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Magic ferritin: A novel chemotherapeutic encapsulation bullet

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Abstract

The dissociation of apoferritin into subunits at pH 2 followed by its reformation at pH 7.4 in the presence of doxorubicin–HCl gives rise to a solution containing five doxorubicin–HCl molecules trapped within the apoferritin. This is the first report showing that ferritin can encapsulate an anti-cancer drug into its cavity.
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1. Introduction

The concept of a magic bullet has been put forward over 100 years ago. Since then, in order to enhance the therapeutic index of drugs and specifically deliver these agents to defined targeted cells, several specific and targeted micro- and nano-delivery systems have been investigated.

The development of new injectable drug delivery systems has received considerable attention over the past few years. This interest has been sparked by the advantages of these delivery systems. These systems possess ease of application, localized delivery for a site-specific action, prolonged delivery periods, decreased body drug dosage with concurrent reduction in possible undesirable side effects, and improved patient compliance and comfort [1].

Initial studies examined delivery systems such as polymers [2], liposomes [3] and biodegradable microspheres [4,5]. Liposomes are not a promising dosage form for long-acting formulations. Local retention of liposomes-entrapped drugs is likely to be longer than that of free drugs, but it may not always be long enough to maintain local therapeutic drug levels, due in part to rapid clearance by macrophages and other cells [6]. Other problems, such as stability issues, sterilization problems and often-low drug entrapment, have played an important role in limiting the utility of liposomes [3]. Targeting of drugs by microspheres is intended to increase selective targeting to specific organs and to reduce these side effects. Microspheres showing targeting ability, prolonged release and sustained action provide a new field in drug delivery to desired target organs [5].

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Iron is an essential element for almost all living organisms but is very toxic in excess. Man is unable to eliminate iron from the body, so that excessive iron accumulates in the liver and other organs, which can lead to tissue damage, organ failure and eventually death [7].

Ferritins are composed of 24 polypeptide subunits packed together to form a nanosphere of diameter ~12 nm, with an internal cavity ~8 nm across (~250 nm^3) where up to 4500 Fe (III) atoms are stored. Human ferritins are encoded by a gene family, which contains 31 ferritin genes, and so far only three different ferritin polypeptides have been identified. These are light (L 19 kDa), heavy (H 21 kDa) and glycosylated (G 23 kDa). Tissue ferritins are composed of variable proportions of H and L subunits. The L subunit-rich ferritins are more abundant in iron-loaded tissues such as liver and spleen, while the H subunit-rich ferritins are found in heart, kidneys and malignant cells [8].

Iron is important for the growth of both normal and neoplastic cells. Epidemiologic and experimental studies have shown that, in humans, increased body iron stores are associated with increased risk of cancer, especially in hepatocellular carcinoma [9]. Serum ferritin levels in these cancers correlate closely with the stage of disease. For example, in Hodgkins disease, serum ferritin increased from 80 ng/ml in stage IA patients to 2000 ng/ml in stage IVB patients [10]. It has been shown that ferritin can be internalized by rat [11] and human liver [12], and the internalization is associated by membrane-specific receptors [13].

In this study, the chemotherapeutic encapsulation properties of ferritin were investigated with doxorubicin–HCl, a chemotherapeutic agent commonly used in cancer therapy, as a model drug.

2. Experimental methods

Horse spleen apoferritin and doxorubicin–HCl (= doxorubicin) were purchased from Sigma.

Loading of doxorubicin into the apoferritin cavity was carried out as follows. An apoferritin solution (2 ml 2.5 mg/ml) was added into 20 mM pH 2.5 glycine-acetate buffer. After 10 min, three different concentrations (50, 400, 850 μM) of doxorubicin were added into the protein solution. The pH of the solution was slowly raised to pH 4 by addition of a 4 M trisma-base solution, using a microsyringe. The resulting solution was dialyzed for a night against 20 mM pH 7.4 trisma-base-acetate buffer. The dialysis reservoir solution was collected and centrifuged at 12,000 rpm to remove denatured proteins. The unbound drug molecules were removed from the protein solution by washing in phosphate buffer at pH 7.4 four times by using concentration tubes. At the same time, protein solutions were concentrated.

