REVIEW

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Evolving approaches in glioma treatment: harnessing the potential of copper metabolism modulation

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The key properties and high versatility of metal nanoparticles have shed new perspectives on cancer therapy, with copper nanoparticles gaining great interest because of the ability to couple the intrinsic properties of metal nanoparticles with the biological activities of copper ions in cancer cells. Copper, indeed, is a cofactor involved in different metabolic pathways of many physiological and pathological processes. Literature data report on the use of copper in preclinical protocols for cancer treatment based on chemo-, photothermal-, or copper chelating-therapies. Copper nanoparticles exhibit anticancer activity *via* multiple routes, mainly involving the targeting of mitochondria, the modulation of oxidative stress, the induction of apoptosis and autophagy, and the modulation of immune response. Moreover, compared to other metal nanoparticles (*e.g.* gold, silver, palladium, and platinum), copper nanoparticles are rapidly cleared from organs with low systemic toxicity and benefit from the copper's low cost and wide availability. Within this review, we aim to explore the impact of copper in cancer research, focusing on glioma, the most common primary brain tumour. Glioma accounts for about 80% of all malignant brain tumours and shows a poor prognosis with the five-year survival rate being less than 5%. After introducing the glioma pathogenesis and the limitation of current therapeutic strategies, we will discuss the potential impact of copper therapy and present the key results of the most relevant literature to establish a reliable foundation for future development of copper-based approaches.

1. Introduction

Copper is an essential micronutrient involved in fundamental processes that are conserved throughout all forms of life. It plays a role in enzyme catalysis, redox reactions, mitochondrial respiration, and free radical scavenging.[1] Copper also contributes to many biological processes such as embryogenesis, growth, and metabolism.[2]

The regulation of the intra-cellular copper quantities is thus fundamental for the orchestration of cell physiology, especially because copper is potentially toxic if found free in the cytosol rather than bound to metallothioneins. Although its redox activity

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is essential for enzymatic reactions, elevated copper levels may damage lipids, proteins, DNA, and other biomolecules by ultimately inducing cell apoptosis.[3] Moreover, copper may interfere with other metal-driven activities. It can impact the correct functioning of proteins containing iron–sulfur clusters[4] or displace zinc (or other metals) from metalloproteins,[5] resulting in inhibition of their activity. This occurs because copper is a critical component of different metalloenzymes as the matrix metalloproteinase (MMP-9), which is essential to the metastatic process.

Imbalances in copper homeostasis have been implicated in the pathogenesis of neurodegenerative disorders that are associated to toxic effects caused by oxidative stress, such as Wilson's disease (WD),[6] Menkes disease,[7] Alzheimer's disease (AD),[8] Parkinson's disease and amyotrophic lateral sclerosis,[9] as well as idiopathic pulmonary fibrosis,[10] rheumatoid arthritis and diabetes mellitus.[11] Furthermore, there is increasing evidence that copper-dependent cell proliferation, known as cuproplasia,[12] plays a fundamental role in the development and/or progression of different types of tumors,[13–16] such as breast, thyroid, cervical, ovarian, lung, pancreatic, prostate, gastric, oral and bladder cancers.[17–25]

Voli *et al.* (2020) demonstrated the role of copper in modulating PD-L1 expression and contributing to cancer immune evasion by inhibiting EGFR and AKT phosphorylation in

neuroblastoma (NB) and glioblastoma (GBM) cells, suggesting that tumors that exhibit increased intra-tumoral copper distribution could be targeted by selectively reducing their levels of copper.[26] An excess of copper may be one of the factors triggering cancer stem-like cells to initiate the tumor and induce its progression. It is well-known, indeed, that copper is involved in the epithelial-mesenchymal (EMT) and the mesenchymal-to-epithelial (MET) transitions within the tumor microenvironment (TME).[1] When this occurs, cell migration, invasion and tumor growth will be promoted through hypoxia-related genes. Under hypoxic conditions, the hypoxia-inducing factor (HIF) genes are deeply involved in the survival of cancer cells by maintaining immature, stem-like tumor cells. Knockdown of these genes would reduce the expression of angiogenesis-associated vascular endothelial growth factor (VEGF), leading to partial sympathetic neural differentiation of tumor stem cells.

In this review, the recent researches investigating the impact of copper in glioma treatment will be discussed, highlighting the possibility to use either copper overload or depletion as a therapeutic strategy. Copper nanoparticles (Cu NPs) will be explored as the approach to provide tumors with excess Cu allowing the production of reactive oxygen species (ROS), while the use of copper chelators will be discussed as the strategy to reduce the Cu concentration. Moreover, the synthetic routes available for the fabrication of highly engineered Cu NPs, as well as the therapeutic outcome of both approaches will be presented, to open the discussion about future developments in the field and the possible effective translation to pre-clinical and clinical studies.

2. Copper nanoparticles for cancer treatment

Currently, the most relevant therapeutic strategies for cancer treatment are based on surgery, chemotherapy, and radiotherapy.[27] Conventional chemotherapy is based on the use of cytotoxic agents mainly acting by blocking DNA synthesis and/

