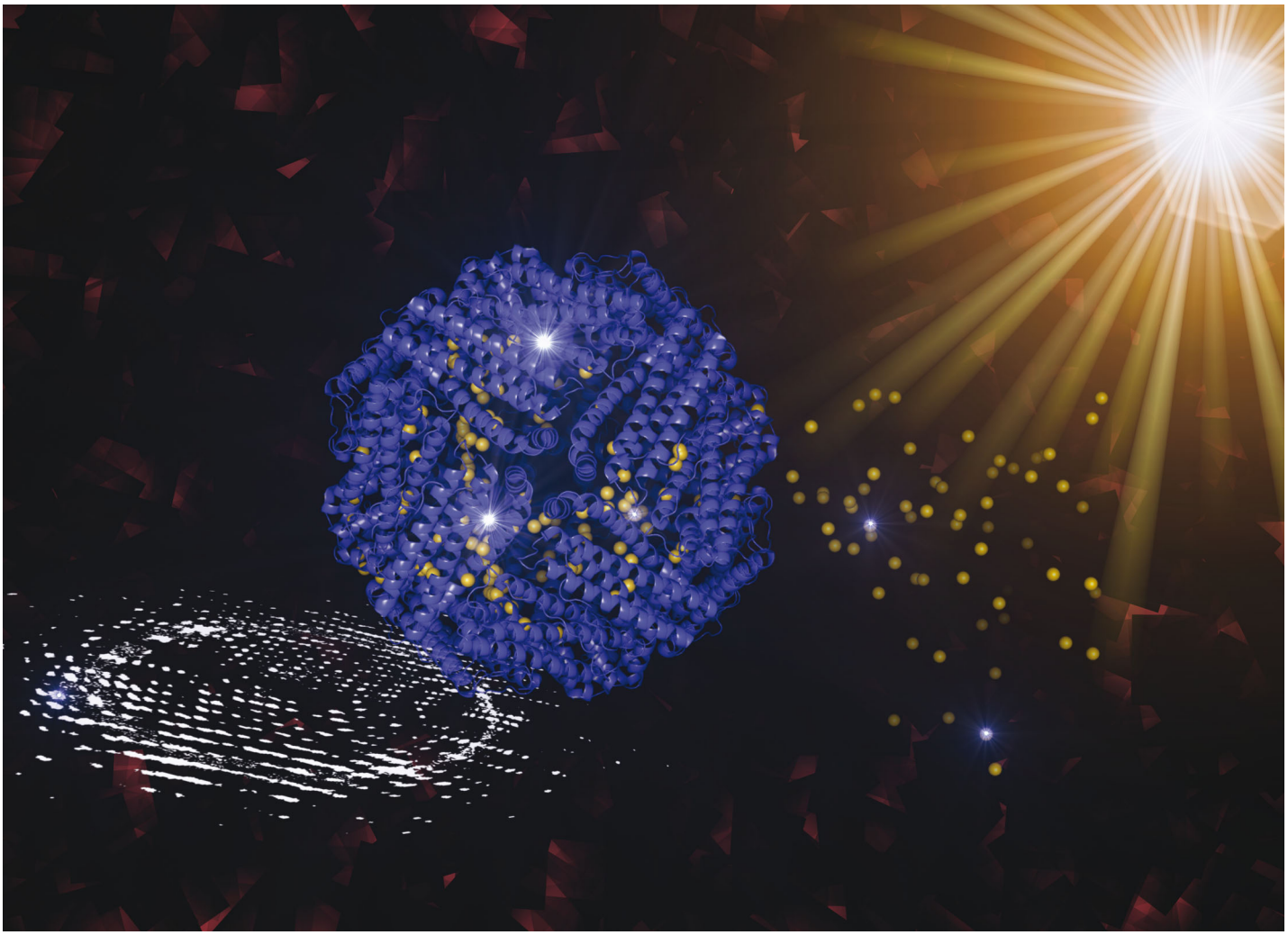
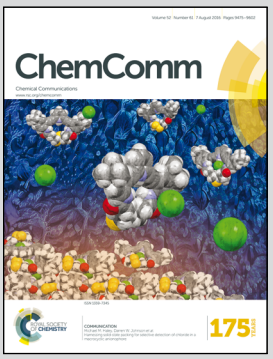


Showcasing research from Prof. Antonello Merlino's laboratory/ Department of Chemical Sciences, University of Naples Federico II, Napoli, Italy

Gold-based drug encapsulation within a ferritin nanocage: X-ray structure and biological evaluation as a potential anticancer agent of the Auoxo3-loaded protein

The authors have encapsulated a cytotoxic gold(III) compound within the ferritin nanocage. Gold binding sites have been identified by X-ray crystallography. The gold-encapsulated nanocarrier exhibits much higher cytotoxicity than free AFt, which is basically non-toxic. The adduct is much more cytotoxic on human cancer cells than on non-tumorigenic cells. These data indicate that encapsulation of gold-based drugs within Ft nanocages is a promising strategy to deliver gold-based drugs to their final targets.

