibly due to insolubilization of the formed complexes with their increasing concentration. In the case of the a_1 peak, the tendency for the first two ratios is different with respect to a_2, since the current and charge decrease with the increasing concentration of thiometallates, possibly meaning that at this potential region, the contribution of residual copper being oxidized is more important than the Cu-thiometallate complex.

For ATT-C6, the current and charge of the c_1, a_1 and a_2 peaks decrease with every thiometallate addition, possibly meaning that the formed complex presents higher stability in this potential region, not being oxidized or reduced, with only the contribution of residual free Cu ions in all of the peaks.

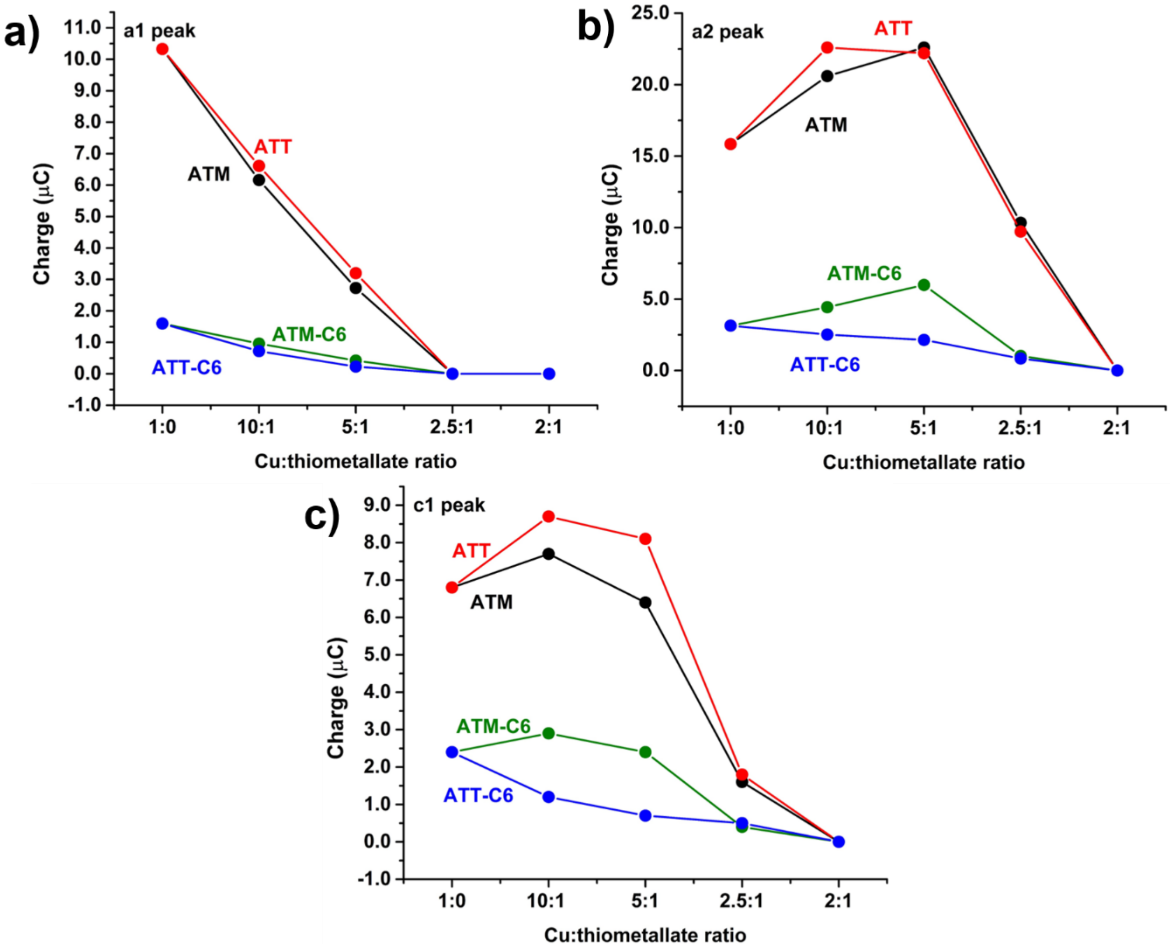

Figure 2. Results of peak integration for a) a_1, b) a_2 and c) c_1 peaks of the cyclic voltammograms with respect to the Cu:thiometallate ratios.

