Protein cage nanostructure as drug delivery system: magnifying glass on apoferritin

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ABSTRACT

Introduction: New frontiers in nanomedicine are moving towards the research of new biomaterials. Apoferritin (APO), is a uniform regular self-assemblies nano-sized protein with excellent biocompatibility and a unique structure that affords it the ability to stabilize small active molecules in its inner core. Areas covered: APO can be loaded by applying a passive process (mainly used for ions and metals) or by a unique formulative approach based on disassembly/reassembly process. In this article, we aim to organize the experimental evidence provided by a number of studies on the loading, release and targeting. Attention is initially focused on the most investigated antineoplastic drug and contrast agents up to the most recent application in gene therapy. Expert opinion: Various preclinical studies have demonstrated that APO improved the potency and selectivity of some chemotherapeutics. However, in order to translate the use of APO into therapy, some issues must be solved, especially regarding the reproducibility of the loading protocol used, the optimization of nanocarrier characterization, detailed understanding of the final structure of loaded APO, and the real mechanism and timing of drug release.

1. Introduction

The production and the development of drug delivery systems (DDS), completely biocompatible, atoxic, and biodegradable is a challenging issue. Thus, modern nanomedicine focused its interest on alternative biomaterials as proteins.

The nature offers different types of multimeric proteins able to self-assemble in protein shell surrounding a hollow interior cavity with shapes and structures within nanometer scale [1]. From a biological point of view, protein cages play numerous functions as the storage, protection and delivery of viral genomes, metal storage, and protein refolding.

Their applicability as DDS depends on the technological formulative aspects as handy, reproducible, scalable methods of formulation and drug encapsulation as well as on the efficient purification process.

In this context, advanced competences in the field of biotechnology help a sustainable large-scale production of proteins using cell factories, making this technology highly desirable and economically advantageous for different technological applications.

One of the most investigated classes of proteins applicable in drug delivery is the ferritin superfamily and particularly apoferritin (APO) [2,3]. In this review, starting from the analyses of the structure of APO, we focus on the mechanism of drug loading in APO cage and the versatility of this protein as DDS in therapy.

2. Ferritin superfamily

Ferritins are a superfamily of proteins which is highly conserved in euakaryotic organisms and ubiquity diffused in humans and other mammals, plants, fungi, and bacteria [4].

The role of ferritin is fundamental for life; it is the first protein involved in iron storage and seizure, thus playing a role of reservoir for metabolic iron.

The crystal structures of many ferritins were deeply characterized since the beginning of 1990s by the combination of transmission electron microscopy (TEM), small-angle X-ray scattering (SAXS), and proteomic techniques [4–7].

In spite of large variations in amino acid sequences from bacteria to humans, ferritins essentially show the same architecture. Focusing on mammalians, the ferritin’s protein cage (450–500 kDa) is composed of 24 polypeptide subunits, with different molecular weights [8] and with specific functions [9]. Heavy (H) units (molecular weight of 21 KDa) play a major role in iron oxidation while light (L) units (molecular of 19 KDa) are more involved in the efficient nucleation and mineralization of iron. Of note, H and L subunits are highly homologous, and they can form a spherical unit in any proportion [10].

The ratio between the two types of chain in ferritin is different with respect to the species and the tissues [11] as in relation to physiological state, as the amount of H chains increases in inflammation and other pathological conditions in response to cytokine stimuli [12,13]. Moreover, as reported in the text below (Chapter 4.1), differences in the type of
receptor binding are present between H/L ferritin/APO as only H-ferritin or H-APO is able to bind TtR1 receptors whereas L-ferritin and L-APO bind SCARA5 receptors.

The subunits self-assemble to form a spherical cage with outer diameter of 12 nm, a 2-nm thick protein shell and an internal cavity with diameter of 8 nm. Overall, the protein molecular mass is 450 KDa and it is able to store up to 4500 Fe(III) atoms [4,14].

The inner and the outer space are connected by pores size of about 0.3–0.5 nm [15,16]: 8 of these pores defined ‘threefold channels’ are hydrophilic and they lead iron to diffuse into (and out of) the core; the other 4 pores defined ‘fourfold channels’ are hydrophobic and their function is still unclear [17,18].

Finally, it is worth to consider that during the protein reassembly, amino acidic content differs from inner to outer cavities. Generally, ferritins have a preponderance of acidic over basic residues with high level of polar groups, thus the isoelectric point is generally in the range 4.5–5.5.

The ferritin surface resulted highly reactive, and some substances can bind to the APO surface through hydrogen bonding (nonionic molecules) or electrostatic interactions (ionic molecules) [19].

3. From ferritin to APO, the applicability as DDS

As described above, from a physiological point of view, ferritin can be described as Fe(III) reservoir. Aiming to exploit APO as DDS, the inner cavity should be free and able to accommodate the cargo, thus the resident iron has to be removed from the cavities. Demineralization of ferritin occurred by reductive dissolution and subsequent chelation of Fe(II) producing APO [20,21], which maintains the same features of ferritin in terms of inner and outer structures.

The octahedral scaffold of APO displays typical hydrophilic and hydrophobic channels connecting inner cavities to the external surface and which are proposed as the route for inward and outward movement of metals and other molecules (therapeutic agents such as imaging agents) [22].

This mechanism of accumulation into the core [23] is named ‘passive loading.’ Unless initially investigated, being a rapid and scalable loading process, it results often less efficient and not able to stabilize therapeutic molecules with large-molecular weights, which result prevalently adsorbed on the surface. Therefore, deeper studies on strategies of improving drug loading with active loading, or at least by means of strong modification in the protocols of ferritin assembly were conducted over the last 10 years. In this contest, a number of studies mostly aimed to elucidate the permeation mechanism of organic molecules into APO and the impact of chemico-physical characteristics on drug-loading efficiency. As example, nuclear magnetic resonance (NMR) relaxation techniques were employed to investigate the diffusion of molecules devoid of net charge and with 18–7500 Da MW (i.e. water, DMSO, glucose, maltose, maltotriose, maltotetraose, maltopentaose, and polyethylene glycol) into the protein cavity [24]. The authors demonstrated that the channel structure is sufficiently flexible to allow the penetration of molecules even larger than their own size (3–4 Å), but with maximum dimensions typical of the maltose (Mw: 342 Da, corresponding to a diameter of 13 Å) [24].