Table 1 Summary of Cu NPs used for glioma treatment: synthetic procedure and main outcome[a]

| NPs | Synthetic route | Mean diameter | | Main outcome | Ref. |
		D	H		
Cu	High-voltage discharge	2.1		Cell membrane depletion nucleus budding	51
CuO CuO–Fe	Flame spray pyrolysis		209	ROS generation	52
CuO	Alkaline precipitation		141	ROS generation	53
CuO	Alkaline precipitation		136	ROS generation	54
CuO	Alcothermal precipitation	30–60		Apoptosis induction	55
UCNs	Alkaline precipitation	20–30		TMZ stabilization	56
Au–CuO ZnO–CuO	Hydrothermal method (*Cnici benedicti* extract)	13 28		Cell cycle blocked at G2-M phase	57
CuS	Hydrothermal method		50	Photothermal ablation	58
Cu$_2$S	Hot injection colloidal approach	15–20		Photothermal ablation Synergistic DOX delivery	59
CuS	Alkaline hydrothermal method		86	Photothermal ablation Synergistic DOX delivery	60
HM-CuS	Reduction + ion exchange		172	Photothermal ablation Synergistic DSF delivery	61
HM-CuS	Reduction + ion exchange	4		Synergistic TMZ delivery Chemodynamic therapy Photothermal ablation Starvation therapy	62
HM-CuS	Reduction + ion exchange		190	Photothermal ablation Macrophage repolarization	63
CdSe/Cu$_2$S	Hydrothermal method	13–17		Photothermal ablation	64
CPNDs	Alkaline precipitation		80	Synergistic PTX delivery (prodrug) Chemodynamic therapy Photodynamic therapy	65
Au@Cu$_{2-x}$Se	Reductive coating	21		Inhibition of autophagy flux	66
Cu$_{2-x}$Se	Reduction method	3		Photodynamic therapy	67
Cu$_{2-x}$Se	Reduction method	3		Synergistic DSF delivery	68
Cu$_{2-x}$Se	Reduction method	3		Synergistic IDX delivery Immunotherapy	69
CuHARS	Redox self-assembly	20		Cell tracking	70
CuHARS	Redox self-assembly	20		Nitrogen oxide production	71
CuHARS	Redox self-assembly	20		Apoptosis	72

[a] CPNDs: copper peroxide nanodots; Cu HARS: copper high-aspect ratio structure; Cu USNPs: copper ultrasmall NPs D: dry; DSF: disulfiram; DOX: doxorubicin; H: hydrated; HM: hollow mesoporous SiO$_2$; IDX: indoximod, NPs: nanoparticles; PTX: paclitaxel; TMZ: temozolomide; UCNs: polymer-templated Cu NPs.

or cell replication.[28] Each approach suffers from some severe limitations, mainly due to the uncomplete removal of cancer cells (surgery),[29] or side effects on healthy cells and tissues (chemo- and radio-therapy).[30,31] Thus, current trends in oncology focus on the design and development of efficient cancer nanomedicines,[32,33] defined as miniature-sized products with ideal properties for interaction with living tissues, including (but not limited to) reduced toxicity, high surface-area-to-volume ratio, and high chemical versatility.[34] Several nanoplatforms are currently in development, including polymer nanoparticles and micelles, liposomes and lipid nanoparticles, inorganic and metal nanoparticles.[35] Each material shows advantages and disadvantages arising from their intrinsic properties, allowing or denying specific applications.[36]

Metal NPs, including noble (e.g. gold, silver, platinum, and palladium) and non-noble (e.g. iron, zinc, titanium, cerium, nickel, copper, magnesium, barium, calcium, and bismuth) metal-based NPs have recently gained significant interest for multipurpose biomedical applications, including cancer treatment.[37–39]

Copper nanoparticles (NPs) possess significant advantages both in terms of synthesis and application within the realm of metal NPs.[40] Notably, the production of copper NPs is more cost-effective than that of other noble metal NPs, owing to the abundant natural availability of the metal.[41] This abundance facilitates efficient large-scale production and straightforward storage and handling of highly engineered nanosystems.[42] The distinctive properties of copper NPs, including their crystallinity, surface strain, and the prevalence of defect sites, contribute to their rapid dissolution.[43] This characteristic leads to noteworthy biological effects, as copper plays a substantial role in various biological functions. Furthermore, copper NPs exhibit versatility in undergoing a wide array of reactions due to their accessible oxidation states (0, I, II, and III).[44] This chemical adaptability not only optimizes the synthetic procedures, but also allows tailored functionalization, thereby enabling precise modulation of biological responses at the tissue and cellular levels.[45]

Over the last decades, the interest over copper NPs is increasing due to their features and associated potential applications in nano-biomedicine,[46] including the possibility to induce copper-dependent apoptosis (cuproptosis) of cancer cells via oxidative stress.[8] Although their potential applications, in vivo studies should better elucidate the toxicity of copper nanoparticles on normal cells since they are not yet fully understood.[47–49]

Within the present review, we will focus on the application of copper NPs in glioma treatment since the hypoxic environment contributes to the development of therapeutic resistance and invasiveness into normal brain tissues,[50] thus increasing the need for a more effective therapeutic regimen. In the following paragraphs, the different types of copper NPs tested for glioma treatment will be discussed, highlighting the synthetic procedure and the main biomedical outcome (Table 1).

3. Synthesis of copper nanoparticles for glioma treatment

The preparation of copper NPs can be accomplished with various methods including physical, wet-chemical, and green synthesis.

As for gold NPs preparation, the wet methods result widely used for their straightforwardness, while physical methods usually require specific equipment. It is worth reporting that several papers describe the synthesis of copper NPs by employing microorganisms, among which plants, algae, bacteria, and fungi, which spontaneously synthesize copper nanoparticles.[73]

3.1. Copper nanoparticles by top-down approaches

Physical syntheses of NPs are techniques which use mechanical or thermodynamic processes. In general, when compared to wet methods, the physical processes often involve large and expensive set-up requiring high energy. On the other hand, the particle distribution can reach an interesting uniformity and the contamination of chemicals can be removed.[74] For example, the solvated metal atom dispersion method allows to avoid purification processes and byproducts formation.[75] This method generates metal atoms through vaporization of the bulk material, subsequently condensed into clusters with a solvent at low temperature (around 70 K). Reaching the room temperature, NPs are separated from the solvent by evaporation. For copper, the nature of the solvent, together with the reaction conditions, have been proved to be crucial for determining the nanoparticle size.[76]

