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RESEARCH PAPER

Dissolution and aggregation of Cu nanoparticles in culture media: effects of incubation temperature and particles size

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Abstract Here, the effects of incubation temperature and particle size on the dissolution and aggregation behavior of copper nanoparticles (CuNPs) in culture media were investigated over 96 h, equivalent to the time period for acute cell toxicity tests. Three CuNPs with the nominal sizes of 25, 50, and 100 nm and one type of micro-sized particles (MPs, ~500 nm) were examined in culture media used for human and fish hepatoma cell lines acute tests. A large decrease in sizes of CuNPs in the culture media was observed in the first 24 h incubation, and subsequently the sizes of CuNPs changed slightly over the following 72 h. Moreover, the decreasing rate in size was significantly dependent on the incubation temperature; the higher the incubation temperature, the larger the decreasing rate in size. In addition to that, we also found that the release of copper ions depended on the incubation temperature. Moreover, the dissolution rate of Cu particles increased very fast in the first 24 h, with a slight increase over the following 72 h.

Keywords Cu nanoparticles · Culture media · Incubation temperature · Aggregation · Dissolution

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Introduction

Over the past decade, an increasing number of manufactured nanomaterials (MNs) have been incorporated into products and manufacturing processes. Currently, copper nanoparticles (CuNPs) have been one of the most widely used MNs (Isomura et al. 2012; Lee et al. 2010). They are used as superconductors, catalysts, and incorporated in a variety of devices as for instance lithium ion electrode materials (Gong et al. 2012; Hwang et al. 2003; Matsushima et al. 2009). In light of their wide usage, the intentional or accidental release to the environment is hence largely unavoidable during the manufacturing process, transport, or use (Glover et al. 2011), which leads to concerns about their potential environmental and health impacts (Handy et al. 2008, 2012; Klaine et al. 2008).

To date, numerous studies have demonstrated the potential toxicity of CuNPs to bacteria (Rispoli et al. 2010; Yoon et al. 2007), phytoplankton (Aruoja et al. 2009), plants (Lee et al. 2008), invertebrates (Shaw et al. 2012; Unrine et al. 2010), and mammals (Prabhu

et al. 2010). In general, copper-containing NPs show a size- and species-dependent toxicity to organisms (Di Bucchianico et al. 2013; Piret et al. 2012). For instance, Chen et al. (2006) found that CuNPs could induce more severely pathological injuries to the kidney, liver, and spleen of mice compared to copper at micrometer size. On the other hand, a few studies have shown that copper ions could induce protein damage, membrane damage, slight DNA damage, and cell death (Li et al. 2013). Therefore, it is essential to determine the changes in physicochemical properties (e.g., size, dissolution, and aggregation) of NPs during toxicological exposure to correctly interpret the dose–response relationships and to avoid inaccurate dose estimation and the misinterpretation of toxicity results.

For the assessment of NPs cytotoxicity, cells are incubated in culture media containing NPs at different temperatures. There are a number of uncertainties about the physicochemical properties of NPs during incubation in a very complex medium as this used for the cell culture (Tejamaya et al. 2012). Of particular importance are the changes in agglomeration and aggregation state of NPs. The tendency of NPs to aggregate/agglomerate would be influenced by incubation temperature, suspension preparation pathway (e.g., ultrasonication), media ingredients, and in particular original particle size as well as solubility. All these properties together would affect the real dose that cells are exposed to. Unfortunately, all these factors have been poorly taken into account in the literature (Alvarez et al. 2009; Auffan et al. 2009; Stone et al. 2010). Some reports have concentrated on silver or titanium dioxide NPs and have described aggregation and dissolution of these NPs in culture media (Ji et al. 2010; Tejamaya et al. 2012). However, there are few data about CuNPs changes in size, aggregation, and dissolution in culture media during incubation period.

In the present study, we aimed to investigate the effects of incubation temperature and particle size on the aggregation and dissolution of Cu particles in culture media. Three different temperatures (20, 30, and 37 °C) that are used for the incubation of a variety of cell lines (20 and 30 °C for fish cells, 37 °C for mammalian cells) were investigated. Three CuNPs and one micron-sized Cu particle (CuMPs), used as reference, were employed to examine their changes in physicochemical properties over acute exposure time scales (96 h). Moreover, culture media normally used for fish and mammalian cell lines were utilized. The data generated through this work would constitute a valuable platform from which to prepare further studies about the health and environment implications of MNs, particularly CuNPs.