Electron paramagnetic resonance spectroscopy analysis were employed to study the diffusion of small and differently charged nitroxide organic probes (7–9 Å of diameter) to the central cavity of the ferritin, showing that the diffusion inside the protein is not purely passive (not only linked to the concentration gradient between the inside and outside of the protein) but also strictly dependent on their respective charge and the polarity. Only positively charged probes penetrate into the protein through the threefold channels, while not polar molecules bind the hydrophobic regions of the protein only on in its outer surface [25,26].

Aiming to extend the number of applications, the research moves through alternative methods to load drug and investigate the dynamic of disassembly and reassembly of the protein nanocage.

Thanks to a large number of intra- and inter-subunit salt bridges and hydrogen bonds, the APO structure is known for its stability (i) over heating condition (up to 85°C) [27]; (ii) amongst a wide pH range (3.40–10) [28]; (iii) in presence of high ionic concentrations; (iv) in presence of high concentrations of denaturing agents [29,30].

Even if highly stable to chemical and physical denaturants, the APO architecture can be disassembled in response to pH variation and then restored, almost completely, by returning the pH back to the ‘physiological’ conditions (pH 7.4). A number of hypotheses to describe the structural changes during disassembly and reassembly of protein were proposed.

One of the first theories hypothesized that APO remains stable in the range of pH 2.8–10.6. On the contrary, at lower pH (1.6–2.8) or higher pH (10.6–13.0), a drastic reduction of MW, which can be related to the presence of disassembled subunits, occurs [31].

A still open question is related to the exact pH at which protein starts its disassembling process. Some authors suggest that a progressive process [32] takes place; this event consists...
of cleavage of non-covalent bonds as a consequence of a conformational rearrangement of the protein [30]. Along with the decrease of pH, the outer surface of APO becomes positively charged (due to the protonation of the carboxylic groups of amino acid), leading to the progressive loosing of subunits forming holes in the spherical structure which switch from the inside to the outside [31]. By combining SAXS data and 3D structural reconstruction, it was shown that more the pH decreases, more the holes become numerous and larger, finally generating rod-like structures at pH 2 [28]. In these conditions, APO is completely disassembled into its subunits and allows molecules/drugs present in solution to interact with the chains.

To lead subunits to reassemble and to form the native conformation, it is pivotal to restore the native electrostatic potential, thus, the solution needs to be buffered to the pH to ‘physiological’ values [33]. Within this reassembly process, drug loading takes place as the drug molecules are entrapped into the spherical structure (Figure 1).

In turn, aiming to expand the drug loading possibilities, some authors chose to move to different pH values, especially in the basic range. Notwithstanding, less conformational data are available regarding the basic dissociation of APO, it was supposed starting over pH 10.6 and caused by the deprotonation and rearrangement of tyrosine residues. A high level of attention should be posed on monitoring pH values, as continuing the basification over pH 12.4 leads to an irreversible alkaline hydrolysis of peptide bonds [34], disabling any possible recovery of native conformation [31].

4. Pharmacokinetics of APO as DSS

After parenteral administration, the pharmacokinetic profile of APO was investigated in tumor-bearing model. Drug-loaded APO is rapidly distributed into the bloodstream reaching small capillaries. These protein DDS do not cross normal vessels, as only molecules smaller than 3 nm are allowed to pass the endothelial cells by means of transcellular route [35], but their low diameter (less than 20 nm) is sufficient to avoid macrophages capture. This drastically reduces aspecific uptake by the reticular endothelial system (RES) [36] and assures to keep the protein longer in the bloodstream circulation.

Membranes of endothelial cells of blood vessels expose receptors that recognize APO and control both iron uptake and transferrin passage from vessel to tissues. H and L subunits show some differences in receptor affinity, as the H subunit specifically binds the receptor of transferrin (TfR1) with a wild distribution in human cells while L subunit binds to the scavenger receptor class A member 5 (SCARAS) first localized in macrophages, retina, and hepatocytes [10,37]. As evident, the protein composition is pivotal to define the APO destiny and, at the same time, the well-known overexpression of TfR1 in many cancer cells (about 100-fold with respect to normal tissue) [38,39] guarantees an intrinsic site-specific targeting potential of APO nanocage for tumor.

Moreover, tumor produces much more growing factors, leading to the presence of blood capillaries that tend to be disorganized, oversized, and leaky. As a consequence, a large number of drug molecules and small structure (as APO) can be

Figure 1. Graphical representation of the mechanisms for drug loading into APO. a) APO formed by H/L subunit can be passively loaded by simple incubation with a drug solution. In function of the MW of the drug, different loading dynamics could be supposed. b) APO structure can be broken down to subunits at low or high pH, incubated with drug and finally the structure can be reassembled once the pH is tuned back to neutral lead the drug molecules to be trapped into the protein inner core.
effectively driven to cancer tissue and stick onto pathological cells.

Also, the mechanism of APO interaction with cells and especially with tumor cells was investigated, in vitro using CACO-2 cells, showing that APO exploits clathrin-dependent endocytosis [40], accumulates into endosomes, and releases the protein cargo into cytoplasm as a consequence of acidification of the compartment, which is responsible for the progressive dissociation of APO [41].

4.1. APO in drug delivery, the tumor case

A number of anticancer drugs suffer poor selectivity for neoplastic cells leading to dose-limiting side effects, which compromise clinical outcomes.

Unfortunately, only limited number of drugs were investigated as well-defined drug properties are needed: (i) controlled molecular weight (typically in the range 300–600 Da); (ii) positive charged at physiological pH thus able to produce electrostatic interaction with the inner negatively charged protein cavity. In this view, DOXO possesses good properties (MW = 544 Da; and pKa = 7.34 (phenol); 8.46 (amine); 9.46 (est)). Different operative conditions were screened in order to optimize its loading into APO, in particular the effect of the buffer, the ionic strength, and the protein concentration during the dissociation process.

During the last decades, the major interest was dedicated to the investigation of the development of loaded APO as DDS for anticancer molecules (Table 1). Among currently used chemotherapeutics with low bioavailability and selectivity, some of them display chemophysical characteristics (low MW and cationic charge at physiological pH) suitable for stabilization into APO. Moreover, exploiting the innate affinity for tumor cells, the encapsulation is supposed to enhance the cell interaction with the tumor cells thus reducing the side and not-target accumulation and side effects. Considering this particular cancer targeting, H-ferritin should be used in order to produce cages able to really and successfully target cancer human cells. Notably, mouse models of cancer should not be taken into consideration, due to the presence of different receptors.

