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Apoferitin nanocage as drug reservoir: is it a reliable drug delivery system?

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Editorial**Title: Apoferritin nanocage as drug reservoir: is it a reliable drug delivery system?****Authors:** Giovanni Tosi^{1*}, Daniela Belletti¹, Francesca Pederzoli¹, Barbara Ruozi¹**Affiliation**

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***Corresponding Author:** Prof. Giovanni Tosi, gtosi@unimore.it, +39.509.2058563**Abstract:**

Apoferritin is a complex protein with a number of possibilities for drug delivery and drug targeting technologies, as it could be considered as the *future self-assembling, not-toxic protein drug delivery carrier*. Few years ago, this concept was a reality; nowadays, after more than 10 years of research, a clear painting of Apoferritin, loaded with drugs, is lacking, in terms of protocols of formulation, characterization, drug release and application. Therefore, a critical evaluation and overall understanding of Apoferritin is due to speed up the possibilities for its translatability into clinical application.

Keywords: Apoferritin, protein, drug carries, drug delivery, translatability

Main Text

In order to reach the ambitious aim of Ehrlich's "magic bullet" of developing drug delivery systems (DDS) selectively binding the site of diseases or even the diseased cells, without further affecting patients' health with side effects, a number of promising approaches were developed in the last decades.

To fit with the ideal design, the "perfect" DDS should be characterized by good properties in:

1. Pharmaceutics (i.e. ability to efficiently load different kinds of therapeutic molecules, drug-release profiles adequate to pathologies treatment, high stability)
2. Safety (i.e. biocompatibility)
3. Selectivity (i.e. specific recognition on targeted site, lowering side effects)

The need to find biocompatible materials for the development of DDS strongly drove the research to a concrete tentative of replacing synthetic materials, as porous hollow silica or not-fully biocompatible polymers, with *natural* materials, endogenously present within the body, thus more acceptable and considered as *self* by the immune defense system.

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3 Looking at this scope, endogenous self-assembling proteins could be a strategic choice, leading to
4 obtain DDS able to efficiently load molecules, considered as non-toxic for the organism and
5 therefore safe.
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7 More recently, the attention of the research focused on Apoferritin (APO), from ferritin family, a
8 uniform regular self-assemblies nano-sized protein, showing excellent biocompatibility and unique
9 architecture able to stabilize small active molecules in its inner core. The low dimension, spherical
10 shape and high homogeneity [1] are among the key aspects that support the wide interest in APO
11 cage. This novel DDS could lead to longer circulation half-life and eventually to better
12 accumulation rates, in comparison with synthetic DDS (i.e. polymeric NPs or liposomes),
13 characterized by higher size (50-200 nm) and less homogeneity. Moreover, APO is fully
14 biocompatible and a-toxic, which is not a common feature for all the conventional nano-DDS.
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18 Beside this aspect on safety/biocompatibility, a major interest in APO research lies in “targeting”
19 abilities. These features are classically addressed by surface conjugation of nano-DDS with ligands
20 (peptide, antibodies...) able to target the diseased sites.
21

22 This inevitably lead to the “over-crowding” of the surface of carriers, with a number of issues to be
23 solved from both *technological* (i.e. production reproducibility, surface characterization) and
24 *biological* points of view (i.e. interaction with bloodstream proteins, protein corona composition,
25 safety and immune reaction), and overall the biocompatibility of the final structure
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27 On the contrary, APO possesses site-specific targeting potential, as they can be recognized and
28 internalized by Ft-binding receptors, such as the transferrin receptor 1 (TfR1). The overexpression
29 of these receptors especially on the surface of malignant cells is one of the most important
30 reasons for the growing interest of APO in the application of field of cancer treatment and
31 diagnosis. This overexpression should be considered as an increased presence of TfR in malignant
32 cells, but not as a unique expression of cancer cells. In fact, transferrin receptors are present onto
33 many other cells, greatly varying among cells depending on tissues, as TfR could be easily found in
34 basal epidermis, endocrine pancreas, hepatocytes, Kupfer cells, testis and pituitary gland [2]. Thus,
35 on the basis of these evidences, the potentiality of APO widens to the application in other fields of
36 nanomedicine as gene therapy, immunology or liver pathology.
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42 *Apoferritin: from the dream to the reality*

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45 Notwithstanding these good premises, some limits emerged in the application of APO as DDS,
46 mainly connected to pharmaceutical formulation, characterization and standardization of process,
47 but also to its biosafety. In particular, a couple of aspects is up-to-date unclear and poorly
48 analyzed and therefore to be strongly investigated and ameliorated to allow APO to play a major
49 role in DDS, namely:
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- 53 1. Pharmaceutical Issues
- 54 2. Biosafety Issues
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57 *The Pharmaceutical Issues*

