TOXICITY OF SURFACE-MODIFIED COPPER OXIDE NANOPARTICLES IN A MOUSE MACROPHAGE CELL LINE: INTERPLAY OF PARTICLES, SURFACE COATING AND PARTICLE DISSOLUTION

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Abstract

Copper oxide nanoparticles (CuO NPs) are used in numerous applications including antimicrobial agents, semiconductors, chemical sensors, catalysts and others. However, their high toxic potential and growing industrial production increases the concern for health and environmental risks. The present study aimed to investigate the toxicity of pristine versus surface-modified CuO NPs in relation to their stability/dissolution in cell culture medium, cellular uptake, cytotoxicity and ROS production in mouse macrophages RAW264.7. Our results showed that NPs modified with different coating agents [citrate (CIT), ascorbate (ASC), polyethyleneimine (PEI) and polyvinylpyrollidone (PVP)] affected the dissolution of the NPs in water but not in cell culture medium. Further, in vitro testing demonstrated that PEI-coated NPs were most cytotoxic while ASC-coated material exerted the lowest cytotoxicity. The anionic coatings CIT and ASC also provided protection against CuO NPs-induced oxidative stress and decreased levels of ROS production. Cellular internalization assessed by TEM was detected in cells exposed to CIT, ASC, PVP-coated and pristine NPs but not PEI-CuO NPs. Intracellular Cu content in cells tended to decrease with time, resulting in reduced cell/total Cu ratios, with the highest reduction noted for cells exposed to uncoated NPs. Overall, the toxicity of the surface-modified CuO NPs could be explained by synergistic interactions between the NPs, their dissolution, and the toxicity of the coating agents.

Keywords: Nanoparticles, surface coating, cytotoxicity, macrophages, oxidative stress

1. INTRODUCTION

Copper oxide nanoparticles (CuO NPs) are used in wide range of applications including antimicrobial agents, wood preservatives, doping materials in semiconductors, chemical sensors, catalysts as well as agents used in biomedicine and many others. However, the growing industrial production of engineered nanomaterials, not only metal oxide nanoparticles, increases the concern for health and environment. Several recent studies demonstrated toxicity in vitro [1,2] and in vivo [3], adverse effect has also been documented in workers exposed to welding fumes containing Cu-based NPs [4].

Although it is generally accepted that toxicity of CuO NPs is attributed to the rapid dissolution in aqueous media and massive release of Cu^{2+} ions [5], recent studies proposed a so-called Trojan horse mechanism by which various metallic nanoparticles are firstly internalized within cell and the subsequent intracellular ion release is elicited by the acidic conditions of the lysosomal cellular compartment [6,7]. Solubility (both extracellular and intracellular) is a function of surface stability of the NPs. Thus, considerable efforts have been devoted to the development of surface modifiers to improve nanoparticle's colloidal properties and enhance their biocompatibility. Surface modification of NPs controls their colloidal stability, surface charge and reactivity and thus may substantially influence their interaction with biomolecules and living organisms.

The present study aimed to investigate the toxicity of pristine versus coated CuO NPs in relation to their stability/dissolution in cell culture medium, cellular uptake, cytotoxicity and ROS production. The surface modifiers were selected to allow for a comparison of anionic, neutral and cationic agents. The molecules ascorbate (ASC) and citrate (CIT) are known for their anti-oxidant properties and are used as reducing agents and stabilizers of NPs. Polyvinylpyrrolidone (PVP) is a bulky, non-toxic neutral polymer used as a dispersing and shape-control agent. Polyethyleneimine (PEI) is a synthetic cationic polymer which provides electrosteric stabilization and enhanced particle cellular uptake.