Materials and methods

Chemicals and materials

All reagents were of analytical grade or higher and used as received without further purification. Three commercial CuNPs with nominal sizes of 25, 50, and 100 nm (designated as CuNPs-25, CuNPs-50, and CuNPs-100) were purchased from IoLiTec, Inc., Germany, and CuMPs with nominal size of 500 nm were purchased from NanoAmor, USA (Houston, TX, USA). The CuNPs-50, as a reference, was measured by X-ray diffraction (XRD) using a PANalytical X'Pert PRO diffractometer with Cu Kα radiation to confirm the crystal structures, confirming that they were Cu particles (data not shown). The pristine Cu particles were analyzed by transmission electron microscopy (TEM, JEOL 2100 HT, JEOL Ltd., Japan). All particles used in this study are uncoated. Ethanol was purchased from Panreac (Barcelona, Spain). Ultraglutamine 1 (200 mM), L-glutamine (200 mM), penicillin and streptomycin (P/S) (10,000 U/mL/10 mg/mL), fetal bovine serum (FBS), non-essential amino acids 100X (NEAA), Eagle's Minimum Essential Medium (EMEM), and Alpha Minimum Essential Medium (α-MEM) were purchased from Lonza (Barcelona, Spain). High purity water (>18 MΩ/cm) obtained from a Milli-Q Element A10 Century (Millipore Iberica, Spain) was used for total reflection X-ray fluorescence (TXRF, Bruker S2 Picofox) analysis.

Preparation of culture media

Two different cell culture media commonly used were prepared using a method reported in our previous study (Song et al. 2014). In the first one (namely culture medium A, CMA), 500 ml of α-MEM received 5 mL P/S, 5 mL L-glutamine, and 25 mL FBS. The second one (namely CMB) was prepared by adding 5 mL P/S, 5 mL ultraglutamine, 5 mL NEAA, and 50 mL FBS to 500 mL EMEM.

Preparation of Cu particles suspension

Suspensions of CuNPs were prepared using a previously developed method (Song et al. 2014). Briefly, 4.0 mg Cu particles were suspended in 20 mL of culture medium and sonicated (S 40H Elmasonic, Elma, Germany) for 10 min at room temperature. It should be noted that the concentration of CuNPs (200 μg/mL) used in the present study is the same one or very similar to the maximal concentration used in a variety of studies based on in vitro tests of engineered nanoparticles (Kunzmann et al. 2011; Song et al. 2014).

Temporal aggregation and dissolution of Cu particles in culture media

The aggregation and dissolution of Cu particles in culture media were investigated at different temperatures (20, 30, and 37 °C). Mean hydrodynamic size of Cu particles suspensions was measured by dynamic light scattering (DLS) using a Zetasizer Nano-ZS instrument (Malvern, Instruments Ltd., UK). These measurements were performed along 96 h incubation. Culture medium without particles was used as a control and to record any background signals arising from components of the medium. Four independent measurements were taken, each consisting of six runs, having each run a duration of 20 s. The size distribution is multimodal, and polydispersity index is between 0.23 and 0.59 for the DLS measurements.

TEM operating at an accelerating voltage of 200 kV was used to characterize the morphology of copper particles. The method of TEM sample preparation has been described in Song et al. (2014). Briefly, 20 μL of Cu particles suspension was dropped on the carbon-coated Ni grid, and then the dried grid was observed by TEM. In order to determine the Cu particles dissolution accurately, the initial concentration of Cu in the particle suspension was measured by graphite furnace atomic absorption spectrometry (GFAAS) after acid digestion. The concentration of copper ions released from Cu particles was determined by TXRF. For that, 1 mL of CuNPs suspension was sampled at time 0, 24, 72, and 96 h after incubation and centrifuged at $13{,}362 \times g$ for 20 min, at 4 °C (5145 R series centrifuge, Eppendorf, Germany) to remove Cu particles from suspension. Before TXRF measurements, the samples (5 μL) were dropped on the silica substrates and then were dried by heating.

Statistical analysis

Results presented are in all cases the mean ± SD of three independent measurements. Statistical analysis was performed using SigmaPlot 12.0 (Systat Software Inc., Chicago, IL, USA). The normality and homoscedasticity of data were checked by the Shapiro–Wilk test and Bartlett's test, respectively. A one-way ANOVA analysis was conducted to examine the effect of incubation temperature on the decrease in particle size. Statistical significance was defined as $p < 0.05$ (Tukey post hoc test). The linear relationship between the decrease in Cu particles size and the increase in copper ions release was tested using Pearson correlation, since these data distributed normally.

Results and discussion

Changes in Cu particles size with time

Previous studies describing NPs toxicity emphasized the importance of NPs size on their bioavailability and toxicity to cultured cells (George et al. 2012; Song et al. 2014). Hence, increasing our knowledge about the factors affecting NPs size in toxicity tests is of paramount importance to understand medium influence and short-term variations in NPs toxicity. In this study, we observed the changes in size of Cu particles incubated in culture media at different temperatures, namely 20, 30, and 37 °C.