As featured in:



See Antonello Merlino *et al.*, *Chem. Commun.*, 2016, **52**, 9518.

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Gold-based drug encapsulation within a ferritin nanocage: X-ray structure and biological evaluation as a potential anticancer agent of the Auoxo3-loaded protein†

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Auoxo3, a cytotoxic gold(III) compound, was encapsulated within a ferritin nanocage. Inductively coupled plasma mass spectrometry, circular dichroism, UV-Vis absorption spectroscopy and X-ray crystallography confirm the potential-drug encapsulation. The structure shows that naked Au(I) ions bind to the side chains of Cys48, His49, His114, His114 and Cys126, Cys126, His132, His147. The gold-encapsulated nanocarrier has a cytotoxic effect on different aggressive human cancer cells, whereas it is significantly less cytotoxic for non-tumorigenic cells.

In recent years, gold-based compounds have emerged as promising alternatives to cisplatin and to other metal-based drugs by displaying specific activities against different cancer cells.[1–3] Yet, the clinical use of gold-based drugs has until now been hampered by a series of factors, including toxicity and low solubility of the compounds in aqueous media.[4] Encapsulation of cytotoxic gold compounds by microcapsules, gels, nanoparticles and micelles has been applied to overcome the limitations associated with poor aqueous solubility and to enhance the selectivity of these potential drugs for tumor cells, improving bioavailability, effectiveness and applicability of these molecules.[4,5]

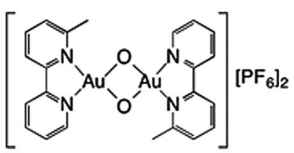
Protein nanocages have attracted intense attention as drug delivery systems due to merits that include high biocompatibility, high solubility and ease of surface modification. Ferritin (Ft) nanocages have been used to encapsulate a variety of drugs and biologically active substances,[6] including gadolinium contrast agents,[7] desferrioxamine B,[8] doxorubicin,[9] inorganic and magnetite nanoparticles,[10,11] β-carotene,[12] a few photosensitizers,[13] cisplatin and carboplatin,[14,15] rhodium[16] and palladium compounds,[17] organometallic CO releasing systems containing Ru and Mn.[18,19] We have recently described the structure of a cisplatin encapsulated apoFt (Aft) nanocage,[20] whose cytotoxicity is well documented;[14,15] our structure has provided hints to explain the reasons why Ft can be efficiently used for the transport of drugs to malignant cells.

Although the use of AFt in the transport of metallodrugs[14,15,20] and in the fabrication of gold nanoparticles[21,22] is well documented, to our knowledge gold-based drugs have never been encapsulated within an AFt nanocage. Here, we have used Auoxo3, a potential gold(III) based drug, a member of a class of gold compounds of medicinal interest (Auoxos), already well characterized both in its cytotoxicity[23–25] and binding to proteins,[26–28] to prepare gold-based drug-encapsulated AFt.

The chemical structure of the *trans* isomer of the drug is reported in Fig. 1.

Previous data indicated that the probable biomolecular targets for Auoxos are histone deacetylase (HDAC), protein kinase C (PKc) or thioredoxin reductase.[23,29] According to mechanistic studies, the interactions between these compounds and the targets are likely dominated by redox transformations. Auoxos behave as prodrugs: they degrade and release gold(I) ions which eventually bind and inhibit the final target(s). It was found that the antiproliferative effects of the various Auoxos and the cytotoxicity profile of cisplatin are rather diverse, implying that their mechanisms of action are very different.

The potential antitumor drug-loaded nanostructure was prepared using the procedure previously described by Huang



Auoxo3

Fig. 1 Chemical structure of the *trans* isomer of Auoxo3.

