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Novel method of doxorubicin–SPION reversible association for magnetic drug targeting

E. Munnier[a,b], S. Cohen-Jonathan[a,b], C. Linassier[a,b,c], L. Douziech-Eyrolles[a,b], H. Marchais[a,b], M. Soucé[a,b], K. Hervé[a,b], P. Dubois[a,b], I. Chourpa[a,b,*]

[a] Université François-Rabelais, Faculté de Pharmacie, "Focalisation magnétique d'agents anticancéreux", Tours F-37200, France
[b] Institut Fédératif de Recherche 135 "Imagerie Fonctionnelle", Tours F-37000, France
[c] CHRU Bretonneau, Service d'Oncologie Médicale, Tours F-37000, France

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ABSTRACT

A new method of reversible association of doxorubicin (DOX) to superparamagnetic iron oxide nanoparticles (SPION) is developed for magnetically targeted chemotherapy. The efficacy of this approach is evaluated in terms of drug loading, delivery kinetics and cytotoxicity *in vitro*. Aqueous suspensions of SPION (ferrofluids) were prepared by coprecipitation of ferric and ferrous chlorides in alkaline medium followed by surface oxidation by ferric nitrate and surface treatment with citrate ions. The ferrofluids were loaded with DOX using a pre-formed DOX–Fe^{2+} complex. The resulting drug loading was as high as 14% (w/w). This value exceeds the maximal loading known from literature up today. The release of DOX from the nanoparticles is strongly pH-dependent: at pH 7.4 the amount of drug released attains a plateau of ~85% after 1 h, whereas at pH 4.0 the release is almost immediate. At both pH, the released drug is iron-free. The *in vitro* cytotoxicity of the DOX-loaded SPION on the MCF-7 breast cancer cell line is similar to that of DOX in solution or even higher, at low-drug concentrations. The present study demonstrates the potential of the novel method of pH-sensitive DOX–SPION association to design novel magnetic nanovectors for chemotherapy.

1. Introduction

The anthracycline antibiotic adriamycin or doxorubicin (DOX, Fig. 1) is a highly efficient antineoplastic agent commonly used in the treatment of a variety of cancers like leukaemia, ovarian cancer and especially late stage breast cancer (Hoke et al., 2005). The clinical use of DOX is limited by the resistance developed by cancer cells and by strong side effects, namely a dose-dependent cardiotoxicity (Bast et al., 2007; Petit, 2004).

Drug targeting, that is drug delivery to the tumor site, helps prevent side effects and increase cytotoxicity of doxorubicin. Several principles of drug targeting are being investigated, for instance molecular coupling of the anticancer agent to specific molecules like low-density lipoproteins (Lo et al., 2002) or monoclonal antibodies (Inoh et al., 2006) which interact with specific receptor(s) in the tumor.

Another possible approach for drug targeting is the delivery using an aqueous suspension of magnetic nanoparticles retained in a tumor by application of an external magnetic field (magnetic drug targeting) (Lübbe et al., 2001; Torchilin, 2006). The superparamagnetic iron oxide nanoparticles (SPION, Neuberger et al., 2005) are particularly interesting, since they are devoid of magnetic remanence due to their very small size (often below 10 nm). In the last years, SPION-based ferrofluids have been developed as contrast agents for magnetic resonance imaging (MRI) and as heating intermediates for magnetic hyperthermia (Alexiou et al., 2006; Corot et al., 2006; Duguet et al., 2006; Moghimi and Kissel, 2006; Neuberger et al., 2005). The enhanced interest of the SPION–drug associates is related to the potentially combined functions of targeted therapy and diagnosis (Duguet et al., 2006; Neuberger et al., 2005). The association drug–SPION for magnetic drug targeting can be realized either by direct binding to the iron oxide surface or by encapsulating both drug and SPION within a biodegradable polymeric matrix (Berry and Curtis, 2003; Duguet et al., 2006; Jain et al., 2005; Neuberger et al., 2005; Ngaboni Okassa et al., 2007).

