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Note

Comparative study of doxorubicin-loaded poly(lactide-co-glycolide) nanoparticles prepared by single and double emulsion methods

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Abstract

This study describes how the control of doxorubicin (DOX) polarity allows to encapsulate it inside poly(lactide-co-glycolide) (PLGA) nanoparticles formulated either by a single oil-in-water (O/W) or a double water-in-oil-in-water (W/O/W) emulsification method (SE and DE, respectively). DOX is commercially available as a water soluble hydrochloride salt, which is useful for DE. The main difficulty related to DE approach is that the low affinity of hydrophilic drugs to the polymer limits entrapment efficiency. Compared to DE method, SE protocol is easier and should provide an additional gain in entrapment efficiency. To be encapsulated by SE technique, DOX should be used in a more lipophilic molecular form. We evaluated the lipophilicity of DOX in terms of apparent partition coefficient (P) and modulated it by adjusting the pH of the aqueous phase. The highest P values were obtained at pH ranging from 8.6 to 9, i.e. between two DOX pK_a values (8.2 and 9.6). The conditions favorable for the drug lipophilicity were then used to formulate DOX-loaded PLGA nanoparticles by SE method. DOX encapsulation efficiency as well as release profiles were evaluated for these nanoparticles and compared to those with nanoparticles formulated by DE. Our results indicate that the encapsulation of DOX in nanoparticles formulated by SE provides an increased drug entrapment efficiency and decreases the burst effect.

Keywords: Doxorubicin; Partition coefficient; PLGA nanoparticles; Single emulsion; Double emulsion; Release kinetics

1. Introduction

Doxorubicin (DOX) is an anthracycline drug commonly used in the treatment of a large spectrum of tumors. However, DOX-related acute cardiotoxicity and multidrug resistance of cancer cells have led researchers to investigate advanced DOX delivery systems. Modification of the DOX biodistribution can be achieved through entrapping it in submicron carriers such as liposomes, polymeric micelles or nanoparticles.

DOX-loaded composite nanoparticles, formulated as a polymeric matrix containing superparamagnetic magnetite nanoparticles, are being studied in our group as a new system for targeted chemotherapy of cancers [1]. After intravascular administration, nanoparticles containing both an anticancer drug and superparamagnetic iron oxide could be accumulated in the tumor by application of an external magnetic field [2].

One of the most employed methods to produce PLGA nanoparticles is emulsification. Commonly, hydrophobic PLGA is dissolved in an organic solvent. If the drug is also hydrophobic, it is dissolved with the PLGA. This organic phase is emulsified with an aqueous phase to make an O/W single emulsion (SE). If the drug is hydrosoluble, it is

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dissolved in an aqueous phase W_1 which is emulsified in the PLGA organic solution to make a W_1/O emulsion. This emulsion is emulsified in a second aqueous phase W_2 to form a $W_1/O/W_2$ double emulsion (DE). In both techniques, removal of the organic solvent results in solid particles. However, an important difficulty related to the DE protocol is that the low affinity of small molecular weight hydrophilic drugs to the polymer can result in low entrapment efficiency.

The DOX molecule (Fig. 1) contains two phenols (C_6, C_{11}) and one amine ($C_{5'}$) ionizable functions. Therefore, pH adjustment should enable to control protonation and polarity of DOX. The neutral molecular form of DOX can be extracted from aqueous to organic phase. The organic phase enriched in both DOX and PLGA should enable to prepare nanoparticles by an *SE* method that is expected to provide an additional gain in entrapment efficiency, while facilitating the formulation protocols compared to DE method.

The primary goal of this work was to investigate the polarity of DOX versus pH in order to find experimental conditions favoring DOX extraction into the organic phase. The resulting DOX-enriched organic phase was used in SE protocols to elaborate DOX-loaded PLGA nanoparticles. The interest of these nanoparticles as vectors of doxorubicin was evaluated in terms of the drug entrapment efficiency and release profiles over 24 h, in comparison with the PLGA nanoparticles obtained by DE that incorporate DOX from an aqueous phase.