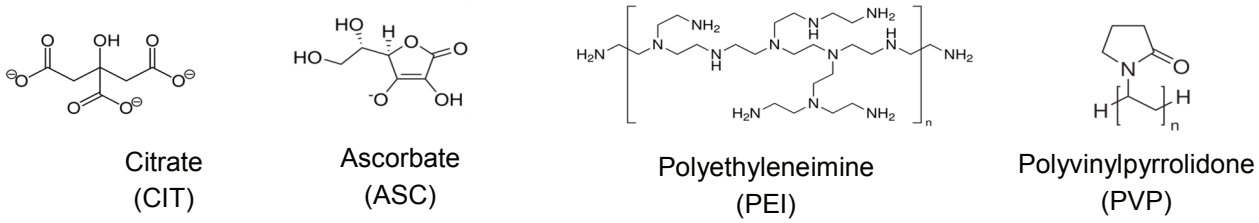

Figure 1 Chemical structure of selected coating agents used in the study

2. MATERIALS AND METHODS

2.1. Nanomaterial characterization

CuO NPS with a primary particle size of 12 nm (± 4 nm) were obtained from PlasmaChem (Germany). Surface of pristine nanoparticles was modified by self-assembly technique, i.e. CuO NPs were dispersed in buffer (PBS, pH 7.4), combined with organic additives at the proportion 10 % m/m relative to CuO and 95h milled with zirconia spheres as grinding media [8]. Particle dispersion in water cell culture medium was achieved through sonication. Dynamic light scattering (DLS) and electrophoretic light scattering (ELS) were used to determine particle hydrodynamic diameter and zeta potential, respectively (Zetasizer nano ZSP ZEN5600, Malvern Instruments). The dissolution of the NPs was estimated as the ratio between Cu^{2+} and CuO determined by suspension ultrafiltration with 10 kDa filters (Millipore), followed by analyzing the filtered solutions through inductively-coupled plasma optical emission spectrometry (ICP-OES) using 5100 Synchronous Vertical Dual View equipment (Agilent).

2.2. RAW264.7 macrophage cell line culture

RAW264.7 cell line was obtained from EACC (UK). Cells were cultured in DMEM (Sigma) supplemented with 10 % FBS, 1 mM sodium pyruvate, penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were maintained in humidified atmosphere with 5 % CO_2 at 37°C.

2.3. Cytotoxicity assessment

RAW264.7 cells were grown in DMEM (Gibco) supplemented with 10 % FBS, 1mM of sodium pyruvate,100U/ml Penicillin and 100 µg/ml Streptomycin. Cells were treated with 18 different concentrations of CuO NPs ranging from 1.41 to 62.5 µg CuO/ml of and incubated 24h in 37°C. $CuCl_2$ was included as and ionic control. For some experiments, N-acteylcysteine was used for pre-incubation of cells. Upon the treatment, Alamar Blue reagent (Life Technologies) was used to detect cellular viability. Dose-response cytotoxicity data were analyzed by the Benchmark dose method in R software with Proast 38.9 package.

2.4. Intracellular Cu content.

Cells were exposed to the BMD20 dose of each NP to achieve equitoxic conditions. After exposure, supernatants (medium) and cells (adherent cells + residual pellet) were collected separately and digested with

65 % HNO_3 for 24h at RT. Cu was determined through inductively-coupled plasma mass spectrophotometry (ICP-MS) using an iCAP Q apparatus. Results were expressed as ratios of intracellular/total Cu.

2.5. Detection of reactive oxygen species (ROS)

Cells were exposed to 25 µg/mL of CuO NPs for 4h, then 1 µM dihydroethidium was added (15 min, 37°C, dark). Afterwards, cells were collected, washed in PBS and DHE signal was analyzed by flow cytometry using a FACScan (Becton Dickenson).

3. RESULTS

3.1. Nanoparticle characterization

Pristine and modified CuO NPs were tested for their stability and solubility in water and cell culture medium (DMEM). According to DLS data indicating the hydrodynamic size of NP's agglomerates, NPs coated with ionic coating agents CIT, ASC and PEI were better dispersed in water than pristine material while neutral PVP worsened the stability so much that no data could be collected. In DMEM, all CuO NPs were better stabilized comparing to water and showed similar hydrodynamic size. Rate of dissolution in water showed slight differences among CuO NPs. The highest rate of dissolution after 24h exerted ASC-coated (1.99 %) comparing to PVP-coated NPs (0.23 %). However, the dissolution rate in DMEM indicating massive release of Cu^{2+} ions (almost 70 %) was similar for all nanomaterials irrespectively to the surface modification. Zeta potential of CuO NPs in water was consistent with the charge given by the coating agents but in DMEM, all CuO NPs gained the uniform negative charge (overview is presented in **Table 1**).

