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Editorial

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

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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 f*uture 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.

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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.

Looking at this scope, endogenous self-assembling proteins could be a strategic choice, leading to obtain DDS able to efficiently load molecules, considered as non-toxic for the organism and therefore safe.

More recently, the attention of the research focused on Apoferritin (APO), from ferritin family, a uniform regular self-assemblies nano-sized protein, showing excellent biocompatibility and unique architecture able to stabilize small active molecules in its inner core. The low dimension, spherical shape and high homogeneity [1] are among the key aspects that support the wide interest in APO cage. This novel DDS could lead to longer circulation half-life and eventually to better accumulation rates, in comparison with synthetic DDS (i.e. polymeric NPs or liposomes), characterized by higher size (50-200 nm) and less homogeneity. Moreover, APO is fully biocompatible and a-toxic, which is not a common feature for all the conventional nano-DDS.

Beside this aspect on safety/biocompatibility, a major interest in APO research lies in "targeting" abilities. These features are classically addressed by surface conjugation of nano-DDS with ligands (peptide, antibodies…) able to target the diseased sites.

This inevitably lead to the "over-crowding" of the surface of carriers, with a number of issues to be solved from both *technological* (i.e. production reproducibility, surface characterization) and *biological* points of view (i.e. interaction with bloodstream proteins, protein corona composition, safety and immune reaction), and overall the biocompatibility of the final structure

On the contrary, APO possesses site-specific targeting potential, as they can be recognized and internalized by Ft-binding receptors, such as the transferrin receptor 1 (TfR1). The overexpression of these receptors especially on the surface of malignant cells is one of the most important reasons for the growing interest of APO in the application of field of cancer treatment and diagnosis. This overexpression should be considered as an increased presence of TfR in malignant cells, but not as a unique expression of cancer cells. In fact, transferrin receptors are present onto many other cells, greatly varying among cells depending on tissues, as TfR could be easily found in basal epidermis, endocrine pancreas, hepatocytes, Kupfer cells, testis and pituitary gland [2]. Thus, on the basis of these evidences, the potentiality of APO widens to the application in other fields of nanomedicine as gene therapy, immunology or liver pathology.

Apoferritin: from the dream to the reality

Notwithstanding these good premises, some limits emerged in the application of APO as DDS, mainly connected to pharmaceutical formulation, characterization and standardization of process, but also to its biosafety. In particular, a couple of aspects is up-to-date unclear and poorly analyzed and therefore to be strongly investigated and ameliorated to allow APO to play a major role in DDS, namely:

1. Pharmaceutical Issues
2. Biosafety Issues

The Pharmaceutical Issues

As evident from the literature outputs, due to the stringent requirements of loading protocols, only a limited number of drugs could be efficiently encapsulated and not always the efficacy of drug/APO complex is higher respect to free drug. Actually, the plethora of experiments on APO formulation did not produce a standardized protocol that is able to clearly furnish a reproducible unfolding/refolding of the protein, and especially when the drug is present and ready to be loaded. Chemico-physical properties of drugs as molecular weight, pKa and charge should be taken into great account in planning their encapsulation in the APO core. In this view, a particular attention should be devoted to pH values; as a matter of fact, the choice of pH values of the starting solution strongly impact on the type and distribution of charges onto the surface of the protein and therefore could determine rearrangement of the protein conformation. Besides, also pH values applied during the disassembly-reassembly protocol become critical to obtain an effective loading. Not only pH values, but also many other variables can affect encapsulation efficiency into APO protein nanocarrier as: i) ionic concentration; ii) interactions between ions in solution and functional groups onto protein surface; iii) temperature, which could limit of stability of the protein by inducing its unfolding and denaturation; iv) protein concentration which can increase the frequency of molecular collisions and can promote aggregation; v) mechanical stress caused by processes such as mixing, stirring, filtration, dialysis, concentration etc. [3].

From this point of view, synthetic DDS offer a larger versatility of formulation as, by simply changing the composition of the carriers or the formulation technique, a greater number of molecules could be encapsulated or even adsorbed. Moreover, synthetic DDS are studied to reach the goal of achieving controlled&targeted drug release, thus limiting well-known side effects of high systemic or off-target exposure. In the case of APO, it is very hard to modulate drug release from the inner cage, especially depending on their chemico-physical properties. That way, ions, allowed to enter/exit through pores, could be modulated, but larger molecules could be released from the inner core only as consequence of protein denaturation once in cellular acidic compartment, thus strongly impacting and often decreasing the possibility for release modulation [4].

Thus, a rationale planning of APO as DDS should be based on the “prediction/evaluation” of the combination of the formulative variables, which will govern both the loading efficiency and drug release kinetics. In fact, the possibility for the drug to penetrate and to escape through the APO inner channels is strongly function of drug/protein electrostatic interactions. These interactions impact also on the structural rearrangement of APO, as, depending on its charges, any electrostatic interaction with the drug will strongly affect the correct reassembly of the protein. This electrostatic interaction between charged drug and charged protein will also play an important role both on the stability of the encapsulation and on the un-wanted absorption of the drug on the protein surface.

Finally, a major issue is surely related to the chemico-physical characterization of APO during all the processes of formulation along with the characterization of the final loaded APO-based DDS. This particular aspect is fully addressed in the review we are proposing in this issue.

The Biosafety Issues

It is a common idea that proteins are generally safe and non-toxic; however the reaction after administration of heterologous APO could lead to (i) activation of immune system against foreign proteins, similar to immune response against pathogens or vaccines; and (ii) breach of B and T cell tolerance to autologous proteins [5,6], finally resulting in the production of anti-therapeutic protein antibodies [known as anti-drug antibodies (ADAs)] able to neutralize or otherwise to compromise the clinical effect of therapeutics [7]. A number of studies use ferritin of animal source, mainly derived from horse [8] and pig [9], or in other cases by means of exploitation of recombinant proteins [10,11] and often, the choice of the APO derived from economic aspects. In fact, horse spleen ferritin is cheaper with respect to human ferritin but frequently the researchers underestimated the impact on biosafety and *in vivo* response.

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

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

Expert Opinion

Several evidences provide for the suitability of APO as protein-based DDS, featured by a number of advantages regarding the biocompatibility and application in pathologies, especially in the case of treatment and imaging of cancer. As any new “application”, the starting point consists of the direct evidence of efficacy in treatment, but, as rule, immediately after the first proofs-of-concept, a complete study on formulation, scale-up, translatability, biocompatibility and biodistribution is strongly required.

It is evident that APO based nano-DDS are up-to-date in the middle of this path. Therefore, especially a multidisciplinary research effort should be given in order to complete the overall “painting” of APO application and formulation and to give strong impulse to the development and clinical translatability of these novel and promising DDS.

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