We found that size of CuNPs and CuMPs measured by DLS was higher than this of provided by the supplier (Figs. 1, 2). This is attributed to not only the intrinsic properties of DLS analyses based on light scattering measurements but also to the aggregation of particles in the culture media. Normally, image analysis based on the TEM micrographs gives the 'true radius' of the particles, and DLS provides the hydrodynamic radius on an ensemble average, resulting in that the particle size observed using DLS is much bigger than the actual single particle size (Lim et al. 2013). In addition, as previously reported in a number of studies (George et al. 2012; Song et al. 2014; Tejamaya et al. 2012), there could be aggregation of particles in the culture media, as shown in Fig. 3, leading to the observed increase in size compared to that of single particles.

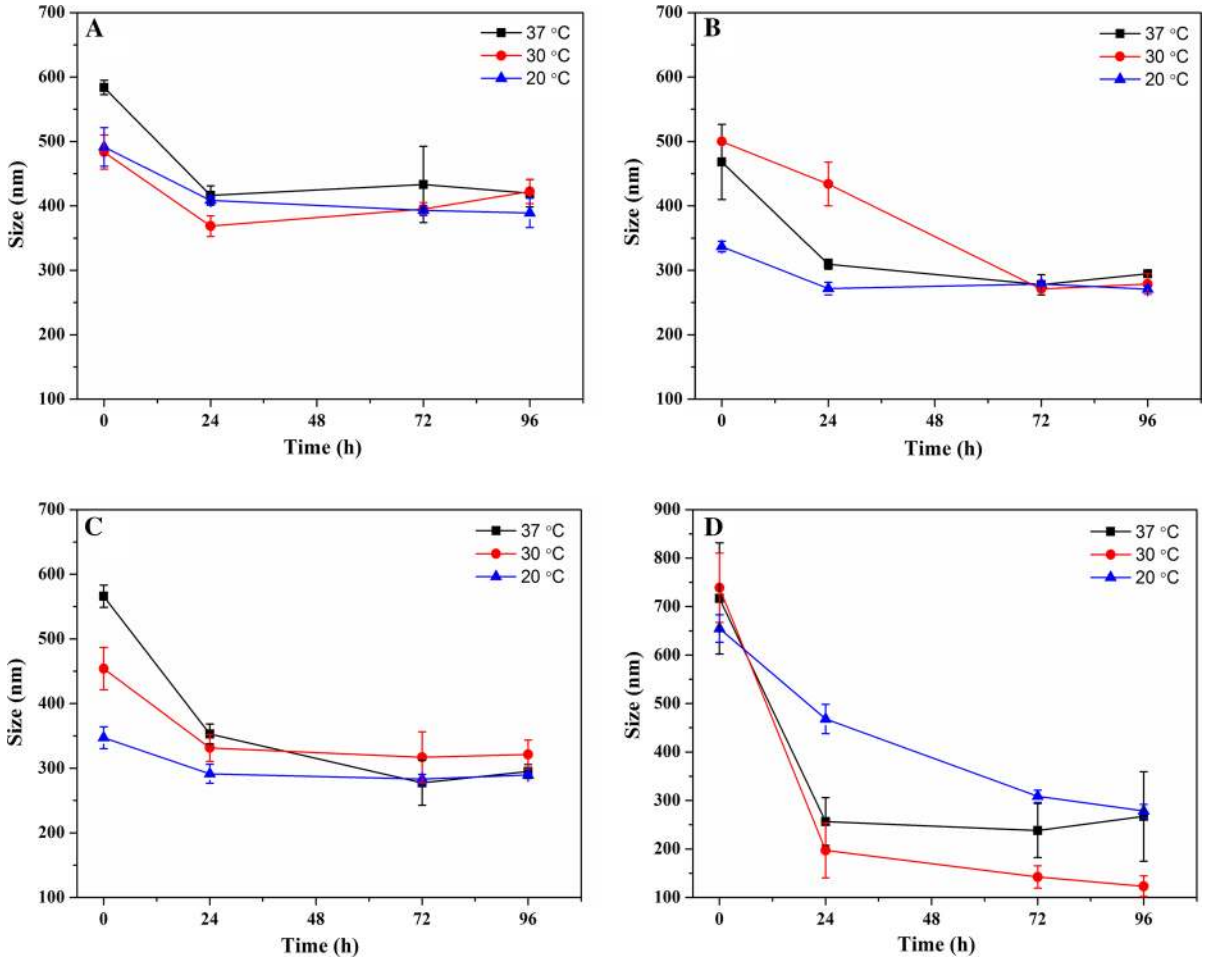

Fig. 1 Size of Cu particles within 96 h in culture media named CMA. **a** CuNPs-25. **b** CuNPs-50. **c** CuNPs-100. **d** CuMPs. Results are expressed as mean ± SD

