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Evaluation of Toxicity with Brine Shrimp Assay

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[Abstract] The *in vivo* toxicity of new metallodrugs either as Small Bioactive Molecules (SBAMs) or Conjugates of Metals with Drugs (CoMeDs) or their hydrogels such as with hydroxyethyl-methacrylate (HEMA) (pHEMA@SBAMs or pHEMA@CoMeDs) are evaluated by the brine shrimp assay. Thus individuals of *Artemia salina* larvae are incubated in saline solutions with SBAMs, CoMeDs, pHEMA@SBAMs or pHEMA@CoMeDs or without for 24 h. The toxicity is then determined in terms of the mortality rate of brine shrimp larvae. Brine shrimp assay is a low cost, safe, no required feeding during the assay, while it requiring only a small amount of the tested agent.

Keywords: Bioinorganic Chemistry, Toxicity, *Artemia salina*, Larvae mortality of brine shrimp

[Background] The approval of cisplatin in the clinical treatment of cancer boosted the development of the field of bioinorganic chemistry or medicinal inorganic chemistry. The discovery of new active metallodrugs requires the elucidation of their mode of action. Thus, for example, the targeted delivery of antiproliferative metallodrugs to malignant cells, the activation of inorganic prodrugs and the advent of nanoscience have prompted the scientists to spotlight the toxicity of them (Metzler-Nolte and Guo, 2016). Especially, the research on the design and development of new metallodrugs (either as Small Bioactive Molecules (SBAMs) or Conjugates of Metals with Drugs (CoMeDs)), includes their *in vitro* testing against numerous cancerous cell types and their *in vivo* toxicity evaluation towards model organisms (Sainis *et al.*, 2016, Stathopoulou *et al.*, 2018; Banti *et al.*, 2016, 2018, 2019 and 2020; Chrysouli *et al.*, 2018a, 2018b and 2020; Latsis *et al.*, 2018, Milionis *et al.*, 2018; Karetsi *et al.*, 2019; Polychronis *et al.*, 2019; Ketikidis *et al.*, 2020; Rossos *et al.*, 2020). For example: When *Artemia salina* larvae were incubated with the copper(II) complex of amantadine ($AdNH_2$), with formula $\{[AdNH_3^+]\bullet[CuCl_3]^-\}$ (**CA**) (Banti *et al.*, 2020) or the corresponding one of the silver(I) with penicillin G (PenH) $[Ag(pen)(CH_3OH)]_2$ (PenAg) (Ketikidis *et al.*, 2020) for 24 h, the percentages of survival of brine shrimp larvae at 30, 60, 90, 120 and 150 μM of **CA** were (78.3±10.2), (85.4±6.5), (87.9±9.6), (82.6 ±10.8) and (76.9±11.9) %, respectively. The survival rate of brine shrimp larvae at the concentrations of 150 μM, is similar with the corresponding one of the non-treated larvae, suggesting its non toxic behavior. In case of **PenAg**, the percentage of survival of brine shrimp larvae at 37, 74.5, 150, 220 and 1050 μM are (94.7±2.5), (87.3±5.0) (82.6±4.7), (63.4±6.1) and (11.0±5.0) %, respectively, indicating toxicity at the concentration of 1050 μM. Moreover, in the case of the hydrogel which derives by the dispersion of the cluster $\{[Ag_6(\mu_3\text{-}HMNA)_4(\mu_3\text{-}MNA)_2]^{2-}\bullet[(Et_3NH)^+]_2\bullet(DMSO)_2\bullet(H_2O)\}$ (**AGMNA**), (H_2MNA= 2-thio-nicotinic acid), in polyhydroxyethyl-methacrylate (pHEMA) (pHEMA@AGMNA-1) (Rossos *et al.*, 2020), no

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mortality rate of brine shrimp larvae was found, upon their incubation with pHEMA@AGMNA-1 for 2, 4, 6, 8 and 24 h, suggesting the non-toxic behavior of the material.

Artemia salina is a zooplanktonic crustacean found in a variety of seawater systems (lakes, oceans) and it is one of the most popular live foods for many fishes and aquatic invertebrates (Zhu *et al.*, 2018). *A. salina* interacts with the aquatic environment and faces high risk exposure to contaminants (Zhu *et al.*, 2018). The nauplii of the brine shrimp are considered as a simple and suitable model system for acute toxicity tests (Trompeta *et al.*, 2019). The nauplii feature a higher sensitivity to toxic agents compared to the adult Artemia (Trompeta *et al.*, 2019).

A. salina is a popular model organism for toxicological tests, due to its short life-cycle, ease of culture, high offspring production, the commercial availability of its cysts, year-round availability, low cost, safety, no required feeding during the assay, requiring only a small amount of the tested agent (Ates *et al.*, 2013; da Silveira Carvalho *et al.*, 2017; Zhu *et al.*, 2018). Moreover, many endpoints can be selected for toxicological evaluation, including hatching, mortality, swimming, morphology and biomarkers (Živković *et al.*, 2016; Zhu *et al.*, 2018).

The assay correlated with the toxicity data of rodents and humans and shows a good correlation with cytotoxicity tests making these measurements suitable as preliminary results (Živković *et al.*, 2016; da Silveira Carvalho *et al.*, 2017). Artemia species have been used in testing acute toxicity of toxic materials, such as heavy metals and pesticides (Ates *et al.*, 2013), nanoparticles (Zhu *et al.*, 2018), bioactive molecules, natural extracts and metal complexes (da Silveira Carvalho *et al.*, 2017).