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c6cc02516a

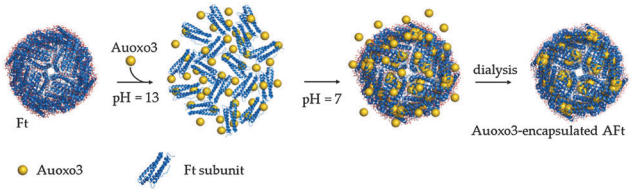

Fig. 2 Encapsulation of Auoxo3 within AFt. Horse spleen ferritin from Sigma (Ft, code F4503, mainly L-subunits) was dissociated at pH 13 using 0.1 M NaOH and reconstituted in the presence of the drugs using sodium phosphate buffer at pH 7.4. A ~ 75 : 1 Auoxo3 to protein molar ratio was used. The gold-compound was encapsulated within the Ft nanocage after its reconstitution.

and coworkers[15] and already used to encapsulate cisplatin and carboplatin.[14,15,20] A schematic picture representing the gold-based drug encapsulation with AFt is presented in Fig. 2.

The protein folding was verified by using far-UV circular dichroism (CD) spectroscopy (Fig. S1, ESI†). Data reveal that Ft can reassemble in the presence of the drug, retaining its native conformation. Inductively coupled plasma mass spectrometry (ICP-MS) was used to verify the potential drug encapsulation. A Ft nanocage can encapsulate about 420 gold atoms (from 350 to 500 atoms of gold depending on the preparation), much more than those found when gold ions from $AuCl_4^-$ were encapsulated in horse liver AFt (<185 gold atoms).[22] The size of Auoxo3-encapsulated AFt was measured in solution by dynamic light scattering (DLS). The average size (*Z*-average) of fresh solutions of Auoxo3-encapsulated AFt is 13.8 ± 0.9 nm, in good agreement with what is found for AFt.

The structure of Auoxo3-encapsulated AFt was refined at 1.85 Å resolution to an Rfactor of 0.180 (Rfree 0.226) (Fig. 3A). The X-ray structure confirms the proper assembly of the 24-mer Ft. The gold binding sites were unambiguously identified by using Fourier difference and anomalous electron density maps (Fig. 3B–E, see also the ESI† for further details and discussion).

As previously reported for the interaction of Auoxo3[28] and other members of the Auoxos series with the model proteins hen egg white lysozyme and bovine pancreatic ribonuclease,[27,28,30] upon encapsulation within AFt, the compound degrades extensively: gold atoms are found bound to the side chains of Cys48, His49, Cys48 and His49, His114 and Cys126, Cys126, His132 and His147 (Fig. 3C–F). The linear geometry close to gold atoms suggests that the metal is in the oxidation state +1 and thus that the original compound Auoxo3 undergoes degradation and reduction. Interestingly, His114 and Cys126 are conserved residues known to play a role in the iron transport to the Ft three-fold channel.[31] Gold ions were found close to the side chains of Cys48, His49, His114 and Cys126 also in the X-ray structure of horse liver apo-Ft with $AuCl_4^-$ (pdb code 3H7G),[22] although the details of the interactions between Au and protein residues are different when compared to those described here (see Fig. S2, ESI† for further details). In our structure, at the Cys48 binding site, a gold ion is located between the SG atom (unrestrained distance = 2.3 Å) and π electrons from the imidazole of His49 (distance from the centroid of imidazole = 3.0 Å), which in turn is also coordinated to another Au atom.