The size of CuNPs incubated in the CMA and CMB shows time-dependent profiles (Figs. 1, 2, respectively), with an important decrease in the first 24 h. Thereafter, only slight changes are observed over the following 72 h. One exception was the CuMPs when incubated in CMA at 20 °C whose size did not stabilize until 72 h incubation (Fig. 1d). Considering these data, two practical considerations must be taking into account when designing in vitro experiments involving NPs. First, a detailed description of the variation in size of the NPs is necessary to be able to establish any relationship between size and toxicity. Second, to avoid misinterpretations, it would be advisable to perform the toxicity tests using suspensions in which the size of the NPs has been stabilized with time, in our case for instance suspensions prepared at least 24 h in advance. The limited changes in the size of the NPs under these conditions would facilitate to reach any conclusion about the possible effects of size.

Interestingly, our findings contrast with results reported by other studies. For instance, Tejamaya et al. (2012) investigated the stability of AgNPs in a variety of ecotoxicity test media at room temperature and showed that the size of AgNPs in the OECD media for *Daphnia* sp. (Test NO. 202, OECD) increased with the increase of incubation time. This might be attributed to the high content of salt in this medium, as Mukherjee and Weaver (2010) reported that ions like Ca^{2+}, Mg^{2+}, K^{+}, and Na^{+} could induce metallic and nonmetallic NPs aggregation in solution. In this study, despite the high contents of salt in both media used, the