The drug loading values obtained with direct drug–SPION binding are generally comprised between 0.5 and 12% (w/w) (Alexiou et al., 2000; Jain et al., 2005; Lübbe et al., 1996a,b; Mykhaylyk et al., 2005; Rudge et al., 2000; Zhang and Misra, 2007). Direct drug binding to magnetic nanoparticles can be achieved by either covalent bonds or ionic interactions (Alexiou et al., 2000; Duguet et al.,

* Corresponding author at: Laboratoire de Chimie Analytique, UFR de Pharmacie, 31 avenue Monge, Tours F-37200, France. Tel.: +33 247 367162; fax: +33 247 367270.
E-mail address: chourpa@univ-tours.fr (I. Chourpa).

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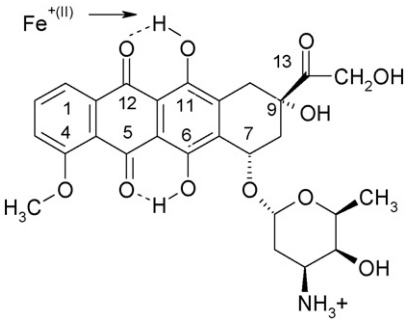

Fig. 1. Structural formula of doxorubicin (DOX) with the site of preferential Fe^{2+} ion binding in an aqueous buffer pH 7.6: iron replaces one phenolic hydrogen at position 11.

2006; Lübbe et al., 1996a,b). In contrast to covalent binding, ionic adsorption is easily reversible since it depends on ionic strength and pH. As a result, the kinetics of drug release from ionic systems are expected to be faster than in the case of covalent binding. Nanoparticular magnetic fluids with ionically adsorbed epirubicin have been reported to increase the drug content in tumor tissue (Lübbe et al., 1996a,b), thus demonstrating magnetic drug targeting feasibility. In that work, epirubicin was adsorbed due to the interaction between the positively charged amino sugar of the drug and the particle surface previously functionalized with anionic phosphate groups.

In the present article, we propose and evaluate a new approach of binding DOX to the SPION surface using a pre-formed complex of the drug with ferrous ion (Fe^{2+}). The ability of anthracyclines to efficiently chelate Fe^{3+} ions has been extensively studied (Fiallo et al., 1999) because it is thought to be implied in this drug cardiotoxicity (Tokarska-Schlattner et al., 2006) and in oxidative destruction of DNA (Eliot et al., 1984). It has been established that, in the Fe^{3+}–DOX complex, the α-ketol group of the drug reduces iron to Fe^{2+} while being itself oxidized to semidione free radicals (Hasinoff, 1989; Malisza and Hasinoff, 1995). The oxidation products are able to stimulate a production of reactive oxygen species (ROS) that are more implied in the cardiotoxicity (Andreadou et al., 2007) than in the anticancer activity of the drug (Keizer et al., 1990). These considerations motivated our choice of ferrous ion rather than ferric ion to play the role of intermediate in the DOX–SPION binding. At the same time, particular care was taken to reduce the presence of free iron in the samples.

We obtained DOX-carrying SPION ferrofluids with properties favorable for magnetic drug targeting, i.e. drug loading of up to $14.6 \pm 0.5\%$ (defined below) and rapid release kinetics at physiological pH 7.4 (~85% DOX released in 1 h). It should be particularly underlined that this release provided free DOX and not DOX–iron complex. Furthermore, the biological evaluation of DOX-loaded SPION demonstrates that their cytotoxicity against MCF-7 cancer cells is not only preserved but, under certain conditions, is even increased compared to DOX solutions.