Protein analysis: After the denaturation–renaturation process, samples were collected and centrifuged at 12,000 rpm for 10 min. The supernatant was collected and used for the protein analysis at 280 nm wavelength using a UV spectrophotometer (Cecil 1020).

Drug analysis: The amount of doxorubicin encapsulated by the protein was determined using fluorescence spectroscopy (Perkin Elmer LS 50B). Doxorubicin is a red-colored, naturally fluorescing agent and is suitable for fluorescence spectroscopy analysis. Maximum excitation wavelength and maximum emission wavelengths of doxorubicin and ferritin were determined (Fig. 1). After the denaturation–renaturation process, the supernatant samples collected after the protein analysis

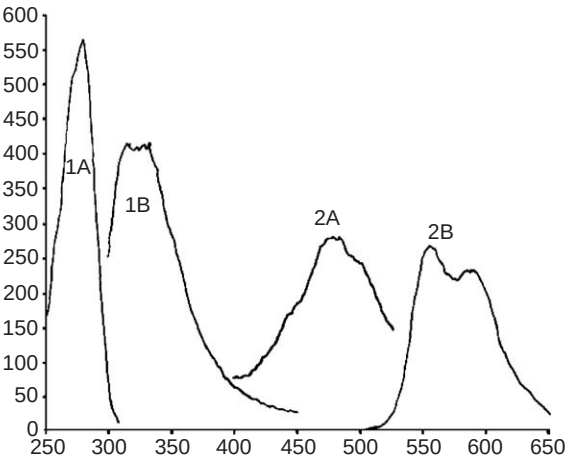

Fig. 1. Maximum excitation of (I-A) ferritin and (II-A) doxorubicin as well as maximum emission of (I-B) ferritin and (II-B) doxorubicin.

were used to determine encapsulated drug concentrations at a wavelength of 480 nm.

3. Results and discussion

An approach to trapping molecules inside apoferritin has been reported [14], consisting of the dissociation at pH 2.0 of apoferritin into its 24 subunits followed by its reconstruction at pH 7.0–9.0, thereby trapping the solution components within its interior. By following a similar procedure it was possible to trap about five molecules of doxorubicin inside the apoferritin.

The initial apoferritin and doxorubicin–HCl concentrations were 2.5 mg/ml and 0.05–1 mM, respectively. After the pH-induced unfolding–refolding process, the final apoferritin concentrations were determined by UV spectroscopy at 280 nm (Table 1). Control experiments showed no aggregation of the protein–doxorubicin complex in glycine-acetate buffer at pH 2.5 or in phosphate buffer at pH 7.4. After the denaturation–renaturation process, however, an average of 48% protein was lost, especially when the pH was raised from 2.5 to 4. The subunits of the ferritin and doxorubicin molecules have many hydrophobic and charged regions, allowing them to interact with each other. We suggest that the loss of protein is related to these interaction because the solution centrifuged at 12,000 rpm to remove unrenaturated protein molecules from the solution resulted in a red-colored pellet collapsed in the bottom of the Eppendorf tube. It was shown that doxorubicin molecules bind to ferritin subunits between a pH of 3–4.

Once the apoferritin was reconstructed in the presence of doxorubicin, two types of doxorubicin molecules could be detected: one type was trapped by the apoferritin and the other was outside the apoferritin. The untrapped molecules present in the solution were separated by dialysis against trisma-base-acetate buffer pH 7.4. It was shown that doxorubicin molecules were completely removed from the buffer, washing it in trisma-base-acetate buffer using concentration tubes four times. Apoferritin solution and three different concentrations of doxorubicin were put into 2 ml of trisma-base acetate buffer at pH 7.4 and centrifuged. Since no residue was observed, the ferritin–doxorubicin complex did not aggregate at pH 7.4. Separation of the doxorubicin from the protein by concentration tubes resulted in a concentration of less than 0.0004 μM. The concentration of doxorubicin bound to ferritin outside was thus neglectable. According to this control experiment, the drug concentration of doxorubicin as determined by fluorescence spectroscopy therefore reflects only the concentration of trapped doxorubicin molecules.