Gefitinib, an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (MW 447 Da), was passively loaded in APO. Drug resulted probably distributed in the inner and outer part of the protein cage (totally 10 molecules for each APO), thus the release starts at physiological pH and maximizes at pH 2 in correspondence with the protein disassembly.

Authors demonstrated that gefitinib/APO induced cytotoxicity in breast cancer cell lines overexpressing both TtR1 and HER-2 receptors with a profile comparable to that of free drug [42].

A strong effort in the research was focused on strategies to force structured molecules to stably enter the APO core. In some particular cases (i.e. doxorubicin (DOXO) and mainly Gd), for molecules with appropriate MWs, some authors proposed a pre-complexation of the molecule with a metal in order to use the natural affinity of metal for the inner core of APO as driving force to maximize the passage through the threefold channels.

DOXO, a wide-spectrum anticancer antibiotic with affinity for different metals was previously pre-complexed with Cu(II), and efficiently loaded by simple incubation in APO systems (Cu/DOXO/APO)[54,55]. This strategy increases the drug-loading efficiency in APO compared by simple DOXO/APO (30:100 DOXO:APo w/w ratio in presence of Cu with respect to 8:100 DOXO:APo w/w ratio in absence of metal) [43]. Cu/DOXO/APO keeps both the dimension and the morphology of DOXO/APO and APO alone and no aggregates were observed. The release of the drug in physiological conditions showed a typical biphasic profile characterized by an initial burst effect (about 80% of drug) and absence of drug release over 48 h. These data could indicate that the metal–drug complex is mostly adsorbed on the surface of protein. Notwithstanding, technological data seem to suggest only a limited ability to protect DOXO; in vitro and in vivo tests on tumor models showed that DOXO/APO nanocages, targeted to integrin Rv83 (a tumor angiogenesis biomarker) by RGD peptide anchored on APO surface, displayed a better therapeutic profile, resulting in a longer circulation half-life, higher tumor uptake, better tumor growth inhibition, and less cardiotoxicity than free DOXO [43] (Table 1).

A second strategy proposed to force the drug into the protein core consists of disassembly–reassembly process. The natural metal binding site present in the inner core of APO can be used as driving force to stabilize some metal-containing compounds. In this contest, platinum-based anticancer drugs could be efficiently encapsulated into APO after dissociation at pH 2 and reconstitution at physiological pH.

Cisplatin and carboplatin were loaded with the aim to reduce the systemic toxicity and high tumor resistance [50]. Through ICP-MS analysis, authors demonstrated that about 2 and 5 molecules of cisplatin and carboplatin, respectively, became part of the protein structure, even if no clear evidences about the rate between internalization and absorption were reported. In a further study, these data were corroborated by a deeper characterization of platinum-loaded APO (oxalplatinum) and evaluating the effect on a cancer cell line overexpressing transferrin receptor. Data highlighted the low toxicity of APO on cancer cells and the rapid decrease in cellular viability as a consequence of internalization of loaded protein, thus suggesting the potentiality of APO to overcome the mechanism of resistance typical of this type of metal-based anticancer drug [35].

As alternative, cisplatin was stabilized into APO after dissociation at more handy and ‘stable’ formulative conditions, as weak alkaline pH [51]. Once tested on cancer cells, cisplatin loaded into APO showed the ability to induce cytotoxicity through the same apoptosis pathway of free drug. Even if it is not clearly proved, the exact localization of the drug within the APO, the evidence that APO slows down the biological response at short time respect to free drug, suggests that the protein plays an important role in controlling the drug release over the time.

Disassembly–reassembly process was also applied to load drugs in APO without pre-complexation with metal. To date, only limited number of drugs were investigated as well-defined drug properties are needed: (i) controlled molecular weight (typically in the range 300–600 Da); (ii) positive charged at physiological pH thus able to produce electrostatic interaction with the inner negatively charged protein cavity. In this view, DOXO possesses good properties (MW = 544 Da; and pKa = 7.34
### Table 1. APO utilization in drug delivery to cancer.

| Drug                  | Method of encapsulation | Surface ligand | Source of APO | Outcome/Short comings                                                                 | Biological end points                                                                 |
|-----------------------|-------------------------|----------------|---------------|----------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|
| Gatifloxin            | Passive loading at pH 7.2 followed by reassembly | Horse spleen | Recombinant | Increased drug stabilization (4 times higher) with VT/VV model                           | VT: cellular internalization mediated by interaction with the TR1 receptor; selective antitumor activity in HER2 overexpressing cells |
| Gatifloxin            | Disassembly in glycine buffer (pH 2.5) followed by reassembly at physiological pH | Human colon adenocarcinoma cell line | Recombinant | Drug loading of 23 molecules of drug.                                                  | VT: rapid cellular uptake and nuclear translocation of loaded APO                    |
| Gatifloxin            | Disassembly at pH 2 followed by reassembly at physiological pH | Human lung carcinoma cell line | Recombinant | To solve: structural conformation                                                       | VT: cellular internalization and cytotoxicity (APO vs. free drug)                    |
| Gatifloxin            | Disassembly at pH 2 followed by reassembly at physiological pH | Human colon adenocarcinoma cell line | Recombinant | Cage stabilizing 28 molecules of drug; long-term controlled drug release (up to 72 h) in PBS pH | VT: reduced tumor size in mice (APO vs. saline or free drug)                         |
| Gatifloxin            | Disassembly in urea buffer followed by reassembly at physiological pH | Pancreatic cancer cells (PANC-1) | Recombinant | Drug loading of 23 molecules of drug.                                                  | VT: rapid accumulation into tumoral cell through interaction with TR1 receptor      |
| Daunomycin            | Pre-complexation with PPLA | Not reported   | Not reported  | Increased drug stabilization (4 times higher) with respect to non-complexed drug.       | VT: vancomycin inactivation of tumor tissue with respect to healthy one.             |
| Daunomycin            | Pre-complexation with PPLA | Recombinant | Recombinant | To solve: chemical and structural characterization                                       | VT: decreased viability of cancer cells (loaded vs. unloaded APO)                    |
| Cisplatin             | Pre-complexation at pH 7 followed by reassembly at physiological pH | Pig pancreas | Recombinant | Cage encapsulating 15 molecules of drug.                                               | VT: induction of apoptosis                                                           |
| Cisplatin             | Disassembly at pH 7 following by reassembly at physiological pH | Antibody directed to CSPG4 | Recombinant | Protein cage encapsulating 12 molecules of drug.                                       | VT: increased antiproliferative effect (modified vs. not modified APO)               |
| Cisplatin             | Disassembly at pH 7 following by reassembly at physiological pH | Human breast cancer cells (MDA-MB-468) | Recombinant | To solve: structural characterization                                                   | VT: redistribution of tumor size in mice bearing melanoma                           |