3.3. *In vitro* cytotoxicity assay (IC_{50})

During the *in vitro* assay, BALB/3T3 cells, known to be an excellent cell model to study biometal-induced cytotoxicity[46], were incubated for 72 h in the presence of ATM, ATM-C6, ATT and ATT-C6 at different concentrations (0.1-1000 μg mL^{-1}). Figure 3 shows cell viability graph after exposure, from which cytotoxicity was evaluated and IC_{50} was determined. Results showed that thiomolybdates and thiotungstates behave differently at low concentrations (<250 μg mL^{-1}). At a 10 μg mL^{-1} concentration, ATT and ATT-C6 presented 100% of cell viability, while the thiomolybdates at the same concentration presented 25% of cell mortality. Up to a concentration of 250 μg mL^{-1}, thiotungstates presented low cell cytotoxicity, however, surpassing this concentration, the tendency is inverted and thiotungstates show higher cytotoxicity in comparison to thiomolybdates.

After the assay, IC$_{50}$ was determined, resulting in 7045, 1953, 1233, and 788 μg mL^{-1} for ATM, ATT, ATM-C6, and ATT-C6, respectively. These results determined that thiotungstates present higher toxicological effects on BALB/3T3 cells when compared to their Mo analogous. It was also found that the thiometallates with incorporated hexyltrimethylammonium ion presented higher cytotoxicity than their non-carbon containing analogous. Nonetheless, all compounds presented high IC$_{50}$ values, indicating low cytotoxicity on BALB/3T3 cells. Our results suggest that the synthesized thiometallates are safe and biocompatible to be used in biomedical applications.

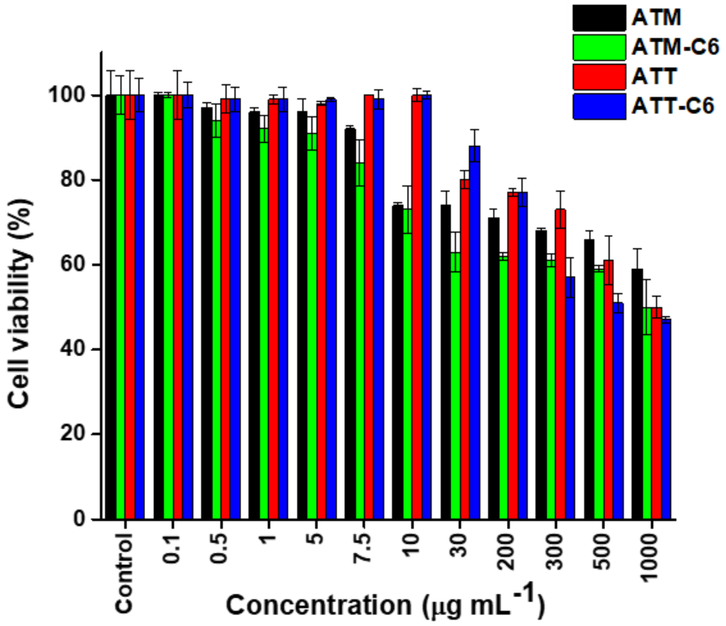

Figure 3. Cytotoxicity assay on BALB/3T3 cell line. Graph of MTT assay after 72 h treatment of BALB/3T3 cells with various concentrations of thiometallates. Positive control shows BALB/3T3 cells without the exposure to thiometallates. The cell viability is expressed as % cell viability in comparison to a positive control.

3.4. *In vivo* zebrafish larvae studies

For *in vivo* testing with zebrafish embryos, thiometallates solutions ranging from 1 to 1000 μg mL^{-1} were prepared using the solution in which the embryos were incubated as solvent (E3 medium) which contains salts of Na, K, Ca, and Mg. Prepared solutions have a coloration, which increases its intensity with concentration; thiomolybdate solutions are stained brown while thiotungstate solutions have a faint yellow coloration. It was also observed that when thiomolybdate solutions (ATM and ATM-C6) were prepared significant precipitation occurs at concentrations over 100 μg mL^{-1}. Unlike thiomolybdates, thiotungstate solutions (ATT and ATT-C6) did not precipitate, except for the highest concentrations, where a faint precipitation occurred. These observations may be related to the capacity of thiomolybdates to form complexes with the ions contained in the solvent (Na, K, Mg, Ca), which tend to precipitate at high concentration.