The maximum emission of ferritin (Fig. 1) changed after the addition of 10 μM doxorubicin. The maximum emission of doxorubicin also changed after the addition of 500 μg/ml ferritin. The results are shown in Figs. 2 and 3. Maximum emission and excitation wavelengths of ferritin were 280 and 330 nm, respectively, and the 280 nm emission wavelength was used for quantitative assays. After adding doxorubicin to the protein solution, the maximum emission wavelength of ferritin was reduced by 28% (Fig. 4).

The multi-subunit construction of the apoferritin shell allows the generation of channels. Eight hydrophilic channels of about 4 Å lead to the protein cavity. Water, metallic cations and hydrophilic molecules of appropriate size would diffuse through these channels from the external solution to the cavity or from the cavity to the external solution, but doxorubicin cannot. Channels in the sphere are formed at the intersections of three or four peptide subunits. These channels are critical to ferritin's ability to release iron in a controlled fashion. Iron atoms interact with these channels. First, ferritin enters through the channel and then binds to the subunits and accumulates in the cavity. We suggest that doxorubicin can also bind

Table 1
Final apoferritin concentrations

Samples	Initial concentration (μM)	Final concentration (μM)	Percentage lost (%)
FR1	2.5	0.8	68
FR8	2.5	1.6	36
FR17	2.5	1.5	40

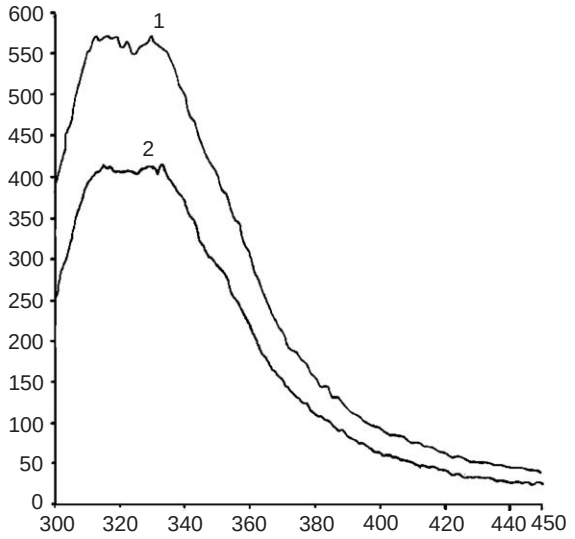

Fig. 2. Maximum emission of ferritin (1) initially and (2) after adding doxorubicin.

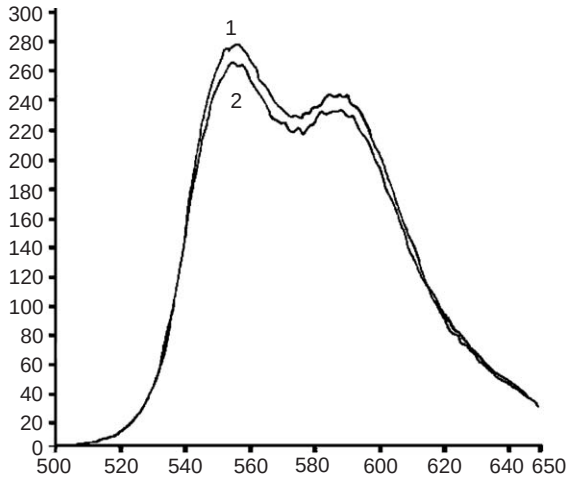

Fig. 3. Maximum emission of doxorubicin (1) before and (2) after adding ferritin.