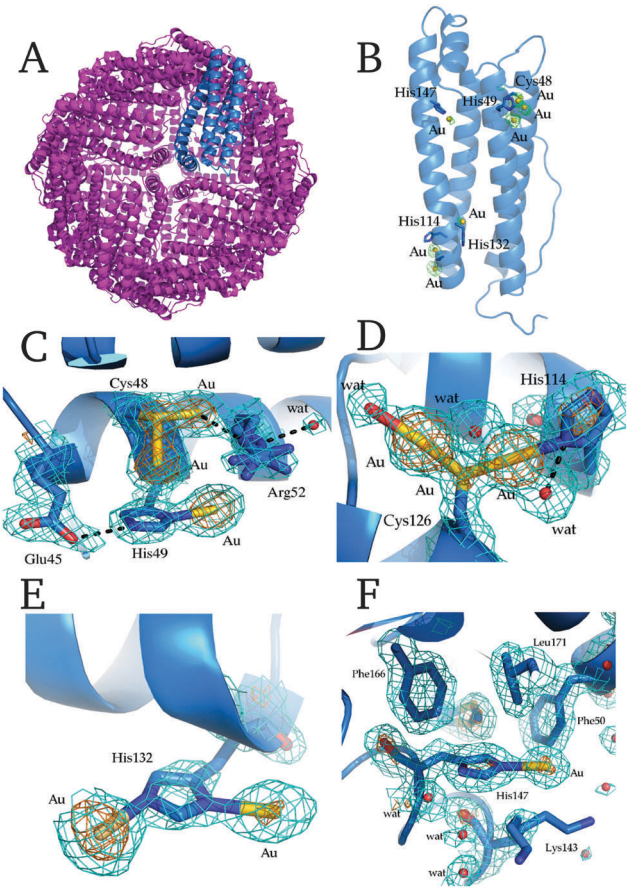

Fig. 3 The X-ray structure of Auoxo3-encapsulated AFt complex (A) expanded to show a single four-helix bundle subunit with Au binding sites evidenced and Bijvoet difference Fourier map calculated with anomalous data reported at 3σ (B). In panels C–F 2Fo-Fc electron density maps at 1σ (cyan) and 4σ (orange) of gold ions coordinated to Cys48, His49, Cys48 and His49 (C) His114 and Cys126, Cys126 (D), His132 (E) and His147 (F) are reported. The gold ions were refined with partial occupancy (0.30, 0.35, 0.45, 0.40, 0.45, 0.20 and 0.25); the average B-factors for gold ions are in the range 20.0–50.3 $Å^2$. The structure was deposited in the Protein Data Bank under accession code 5IX6.

The side chain of His49 is maintained in its position by the salt bridge that forms with the side chain of Glu45. At 3.1 Å from this Au ion, a second gold atom is bound to the SG of Cys48. This metal centre is in contact with the side chain atoms of Arg52 (Fig. 3C). A peculiar arrangement of Au ions is also found at the His114 and Cys126 binding sites. Here, the electron density map suggests the possibility that a water molecule could be accommodated in the cavity formed between the two gold centres that are at a distance of 3.7 Å. These atoms are bound to the SG atom of Cys126 (distance = 2.2 Å) and to a water molecule (distance = 2.5 Å), and to the SG atom of Cys126 (distance = 2.2 Å) and to the ND1 atom of His114 (distance = 2.2 Å), respectively (Fig. 3D). A detailed description of the interaction that Au atoms form with protein residues or solvent molecules is reported in Table S1 (ESI†).

With the exception of the Au ions bound to side chains of His114 and Cys126, which are buried at the interface between two AFt subunits, all of the Au binding sites identified in the

Auoxo3-encaspulated-AFt structure are located on the inner surface of the nanocage. This indicates that the structure and electrostatic potential of the outer surface of AFt are not affected by drug encapsulation. Thus, Auoxo3-encapsulated AFt retains the physico-chemical features that make this protein an ideal nanocarrier for drug delivery to target sites (*i.e.* biocompatibility, solubility and possibility to be recognized by specific receptors).

The effects of Auoxo3-encapsulated AFt were then tested on three human tumor cell lines (MCF-7, breast cancer cells, HeLa, cervical cancer cells and HepG2, hepatic carcinoma cells) and three non-tumorigenic cell lines (HRCE, human renal cortical epithelial cells, HaCaT, human keratinocyte cells and H9c2, rat cardiomyoblast cells) by using a cell viability assay.[32] Native Ft was used as control (Fig. 4 and Table 1). From the MTT assay, it was observed that native Ft had no effect on cancer cells (Fig. 4A), whereas the encapsulated nanocage showed a significant cytotoxic effect on all the analysed human cancer cell lines (Fig. 4B). Our data on free AFt are in agreement with previous studies, demonstrating that native Ft is non-toxic for Gastric Cancer (GCC) and HeLa cells.[14,15] Interestingly, Auoxo3-encapsulated AFt exerts lower effects on normal cells, as indicated by IC_{50} values reported in Table 1.