2. Experimental

2.1. Materials

DOX hydrochloride salt was purchased from DAKOTA Pharm (France). Poly(D,L-lactic-co-glycolic acid), having a lactic/glycolic molar ratio of 50/50 and a weight average molecular weight ($\overline{M}_w$) of 40–75 kDa, was obtained from Sigma Chemicals (Germany). Other chemicals were of reagent grade and obtained from Sigma Chemicals (Germany).

2.2. Apparent partition coefficient measurement

DOX lipophilicity was estimated by measuring its apparent partition coefficient (P) between an organic phase composed either of ethyl acetate (EA) or methylene chloride (MC) and an aqueous buffer (citrate–phosphate or borate) of various pH values comprised between 4 and 9. P was defined as the ratio of DOX concentrations in the organic and aqueous phases at equilibrium. The partition was obtained by vigorously shaking 2 ml of aqueous DOX solutions (8.5×10^{-5} M) with 2 ml of organic phase (MC or EA). Shaking was performed during 24 h in a water bath tempered at 25 °C. We checked that within this time, equilibrium was attained. DOX concentration in the organic phase was calculated as difference between the initial and final drug concentrations in the buffer. Concentration of DOX in the aqueous phase was measured by HPLC with fluorescence detection (LC 10A system, Shimadzu, Japan). The excitation wavelength was 470 nm and emission was monitored at 590 nm. Samples were injected automatically onto a C18 column (Kromasil 5 μm C18, Interchim). The mobile phase composed of methanol/sodium acetate 0.01 M/acetic acid (70:30:1.3) was filtered and pumped at a flow rate of 1.5 ml/min. Experiments were carried out three times for each sample.

2.3. Formulation of poly(lactide-co-glycolide) nanoparticles

We formulated two kinds of DOX-loaded PLGA nanoparticles using single and double emulsion methods as detailed below. In the first method (single emulsification-solvent removal technique), we used a MC solution of DOX prepared by partition from borate buffer pH 8.6. In the second method (double emulsification technique), we used an aqueous solution of DOX obtained by dissolving the DOX hydrochloride.

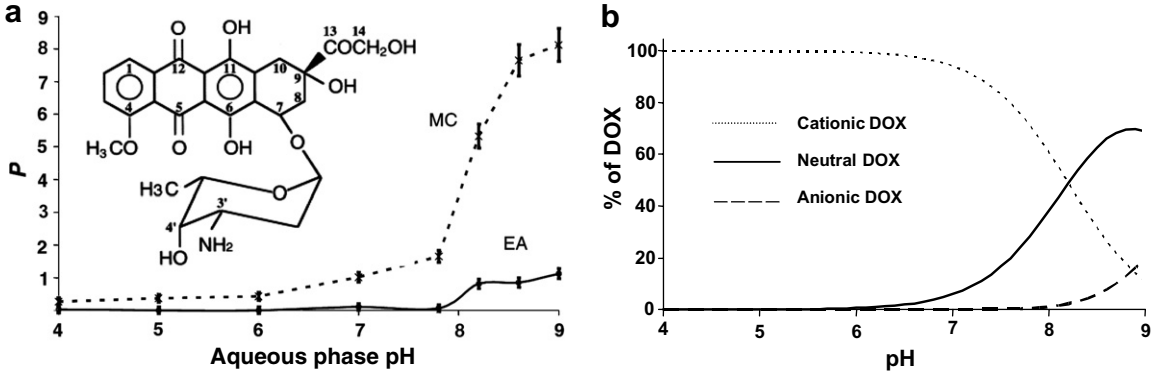

Fig. 1. (a) Experimentally established partition coefficients P for doxorubicin (DOX, structural formula in insert) in methylene chloride (MC) and ethyl acetate (EA) versus pH of initial aqueous phase. Initial DOX concentration in the aqueous phase: 8.5×10^{-5} M. Temperature: 25 °C. Equilibrium time 24 h. (b) Theoretical plot of ionization state of DOX versus pH.