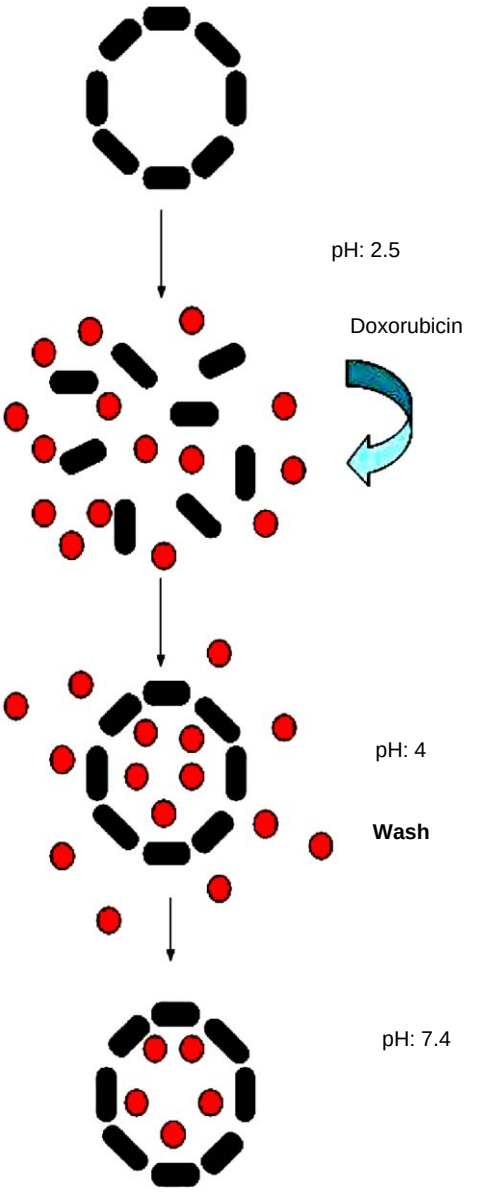

Fig. 4. Schematic representation of the pH-induced dissociation–reformation process of apoferritin in the presence of doxorubicin.

to the subunits of ferritin and accumulate in the cavity in a similar way. Alternatively, doxorubicin might be present in the cavity in free form, but confirmation will require further experiments.

The trapping of doxorubicin molecules within the apoferritin must be a consequence of their size, because doxorubicin is too large to diffuse through the channels and leave the apoferritin. For this reason, only the untrapped doxorubicin molecules present in the external solution are removed by dialyzing, whereas the trapped doxorubicin molecules are not.

Table 2
Encapsulated doxorubicin concentrations

Initial drug concentration (μM)	Amount of encapsulated drug (μM)
50	0.05–0.1
400	0.1–1
850	1–5

The initial ferritin concentration was 2.5 μM.

Three different concentrations (50, 400, 850 μM) of doxorubicin were used. The highest drug encapsulation was found at a ratio of 1:5 (ferritin:doxorubicin) (See Table 2).

Maximum emission and excitation wavelengths of doxorubicin were 555 and 480 nm, respectively. After adding ferritin to the doxorubicin solution, the maximum emission wavelength of doxorubicin was reduced by 3%.

Doxorubicin was previously encapsulated in other carrier systems, such as liposomes. In cell culture [15], IC_{50} values of 6.4 and 0.78 μM were found for free doxorubicin and hyaluronan-targeted liposomes, respectively. Comparing our reached encapsulation of up to 5 μM to these concentrations, it seems possible that our ferritin nanoencapsulation system can deliver effective drug concentrations for therapeutic applications.

Since ferritin is stable at the physiological pH of 7.4, we suggest that it will not precipitate in blood. Since it is the natural human ferric iron storage protein and iron atoms can be easily trapped into ferritin cavity, we also do not expect aggregation of the drug-loaded iron containing ferritin in the blood.

In conclusion, the present work has demonstrated that it is possible to trap five molecules of doxorubicin inside an apoferritin molecule. This is the first report showing that ferritin can encapsulate an anti-cancer drug into its cavity.

Acknowledgements

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