**Footnotes:**
- AUC: analytical ultracentrifugation; CAP: cationic peptides; CD: circular dichroism; CSPG4: chondroitin sulfate proteoglycan 4; CTP: cell-penetrating peptide; DLS: dynamic light scattering; FACS: fluorescence-activated cells sorting; HA: hyaluronic acid; HER2: epidermal growth factor receptor 2; HPLC-UV: high-performance liquid chromatography-UV; ICP-EAS: inductively coupled plasma atomic emission spectroscopy; ICP-MS: inductively coupled plasma mass spectrometer; LSCM: Laser-scanning confocal microscopy; MALDI TOF/TOF: Matrix-assisted laser desorption/ionization; MITT: 3-(4,5-dimetiltiazol-2-il)-2,5-difenilitetrazolio; NMR: nuclear magnetic resonance; PLLA: poly-L-aspartic acid; RGD: peptide with affinity for integrin receptor upregulated on tumor endothelial cells; RT-PCR: reverse transcriptase-polymerase chain reaction; SEC: size-exclusion chromatography; TEM: transmission electron microscopy; TR1: transferrin receptor; UV-FTIR: Fourier transform infrared spectroscopy; UV-VIS: ultraviolet-visible spectroscopy; WB: western blot.
Different operative conditions (phenol); 8.46 (amine); 9.46 (est). Different operative conditions were screened in order to optimize its loading into APO, in particular the effect of the buffer, the ionic strength, and the protein concentration during the dissociation process.

Interestingly, the use of glycine-acetate buffer (pH 2.5) is one more efficacious approach in disassembling APO without any effect related to the initial protein concentration [44].

Since only H-chain showed affinity for cancer cells through the binding with transferrin receptor, by means of an elegant combination of chemical engineering and fermentation technology, different types of mammalian ferritin variants were formulated by self-assembling by only H subunits (H-APO) [69]. Thus, H-APO was loaded with DOXO following both the previously reported acidic [45,46] or basic dissociation protocols [47]. The complex DOXO-loaded H-APO (DOXO/H-APO), tested in vitro, was compared in terms of efficacy with free DOXO and with clinical-approved liposome-loaded DOXO formulation (Doxil®).

The ‘innate’ targeting ability of the protein cage was confirmed as DOXO/H-APO showed better ability to mediate efficient and rapid interactions with cancer cells than the other control formulations [45,46]. Some conflicting data were derived from in vitro distribution studies; DOXO/H-APO rapidly entered in a colon cancer cell line accumulating in lysosomes where DOXO is gradually released and subsequently translocated into nucleus [47]. Differently, in a liver carcinoma cellular line, DOXO/H-APO accumulated rapidly into nucleus through a specific interaction of H subunit with receptor exposed on nuclear membrane. Accordingly, DOXO intercalates DNA causing higher and more rapid cytotoxicity in cancer cells with respect to controls (DOXO or Doxil) [45,46]. We could speculate on the different biological response and mechanism of cellular accumulation as consequence of multiple aspects, especially relating to the sensibility of tumor type and to the effect of the architecture and surface proprieties of loaded APO formulated applying different protocols. As evident, more efforts are needed to completely characterize these new entities.

To go further, DOXO/H-APO, obtained by means of basic dissociation protocol, was in vivo tested aiming to investigate pharmacokinetics profile and toxicity in mice bearing tumor (colon cancer cells). Namely, DOXO loaded into APO showed 10-fold higher AUC and tumor accumulation with respect to free drug and Doxil and reduced the drug concentration in healthy tissues (muscle, lung, kidney, spleen, and most importantly heart). In addition, DOXO/H-APO led to a significant reduction in tumor growth twofold higher than Doxil® [47].

The protocols for encapsulation of DOXO are now used as a template. Recently, atropine and carbachol, two molecules active on muscarinic receptors, involved in the development of pancreatic tumor, was loaded into APO cage. All studied molecules show similar MW (carbachol = 183 Da; atropine = 290 Da) and pKa (carbachol = 12.5; atropine = 4.5), suggesting loading efficiencies similar to DOXO case. Authors described an efficient accumulation both in vitro and in vivo at the tumor site, a partial suppression of tumor development and a reduction of toxicity if compared with the treatment with free drugs [48]. To ameliorate the affinity of APO for cancer cells, some authors proposed the surface modification by the insertion of target moieties as antibodies or peptides.

APO has a fair versatility regarding chemical reactivity: in fact, a variety of chemical groups, e.g. primary amines, carboxylates, and thiol present on the external and internal surface, can be linked either genetically [29,43] and/or chemically [70,71]. Interestingly, after surface modification, APO diameter generally increased and the surface proprieties change (zeta-potential, hydrophilicity). As a consequence, the cellular interaction appeared to be more driven by the presence of ligands exposed onto the surface than by the ‘innate’ tumor affinity of APO.

That way, a complex of daunorubicin (DAUNO) with negatively charged polypeptide PLLA (poly-L-aspartic acid) [24,49] was incorporated into APO after a partial disassembling at pH 5 and reassembly at physiological pH. To target solid tumor cells, the surface of APO was engineered (by means of NHS/EDC protocol) with hyaluronic acid able to specifically bind CD44. The efficacy of this engineered nanocage was in vitro tested on lung cancer cells overexpressing CD44 and a control cell line, not expressing any antigen. Modified nanocages were safe and tolerable for both cell lines, but it was clearly demonstrated that the cellular uptake was strongly driven by the binding with the cellular antigen. Briefly, DAUNO/APO accumulated only in CD44-positive cell line and both the cellular uptake and cytotoxicity were sensible to the preincubation with free antigen (which means inhibiting the cell-specific binding) [49].

In a second example, APO loaded with cisplatin proposed by Xing and collaborators [34] was conjugated through a NHS-PEG-Mal cross-linker with an antibody selective to a proteoglycan expressed on the surface of melanoma cells (CSPG4). The new modified vectors showed a significant increase in both their molar mass and diameters (33 nm vs. 13 nm of native APO). In vitro studies confirmed that modified APO specifically bound melanoma cells expressing CSPG4(+), but not CSPG4(−) breast cancer cells and, accordingly, in vivo study showed a delayed tumor growth in model mice of melanoma. On the contrary, the tumor size of breast tumor, which does not overexpress the specific antigen, was only marginally affected [52].