Table 1 Physicochemical properties of CuO NPs

	d_{DLS} (nm)		**Zeta potential $_{ELS}$ (mV)**		**Dissolution after 24h (Cu^{2+}/Cu mass (%))**	
	MilliQ	DMEM	MilliQ	DMEM	MilliQ	DMEM
Uncoat. CuO	2052.3 ± 347	55.1 ± 16	-9.1 ± 0.4	-8.2 ± 0.4	0.1	67.41
CIT-CuO	313.4 ± 8	37.4 ± 2	-18.0 ± 0.3	-9.7 ± 0.6	1.98	69.19
PVP-CuO	nd	52.9 ± 25	-17.4 ± 0.3	-9.2 ± 0.2	2.08	65.39
PEI-CuO	383.5 ± 22	44.6 ± 14	-8.1 ± 2.3	-9.4 ± 0.8	0.23	66.93
ASC-CuO	109.7 ± 2	72.8 ± 21	+28.3 ± 0.7	-10.1 ± 0.7	1.68	66.01
DMEM		17.4 ± 0.3				

3.2. Cytotoxicity assessment

The cytotoxic response was assessed by Alamar Blue assay and dose-response effect was analysed by benchmark mark dose (BMD) method. BMD_{20} values of modified and pristine CuO NPs were inferred from dose response curves and could be ranked as follows: PEI-coated > uncoated > PVP-coated > ASC-coated > CIT-coated (**Table 2**).

Table 2 BMD_{20} estimates (given as µg/mL and 95 % confidence intervals) in RAW264.7 macrophages.

CuO NPs	**BMD_{20}**	**Coating agent**	**BMD_{20}**
Uncoated NPs	3.43 (2.67 - 4.28)	-	-
CIT-coated NPs	13.2 (11.3 - 15.3)	Citrate	52.5 (49.38 - 64.32)
ASC-coated NPs	14.6 (12.4 - 16.8)	Ascorbate	1.14×10^{19} (ND)
PVP-coated NPs	6.32 (5.43 - 7.27)	Polyvinylpyrrolidone	436 (132.4 - 8805)
PEI-coated NPs	2.06 (1.4 - 2.91)	Polyethyleneimine	13.4 (11.6 - 15.3)
Ionic control ($CuCl_2$)	55.6 (49.2 - 58.9)		

The cytotoxicity of ionic control $CuCl_2$ and coating agents themselves was also tested. $CuCl_2$ exerted considerably lower cytotoxicity and also the cytotoxicity of the coating agents, excepting PEI, was much reduced comparing to respective modified CuO NPs (**Table 2**).

3.3. Cellular internalization

The intracellular Cu content was determined by using ICP-MS after 4h and 24h exposure. Overall, Cu concentrations were consistently higher in medium than in cells regardless to the treatment an exposure time, Cu concentrations tended do decrease with time. After 4h exposure, differences in the internalization of NPs (expressed as ratio Cu in cells/total Cu) were found and ratios could be ranked as follows: uncoated > ASC-coated > PEI-coated > PVP-coated > CIT-coated. Twenty-four hour exposure resulted in decrease of Cu in all samples of NP-exposed cells on a similar level, the highest reduction was noted for uncoated and PVP-coated CuO NPs (**Figure 2**). Interestingly, the absolute concentration of Cu in cells did not correlated with the Cu ratio.