The voltage discharge method belongs to the top-down methodologies and it is often referred as a simple and cheap physical method for nanoparticle synthesis.[77] Briefly, the bulk material of two electrodes is eroded by an increase of temperature associated with a current flow.[78] Vodyanoy et al. used two metal electrodes immersed in water and connected to a high-voltage generator.[51] Applying an AC voltage (15 kV), an electric discharge was generated between the electrodes for 1 hour. Depending on the distance between the electrodes, the generated plasma produced a satisfactory dispersion of the metal. After 12 h sedimentation and 2 h centrifugation, 2 nm NPs were recovered from the supernatant. Size and distribution were measured with atomic force microscopy, revealing a quite narrow distribution with a standard deviation of 0.6 nm and a polydispersity index of 0.082. A 15% of the atoms were oxidized and the material was investigated on rat brain glioma cancer cells RG2.

The ablation of copper can also be accomplished by employing a pulsed laser in a liquid environment. Tilaki et al. investigated the influence of the medium on the size and shape of the obtained NPs. They used a Nd:YAG laser in water and acetone.[79] TEM images of the resulting particles showed an average diameter of 30 and 3 nm in water and acetone, respectively, with a more regular shape in the second medium. Colloidal copper in acetone had a longer shelf life compared to the particles in water (10 months vs. two weeks). Furthermore,

the degree of oxidation of copper NPs in water was higher due to the oxygen dissolved into the medium. The presence of copper oxide is a significant information since it can crucially influence the bio-toxicity of the particles. CuO NPs, indeed, may improve the production of ROS, which can alter the metabolism of cells.[40] Iron-doped CuO NPs were used by Joshi et al. on C6 glioma cell line.[52] The synthesis was accomplished through flame spray pyrolysis, a physical method where a solution containing precursors (usually metal salts) is sprayed into a flame. The formed droplets, after the evaporation of the solvent and pyrolysis of the precursor, result in the production of the metal oxide NPs. Combustion process, aerosols technology and materials of precursors determine the characteristics of the particles in terms of morphology, homogeneity, and size.[80] In particular, CuO-NPs and 10% iron-doped CuO-NP (CuO-Fe-NPs) ultrafine powders were produced using copper naphthenate and iron naphthenate precursor solutions in xylene, a methane/oxygen flame and a glass fiber to collect the NPs.[52] Flow rate, flame precombustion characteristics and precursor concentration were finely tuned to obtain spherical 50 nm particles, stable in water after functionalization with dimercaptosuccinate (ζ-potentials of -40.3 and -37.2 mV).

3.2. Copper nanoparticles by bottom-up approaches

Wet-chemical synthesis procedures are referred to as bottom-up approaches, and they allow a better tuning of size, shape, and chemical composition of the final metal NPs compared to the top-down approaches.[81] NPs have to be purified from the chemical and biological unreacted precursors, which could limit the biocompatibility of the NPs, especially for the non-green procedures. The persistence of toxic reactants, indeed, may affect both the biological investigation and the potential applications. Reduction of the metal ions in solution is one of the preferred methods to produce metal NPs due to the versatility of the process. Stabilizing agents of low or high molecular weight can be employed to protect the metal NPs from aggregation and environmental stress factors.[82] It is worth to remember that the conjugation of stabilizing agents on the surface of metal NPs can modify the biological interactions of the nanostructures.

Copper NPs (CuNPs) and copper-oxide NPs (CuO NPs) for the treatment of gliomas can be prepared by reduction of copper salts. Joshi et al. synthetized CuO NPs of around 141 nm of hydrodynamic diameter by a wet chemical method.[53] Following the procedure proposed by Bulcke et al., and by Kobayashi et al., $Cu(NO)_3$ was reduced in an alkaline solution (NaOH) to induce the NPs formation at 75 °C.[54,83] Dimercaptosuccinic acid (DMSA) was used to coat CuO NPs, and a bovine serum albumin (BSA) coating was used to improve the stability of the resulting CuO NPs. The protein coating increased the hydrodynamic diameter to 175 nm, with an increase of the zeta potential from -42.5 mV to -14.1 mV for CuO NPs. Precipitation by addition of high salt buffer allowed their purification. The copper content was quantified photometrically using bathocuproine disulfonate by absorption at 405 nm against a calibration curve of $CuCl_2$, and atomic absorption

spectroscopy was used for validation.[53] Joshi et al. suggested that the toxic effects observed in C6 glioma cells was due to the release of copper ions from the NPs. Kukia et al. produced Cu NPs of 30 and 60 nm (as per scanning electron microscopy – SEM analysis) through an alcothermal method.[55] $CuSO_4$ was used as a Cu^{2+} source, and sodium borohydride ($NaBH_4$) reduced the ions. The reduction of Cu^{2+} is followed by the change of the colloid solution color from blue to green, and eventually to brown. Polyvinylpyrrolidone K30 (PVP) was selected as stabilizing agent, and further stabilization was provided by ascorbic acid added at 60 °C to avoid Cu oxidation to CuO.[84] Cu NPs were purified through precipitation and washing in ethanol. These Cu NPs exerted toxicity to a glial rat cancer cell line (B92) by stimulating apoptosis, especially for the smaller NPs at higher concentrations.

Wang et al., proposed a biomimetic polymer-templated Cu NPs (UCNs) to stabilize a temozolomide intermediate[56] (Fig. 1).