Recently, APO was proposed as nonviral system to stabilize and protect small-interfering RNA (siRNA). siRNAs are characterized by high MW and negative charges, features that hampered a stable internalization into the protein core. Taking advantage of the protein recombinant technology, APO was modified aiming to orderly expose cationic peptides on its surface. Thus, protamine-derived peptides were inserted as moieties able to complex and capture siRNA; besides, APO was also modified with penetrating peptides to enhance the target delivery to tumor cells.

The experiments clearly highlighted that after modified APO/siRNA complexation, the targeting peptides exposed on the protein surface drove the complex cellular interaction and siRNA accumulated into the cell cytoplasm, thus opening to a great potential for further applications of APO in gene therapy applied to cancer treatment (Figure 2 and Table 1) [53].
4.2. Application of APO in diagnosis

Imaging agents are among those compounds that can be successfully loaded into the interior cavity of APO protein (Table 2).

Magnetic resonance imaging (MRI) is a noninvasive imaging method for tumor diagnosis; however, most of the diagnostic agents suffers from a lack of selectivity, low proton relaxivity, and higher toxicity after administration [56].

APO could act as dual-functional tools allowing both the use of protein shell to target tumors and the ability in loading probes within the internal core to image tumors.

Gadolinium (GD) is one of the most used contrast agent in clinical and experimental settings; thus, a number of authors proposed the formulation of water suspended APO-GD formulation with high NMR longitudinal relaxivity similar to clinically approved GD chelates [56–59]. The technology employed in order to stabilize this contrast agent, described for the first time more than 10 years ago, was based on a well-described acidic protocol (dissociation at pH 2) and the use of a neutral chelates (GD-HPDO3A) [56].

To improve tumor targeting, the surface of APO/GD-HPDO3A was engineered via streptavidin-biotin technology with an antibody directed to neural cell adhesion molecules (NCAMs), overexpressed during the angiogenesis in endothelial cells. After in vivo administration, this new contrast agent was stable for few hours with a relaxation efficiency fivefold higher with respect to the free probe, showing an increase in selectivity for tumor vessels [59].

In a more recent work, in order to maximize the loading efficiency, GD-HPDO3A was replaced with a positively charged GD-Me2-DO2A. With the aim to assure a stable insertion into the cavity, the process was modified by adding GD complex during the acidification stage (at pH 4). Finally, the surface decoration of GD-loaded APO with dextran lead to the accumulation of APO in transplanted tumor region in mice due to the exploitation of EPR effect [57].

Also, Mn ions could be used as contrast agent in MRI. Through the stabilization into APO, it has been demonstrated the improving in relaxitivities [65,66]. These noninvasive imaging tools were applied as MRI sensor for different kinds of tumor. Taking advantage of the reduced expression of SCARA5 (APO cellular receptor) into hepatoma cells with respect to health hepatocytes, Geninatti and coworkers demonstrated the possibility to discriminate between tumor lesion and monitor the hepatic tumor progression with a sensitivity similar to commercial probe (Gd-BOTA) [67]. A similar structure was also developed for the early diagnoses and imaging of melanoma cells. The increased melanin production in cancer cells corresponds to an increased reduction of Mn (III) to Mn (II) (by the oxidation of L-DOPA to melanin), thus producing high relaxivities and contrast during MRI investigation [68].

Also, iron is another optimal contrast agent for MRI. Autologous ferritins have been successfully investigated as an MRI contrast agent. One of the first example reported described the use of a cationized ferritins for MR imaging of kidneys obtained after coupling with N, N dimethyl-1,3 propanediamine (DMPA) that after iv administration accumulated in the kidneys because the glomerular basement membrane is negatively charged [73]. More recently, ferritin was investigated as MRI contrast agent in neurodegenerative disorders because of the documented altered iron metabolism in many neurodegeneration (particularly Alzheimer’s disease and Huntington’s disease) [74]. As example of this application in vivo experiments in mice demonstrated that it is possible to increase the iron expression (thus the MRI signal) in nerve cells of mice after transfection of nerve cells using lentiviral and adenovirus vectors expressing transferrin [75] (for a complete review on this point, see ref [76]. Unless some application reported, generally endogenous ferritin shows low relativity index if compared to synthetic iron oxide nanoparticles [77]. Human recombinant H-APO offers the possibility to act as suitable platform for synthesis and encapsulation of magnetite nanoparticles...
| Drug                  | Method of encapsulation                        | Surface modification | Source of APO | 1. Chemico-physical and technological studies | 2. In vitro (VT)/in vivo (VV) model test adopted | Structural/technological end point | Biological end points | REF |
|----------------------|------------------------------------------------|----------------------|---------------|-----------------------------------------------|-----------------------------------------------|----------------------------------|---------------------|-----|
| Gadolinium           | Passive loading                                 | Not reported         | IR, TEM, UV-Vis, ICP, SDS-PAGE | Small cage stabilizing GD (III); increase GD longitudinal and transverse relaxivities | To solve: structural characterization; localization of the drug | VV: massive accumulation in tumor site and high proton relaxivity | [58]               |
| GD-Me$_2$DO2A        | Disassembly at pH 2 followed by reassembly at physiological pH Drug was added during the basification process | Dextran              | Not reported | TEM, UV-VIS, ICP-EAS | W: mice bearing HeLa cells. MRI, NIRF | Spherical 30 nm modified cage encapsulating 36 molecules of contrast agent. To solve: structural characterization; localization of the drug | VV: efficient cellular internalization (loaded/modified APO vs. loaded APO) | [57] |
| GD-HPDO3A            | Disassembly at pH 2 followed by reassembly at physiological pH | NCAM                 | Not reported | VT: renal cell carcinoma cells (TECs), ICP-MS, MRI W: SCID mice bearing TEC cells. MRI, histology, immunofluorescence | Modified cage encapsulating 8–10 molecules of contrast agent with high relaxivity (5 times higher with respect to free molecule) To solve: structural characterization; localization of the drug | VT: accumulation into tumor site (modified/loaded APO>loaded APO) | [59]               |
| GD-HPDO3A            | Disassembly at pH 2 followed by reassembly at physiological pH | Not reported         | Not reported | Cage encapsulating 10 molecules of contrast agent. Relaxivity constant for several days. To solve: structural characterization; localization of the drug | Absence of structural analyses and quantification of exposed ligands | VV: massive tumor accumulation mediated by RGD-integrin interaction | [56]               |
| 64Cu+Cy5             | Dissociation at pH 2 followed by reconstruction at physiological pH | RGD4 C               | Not reported | DLS, TEM | W: tumor-bearing U87 MG glioma. NIRF, PET, LCSM | Cage of 12–13 nm encapsulating 5000 Fe. To solve: structural characterization | VV: uptake of APO into cells and accumulation of 116 μg Fe/cell within 72 h | [64] |
| Fe3O4                | Mineralization procedure                        | None                 | Recombinant (H-human) | DLS, TEM, MR | VT: murine macrophage cells. MR, ICP-MS | Spherical surface modified cage of 14 nm encapsulating 5000 molecules of Fe | VT: specific cellular uptake mediated by interaction with integrin | [60] |
| Fe3O4                | Mineralization procedure                        | RGD4G               | Recombinant (H-human) | DLS, TEM, magnetic measurement | VT: human melanoma cell line (C32). FACS, TEM | VT: enhanced cellular uptake (modified vs. not modified APO) | VV: high accumulation in carotid arteries (modified vs. not modified APO) | [61] |
| Fe3O4                | Mineralization procedure                        | RGD                 | Recombinant (H-human) | VT: bovine aortic endothelial cells. Fluorescence microscopy | W: WT mice. Fluorescence microscopy, histology | VT: specific cellular uptake mediated by interaction with integrin | [62]               |