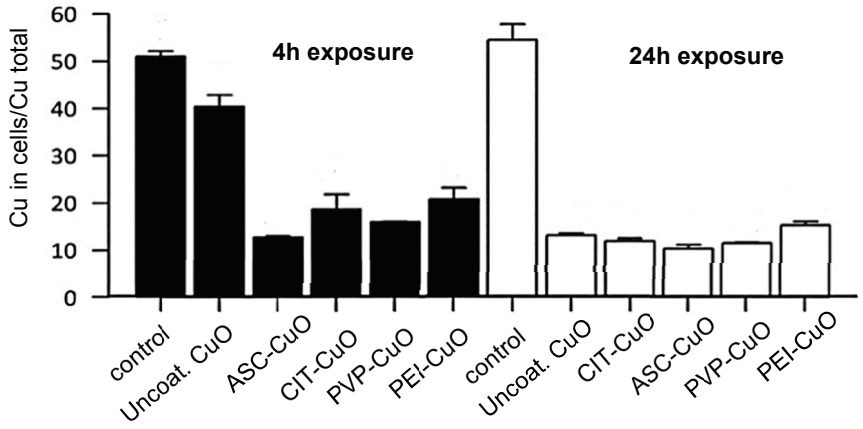

Figure 2 Ratios of Cu between cells and cells + medium (total Cu). Taken and modified from [9].

3.4. Cellular ROS production and the effect of ROS

Intracellular ROS generation in response to coated and uncoated CuO NPs was analysed by flow cytometry using DHE assay. Anionic coating agents clearly lowered levels of ROS when compared to uncoated CuO NPs while PVP-coated NPs produced the opposite effect (**Figure 3**). The rank of NPs according to their pro-oxidant properties is following: PVP > uncoated ~ PEI > CIT > ASC. On the other hand, no significant effect of ROS reduction on cytotoxicity was observed when cells were pre-incubated with 10 mM N-acetylcysteine before the exposure to uncoated CuO NPs (**Figure 4**).

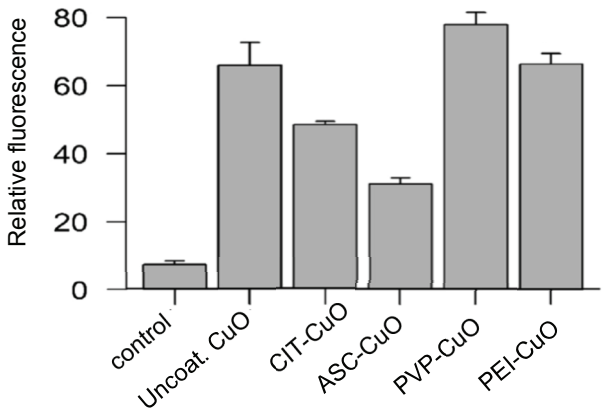

Figure 3 ROS formation after 4h exposure analyzed using redox-sensitive fluorescent probe DHE. Taken and modified from [9].

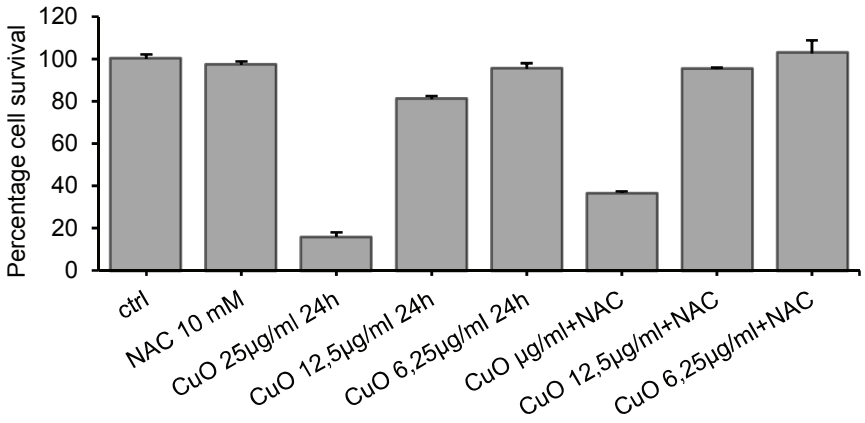

Figure 4 Cytotoxicity of selected toxic concentrations of uncoated CuO NPs after 4h exposure and the effect of NAC pre-treatment. Taken and modified from [9].