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3 As evident from the literature outputs, due to the stringent requirements of loading protocols,
4 only a limited number of drugs could be efficiently encapsulated and not always the efficacy of
5 drug/APO complex is higher respect to free drug. Actually, the plethora of experiments on APO
6 formulation did not produce a standardized protocol that is able to clearly furnish a reproducible
7 unfolding/refolding of the protein, and especially when the drug is present and ready to be
8 loaded. Chemico-physical properties of drugs as molecular weight, pKa and charge should be
9 taken into great account in planning their encapsulation in the APO core. In this view, a particular
10 attention should be devoted to pH values; as a matter of fact, the choice of pH values of the
11 starting solution strongly impact on the type and distribution of charges onto the surface of the
12 protein and therefore could determine rearrangement of the protein conformation. Besides, also
13 pH values applied during the disassembly-reassembly protocol become critical to obtain an
14 effective loading. Not only pH values, but also many other variables can affect encapsulation
15 efficiency into APO protein nanocarrier as: i) ionic concentration; ii) interactions between ions in
16 solution and functional groups onto protein surface; iii) temperature, which could limit of stability
17 of the protein by inducing its unfolding and denaturation; iv) protein concentration which can
18 increase the frequency of molecular collisions and can promote aggregation; v) mechanical stress
19 caused by processes such as mixing, stirring, filtration, dialysis, concentration etc. [3].
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27 From this point of view, synthetic DDS offer a larger versatility of formulation as, by simply
28 changing the composition of the carriers or the formulation technique, a greater number of
29 molecules could be encapsulated or even adsorbed. Moreover, synthetic DDS are studied to reach
30 the goal of achieving controlled&targeted drug release, thus limiting well-known side effects of
31 high systemic or off-target exposure. In the case of APO, it is very hard to modulate drug release
32 from the inner cage, especially depending on their chemico-physical properties. That way, ions,
33 allowed to enter/exit through pores, could be modulated, but larger molecules could be released
34 from the inner core only as consequence of protein denaturation once in cellular acidic
35 compartment, thus strongly impacting and often decreasing the possibility for release modulation
36 [4].
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41 Thus, a rationale planning of APO as DDS should be based on the “prediction/evaluation” of the
42 combination of the formulative variables, which will govern both the loading efficiency and drug
43 release kinetics. In fact, the possibility for the drug to penetrate and to escape through the APO
44 inner channels is strongly function of drug/protein electrostatic interactions. These interactions
45 impact also on the structural rearrangement of APO, as, depending on its charges, any
46 electrostatic interaction with the drug will strongly affect the correct reassembly of the protein.
47 This electrostatic interaction between charged drug and charged protein will also play an
48 important role both on the stability of the encapsulation and on the un-wanted absorption of the
49 drug on the protein surface.
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54 Finally, a major issue is surely related to the chemico-physical characterization of APO during all
55 the processes of formulation along with the characterization of the final loaded APO-based DDS.
56 This particular aspect is fully addressed in the review we are proposing in this issue.
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59 *The Biosafety Issues*
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3 It is a common idea that proteins are generally safe and non-toxic; however the reaction after
4 administration of heterologous APO could lead to (i) activation of immune system against foreign
5 proteins, similar to immune response against pathogens or vaccines; and (ii) breach of B and T cell
6 tolerance to autologous proteins [5,6], finally resulting in the production of anti-therapeutic
7 protein antibodies [known as anti-drug antibodies (ADAs)] able to neutralize or otherwise to
8 compromise the clinical effect of therapeutics [7]. A number of studies use ferritin of animal
9 source, mainly derived from horse [8] and pig [9], or in other cases by means of exploitation of
10 recombinant proteins [10,11] and often, the choice of the APO derived from economic aspects. In
11 fact, horse spleen ferritin is cheaper with respect to human ferritin but frequently the researchers
12 underestimated the impact on biosafety and *in vivo* response.

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14 Thus, the protein source, its purity and its final rearrangement could deeply affect the safety
15 profiles of the protein and the final applicability of the DDS.

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17 Moreover, the advantage of use a natural molecule as starting material for DDS is drastically
18 affected when *ex vivo* engineering processes are performed in order to link targeting moieties
19 onto the DDS surface. The use of solvent, chemical reagents and ligands obviously impact on the
20 biocompatibility of the formulation as the protein conformation deeply suffers in terms of stability
21 and maintenance of the tertiary/quaternary structures of the modified proteins. Moreover, the
22 presence of ligand could interfere with the surface integrity, governing or altering the overall
23 biodistribution and body-distribution.
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30 **Expert Opinion**

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32 Several evidences provide for the suitability of APO as protein-based DDS, featured by a number of
33 advantages regarding the biocompatibility and application in pathologies, especially in the case of
34 treatment and imaging of cancer. As any new “application”, the starting point consists of the
35 direct evidence of efficacy in treatment, but, as rule, immediately after the first proofs-of-concept,
36 a complete study on formulation, scale-up, translatability, biocompatibility and biodistribution is
37 strongly required.
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40 It is evident that APO based nano-DDS are up-to-date in the middle of this path. Therefore,
41 especially a multidisciplinary research effort should be given in order to complete the overall
42 “painting” of APO application and formulation and to give strong impulse to the development and
43 clinical translatability of these novel and promising DDS.
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