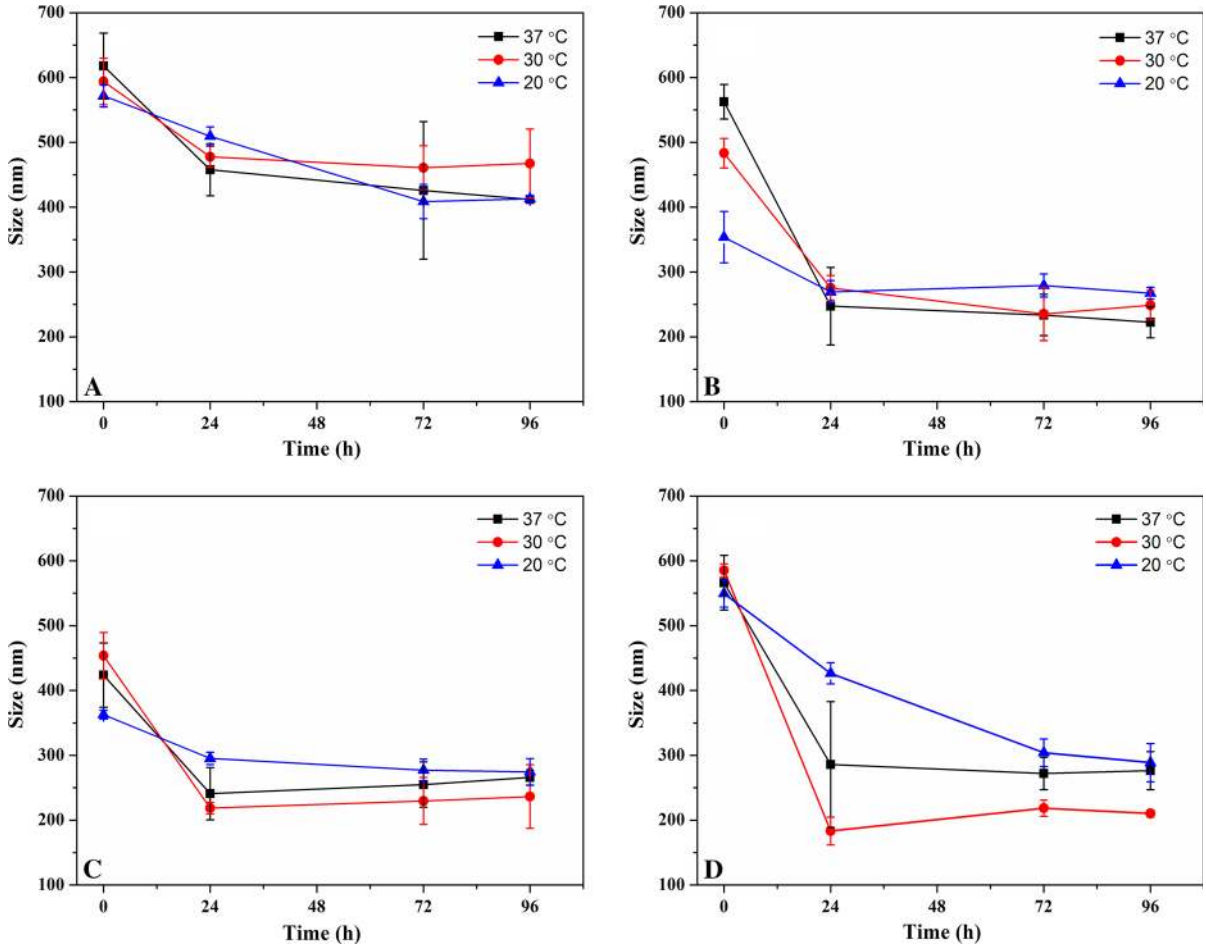

Fig. 2 Size of Cu particles within 96 h in culture media named CMB. **a** CuNPs-25. **b** CuNPs-50. **c** CuNPs-100. **d** CuMPs. Results are expressed as mean ± SD

presence of FBS could have increased the stability of CuNPs. For instance, FBS could favor the dispersion of TiO_2 NPs reducing the aggregated size of TiO_2 NPs in culture media (Ji et al. 2010). In our previous studies, the addition of FBS to culture media also led to a stable dispersion of graphene nanoplatelets (Lammel et al. 2013).

Moreover, we also found that the sizes of stabilized CuNPs-25 were much bigger than those of the other three types of Cu particles (Table 1), which is consistent with the report by Kobayashi et al. (2005) who found that smaller NPs could show a higher tendency to aggregation in solution. According to the theory of Kallay and Zalac (2002), we presumed that the CuNPs-25 could aggregate more easily than the other three types of Cu particles due to their higher number concentration under the same mass concentration conditions.

In addition to the size measurement, TEM images of the stabilized Cu particles were taken to observe the state of the CuNPs in culture media (Fig. 3). Normally, TEM measurements provide direct information about the morphology of particles. Since the previous observations produced similar results in CMA and CMB, we performed TEM analysis only in CMB as a reference. We found that Cu particles in ethanol look aggregated, as described in previous studies (George et al. 2012; Song et al. 2014), while Cu particles except CuNPs-25 dispersed very well in the CMB. As we mentioned above, the good dispersion might be attributed to the presence of FBS in the culture media. Dominguez-Medina et al. (2013) also found that FBS