The general effect of the administration of these thiometallates to the embryo medium is size reduction of the embryo, hatching delay and lethality at high concentrations. In principle, we focused our attention to the latter effect analyzing the process in a zebrafish embryo acute toxicity (ZFET) assay.

3.5. Zebrafish embryo toxicity assay (ZFET) (LC_{50})

We have carried out a ZFET assay to evaluate *in vivo* toxic effects of thiometallate solutions. Four-hpf embryos were incubated in increasing concentrations of thiometallate solutions in E3 embryo medium (1, 10, 100, 250, 750 and 1000 μg mL^{-1} ATM, ATM-C6, ATT and ATT-C6) at least three times. Viability and phenotypes of embryos were daily studied until 120 hpf stage. Based on the findings, we graphed the survival rate and calculated the LC_{50}.

The LC_{50} ($\mu g\ mL^{-1}$) was determined following a log-linear approach for high concentrations and was found to vary with the embryonic stage and the thiometallate studied (Table 3). In general, LC_{50} decreases ~~with the progress of the study and embryonic stages,~~ which is due to the cumulative effect of salts (compare the results for 72 and 48 hpf in Table 3). In our study, the hexyltrimethylammonium ion significantly increases the LC_{50} of ATT (ATT-C6 versus ATT), suggesting a lower toxicity. Similar findings were observed in an earlier study from our group, where the introduction of the octyltrimethylammonium ion (ATT-C8) into ATT yielded comparable results regarding cell viability/cytotoxicity[6]. Notably, our results display lower LC_{50} than prior studies by our group where thiotungstates were administrated at 24 hpf. This suggests a significant effect of these compounds over early stages of embryogenesis in this species. Anyhow, our study further supports previous results on the lower toxicity provided by incorporation of the hexyltrimethylammonium ion in the structure of the ATT.

Nevertheless, when compared to ATM, the obtained results are different, as the hexyltrimethylammonium ion decreases the high LC_{50} observed for the non-carbon containing analogous.

Table 3. LC_{50} ($\mu g\ mL^{-1}$) of thiometallates calculated at 48 and 72 hpf.

Thiometallate	48 hpf	72 hpf
ATM	1 E^{+7} ± 2 E^{+7}	1107 ± 501
ATM-C6	334 ± 34 *****	304 ± 28
ATT	309 ± 179	201 ± 100
ATT-C6	529 ± 438	374 ± 244

Footnote: ~~XX ±YY is mean ± SD of three independent replicates~~.

***** is difference significance between the thiometallate and its analogue with the hexyltrimethylammonium ion under the equal slopes test[47].

We have further displayed these results in survival rate plots (Figure 4). Whereas LC_{50} of ATT-C6 is higher than the one obtained for ATT, both show similar survival profiles for concentrations over 250 μg mL^{-1}. Below this concentration, the survival rates are significantly different, avoiding or significantly reducing embryo lethality at any ATT-C6 concentration used (Figure 5). This suggests that the LC_{50} increase provided by the hexyltrimethylammonium ion of this compounds resides in an effect preferentially occurring at 100 μg mL^{-1} concentrations or below. This delimits a potential therapeutical dose of the thiometallate.

Thiomolybdates showed, in turn, a very different behaviour with respect to their tungsten analogous. ATM presented extremely low toxicity at stages prior to 96 hpf, in contrast to ATM-C6. This is reflected by the high LC_{50} observed for ATM and the much lower LC_{50} shown by ATM-C6 (Table 3). Nevertheless, ATM and ATM-C6 toxicities vary when embryos over 96 hpf are studied, being ATM/ATM-C6 survival rates comparable to those of ATT/ATT-C6 (Figure 4). At this stage, ATM-C6 toxicity shows a significant reduction when compared to ATM, increasing its viability rate at concentrations below 100 μg mL^{-1}, as ATT-C6. This suggests that under long treatments, ATM lethality is reduced by the presence of the hexyltrimethylammonium ion, as in the case of ATT (Figure 4).