(Continued)
| Drug | Method of encapsulation | Surface modification | Source of APO | Chemicophysical and technological studies | Structural/technological end point | Outcomes/Short comings | Biological end points | REF |
|------|------------------------|----------------------|---------------|---------------------------------|----------------------------------|-----------------------------|----------------------|-----|
| Fe3O4 | Mineralization procedure | None | Recombinant (H-human) | VT: breast epithelial cells (MCF-10A cells), breast cancer cells (MCF-7). FACS, MTT | Spherical cage of 19 nm | To solve: structural characterization | VT: absence of toxicity at high APO concentration. Good targeting through cancer cell with respect to normal cells (interaction with EGFR). | [72] |
| Mn   | Incubation with MnCl₂ at pH 9 followed by reduction | None | Horse spleen | TEM, UV-VIS, size exclusion | Protein shell with a mineral core. Encapsulation of about 1090 Mn atoms. To solve: structural characterization and localization of the contrast agent | Protein shell encapsulating mineral core | To solve: structural characterization | [65] |
| Mn   | Incubation with MnCl₂ at pH 9 followed by reduction | None | Horse spleen and recombinant (H- and L-human) | TEM, UV-VIS, EDXA | Protein structure encapsulating 3000 Mn(II) ions | To solve: structural conformation | VT: high accumulation in healthy cells with respect to hepatoma. VV: detection of tumor lesion and improved sensitivity with respect to commercial probe for MRI | [66] |
| Mn   | Incubation with MnCl₂ at pH 9 followed by reduction | None | Horse spleen | ICP-MS, Bradford quantification | VT: melanogenic cells (B16-F10 m) and non-melanogenic cells (B16-F10non-m) UV-VIS; ICP-MS | To solve: discrimination between melanogenic and non-melanogenic cells; high cellular uptake in melanogenic cells. VV: APO accumulation into tumor site | VT: discrimination between melanogenic and non-melanogenic cells; high cellular uptake in melanogenic cells. VV: APO accumulation into tumor site | [67] |

EDXA: energy dispersive X-ray analysis; LSCM: laser-scanning confocal microscopy; ICP-MS: inductively coupled plasma mass spectrometry; NMR: nuclear magnetic resonance; MR: magnetic resonance; NCAM: neural cell adhesion molecule; NIRF: near-infrared fluorescence; PET: positron emission tomography; IR: infrared spectroscopy; TEM: transmission electron microscopy; UV-VIS: ultraviolet–visible spectroscopy; SDS-PAGE: sodium dodecyl sulfate – polyacrylamide gel electrophoresis; ICP-EAS: inductively coupled plasma atomic emission spectroscopy; DLS: dynamic light scattering; MRI: magnetic resonance imaging; ICP-MS: inductively coupled plasma mass spectrometer; PET: positron emission tomography; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; FACS: fluorescence-activated cells sorting; RGD: arginylglycylaspartic acid; EGF: epidermal growth factor.
in vivo study, the enhanced permeability and retention (EPR) effect targets the tumor by means of RGD conjugation with the surface amines. This structure was able to with RGD4C peptide anchored to the ferritin surface by conjugation, thus enabling also a pH-controlled release of the drug and opening the pave to a simultaneous both drugs and imaging agents. The combination of therapy and diagnoses in a single DDS is now emerging as a theranostic approach.

This strategy can find applications for various different purposes, as to monitor the biodistribution and the target site accumulation of nanomedicines, to visualize and quantify drug release, and to assess the efficacy of the therapeutic treatment. The design of a theranostic nanosystems could be obtained by following different protocols in terms of drug-loading processes and insertion of the diagnostic agents. The DOXO/APO systems characterized by a natural affinity for tumor were largely studied and recently were modified onto their surface with magnetic nanoparticles by means of streptavidin-biotin technology. This approach preserved the natural loading ability of APO and it avoided any possible incompatibility between drug and diagnostic agent. Authors demonstrated that modified DOXO/APO was sensible to the application of a magnetic field, thus enabling also a pH-dependent release of the drug and opening the pave to a possible in vivo application in the treatment and diagnoses of tumors.

More recently, a DOXO/APO complex was specifically designed as theranostic agent for prostate cancer. Briefly, the surface was double engineered with an antibody direct to anti-prostate specific membrane antigen (PMSA) and with gold nanoparticles. The complex coverage of protein seems to protect the cargo from undesired premature release in the bloodstream; moreover, once tested in vitro, this new carrier assured a nice selectivity for prostate cancer cells preserving healthy cells from the drug toxicity.