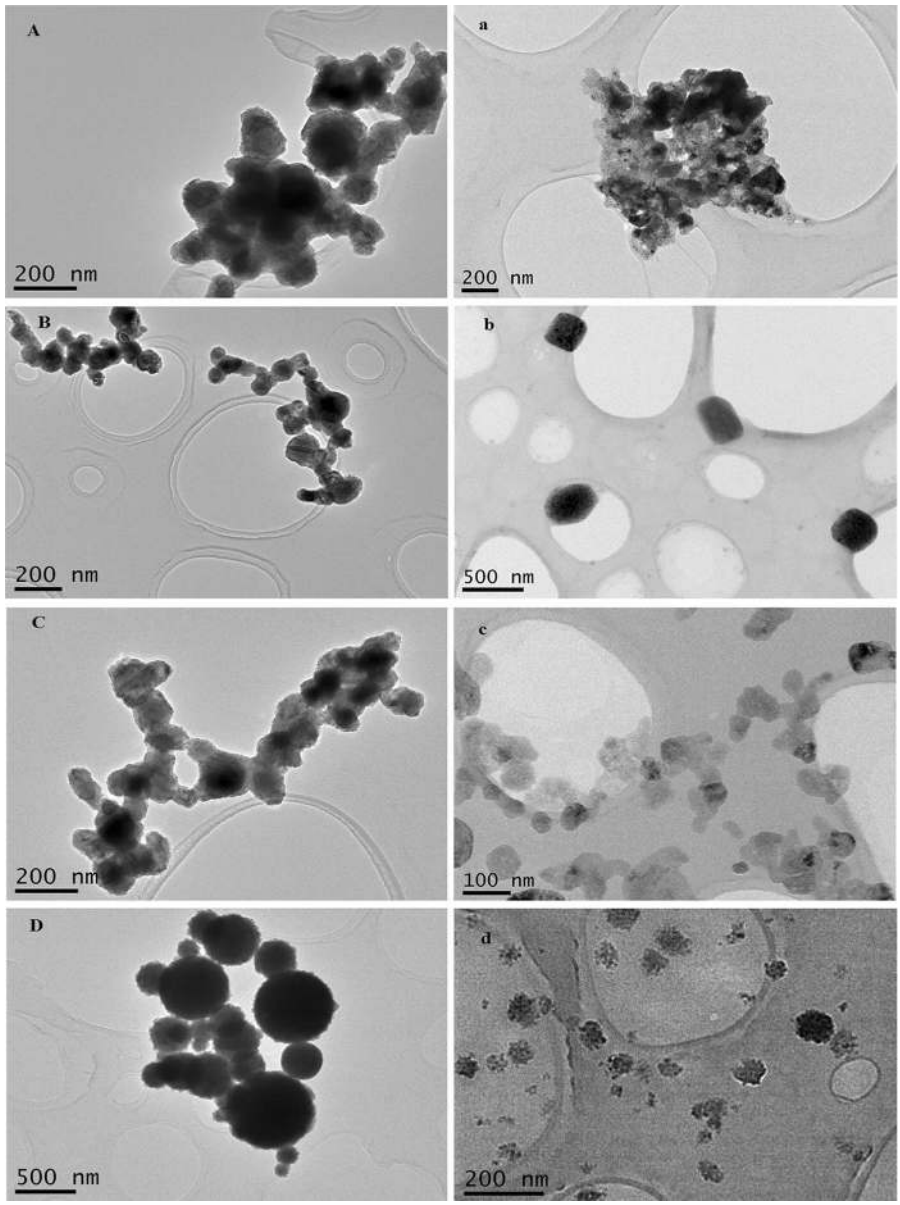

Fig. 3 TEM images of Cu particles in their pristine form (prepared in ethanol, images **a**, **b**, **c**, and **d**) and following 24 h incubation in culture medium B, CMB, under culture conditions (37 °C, images *a*, *b*, *c,* and *d*). *Scale bars* indicate size (nm). **a** and *a*: CuNPs-25. **b** and *b*: CuNPs-50. **c** and *c*: CuNPs-100. **d** and *d*: CuMPs

could prevent NPs from aggregating in high ionic environments.

Effect of incubation temperature on Cu particles size in culture media

Previous studies have shown that temperature is one of the important factors affecting NPs size in the case of NPs synthesis (Jiang et al. 2011; Sahu and Prasad 2013). Therefore, we determined any possible effect of temperature on the evolution of NPs size with time. Fish cell lines are normally maintained at 20 or 30 °C and mammalian cells at 37 °C. For comparative purposes, the three temperatures were used in the present work.

At the beginning of the measurement (time 0), Cu particles exhibited clear differences in size at different

Table 1 The stabilized size (nm) of Cu particles after 24 h incubation in the culture media

	CMA			CMB		
	37 °C	30 °C	20 °C	37 °C	30 °C	20 °C
CuNPs-25	416.2 ± 14.9	368.7 ± 16.1	408.7 ± 6.9	457.9 ± 40.1	477.7 ± 16.2	509.4 ± 14.5
CuNPs-50	309.4 ± 7.8	434.0 ± 34.0	271.7 ± 9.8	247.3 ± 59.8	275.5 ± 19.1	269.3 ± 17.5
CuNPs-100	353.0 ± 15.3	331.4 ± 20.9	291.4 ± 14.8	240.9 ± 40.4	218.6 ± 9.0	295.2 ± 9.4
CuMPs	256.7 ± 49.3	197.1 ± 56.6	308.7 ± 12.5[a]	285.8 ± 97.2	183.2 ± 21.6	426.5 ± 16.3

[a] The stabilized size of CuMPs incubated in CMA at 20 °C was measured after 72 h incubation

Table 2 The decrease in size (nm) of Cu particles after 24 h incubation in the culture media

	CMA			CMB		
	37 °C	30 °C	20 °C	37 °C	30 °C	20 °C
CuNPs-25	167.5 ± 26.1	114.8 ± 42.4	82.9 ± 36.9**	159.8 ± 90.6	116.3 ± 51.9	62.2 ± 31.2**
CuNPs-50	158.9 ± 65.8	66.0 ± 34.4	65.1 ± 18.1**	315.0 ± 86.3	207.8 ± 41.7	84.3 ± 56.9**
CuNPs-100	213.0 ± 32.6	122.6 ± 53.4	55.8 ± 31.6**	182.8 ± 89.4	235.1 ± 44.5	67.5 ± 16.7**
CuMPs	460.2 ± 163.2	541.7 ± 127.9	345.9 ± 58.4[a, **]	280.5 ± 139.6	402.2 ± 31.4	123.0 ± 37.2**

[a] The decrease in size of CuMPs incubated in CMA at 20 °C was calculated after 72 h incubation