In this assay, we have not found an increase of osmolar effects, such as cardiac oedema, or inflammation signs. Only a significant delay of embryonic growth has been observed when ATM or ATM-C6 treated embryos are studied. In general, our results limit the potential use of these thiometallates to concentrations below 100 μg mL^{-1} where zebrafish embryo mortality is completely avoided.

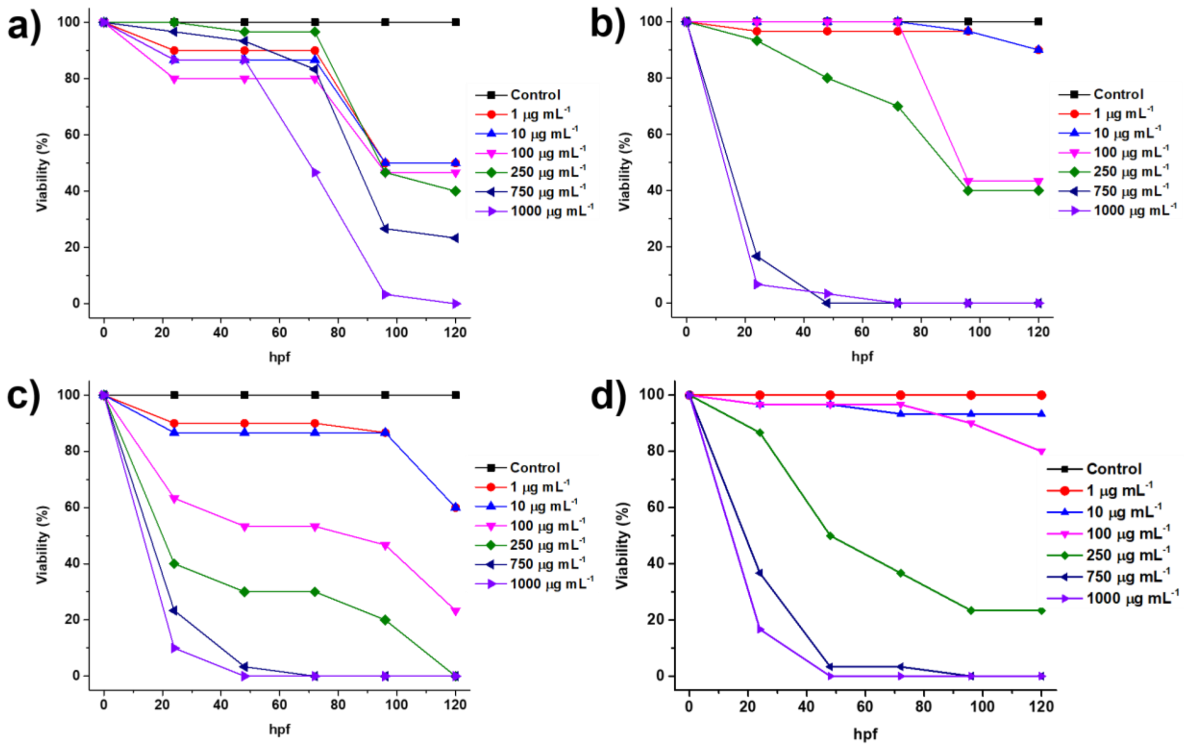

Figure 4. Survival rates of zebrafish embryos grown under different concentrations of thiometallates. a) ATM; b) ATM-C6; c) ATT and d) ATT-C6. All experiments were done in triplicate.

During the ZFET assay, it was observed that ATM and ATM-C6 form a brown precipitate, potentially as complexes formed with the ions found in the E3 medium. This same phenomenon was also observed in preliminary voltammetry tests where the used electrolytic medium was PBS, containing sodium and potassium salts (data not shown). The brown precipitate can also be found over the chorion surface, which hardens up, avoiding embryo hatching. ATT and ATT-C6 form, in turn, a faint yellowish precipitate, which becomes more evident as concentrations increase in the embryo medium. These thiotungstates do not induce any chorion hardening and the hatching rate of embryos resembles control.