Temozolomide is the first-line treatment for glioblastoma, and it was designed as a prodrug: temozolomide crosses the blood–brain barrier and converts spontaneously into the active 5-(3-methyl-1-triazeno)imizadole-4-carboxamide (MTIC) molecule.[85] This prolongs the short half-life of MTIC, but the conversion kinetics is slow and at the same time MTIC can be released extracellularly. UCNs were designed as delivery system of the active form MTIC. First, Cu^{2+} ions from $CuSO_4$ were complexed by a DNA-mimetic polymer, and then reduced to Cu using hydroxylamine chloridrate in an alkaline environment (pH 11.5). After stirring for 2 hours, UCNs were purified through dialysis using 10 kDa MWCO membrane against pH 9.6. Copper nanoclusters stabilized by these biomimetic polymers were 20–30 nm (TEM). The reduction of Cu was not complete, as an autoxidation took place.

Moreover, the polymer avoided Cu escape into the solution. Copper was quantified spectrophotometrically by detection of the complex between Cu^{2+} and diethyldithiocarbamate at 450 nm. The persistence of Cu^{2+} is crucial as only this ion can complex MTIC.[86] Finally, temozolomide was added to UCNs and
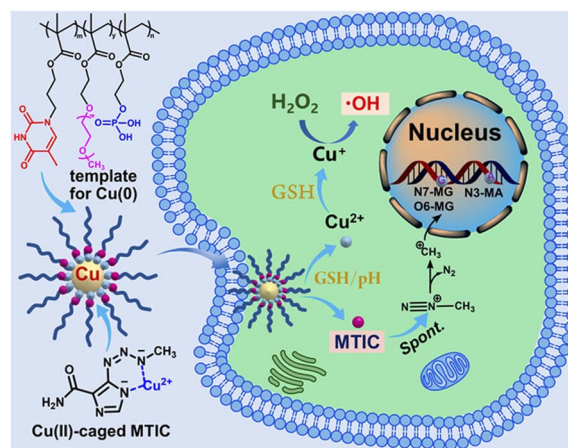

Fig. 1 Schematic representation of UCNs for TMZ stabilization. Reproduced with permission from ref. 56. Copyright© 2021, American Chemical Society.
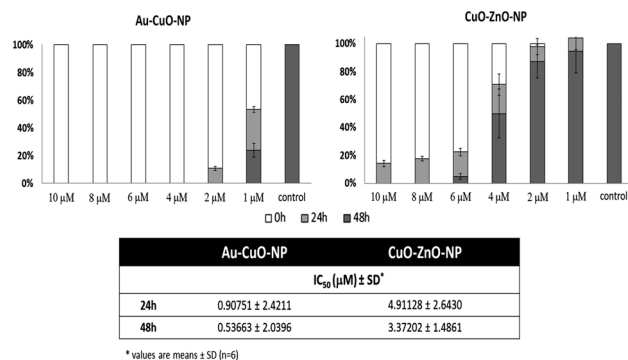
Fig. 2 Cytotoxicity of biosynthesized Au–CuO and CuO–ZnO nanoparticles on C6 cells. Reproduced with permission from ref. 57. Copyright© 2018 Production and hosting by Elsevier B.V. on behalf of King Saud University.

the drug was spontaneously hydrolyzed to MTIC, which in turn complexed Cu^{2+}. These delivery systems were tested on temozolomide-resistant and -sensitive glioblastoma cells (T98G and U-87MH, respectively). Interestingly, authors conjugated the effect of CuNPs to the therapeutic effect of MTIC paving the way for the reduction of temozolomide resistance burden.

New trends in the bottom-up approach for the synthesis of CuNPs and CuO NPs involve the use of green reducing agents. For example, Elemike *et al.* used the *Alchornea cordifolia* aqueous leaf extract to reduce $CuSO_4$ and obtain a dark green colloidal suspension of 16 nm Cu_2O/CuO NPs tested against HeLa cancer cells (cervical adenocarcinoma).[87] *Wringhtia titoria* extract was used by Rajagopal *et al.* to reduce $CuSO_4$ to obtain 15 nm CuNPs applied to breast cancer cell line (MCF-7).[88] Sankar *et al.* discovered the capability of *Ficus religiosa* leaf extract to reduce $CuSO_4$ and produce CuNPs with a hydrodynamic diameter of 577 nm.[89] These NPs had anticancer activity and were tested by Kalaiarasi *et al.* in A549 lung cancer cells, in which they stimulated apoptosis and anticancer activity *via* inhibition of the histone deacetylase.[90] As far as glioma is concerned, *Cnici benedicti* water extract were used for the green synthesis of bimetallic nanoparticles such as Au–CuO and CuO–ZnO NPs, obtaining higher efficiency in the presence of Au rather than ZnO (Fig. 2).[57]

3.3. Copper sulfide nanoparticles

Over the past few years, considerable attention has been paid to biomedical applications of copper sulfide nanostructures (Cu_xS_y) as multimodal imaging, due to the paramagnetic behavior and to the possibility to vary the stoichiometries in order to tune the absorption above 900 nm.[91] In this spectral region, almost no interference of biological tissues occurs, thus it is possible to significantly reduce background noise and enhance the spatial imaging resolution,[92,93] including magnetic resonance imaging (MIR), upconversion luminescence imaging, and photoacoustic imaging.[94,95]

Moreover, copper sulfide NPs are recognized as an effective alternative to Au NPs for photothermal therapy, which originates from a d–d electronic transition,[96] whereas surface
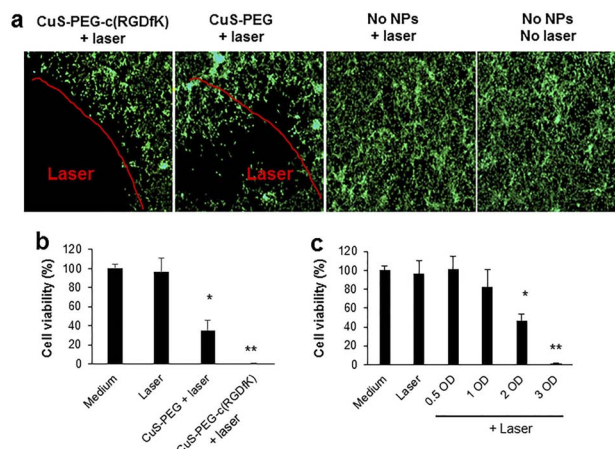