2. Experimental

2.1. Chemicals

Doxorubicin hydrochloride was purchased from TEVA Pharmaceuticals Ltd. (Puteaux, France). Potassium hydroxide solution was purchased from Prolabo (Fontenay-sous-Bois, France). Dimethylsulfoxide (DMSO), ferric nitrate, MTT and penicillin-streptomycin solution were furnished by Sigma–Aldrich (Saint-Quentin-Fallavier, France). Sodium acetate and tris-(hydroxymethyl)-aminomethane (Tris) were provided by Merck (Fontenay-sous-Bois, France). Anhydrous ferrous chloride, citric acid, Dulbecco's modified Eagle medium (DMEM), iron standard solution (titrisol), hydrochloric acid solution (trace analysis or analytical grade) and ferrous chloride ($FeCl_2{\cdot}4H_2O$) were purchased from Fisher Bioblock Scientific (Illkirch, France), and ferrous ammonium sulphate ($(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$) from Carlo Erba (Val de Reuil, France).

2.2. Preparation and characterization of citrate-stabilized ferrofluids of SPION

SPION were synthesized as aqueous ferrofluids by a coprecipitation of ferric and ferrous chlorides in alkaline medium followed by a surface oxidation by ferric nitrate and finally peptized in water, as described elsewhere (Chourpa et al., 2005). To increase the stability of the ferrofluid suspension at neutral pH, the SPION surface was treated with citrate ions by incubation in a 1.5-g/L citric acid solution under vigorous agitation for 2 h. Following this treatment, the ferrofluid pH was readjusted to 7.0 by addition of potassium hydroxide. The particles were then purified from excess citrate by 48 h dialysis (Float-a-Lyzer dialysis membrane, MWCO 8000, Interchim, France) against a 150-fold acceptor volume of distilled water.

The SPION concentration in ferrofluids was 0.56 g/L as estimated based on the measured iron concentration and considering that iron represents 71.5% of SPION (composed of 60% magnetite and 40% maghemite; Chourpa et al., 2005). The overall iron content ($Fe^{2+} + Fe^{3+}$) in the ferrofluids was 0.4 g/L as measured by atomic absorption spectrophotometry (SpectrAA-10 Plus spectrometer, Varian, France) using a calibration curve obtained with titrisol standard solution. Prior to these measurements, the particles were dissolved in HCl 6 M.

The hydrodynamic diameter of the particles was determined by DLS (dynamic light scattering) technique with an Autosizer 2c (Malvern Instruments, Orsay, France). The fine particle morphology was analysed by transmission electron microscopy (TEM) using a JEOL 1010 microscope (Jeol, Japan) at 88 kV. The samples were placed on a carbon coated copper grid and stained with 3% (w/v) uranyl acetate for TEM viewing.

2.3. Preparation and characterization of DOX–Fe^{2+} complex and of DOX–SPION ferrofluids

DOX–Fe^{2+} complex solutions of variable drug/iron molar ratio were obtained by adding an aqueous solution of ferrous ammonium sulphate ($(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$) to DOX in Tris buffer pH 7.6. The drug–iron complex used to load the nanoparticles was prepared with a 1.5-fold molar excess of Fe^{2+} over DOX. Then the DOX–Fe(II) complex was incubated in the dark with citrate-treated ferrofluid (total iron concentration 0.05 g/L), varying the mass ratio of DOX/SPION from 0.16 to 0.95 (w/w). After 15 min incubation, the drug-loaded SPION were harvested by centrifugation at $15{,}000 \times g$ for 15 min (4 °C). Finally, the nanoparticles were washed with ice-cold fresh aqueous buffer pH 7.6 and used immediately afterwards.

The DOX loading (%) was defined as the weight fraction of the drug in the final drug-loaded SPION. To measure the loaded drug, the particles were re-suspended for 20 min in acetate buffer pH 4.0, conditions that lead to dissociation of the DOX–Fe^{2+} complex and therefore to release of 100% of DOX (discussed below). The sample was then centrifuged and the DOX concentration in the supernatant was measured by means of UV–vis spectrophotometry (Anthélie Advanced Spectrophotometer, Secomam, France), using the DOX absorbance at 480 nm ($\varepsilon = 11{,}500\ cm^{-1}\ mol^{-1}\ L$, Fiallo et al., 1999). Each determination was performed in quadruplicate.