Interestingly, the brown coloration of the precipitate induced by the ATM is also accompanied by a significant shift to a rose-to-brown colored yolk displayed by embryos incubated with increasing concentrations (Figure 5). As stated above, these embryos also show reduction of size suggesting a growth arrest which deviates them from the expected body plan of early developmental stages. This growth retardation was also observed in an assay performed with 24 hpf dechorionated embryos (data not shown). These results rule out a causal relationship between chorion hardening and embryo growth.

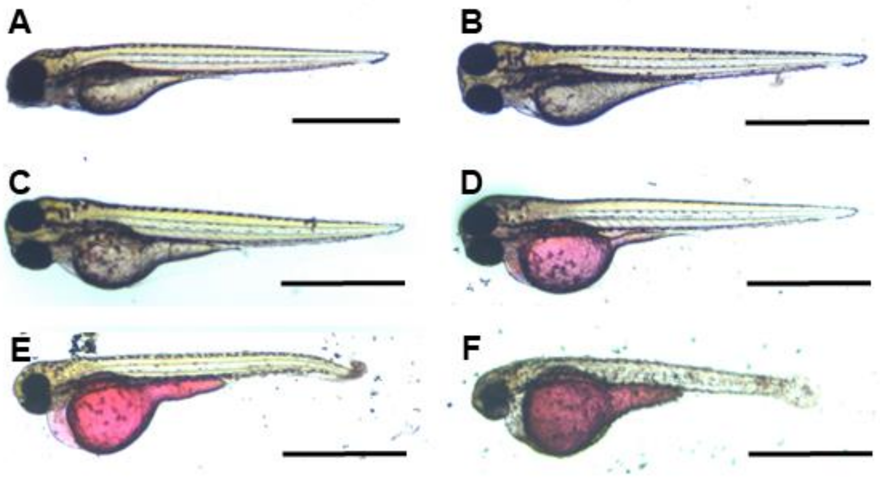

Figure 5. Increase in yolk colour and embryo growth reduction with increasing ATM concentration. 72 hpf zebrafish larvae after 48 h of ATM solution exposure; A) E3 medium; B) 1 μg mL^{-1} ATM; C) 10 μg mL^{-1} ATM; D) 100 μg mL^{-1} ATM; E) 250 μg mL^{-1} ATM; F) 1000 μg mL^{-1} ATM; Scale bar: 1000 μm.

Chorion hardening is an interesting result observed in these ZFET assays. This was further investigated the correlation between the formation/deposition of thiomolybdate crystals or thiomolybdate complexes with the salts present in the E3 medium, specifically in the context of the hardened chorion. For these studies we performed XRD analysis of chorions treated with 250 μg mL^{-1} ATM and ATM-C6 for three days as well as for a non-treated chorion.

All normalized and indexed XRD diffractograms are shown in Figure S3. In general, these diffractograms are similar, displaying a broad peak at $2\theta \approx 27°$, pointing out the amorphous nature of the chorion structure. This main signal coincides with important diffraction peaks of

$Na_2SO_4{\cdot}10H_2O$ (JCPDS-ICDD 74-0937), possibly overlapping with this signal. The diffractograms also show two additional incipient peaks at 2θ: 16 and 42°, which coincide with the main diffraction peaks of $Na_2SO_4{\cdot}10H_2O$ and NaCl (JCPDS-ICDD 04-020-4597), respectively. The presence of these salts suggests the crystallization of the components of the E3 medium. When the non-treated chorion pattern is compared to those obtained from thiomolybdate-treated chorions, no significant changes are found. Nevertheless, the 2θ signal at 42° is significantly stronger for the ATM-treated chorion, suggesting an increase of crystallized NaCl. Additionally, there is a slight shift of the main diffraction signal ($2\theta \approx 27°$) for both thiomolybdate-treated chorions, which could be due to the relative increase of the NaCl concentration, or to the presence of other types of salts in the chorion. According to these XRD results, the presence of thiomolybdates may promote the NaCl crystallization or form complexes with other salts in the E3 medium that cannot pass through chorion channels gradually sticking to its external surface, inducing chorion hardening.

It is noteworthy to state that the chorion diffractograms do not indicate the presence of molybdenum crystals. This suggests that these substances may be in an amorphous state or as very small crystals, undetectable with this technique.