Fig. 3 (a) Photothermal ablation of U87 cells mediated by CuS-PEG-c(RGDfK) or CuS-PEG NPs. (b) U87 viability upon treatment with CuS-PEG-c(RGDfK) or CuS-PEG NPs. (c) U87 viability upon treatment with increasing concentrations of CuS-PEG-c(RGDfK) NPs. Reproduced with Permission from ref. 58. Copyright© 2018, American Chemical Society.

plasmon resonance is involved in the case of Au NPs.[97] Interestingly, the acidic environment of tumor tissues induces the Cu^{2+} release from CuS NPs, further promoting the ROS production *via* Fenton-like reaction with H_2O_2.[98] By combining MIR and PTT properties of CuS, theranostic platforms for image-guided ablation therapy can be designed, with possibility to target glioma cells by conjugation with PEGylated c(RGDfK) peptide (Fig. 3).[58]

Poulose *et al.* reported on the anticancer efficiency of Cu_2S NPs synthesized by hot injection colloidal approach involving the use of Cu and S precursors in the absence of any cytotoxic ligands. NPs were coated with 1,2-distearoyl-*sn-glycero*-3-phosphoethanolamine-*N*-[folate(polyethylene glycol)-2000] (DSPE-PEG-Fol) for targeting the glioma cancer cells and taking advantages from a targeted NIR-responsive photothermal ablation in synergism with the chemotherapeutic action of a loaded cytotoxic agent such as DOX.[59] A similar chemo- and photothermal co-therapy was obtained by loading DOX into the mesoporous SiO_2 coated CuS NPs.[60] In details, CuS NPs were capped with cetyltrimethylammonium bromide to confer the positive charge suitable for the growth of the mesoporous silica shell.

In a different approach, Lan *et al.* synthesized hollow mesoporous CuS NPs (HM-CuS NPs) as disulfiram delivery vehicle.[61] At first, Cu_2O NPs were prepared by reduction of $CuCl_2$ with hydrazine, and then subjected to ion exchange in the presence of Na_2S. Then, disulfiram was loaded by exploiting its ability to chelate copper, and a targeting effect was obtained by coating with transferrin. The final nanosystem was found to selectively kill glioma cells both *in vitro* and *in vivo* as a result of CuS acid-responsive dissolution and copper ions release characteristics (Fig. 4).

Hollow mesoporous copper sulfide NPs (HM-CuS NPs) were used for a chemotherapy (CT)/starvation therapy (ST)/ chemodynamic therapy (CDT)/photothermal therapy (PTT) of glioblastoma.[62] A complex HM-CuS NPs nanosystem was
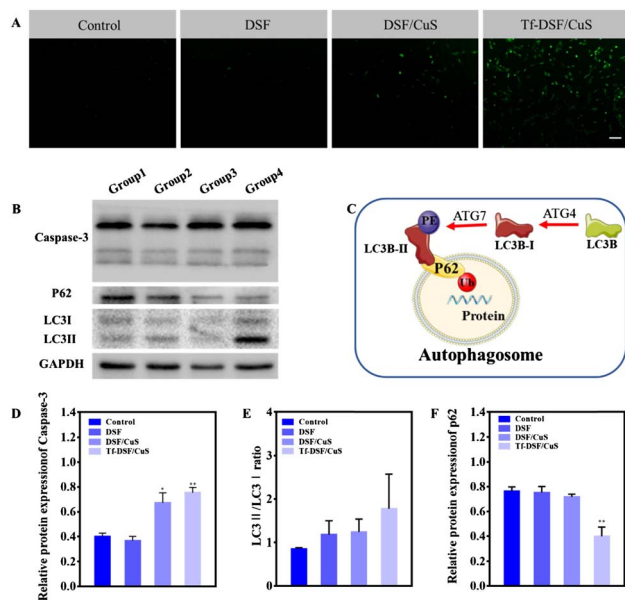
Fig. 4 ROS generation autophagy induction by Tf-DSF/CuS. (A) H2DCFDA (green) staining indicates ROS generation. Scale bar = 100 μm. (B) Western blot analysis of apoptosis-related protein (caspase-3) and autophagy markers (LC3I/II, P62). (C) Schematic process of autophagy. Densitometry of caspase-3 (D), LCII/LC3I (E), and p62 (F) in western blots. Reproduced with permission from ref. 61. Copyright© 2021 Elsevier B.V.

prepared by loading with temozolomide as a chemotherapeutic, while hyaluronic acid was used as a coating material for inducing targeting efficiency and preventing drug from premature leakage. Glucose oxidase (GOx) was also inserted on the NPs shell to

Fig. 5 (a) SEM and (b) TEM image of HM-CuS NPs. (c) TEM images of CTHG-Lf NPs. (d) STEM image and (e) and (f) elemental mapping of Cu and S of CTHG-Lf NPs. (g) XRD of CTHG-Lf NPs. (h) UV-vis-NIR absorption spectra of TMZ, HM-CuS, HM-CuS(TMZ), GOx and CTHG NPs. (i) FT-IR of HA, GOx, HM-CuS NPs and CTHG NPs. Reproduced with permission from ref. 62. Copyright© 2021 Elsevier B.V.

reduce the intracellular glucose level thus inducing ST, and to generate H_2O_2 (acting as a substrate of Cu based Fenton-like reaction) upon production of gluconic acid further promoting the copper ions release due to pH reduction. NPs surface was further modified with lactoferrin (Lf) to enhance the blood–brain barrier penetration (Fig. 5).