4. DISCUSSION

Numerous studies examined the influence of surface coating of diverse nanoparticles in order to prevent their adverse effects towards biological systems and environment [10, 11]. The present study demonstrates that surface coating of CuO NPs significantly affect the NP stability and physicochemical properties thus contributing to their toxicity in RAW264.7 macrophage cell line. Colloidal stability of CuO NPs in water was improved by ionic surface modifiers (CIT, ASC and PEI) but worsened by neutral PVP in comparison with pristine CuO NPs. This can be explained by depletion-flocculation phenomena. Negative ζ potential of uncoated CuO NPs was probably given by PO_4^{3-} from buffer specifically adsorbed on NP surface. Coated NPs diluted in water gained the ζ potential consistent with the charge of the coating agent confirming the preferential interaction with the surface modifier. In contrast, DMEM culture medium with FBS substantially changed properties across all NPs irrespective of the coating agent; all CuO NPs displayed similar Z-potential, mean hydrodynamic diameter and high rate of dissolution. The ability of serum proteins to change the agglomeration or surface chemistry thus reducing the toxicity of nanoparticles was demonstrated in several relevant studies [12, 13]. The cytotoxicity results identified positively charged PEI as the most toxic modifier contributing to the cytotoxicity. PEI alone also exerted a considerable cytotoxic effect as also reported in the study of Wightman 2011. The least cytotoxic were CIT- coated and ASC-coated CuO NPs. In our study, citrate and ascorbate have been shown to reduce pro-oxidant properties of CuO NPs, however, we also demonstrated that the effect of ROS on cytotoxicity was marginal only. Cellular uptake evaluated by transmission electron microscopy (data not shown) indicated complete intracellular dissolution of PEI-coated and uncoated nanoparticles since no particles could be detected after 4h exposure to BMD20. This may imply a Trojan-horse mechanism of toxicity of CuO NPs. Relatively low cytotoxicity of Cu^{2+} ions could further support this hypothesis. Cu concentration in cells measured by ICP-MS decreased after 24h exposure indicating exocytosis or passive release of nanoparticles. On the other hand, intracellular Cu burden did not corelated with the cytotoxicity; the proportion of bioavailable Cu in cells appeared to be more important factor.

5. CONCLUSIONS

Overall, our study suggests that coating agents have a considerable impact on stability of CuO NPs. However, dissolution of the NPs, particularly in cell culture medium, was not affected by different surface modifications. In vitro toxicity testing demonstrated that cytotoxicity of CuO NPs in RAW264.7 was not directly linked to nanoparticle dissolution and Cu burden, nor to cellular ROS production. The interplay between NP cytotoxicity, their dissolution, bioavailability, coating agents toxicity and time could explain the resulting toxic effect in RAW264.7 cells.

ACKNOWLEDGEMENTS

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REFERENCES

[1] KARLSSON Hanna L., CRONHOLM Pontus, GUSTAFSSON Johanna, MOLLER Linnart 2008. Copper oxide nanoparticles are highly toxic: a comparison between metal oxide nanoparticles and carbon nanotubes. Chemical Research in Toxicology. 2008. vol. 21, no. 9, pp. 1726-1732.

[2] DI BUCCHIANICO Sebastiano, FABBRIZI Maria R., MISRA Superb K., VALSAMI-JONES Eugenia, BERHANU Deborah, REIP Paul, BERGAMASCHI Enrico, MIGLIORE Lucia. Multiple cytotoxic and genotoxic effects induced in vitro by differently shaped copper oxide nanomaterials. Mutagenesis. 2013. vol. 28, no. 3, pp. 287-299.