In some of the experiments, ATM was dissolved in DMSO prior to the final E3 dilution (up to 0,5%) trying to prevent the precipitate. In these cases, although the precipitate also formed, the yolk remained colorless. Furthermore, the LC_{50} was reduced to 644.5 $\mu g\ mL^{-1}$ at 48 hpf. Whereas embryos incubated in ATM-C6 also show growth depletion, their yolks are completely uncolored in both 0.5% DMSO and non-DMSO dilutions. This suggests that the low toxicity of ATM over embryos below 96 hpf in the survival rate plots (Figure 4a) could be due to the accumulation of this compound in the yolk. The over accumulation of ATM in the yolk at 96 hpf, could then affect the viability of the embryo. The incorporation of the hexyltrimethylammonium ion in ATM may prevent the interaction with the yolk, avoiding its pigmentation and granting a lower LC_{50} at 48 and 72 hpf. However, at 96 hpf embryos treated with ATM show higher cytotoxicity compared to ATM-C6, suggesting that ATM-C6 is less toxic in longer treatments. Growth retardation has not been significant in ATT and ATT-C6 treated embryos.

Even though no chorion hardening, or yolk coloration, were observed for zebrafish embryos incubated with thiotungstates, 72 hpf larvae treated with 250 μg mL^{-1} ATT and ATT-C6 were analyzed using XPS to determine the presence of tungsten accumulation. For this we freeze-dried larvae, opened their yolks with fine tweezers, and subjected their yolk content surfaces to XPS. No sign of tungsten was observed. This indicates the absence of tungsten accumulation in the yolks of treated larvae (Table S1).

3.6. Zebrafish chelation assay

The chelating properties of this type of compounds were further studied in a copper chelation assay in zebrafish embryos, building upon a prior test conducted by our research team[6]. In this work, 15 embryos per well were cultured in 6 well plates in combinations of $CuCl_2$ at three concentrations (1, 5, and 10 μg mL^{-1}) and 250 μg ml^{-1} thiometallate. Viability of embryos, body deformities and hatching were documented each day after administration of the combined medium. More than three replicas were performed for each combination.

Figure 6 displays the viability results at 48 hpf of the zebrafish embryos in the chelation assay. ~~Whereas no consistent results were observed for the study of body deformities~~, the presence of $CuCl_2$ and thiometallate significantly pertubed the frequency of larva viability. The results show that $CuCl_2$ at 10 μg mL^{-1} induces complete lethality (zero viability). As evidenced in the ZFET assay, this test reveals that thiometallates at 250 μg mL^{-1} lead to a decrease in the viability of zebrafish embryos. However, this effect shows some recovery when combined with 1 μg mL^{-1} of $CuCl_2$. These observations suggest an interaction between copper and thiometallate, resulting in a reduction in the bioavailable thiometallate amount. With the exception of ATM-C6, 5 and 10 μg mL^{-1} $CuCl_2$ reduced viability synergistically with thiometallates, supporting the absence of adequate chelation. Only a slight recovery was observed in embryos treated with 5 μg mL^{-1} $CuCl_2$ + 250 μg mL^{-1} ATT-C6 (approximately 5%) significantly higher than the 0% observed with ATT.

Significant results for viability were found with at 10 μg mL^{-1} $CuCl_2$ + 250 μg mL^{-1} ATM-C6 compared to 10 μg mL^{-1} $CuCl_2$ control. $CuCl_2$-induced lethality is avoided when ATM-C6 is combined (around 80% viability). Embryos cultured with 10 μg mL^{-1} $CuCl_2$ and 250 μg mL^{-1} of any other thiometallate remains zero. This supports a strong chelation of copper ions exerted by ATM-C6 something not found with ATM. These results stress the importance of the hexyltrimethylammonium ion in the stabilization of thiometallate-copper complexes, which decreases the bioavailability of thiometallate and copper in solution.