The surface of HM-CuS NPs was modified with Lauric acid and a PEGylated peptide acting as a gatekeeper and pH-responsive element enabling translocation within cancer cells, respectively, to fabricate a carrier for the ISRIB stress granules inhibitor.[63] The activity of the nanosystem was related to a combined PTT and remodeling of the immunosuppressive microenvironment of brain metastases.

CuS was finally used as a component of quantum dots platforms (QDs). Mohamed *et al.* developed a strategy to encapsulate Cu_2S within CdSe QDs by hydrothermal treatment in the presence of Jatropha curcas oil as a capping coordinating and stabilizing reagent.[64] The ultimate aim was to combine the property of Cd as X-ray contrast agent with the Cu_2S induced PTT, and the system was further engineered by conjugation with PEG and folic acid as biocompatible and targeting elements, respectively.

3.4. Other copper nanoparticles

A different class of copper NPs was proposed by Lin *et al.*, who prepared copper peroxide nanodots (CPNDs) as chemodynamic therapy agent boosting the production of ROS within the cancer cells.[99] Authors exploited the well-known ability of metal peroxides to act as a H_2O_2 source to promote ROS production *via* Fenton-like reactions.[100] They synthesized 16.3 nm CPNDs by the reaction of $CuCl_2$, H_2O_2, and sodium hydroxide in an aqueous solution containing polyvinyl pyrrolidone at room temperature for 30 min. The CPNDs were effectively internalized within cancer cells by enhanced permeability and retention effect, and then decomposed in the acidic endo/lysosomal compartments, with the release of large amount of H_2O_2 (Fig. 6).

A further development of this approach consisted in embedding CPNDs within a hydroxypropyl chitin hydrogel together with RGD-peptide-modified paclitaxel prodrug nanoparticles for a combined photodynamic/chemodynamic/chemotherapy treatment of postoperative glioblastoma, reaching a consistent extent of the survival time of postoperative glioma mice.[65]

To overcome the radioresistance of glioblastoma, Xu *et al.* proposed core–shell copper selenide coated gold NPs ($Au@Cu_{2-x}Se$ NPs) that inhibited DNA repair mechanisms and protective autophagy on U-87MG cancer cells.[66] Inner AuNPs of 13 nm were produced following Turkevich method, while the protective layer was produced by reduction of SeO_2 and $CuSO_4$ with ascorbic acid.[101] This step changed the color of the colloid suspension to dark green. Dithiol PEG (5 kDa) was used to modify the surface of the NPs.

The purification was performed *via* dialysis with a molecular weight cut-off (MWCO) of 30 kDa. The final size was around 21 nm, and it was assessed by TEM. Copper was quantified by using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES).
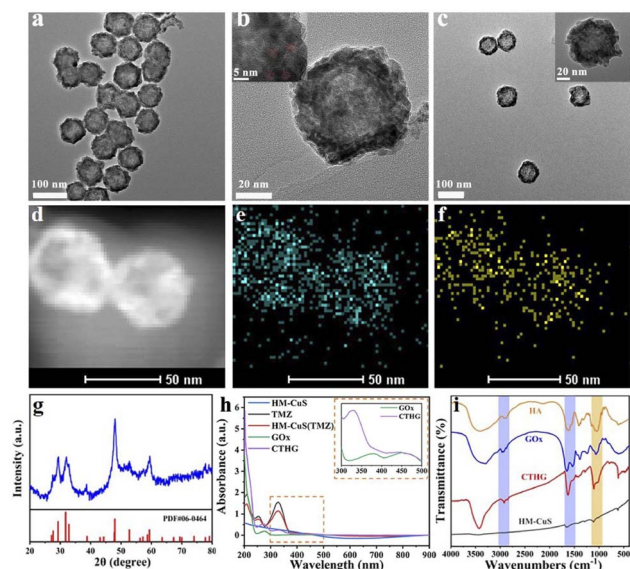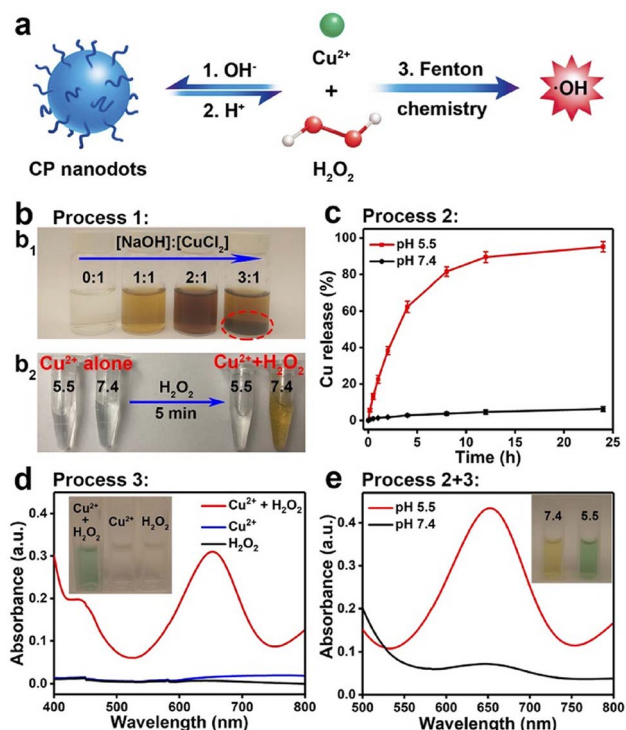
Fig. 6 (a) Schematic representation of formation and dissociation of CP nanodots for ˙OH production. (b$_1$) Photograph of CP materials obtained in the presence of PVP at different molar ratios of NaOH to CuCl$_2$. (b$_2$) Photograph of CuCl$_2$ solutions with different pH values before and after the addition of H$_2$O$_2$. (c) Cumulative Cu release from CP nanodots in different pH conditions. (d) UV–vis spectra and photographs (inset) of TMB aqueous solution treated with H$_2$O$_2$, Cu^{2+}, or Cu^{2+} plus H$_2$O$_2$ for 30 min. (e) Colorimetric detection of ˙OH generated by CP nanodots at different pH values. Reproduced with permission from ref. 99. Copyright© 2019 American Chemical Society.