A different approach is based on a contemporary loading into the inner cavity of the protein of diagnostic and drug molecules. The cytostatic drug 5-fluorouracil (5-FU), being characterized by a low-molecular weight, rapidly leak from the APO cavity; thus to better stabilize the encapsulation, proposed a simultaneous loading with gold nanoparticles (AU-NPs, 3 nm of diameter) known for their optical and photo-thermal proprieties. 5-FU enters through hydrophilic protein pores and once into the protein core binds AU-NPs via electrostatic interaction. This strategy avoided the leaking of 5-FU under physiological condition and promotes the drug release only after accumulation of APO inside the intracellular acidic compartments. In a very complete and worthy in vitro study, the complex was demonstrated to be able to increase the cytotoxic profile of the drug in different tumor cell lines, pointing out the wide applicability of the new therapeutic entities.

Similarly, curcumin, a multi-target drug with potent antioxidant proprieties, was loaded into APO simultaneously with GD through an acidic dissociation protocol, obtaining about 9 and 0.4 molecules of curcumin and GD, respectively, loaded into APO, with a consequent increase in APO diameter of about 66% (from 12 to 19 nm). In presence of curcumin, the GD complex conserves its typical relaxivities and rapidly accumulates into liver (passive targeting). Based on this peculiar distribution, authors proposed the vector as a theranostic agent for a variety of liver diseases.

### 4.3. Application of Apo as theranostic tool

The growing knowledge and advances in nanomedicine drive the recent research in formulating systems containing simultaneously both drugs and imaging agents. The combination of therapy and diagnoses in a single DDS is now emerging as a theranostic approach.

This strategy can find applications for various purposes, as to monitor the biodistribution and the target site accumulation of nanomedicines, to visualize and quantify drug release, and to assess the efficacy of the therapeutic treatment. The design of a theranostic nanosystems could be obtained by following different protocols in terms of drug-loading processes and insertion of the diagnostic agents (Table 3).

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### 4.4. A look over cancer, other possible applications of Apo as DDS

The versatility of APO was recently exploited for the potentiality of antigen presentation onto the surface and immune stimulation in vaccines formulation.

Generally, vaccines for immunization standard schedules are stably prepared and protocols of production are well standardized, but, for rapidly mutating pathogens (as the influenza virus), it is necessary to prepare new complex vaccines on an annual basis to avoid possible circumvention of the immune system by mutated pathogen.

The subunits of APO offer different regions tolerant to peptide insertion; these regions were therefore modified to obtain proteins expressing exogenous components that are able to conserve the ability to reorganize into spherical nanocages.

Following this approach, APO was synthetized in a controlled manner in terms of quantity and location of substituent. By selecting the site for protein insertion, it is possible to expose the antigens on the outer surface or both inner and outer. From a technological point of view, new vaccines were characterized by using PCS ant TEM to prove spherical shape and presence of the exposed antigen.

To evaluate the real efficacy of these new entities in comparison with licensed inactivated vaccines, different in vitro and in vivo tests were performed. Generally, after administration in mice, those APO exposing the antigen protein onto the outer surface induced a higher CD8+ and CD4 + T cell proliferative response and therefore their transformation in functional T cells (Table 4).

In view of expanding the applicability of APO in therapy, Zhang and coworkers exploited the applicability of APO in...
| Drug | Method of encapsulation | Surface modification | Source of APO | Outcomes/Short comings |
|------|------------------------|---------------------|--------------|------------------------|
| **Curcumin + Gd-HPDO3A** | Disassembly at pH 2 followed by reassembly at physiological pH | Horse spleen | SEC, UV-VIS, ICP-MS | Protein cage of 19 nm encapsulating 9.5 and 0.4 molecules of curcumin and Gd-HPDO3A, respectively. Ability to protect drug from degradation. To solve: structural characterization. **VV**: preferential uptake by the liver (about 19% of injected dose). Reduction of ALT, leukocyte infiltration and hepatocyte apoptosis levels (loaded vs. native APO) | **W**: male mice (C57BL), mice model of liver injury. **MR**, histology |
| **AU-NP and 5-FU** | Syntheses of AU-NPs inside the protein cage (AU reduction) followed by a passive loading of 5-FU | Not reported | TEM, EDXA, PAGE, CD, UV-VIS, 19F-NMR | Regular protein cage encapsulation 45 molecules of drug. pH-dependent drug release. To solve: localization of the drug. **VT**: noncancer cells (HKC); cancer cells (HeLa, HepG2, Caco-2, human hepatic L02), CCK-8, CLSM, TEM, ICP-MS, FACS, BrdU, WB | **VT**: accumulation of APO into lysosomes; low toxicity in control cells; decreasing of IC50 value (15 times vs. free drug) and increase of the antiproliferative effect in cancer cells |
| **Doxorubicin** | Disassembly at pH 2 followed by reassembly at physiological pH | Magnetic nanoparticles | Not reported | Partial encapsulation of drug into protein cage and pH-triggered drug release. To solve: structural characterization; localization of the drug. | **VT**: human umbilical vein endothelial cells (HUVEC), human prostate adenocarcinoma cells (LNCaP). **FACS** |
| **Doxorubicin** | Disassembly at pH 2 followed by reassembly at physiological pH | HWR peptide and anti-PSMA antibodies; AU-NP | Horse spleen | Complex protein cage of 22 nm, pH-dependent drug release. | **VT**: specific uptake in tumoral cells and time-dependent inhibition of cell growth. Excellent hemocompatibility |

AU-NPs: gold nanoparticles; BrdU: bromodeoxyuridine; CCK-8: cell counting kit; CD = circular dichroism; CE: capillary electrophoreses; CLSM: confocal laser scanning microscopy; DLS: dynamic light scattering; EDXA: energy dispersive X-ray analysis; FACS: fluorescence-activated cells sorting; FT-IR: Fourier transform infrared spectroscopy; ICP-MS: inductively coupled plasma mass spectrometer; MR: magnetic resonance; NMR: nuclear magnetic resonance; PSMA: prostate-specific membrane antigen; SEC: size-exclusion chromatography; TEM: transmission electron microscopy; UV-VIS: ultraviolet-visible spectroscopy; WB: western blot; 5-FU: 5-fluorouracil.
enzyme stabilization. Considering the large MW and steric hindrance of the molecule to be loaded, the internalization into the protein core appeared difficult, thus the enzyme glucose oxidase (GOx) (chosen as model enzyme) was immobilized onto the protein surface. Authors demonstrated that GOX linked on the surface through a biotin-streptavidin approach retained most of its activity up to 2 weeks and resulted stable to exposition to high temperatures (up to 50°C). This study presents a novel approach to enhance stability and activity of enzyme with promising application in different therapy but also in biological assay [87].