[3] CHO Wan S., DUFFIN Rodger, POLAND Craig A., DUSCHL Albert, OOSTINGH Gertie J., MACNEE William, BRADLEY Mark, MEGSON Ian L., DONALDSON Ken. Differential pro-inflammatory effects of metal oxide nanoparticles and their soluble ions in vitro and in vivo; zinc and copper nanoparticles, but not their ions, recruit eosinophils to the lungs. Nanotoxicology. 2012. vol. 6, no. 1, pp. 22-35.

[4] ANTONINI James M., LEWIS Anthony.B., ROBERTS Jenny R., WHALEY David A. Pulmonary effects of welding fumes: review of worker and experimental animal studies. *American Journal of Industrial Medicine.* 2003. vol. 43, no. 4, pp. 350-360.

[5] STARK Wendelin J. Nanoparticles in biological systems. Agnewande Chemie International Edition. 2011. vol. 50, no. 6, pp. 1242-1258.

[6] CRONHOLM Pontus, KARLSSON Hanna L., HEDBERG Jonas, LOWE Troy A., WINNBERG Linna, ELIHN Karine, WALLINDER Inge O., MOLLER Lennart. Intracellular uptake and toxicity of Ag and CuO nanoparticles: a comparison between nanoparticles and their corresponding metal ions. Small. 2013. vol. 9, no. 7, 970-982.

[7] STRAUCH Bettina M., NIEMAND Rebeca K., WINKELBEINER Nicola L., HARTWIG Andrea. Comparison between micro- and nanosized copper oxide and water soluble copper chloride: interrelationship between intracellular copper concentrations, oxidative stress and DNA damage response in human lung cells. *Particle and Fibre Toxicology*. 2017. vol. 14, no. 1, pp. 28.

[8] ORTELLI Simona, COSTA Anna L., BLOSI Magda, BRUNELLI Andrea, BADETTI Elena, BONETI Alessandro, HRISTOZOV Danail, MARCOMINI Antonio. Colloidal characterization of CuO nanoparticles in biological and environmental media. *Environmental Sciences Nano*. 2017. vol. 4, no. 6, pp. 1264-1272.

[9] LIBALOVA Helena, COSTA Pedro M., OLSSON Magnus, FARCAL Lucian, ORTELLI Simona, BLOSI Magda, TOPINKA Jan, COSTA Anna L., FADEEL Bengt. Toxicity of surface-modified copper oxide nanoparticles in a mouse macrophage cell line: Interplay of particles, surface coating and particle dissolution. *Chemosphere*. 2018. vol. 196, pp. 482-493.

[10] BASTOS V., FERREIRA DE OLIVEIRA José M., CAROLLA Joana, DANIEL DA SILVA Ana L., DUARTEM Iola F., SANTOS Conceição, OLIVEIRA Helena. Coating independent cytotoxicity of citrate- and PEG-coated silver nanoparticles on a human hepatoma cell line. Journal of Environmental Sciences. 2017. vol. 51, pp. 191-201.

[11] GLIGA Anda R., SKOGLUND Sara, WALLINDER Inger O., FADEEL Bengt, KARLSSON Hanna L. Size-dependent cytotoxicity of silver nanoparticles in human lung cells: the role of cellular uptake, agglomeration and Ag release. *Particle and Fibre Toxicoogy*. 2014. vol. 14, no. 11, pp. 11.

[12] KWON Dongwook, PARK Jonghoon, PARK Jaehong, CHOI Seo Y., YOON Tae H. Effects of surface-modifying ligands on the colloidal stability of ZnO nanoparticle dispersions in in vitro cytotoxicity test media. *International Journal of Nanomedicine.* 2014. vol 9, no. 2, pp. 57-65.

[13] MURDOCK Richard C., BRAYDICH-STOLLE Laura, SCHRAND Amanda M., SCHLAGER John J., HUSSAIN Saber M. Characterization of nanomaterial dispersion in solution prior to in vitro exposure using dynamic light scattering technique. *Toxicological Sciences*. 2008. vol. 101, no. 2, pp. 239-253.