Cyclodextrin (CD)-modified Cu$_{2-x}$Se NPs were proposed for a photodynamic chemotherapy (Doxorubicin as cytotoxic drug), by taking advantages of the ability of Near Infrared Radiation (NIR) irradiated NPs to produce a huge amount of ROS by means of both electron transfer and energy transfer mechanisms.[67] Spherical NPs (3.3 nm as per TEM analysis) were synthesized by adding CuCl$_2$ to a selenium precursor obtained by reduction of Se powder with NaBH$_4$ and removing the excess copper by ultracentrifugation. Afterward, NPs were modified with CD and HS-PEG-SH to allow a pH-controlled release of the cytotoxic agent after delivery into malignant glioblastoma by opening the blood–brain-barrier with the assistance of focused ultrasound. The same material was tested as a vehicle for boosting the anticancer performance of orally administrated disulfiram after loading with hypoxia-inducible factor-1α (HIF-1α) inhibitor and coating with tumor cell membrane.[68] Noteworthy, the same research group explored the possibility to use such cell membrane-coated NPs in an immunotherapy protocol by selectively targeting the tumor-associated macrophages instead of glioma cells. Authors were able to re-activate the immune responses through remodeling the tumor immunosuppressive microenvironment by loading an inhibitor of

indoleamine-2,3-dioxygenease (indoximod) and an inhibitor for reducing the expression of PD-L1 (JQ1).[69]

Along with copper nanospheres, materials with different morphologies have been proposed for glioma applications, as the biocomposite of copper and cysteine suggested by Kelly *et al.*[102,103] These copper structures (CuHARS) include copper and L-cysteine and demonstrated high stability with no aggregation in liquid media. L-Cys was dissolved in a NaOH solution, and CuSO$_4$ was added as a source of copper ions. The excess of copper was removed by a short HCl treatment, and the product was purified by precipitation and washings with water.[70,71] CuHARS sizes ranged from about 20 nm in diameter and hundreds of nm in length. Karan *et al.* integrated CuHARS in cellulose discs to test them on CRL2303 glioma cells.[70] CuHARS were slowly but completely biodegraded in biological media, probably because of copper complexation to enzymes and proteins. The entrapment in cellulose scaffold prolongs the persistence of CuHARS and allows the interaction with cells to exert their action. Karekar *et al.* tested the toxicity of CuHARS on CRL2303 glioma and SH-EP1 neuroblastoma cells.[72] CuHARS were more toxic than the silver analogue to these cell lines. The same group evaluated the immunomodulatory potential of CuHARS on CRL2303 glioma cells, in which they increased nitrogen oxide production while reducing the viability of glioma cells and avoiding harming healthy cells (brain microvascular endothelial cells, BMVECs).[71]

Interesting anticancer applications have been demonstrated for ultrasmall-in-nanoarchitectures (NAs) that comprise copper ultrasmall NPs (USNPs) of <2 nm.[104] NAs are hybrid nanomaterials in which ultrasmall metal NPs are entrapped in polymeric aggregates and protected by a silica shell.[105,106] The final size is around 100–150 nm, with a silica shell of approximately 20 nm. NAs are promising tools in anticancer therapy because they do not accumulate in the body nor harm the organs, moreover NAs can combine different therapeutic and diagnostic approaches in a single system.[107–113] Cu USNPs are prepared by reduction of glutathione-complexed copper ions from CuSO$_4$ using NaBH$_4$. Cu content in the whole NAs was quantified by Inductively Coupled Plasma Mass Spectrometry (ICP-MS).[104] CuNAs favored the recovery from burnt skin in *in vivo* models after a single topical application, with a significant anti-inflammatory activity and without systemic toxicity.[104] On the other side, CuNAs also showed to slow down the metastatic cascade in pancreatic chorioallantoic membrane (CAM) cancer models of BxPC-3.

4.   Copper chelation therapy as a therapeutic strategy against glioma

The notion that copper is a key limiting factor for tumor progression has encouraged the development of copper-specific chelators as therapies to inhibit this process.[10] Copper chelators are small molecules that bind to copper ions and reduce their availability in the body. These compounds mimic the regulatory chaperones that manage cellular copper influx and efflux across cell membranes in physiological conditions. They can directly

induce apoptosis through the generation of ROS and the inhibition of cytochrome C oxidase, which would decrease the level of ATP produced. These phenomena are directly linked to the role played by copper in essential cellular processes such as energy production. Additionally, copper chelators can prevent the recruitment of bone marrow-derived endothelial progenitor cells (EPC). This results in the inhibition of angiogenesis and tumor progression by affecting signaling pathways such as PI3K/AKT and MAPK/ERK, and/or via the regulation of epigenetic changes, also during late-stages.[7]