Materials and Reagents

Brine shrimp assay

1. Latex gloves (KCWW, Kimberly-Clark, catalog number: 57330)
2. 24-well plate, sterile and tissue-treated (Corning, catalog number: 3526)
3. Pipette tips
4. Brine shrimp eggs (*Artemia salina*) (were purchase from Ocean Nutrition) and the Pure Artemia Cysts are coming from the Great Salt Lake and therefore geographical variations are not affecting the assay
5. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
6. Sea salt (Tropic Marin)

Equipment

1. 2 L separating funnel with 24/29 joint
2. Stereo microscope Stemi 2000 (ZEISS)
3. Air Pump (Mouse M-106, two outputs 4w)
4. Soft light source such as fluorescent lamps 7w
5. Incubator

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Note: Any incubator is fine if it maintains 25 °C to 37 °C, 50-60% humidity and 12 h day lighting/12 h dark for 24 h.

Software

1. Microsoft Office Excel

Procedure

1. 1 g cysts of *Artemia salina* are soaked in 500 ml natural fresh water for one hour in a 2 L separating funnel. The funnel should not be covered on top.
2. 17 g of sea salt are dissolved in the 500 ml natural freshwater water above.
3. Facilitate the funnel with good aeration using an air pump at room temperature and under continuous illumination for 48 h (Figure 1).

Figure 1. The nauplii incubation apparatus

4. After 48 h hatching, the nauplii released from the egg shells.
5. Collect nauplii at the bright side of the funnel (near the light source) by using a micropipette.
6. Transfer the separated larvae above in a small beaker containing NaCl 0.9%.
7. Introduce an aliquot (0.5 ml) from a small beaker about 10 to 20 nauplii to each well of 24-well plate. The total volume of the well of 24-well plate will be 1ml NaCl 0.9%.
8. Test the metallodrug at three concentrations, with a range of, for example 0.05, 0.5 and 5 μM with three replicates per concentration. The IC_{50} value of the metallodrug towards the cancerous cell lines should be in this range of the tested concentrations. When an antibacterial or a fungicidal compound is tested, then the tested concentration should be the MIC and 2× MIC, 4×

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MIC values. In the case of hydrogel materials, discs with a diameter of 9 mm were added to each well.

9. The final volume of each well is 1 ml with NaCl 0.9% and the presence of the metallodrug.
10. Maintain the plates at 25°C in an incubator.
11. Examine, after 24 h the brine shrimps, using a stereoscope.
12. Larvae were considered alive if they exhibit internal or external movement during 10 s of observation.
13. Repeat each experiment three times.

Note: During the decapsulation of Artemia cyst, continuous aeration, using an air pump, should be done for proper hatching of the embryo, at 25 °C with simultaneously continuous illumination. For the acceptability of the test, up to 10% of mortality was admitted in the control.

Data analysis

Representative data

Selected icons of hatching eggs, live and dead nauplii brine shrimps which were treated with an agent are shown in Figure 2.

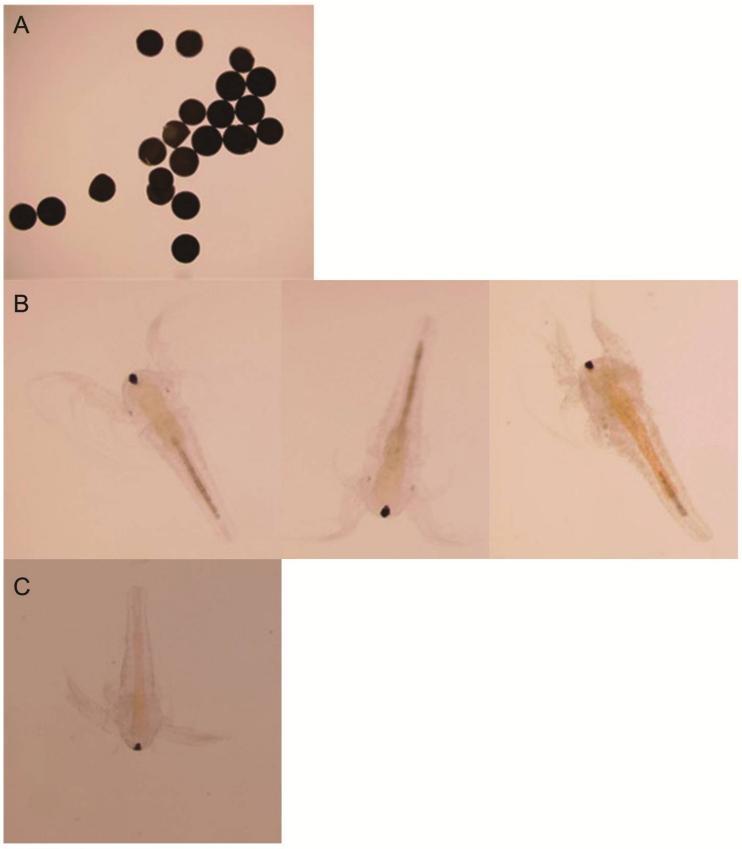

Figure 2. Selected icons of hatching eggs (A), alive (B) and dead (C) nauplii brine shrimps which were treated with a metallodrug

1. Observed larvae, and the dead larvae are considered those that did not exhibit any internal or external movement in 10 s of observation.
2. Count numbers of dead larvae.
3. The (%) mortality of *Artemia salina* larvae was calculated according to Abbott 1987:

$$M(\%\ vs.\ control)=[(L_C-L_T)/L_C]\times 100$$

where M is mortality; L_C, living nauplii in the control after 24 h; L_T, living nauplii with the tested agent after 24 h.

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Competing interests

The authors declare no conflicts of interest or competing interests.

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