** Denote statistically significant differences ($p < 0.01$) in the decrease in size observed at 20 °C and those observed at 30 and 37 °C

temperatures (Figs. 1, 2). However, with time and the tendency of particles to reduce their size, these differences were decreasing until almost disappearing. As shown in Table 2, CuNPs incubated at 37 °C show the largest decrease in size, followed by those at 30 and 20 °C, which might be attributed to the larger amount of ions release from Cu particles at higher temperature (see below). Statistical analysis showed that the decrease in size observed at 20 °C was significantly different ($p < 0.01$) from those observed at 30 and 37 °C (Table 2), which did not show significant differences between them ($p > 0.05$). In addition to that, we also found that the CuMPs showed the largest size reduction compared to the other three CuNPs independently of the incubation temperature (with the only exception of CMB and 37 °C incubation temperature, in which the reduction of size in CuNPs-50 is higher), which might be attributed to the slight sedimentation of MPs during the incubation period (Hinderliter et al. 2010; Teeguarden et al. 2007). In the case of CuMPs, the reduction in size is higher at 30 °C, but as for CuNPs no statistical differences were observed with respect to the reduction in size at 37 °C ($p > 0.05$), being in both cases significantly ($p < 0.01$) different from the reduction observed at 20 °C.

Copper ions release in culture media

Previous studies have shown that the release of copper ions from CuNPs has a deep influence on the general toxicity caused by these NPs (Gomes et al. 2011; Song et al. 2014). Since there was a strong change in the size of NPs in the first 24 h of incubation, we wanted to observe if such a reduction was related with copper ions release. The measurement of copper ions in both media was performed by means of TXRF. Since a clear trend in variation of size depending on the temperature was previously observed, measurements were done at only the two most different temperatures: 37 and 20 °C. It should be noted that Fig. 4 illustrated the percentage of copper ions release from Cu particles calculated as a percentage of the initial copper concentration.

As shown in Fig. 4, there are marked differences in release rates of copper ions at the different incubation temperatures. We found that the release rates at 37 °C were much higher than those at 20 °C (Fig. 4), which

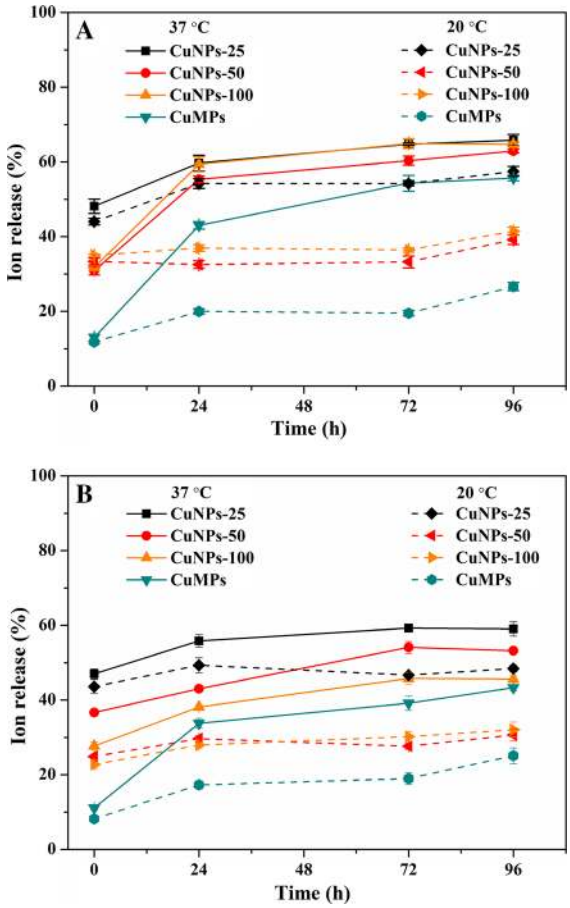

Fig. 4 Copper ion release profiles for the Cu particles incubated in culture media over 96 h under different temperature conditions. **a** Culture medium A, CMA. **b** Culture medium B, CMB. The data are expressed as percentage of the initial Cu particles concentration. Results are expressed as mean ± SD

might be attributed to the temperature dependence of ionic diffusion coefficient. Moats et al. (2000) documented that the diffusion coefficient of copper ions increased with increasing temperature, due to the increase of the amount of energy available. Clearly, our finding is in good agreement with previous studies which presented that dissolution of metal or metal oxide NPs would take place rapidly at elevated temperatures (Liu and Hurt, 2010; Zhang et al. 2011). For instance, dissolution of PVP-coated AgNPs as percent of the initial silver mass changed from 50 % at 25 °C to 90 % at 37 °C (Kittler et al. 2010).