Copper chelating agents such as tetrathiomolybdate (TM), D-penicillamine (DPA) and triethylenetetramine dihydrochloride (trientine/TETA), alone or in combination therapies,[114] are already employed for the treatment of Wilson's disease.[115,116] Clinical trials revealed that these therapies are generally well tolerated, since selectively targeting cancer and exerting little toxicity on normal cells.[117,118] Based on their efficacy, these compounds and other similar drugs are currently being investigated for their adaptability to cancer therapeutics.[7] As a matter of fact, TM has been shown to inhibit tumor growth and angiogenesis in animal models of breast cancer by reducing the expression of the highly-angiogenic factor Lysil oxidase (LOX) and the metalloproteinases MMP-2 and MMP-9, which are involved in the generation of metastases.[119,120] This compound is also currently used in phase I or II clinical trials for the treatment of BRAF V600E mutated tumors,[121–123] head and neck carcinoma,[124] mesothelioma[118] and pancreatic duct adenocarcinoma.[125] Similarly, D-penicillamine treatment reduces metastatic melanoma cells.[126] Furthermore, it was able to delay progression of glioblastoma by inhibits LOX activity and reducing VEGF expression.[127] Despite reducing excessive copper levels in the system by secretion through the urine, it was also demonstrated that DPA chelation leads to the generation of hydrogen peroxide (H_2O_2) and other ROS, resulting in copper-dependent cytotoxic effects.[128] Consistently, even though DPA was involved in different clinical trials for the treatment of Wilson's[129] and Alzheimer's disease,[8] as well as glioblastoma,[15] DPA chelation of copper in brain (where copper concentration is usually higher) did not ameliorate patients' neurological symptoms, due to ROS-dependent toxicity.[130] However, around 60% of patients showed an improvement after 4 years of therapy.[131] The combination of DPA with inhibitors of hydroperoxide metabolism is thus more common than the treatment involving the single agent. Alternatively, despite having a reduced effect compared to DPA, TETA shows an improved safety profile. This drug was originally introduced for the treatment of DPA-intolerant patients with Wilson's disease[132] and its efficacy to hinder tumor growth in vivo, with a molecular mechanism involving inhibition of IL-8 production, was first demonstrated in hepatocellular carcinomas.[133] In addition, it was shown that TETA exerts an anti-tumor effect not only via the regulation of copper transporters' flux, but also by the interaction with multiple anticancer targets, which results in the reduction of oxidative stress and polyamine metabolism, an important energy source for cell's growth.[134] Remarkably, it was demonstrated how TETA can cross the blood–brain-barrier (BBB) and target the central nervous system (CNS) by surface modified liposomes, which enable a higher-dose drug delivery and successfully delays

neurodegeneration,[135] resulting in the most promising copper-chelating agents to target brain tumors.

A similar molecular mechanism is exerted by the FDA-approved tetraethylenepentamine (TEPA), an analogue of trientine. Recently, it was reported how TEPA can increase immune cell infiltration in the TME, downregulating STAT3, EGFR, AKT, and GSK3b phosphorylation, which inhibits the transcription of PD-L1 and thus reduces tumor growth in NB and GBM mouse models, improving survival.[26] Furthermore, another study showed that TEPA downregulates EMT-associated cancer invasion in vitro by directing reducing the levels of TGF-β and downstream signaling pathways in three tumor types: human triple-negative breast cancer (TNBC), diffuse intrinsic pontine glioma (DIPG) and NB. In the same study, it was also shown how metastases are suppressed in vivo, even if small doses of TEPA were used, making it stand out against other copper chelating agents, or other therapies directly targeting TGF-β, as an attractive non-toxic alternative.[136]

The number of ongoing clinical trials for copper chelators as single agents is much bigger than the number of trials concerning combination therapies. A deeper investigation of the molecular mechanisms acting in synergy during treatment would boost consideration of these novel agents as an effective therapeutic strategy against cancer.

5. Conclusions and perspectives

The involvement of copper in the development and/or progression of different types of tumors suggests new therapeutic opportunities, with three main routes evoked for explain the biological mechanisms to kill cancer cells by using copper, namely (i) the use of Cu NPs as ROS inducers; (ii) Cu chelation by suitable chemical species, and (iii) induction of Cu overload by local release of copper in the tumor area followed by ROS production and cuproptosis. Each strategy shows great potentiality allowing hypothesizing effective translation to the pre-clinical and clinical practice, although some key issues should be addressed. Cu NPs, indeed, may lead to a moderate risk of off-target toxicity; copper chelators are characterized by safer profile and much less toxicity but often they need to be used in combination with other therapeutic(s) for an effective cancer treatment; while fewer groups tested Cu overload as a therapeutic regimen.

In this review, we mainly discussed the recent development in using Cu NPs, since they showed the key advantage to allow customizing the particles physico-chemical properties, loading Cu chelators and drugs on the same structure, including ROS inducers and conventional chemotherapeutics. Open questions and possible improvements are in the area of specificity, immune system clearance of the particles, ADMET, and off target toxicity. Solving these challenges will contribute to enhance the translation of copper overload/depletion in oncology, even if additional investigations are required to improve the rational design of nanoparticle architectures to tailor the features for the desired therapeutic activity.

We expect that in the future many clinical trials will start to test combinational therapies more than single agent ones, Cu-related treatments are often wide range and multitargeting,

i.e. TEPA downregulation of kinases and mitochondrial activity. These wide spectra therapeutics are suitable for combination with highly specific drugs, such as EGFR inhibitors for example, resulting in a stronger and more articulated inhibition of tumor growth. A multitarget therapy combined with a highly specific one can tackle the cancer cells ability to grow resistance upregulating other pathways, *i.e.* mTOR upregulation as resistance to MAPKs inhibition.

Moreover, the design of nanoparticle from an industrial perspective is urgently required to hypothesize an effective clinical translation of Cu NPs. Although these issues still need to be solved, we hope that this review can open a discussion among the scientific community and show the potential applicability of Cu NPs for the treatment of brain cancer.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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