IC_{50} values were also derived for cisplatin and Auoxo3, used as positive controls, on the same cell lines (Table 2). Although a direct comparison between the IC_{50} values of these drugs and those obtained using Auoxo3-encapsulated AFt is not possible, since it is clear from previous experiments that drug-encapsulated Ft nanocages exert cytotoxicity by a mechanism of action different from free drugs,[15] it is interesting to note that Auoxo3 is rather toxic even on normal cells, contrary to Auoxo3-encapsulated AFt. The mechanism of Au release from Auoxo3-encapsulated AFt has not yet been studied in detail, but based on the literature data on other drug-encapsulated AFt adducts, it can be postulated that Auoxo3-encapsulated AFt can efficiently transport drugs to tumors through interactions with integrin or with specific receptors overexpressed on the surface of tumor cells.[15,33] Free Au(I) ions are probably released inside the cell upon protein degradation *via* a lysosome/proteasome pathway.[34] In order to obtain preliminary results on this issue, we quantified the gold uptake inside MCF-7 cells upon 24, 48 and 72 h of incubation with Auoxo3-encapsulated AFt using ICP-MS. The Au uptake ratio associated with Auoxo3-encapsulated AFt is 9.0, 11.9 and 21.6% after 24, 48 and 72 h, respectively (see the ESI† for further details). Furthermore, the apoptosis level in these tumor cell lines was examined by western blotting analyses using a specific antibody against Caspase 3, an effector Caspase. The results revealed that, after 24, 48 and 72 h incubation, no activation of Caspase 3 was observed in the presence of Auoxo3-encapsulated-Aft (Fig. S4, ESI†).

In conclusion, we have encapsulated a gold-based anticancer compound within a ferritin nanocage and have characterized the formed adduct from a structural viewpoint. The X-ray structure of Auoxo3-encapsulated AFt, refined at 1.85 Å resolution, reveals that naked gold(I) ions bind close to Cys or His side chains, mainly at the inner surface of the nanocage. The cytotoxicity of this adduct has been evaluated for three human cancer cells (HepG2, MCF-7, HeLa) and three non-tumorigenic cell lines (H9c2, HRCE and HaCaT). It exhibits much higher cytotoxicity than free AFt,

Table 1 Cytotoxic activity of Auoxo3-encapsulated AFt on different cancer (HepG2, HeLa and MCF-7) and normal cells (H9c2, HRCE and HaCaT). Cytotoxicity is expressed as IC_{50} values in μg mL^{-1}, *i.e.* the protein concentration inducing 50% cell death

	Auoxo3-encapsulated AFt	AFt
HepG2	35 ± 1	>1000
MCF-7	41 ± 9	>1000
Hela	42 ± 1	>1000
H9c2	59 ± 10	>1000
HRCE	61 ± 5	150 ± 55
HaCaT	69 ± 11	>1000

Table 2 Cytotoxic activity of Auoxo3 and cisplatin on the studied cell lines. Cytotoxicity is expressed as IC_{50} values in μg mL^{-1}, *i.e.* the compound concentration inducing 50% cell death

	Auoxo3	Cisplatin
HepG2	16 ± 6	2.8 ± 0.9
MCF-7	8 ± 2	21 ± 4
Hela	3 ± 1	2 ± 1
H9c2	4 ± 1	5 ± 2
HRCE	2.2 ± 0.4	2.0 ± 0.6
HaCaT	14.2 ± 0.7	2.1 ± 0.4

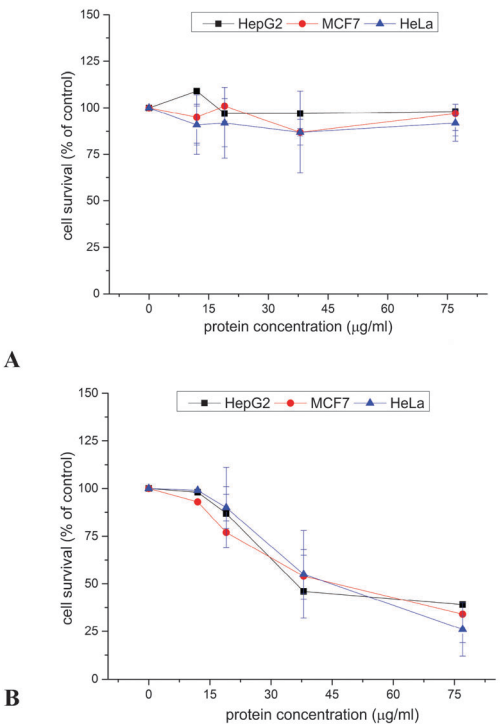

Fig. 4 Effects of free AFt (A) and Auoxo3-encapsulated AFt (B) on human cancer cells. HepG2, HeLa and MCF-7 were treated with increasing amounts of proteins for 72 hours. Cell viability was assessed by the MTT assay and expressed as described in the materials and methods section. All values are given as means ± SD ($n \geq 3$).

which is basically non-toxic. The adduct is much more toxic to tumor cells than to non-tumor cells.

Altogether these data indicate that encapsulation of gold-based drugs within Ft nanocages is a promising strategy to deliver these molecules to their final targets.

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