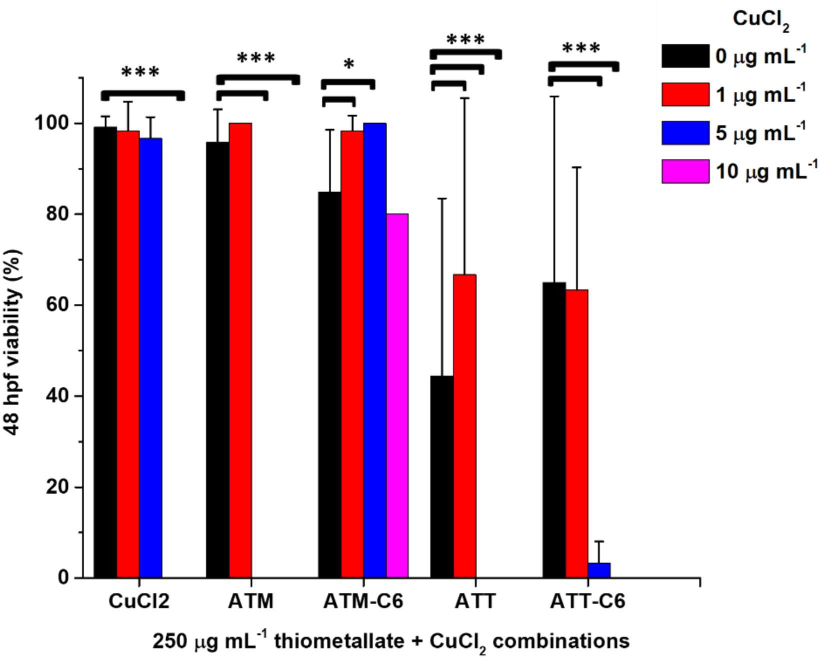

Figure 6. Viability percentage of 48 hpf zebrafish embryos under different culturing $CuCl_2$ + 250 μg mL^{-1} thiometallate combinations. Only the combination with ATM-C6 recovers 10 μg mL^{-1} $CuCl_2$ lethality.

During the assay, changes in the hatching time of zebrafish embryos were observed, which tupically hatch between 48 and 72 hpf. The results are presented in Figure 7, which shows hatching at 72 hpf of the treated embryos. It is well known that the zebrafish hatching enzyme (ZHE1) catalyzes the digestion of the chorion[48], and its activity is limited or inhibited by the presence of Cu[49]. This effect depends on the copper concentration and the stage of the zebrafish embryo and has been previously reported by our group and other research groups[6], [50]–[53]. These results are consistent with this study, as it was found $CuCl_2$ reduces hatching at 72 hpf at a concentration of 1 μg mL^{-1}. Although embryos cultured in $CuCl_2$ at 5 μg mL^{-1} are alive at 72 hpf, hatching is completely inhibited. As previously stated, 10 μg mL^{-1} is a concentration of $CuCl_2$ lethal for zebrafish embryos, therefore, there is no hatching.

The impact of thiometallates on embryo hatching varies between those containing molybdenum and those with tungsten. Thiotungstates at 250 μg mL^{-1} do not significantly affect hatching compared to the control. This observation aligns with previous research from our group, where it was noted that thiotungstates did not cause a delay in embryo hatching [6]. Nevertheless, there is a noticeable delay in the hatching of zebrafish embryos cultured in 250 μg mL^{-1} of thiomolybdates. However, significant recovering was observed in embryos incubated in 1 and 5 μg mL^{-1} $CuCl_2$ + 250 μg mL^{-1} ATM-C6. This supports the notion that ATM-C6 and copper interact to form a complex mitigating the effects observed when copper and ATM-C6 when administered separately on embryo hatching. This also suggests that the complex formed reduces the precipitation on the chorion surface of ATM-C6 that had been observed in the ZFET assay.

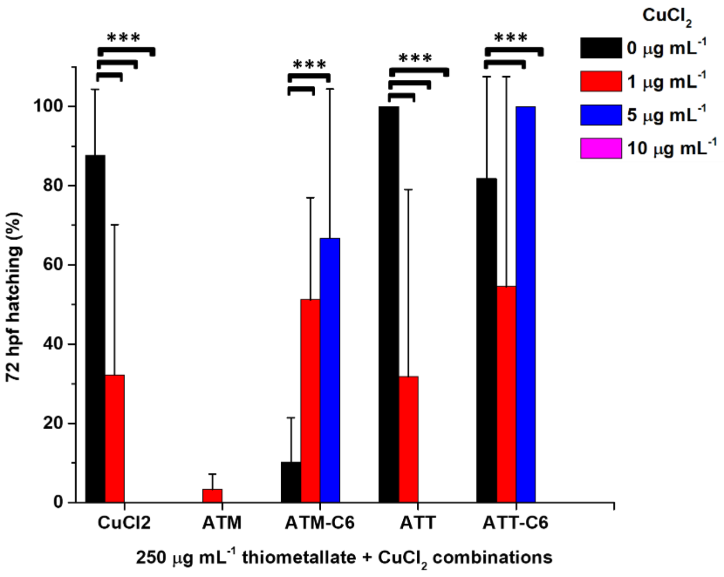

Figure 7. Hatching percentage of 72 hpf zebrafish embryos under experimental combinations.