Finally, in a recent paper aiming to explore the applicability of APO in brain disease therapy, H-Apo was tested on a cerebellar organotypic culture demonstrating high affinity for astrocyte cell and a rapid and massive accumulation into nuclei [45].

5. Conclusion

APO, featured by high stability, special and reproductive structure, auto-assembly ability and biocompatibility, is certainly one of the most investigated and interesting structures proposed in the modern scenario of nanomedicines.

From the beginning of its history (less than 15 years) as material for drug delivery, its applicability was investigated within the field of cancer medicine due to its natural affinity for iron receptor, largely distributed on cells in rapid division. Unfortunately, based on its specific conformation and small internal spaces, only a limited number of chemotherapeutics (mainly belonging to anthracycline family as daunomycin and DOXO or contrast agent for tumor diagnoses) resulted suitable for successful loading into APO cage. Aiming to broaden the field of APO application, two main technological innovations were proposed: (i) the engineering of the protein surface by means of linkage of molecules able to drive the destiny of the protein cage; (ii) the use of protein reactivity to link the drug or the active molecules directly on the surface. That way, APO became object of investigations in other fields of nanomedicine as gene therapy, immunology, or liver pathology (Table 5).

6. Expert opinion

Despite extensive promises, much work is needed before clinical translation of APO. As example, analyzing literature data, even if interestingly biological data are reported supporting the efficacy of this DDS, the study of the architecture and the structure of loaded APO are still at early stages. This situation, which represents the major limitation is translatable of APO to become DDS, is principally caused by unclear protocols for APO formulation and unclear chemophysical, morphological, and technological characterization and it frequently generates conflicting outputs.

Points to be focused with major attention are related to the nature and narrow size of APO complex and to the evidence that the analytical technique commonly used to characterize synthetic DDS as liposomes or nanoparticles (TEM, AFM, SEM, PCS, DSC, etc.) are unable to completely demonstrate structure, architecture and, in particular, the presence of drug or ligands onto APO surface.

These data relating to chemophysical and morphological characterization of APO formulations are essential to determine the fate and the perspective of any DDS, as any change in surface proprieties, even small differences in chains rearrangements could strongly affect the interaction with serum proteins, recognition by immune systems, impacting on the cage biocompatibility, and also on the ability to transport drug to the site of action.

Currently, within their experimental plan, only few studies are considering the evaluation of the effective recovery of conformation after the loading and disassembly process. Generally, when investigated, this issue on the final APO structure is approached by applying far-UV circular dichroism (CD). CD permits to evaluate the presence and the distribution of helical structures into the new protein entities. Despite these data are surely reliable,
Table 4. APO utilization in therapy.

| Drug          | Method of encapsulation                          | Surface modification | Source of APO               | 1. Chemico-physical and technological studies | 2. In vitro/in vivo test | Structural/technological end point                                                                 | Biological end points                                                                 | REF  |
|---------------|------------------------------------------------|---------------------|-----------------------------|---------------------------------------------|-------------------------|---------------------------------------------------------------------------------------------------|--------------------------------------------------------------------------------------|------|
| OT-1 and OT-2 | Recombinant (bacterial)                         | DLS, TEM, MS        | VT: splenocyte from C57BL/6 mice. FACS. W: C57BL/6 mice. Cytokine assay | Spherical cage of 13 nm exposing antigen in the inner or outer surface. To solve: structural characterization | VT: increase of T cell proliferation after APO exposure. W: effective differentiation of activated T cells in functional effector T cells | [85] |
| Glucose oxidase | Incubation of recombinant protein in TRIS buffer at physiologic pH and purification | HA                  | VV: female BALB/c mice | Smooth spherical cage exposing antigens To solve: structural characterization | W: inhibition of hemagglutination | [86] |
|               | Surface attachment through biotin-streptavidin technology | Not reported         | TEM, UV-VIS                | Cage of 16 nm with an irregular morphology and largely aggregated stabilizing 8 molecules of enzyme. To solve: structural characterization | [45] |

DLS: dynamic light scattering; FACS: fluorescence-activated cells sorting; HA: influenza virus hemagglutinin; MS: mass spectrometry; OT-1/OT-2: antigen peptides derived from ovalbumin; SEC: size exclusion chromatography; TEM: transmission electron microscopy; UV-VIS: ultraviolet–visible spectroscopy.
the results are not representative of the real integrity of protein after loading or disassembly/reassembly process.

To overcome these limits, X-ray diffraction characterization, even if expensive and sometimes difficult to be rendered, could be considered as the best choice for the characterization of protein structural conformation. As an example, a study performed by SAXS technique showed clearly that protein reassembly process could not be totally complete after strong acidification; under pH 2.4, the protein completely loses its spherical structure that cannot be recovered. The pH limit for pseudo-reversible restoration of the quaternary structure was set at 2.66 [28].

One of the best example of an almost complete study of APO formulation is represented by a recent work [88] in which cisplatin-loaded APO were formulated by applying the same method proposed by Ji and coworkers [51] and were submitted to a deep structural characterization by means of X-ray analyses and elaboration of data. By comparing loaded and unloaded samples with native APO, the authors confirmed that the overall structure (shape, polarity/hydrophobicity, volume, and electrostatic potential of the surface) remained stable after the formulation process. Notably, comparing native and cisplatin-loaded APO, the most relevant structural differences were detected close to the site of drug loading and in particular at the His 132, found to be the metal-binding site.

This work represents an important keystone for further investigations aiming to confirm the maintenance of the structural integrity of the protein, in particular regarding the encapsulation of nonmetal drug with large MW.

Furthermore, another point to be investigated aiming to improve the chances of translatability of APO as DDS is related to the drug release and in particular to the changes in morphology and architecture of APO cage during and after the release of the drugs. To our knowledge, poor information are given on this topic which obviously impacts on several technological key features of APO DDS, as the efficiency in drug release, the possibility of governing/modifying the drug release and also the biocompatibility of APO after the release of the drugs. These aspects are still far from an overall evaluation and therefore need to be considered with a major interest.

As final consideration, it seems almost clear that the improvement and the consecration of potentialities of APO application in different fields of drug targeting must be obtained by an extensive collaboration among researchers in the field of technology, chemophysical characterization and physicians.

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Declaration of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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**The first complete chemical, physical, and crystallographic characterization of APO loaded with a metal drug.**