Copper ions release profiles were different among the four types of Cu particles (Fig. 4). We found that the presence of copper ions in medium increased with time. Interestingly, at time 0, the percentage of copper ions released from the biggest particles, namely CuMPs, was the lowest one (12.4 ± 0.6 %), and the highest percentage, namely 48.1 ± 1.9 %, corresponded to the smallest CuNPs (CuNPs-25). NPs with intermediate size (CuNPs-50 and CuNPs-100) presented at time 0 also an intermediate value (about 31.6 ± 1.0 % for CuNPs-50 and 29.5 ± 1.5 % for CuNPs-100) in the release percentage of ions. At time 24 h, the percentages of copper ions release from the CuMPs in CMA and CMB incubated at 37 °C increased to 43.1 ± 1.0 and 33.8 ± 1.3 %, respectively. Compared to CuMPs, the other three CuNPs incubated in CMA and CMB at 37 °C increased to much higher percentages of copper ions release (e.g., 59.7 ± 2.1 % for CuNPs-25). In addition, the percentages of Cu particles dissolution increased slightly over the following 72 h. Clearly, the dissolution rate increased very fast in the first 24 h, with the majority of copper ions being released in the culture media. All these findings are in very good agreement with the observation of changes in Cu particles size. Moreover, this phenomenon is consistent with the finding of Song et al. (2014), which reported that the majority of copper ions could be released from CuNPs in the first 24 h.

Interestingly, for CuMPs, the percentage of copper ions release increased most quickly over the first 24 h incubation, while after 96 h, they still showed the lowest dissolution rate compared to the other three CuNPs when finally the Cu particles stabilized in the culture media. For instance, stabilized (after 96 h) CuNPs-25 showed 67 ± 2 % copper ions release in the CMA at 37 °C, followed by CuNPs-100 (63 ± 1 %), CuNPs-50 (61 ± 0.6 %), and CuMPs (52 ± 2 %) under the same conditions (Fig. 4a). A similar phenomenon was observed in CMB (Fig. 4b). This might be related to the big size of the CuMPs. Normally, smaller particles have larger surface area on an equivalent mass basis. In other words, for smaller particles, more surface atoms are exposed, so more reaction sites are available for oxidation and subsequent dissolution (Liu et al. 2010). In addition, the same mass concentrations of the NPs and MP were used for comparison in this study. Accordingly, the higher effect on the changes in particle size and dissolution could be also related to a higher particle number concentration of small CuNPs (Böhme et al. 2014).

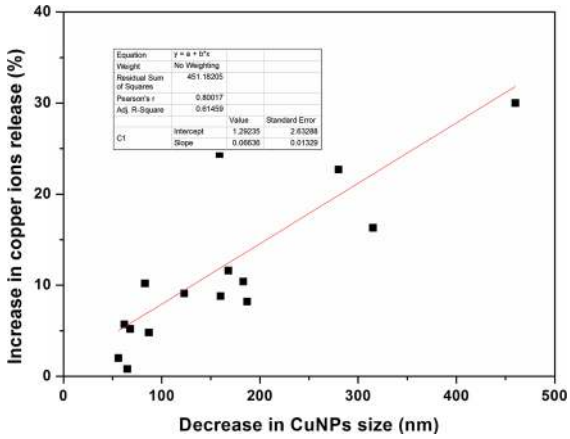

Fig. 5 Relationship between Cu particles size and copper ions release

In order to corroborate the relationship between the reduction in size and the release of ions, a Pearson correlation analysis was performed between the increase in copper ions release and the decrease in particles size, considering globally all the data generated in all the time points for all particles. As it can be observed in Fig. 5, a correlation coefficient of 0.61 was obtained. Therefore, although other factors, as for instance disaggregation could be influencing the observed reduction of size with time, the obtained results appear to indicate that simple dissolution of NPs is playing a key role at this level.

Conclusions

An investigation of CuNPs aggregation and dissolution in culture media is very important to understand the accurate dose–response relationship in CuNPs toxicity studies. However, the effects of incubation temperature and particle size on the aggregation and dissolution of CuNPs in culture media were ignored. This study reported the marked effects of incubation temperature on the size and dissolution of CuNPs in the first 24 h. The decrease in size of CuNPs was dependent on the incubation temperature; the higher the temperature of incubation, the larger the decreasing rate in size. Moreover, the release of copper ions depended on the incubation temperature; the rates of ions release increased with the increase of incubation temperature. In addition to that, the particle size also affected the dissolution of CuNPs. In total, our results suggest that incubation temperature and particle size are very important factors affecting the aggregation and dissolution of CuNPs in culture media.

Evidently, it has been clear that the incubation temperature and particle size could affect the aggregation and dissolution of CuNPs in culture media. However, the behavior of CuNPs in culture media is a complex process which cannot be completely described by a single process. Ongoing research in our laboratory aims at extending this work to examine the behavior of CuNPs in culture media with the presence of cells. Overall, an improved understanding of CuNPs aggregation and dissolution in culture media would allow better predictions of dose–response relationship in CuNPs toxicity studies.

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Conflict of interest The authors declare that they have no conflict of interest.

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