These results suggest a potential inter-relationship of thiometallates and ions in the medium that influence several biological properties of zebrafish embryos in standard culture conditions.

4. Conclusions

Ammonium and hexyltrimethylammonium thiometallates of molybdenum and tungsten were synthesized. The effect of the hexyltrimethylammonium ion on the viabilidad/cytotoxycity and copper-chelating properties of ATM and ATT was studied *in vitro* and *in vivo* and by cyclic voltammetry. The study of the copper-chelating properties of the compounds via cyclic voltammetry showed that the studied thiometallates are able to completely chelate Cu ions at a 2:1 Cu:thiometallate ratio. Both *in vitro* and *in vivo* assays show low toxicity for BALB/3T3 cells and zebrafish embryos. For all thiometallates, high IC_{50} and LC_{50} were found, with ATM standing out.

Other conclusions we can draw from the ZFET assay: firstly, thiotungstates do not affect the growth of embryos nor do they accumulate in embryos. On the other hand, thiomolybdates harden the chorion and delay growth at high concentrations. ATM appears as non-toxic after three days of incubation due to its storage in the yolk, on the other hand, ATM is sensitive to DMSO (absence of yolk color in embryos treated with ATM dissolved in DMSO). The incorporation of the hexyltrimethylammonium ion into the ATM prevents it from accumulating in the yolk. Furthermore, the ZEFT assay highlighted a promising outcome – the incorporation of the hexyltrimethylammonium ion resulted in a reduction of toxicity for both ATM and ATT during prolonged treatments.

Finally, the copper chelation assay in zebrafish embryos indicates that thiometallates at 250 μg mL^{-1} decreased the viability of the zebrafish embryo, but recovery was observed when combined with 1 μg mL^{-1} of $CuCl_2$, suggesting an interaction between copper and thiometallate. This effect was more evident for embryos cultured in 10 μg mL^{-1} $CuCl_2$ + 250 μg mL^{-1} ATM-C6, which exhibited significantly greater viability, highlighting a strong chelation of copper by ATM-C6. Furthermore, embryos treated with 10 μg mL^{-1} of $CuCl_2$ and 250 μg mL^{-1} of other thiometallates showed zero viability, emphasizing the unique chelating properties of ATM-C6. The results highlight the importance of hexyltrimethylammonium ion in stabilizing thiomolybdate-copper complexes, reducing bioavailability and preventing $CuCl_2$-induced lethality. The hatching assay revealed a delay and inhibition of hatching induced by thiomolybdates and $CuCl_2$, respectively. But recovery was observed with 1 and 5 μg mL^{-1} $CuCl_2$ + 250 μg mL^{-1} ATM-C6, suggesting the formation of complexes that mitigate individual effects. In conclusion, our results highlight the potent copper-chelating properties of ATM-C6 and its potential applications in anti-copper therapies.

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6. Bioethical approvals

Research was under A/ES/12/I-22 and A/ES/12/24 notifications (Spanish Ministry of Ecological Transition and Demographic Challenge) and 100-2018-T approval (UMA Bioethics commission).

7. Conflicts of interest

Authors declare that they have no competing financial interests or personal relationships that may have influenced the work reported in this article.

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Sample CRediT author statement

KV-G: Investigation, Writing-Reviewing and Editing. PE-I, KP-V: Methodology, Validation, Data curation. JC-R: Conceptualization, Visualization, Resources. SL-R, CC-S, PV-G have contributed during zebrafish research, data analysis, and draft reviewing. MM-B has conceptualized/supervised zebrafish studies, acquired/administrated zebrafish fundings, written zebrafish texts, and reviewed/edited the manuscript. LBR-S: Writing-Original draft preparation, supervision, formal analysis, project administration.