2.3.1. Single emulsion (SE) method

DOX extraction in MC was carried out as follows. One milligram of DOX was dissolved in 2 ml of 0.05 M borate buffer pH 8.6. The aqueous phase was shaken with 50 ml of MC during 24 h at 25 °C. Then, MC was evaporated under vacuum until a final volume of 1 ml was reached. One hundred micrograms of PLGA was dissolved in 1 ml of MC and mixed with the 1 ml of DOX solution. This phase was emulsified in 4 ml of aqueous poly(vinyl alcohol) (PVA) solution at 3% (w/v) by sonication (Branson Sonifier® 150) for 30 s (15 W) in an ice bath, to obtain an O/W emulsion. This emulsion was then diluted in 50 ml of aqueous solution containing 0.3% (w/v) PVA and left under mechanical stirring for 10 min. Then, MC was removed by evaporation under vacuum at 40 °C. Nanoparticles were washed three times with deionized water by centrifugation at 20,000 *g* for 20 min. Finally, nanoparticle suspensions were freeze dried.

2.3.2. Double emulsion (DE) method

Aqueous DOX solution W_1 (1 mg DOX in 200 μl of deionized water) was emulsified with 2 ml of MC solution containing 100 mg of PLGA, by sonication for 15 s (15 W) in an ice bath. Then, 4 ml of 3% (w/v) PVA solution W_2 was added and sonicated for 30 s to make a $W_1/O/W_2$ emulsion. The double emulsion was diluted into 50 ml of 0.3% (w/v) PVA aqueous solution and MC was evaporated under vacuum. The obtained nanoparticles were collected, washed and freeze dried.

2.4. Characterization of nanoparticles

Nanoparticle morphology was analyzed using a transmission electron microscope (JEOL 1010, Jeol, Japan) at 88 kV under various magnifications. The samples were placed on a carbon coated copper grid and stained with 3% (w/v) uranyl acetate.

The mean hydrodynamic diameter of particles in volume was determined by photon correlation spectroscopy (PCS) using a Malvern Autosizer® 4700 (Malvern Instruments Ltd., Malvern, UK) with photodetector perpendicular to the laser beam.

2.5. Assay of doxorubicin loaded into nanoparticles

The resulting nanoparticles were evaluated in terms of drug encapsulation efficiency (EE, established as ratio between encapsulated over initial drug amount). For the EE determination, a known amount of freeze dried doxorubicin-loaded nanoparticles was completely dissolved in dimethyl sulfoxide (DMSO) and then the drug absorbance was measured at 480 nm using a UV–visible spectrophotometer (Secomam, model Anthelie advanced). Prior to the absorption measurements, acetic acid was added to the DMSO solution, to compensate for basicity of this solvent. DOX concentration was calculated from the absorbance using a calibration curve established previously. The experiments were performed in triplicate for both types of nanoparticles.

2.6. Release of DOX from the nanoparticles

DOX release experiments were performed using a donor dialysis volume of 2 ml containing DOX-loaded nanoparticle suspension (equivalent to a DOX concentration of 250 μg/ml). Dialysis was achieved through a Float-A-Lyzer® (Spectrum Laboratories Inc.) membrane (MWCO 8000) into a 20 ml acceptor volume of 0.05 M phosphate buffer pH 7.4, stirred and thermostated at 37 °C. At regular time intervals, 100 μl of the acceptor solution was sampled and subjected to HPLC assay. Experiments were performed in triplicate for both types of nanoparticles.

3. Results and discussion

3.1. Increase in the apparent doxorubicin partition coefficient

MC and EA are solvents frequently used in formulation protocols because of their capacity to easily dissolve PLGA. It is noteworthy that MC is more toxic than EA [3]. We determined DOX partition coefficient *P* between each of these organic solvents and aqueous phase, as function of initial pH. As described in Section 2, after 24 h of partition between the two phases, the equilibrium concentrations of DOX in the aqueous phase were measured and used to calculate *P*.

Fig. 1a shows *P* values established from DOX in aqueous buffer with pH between 4 and 9. Experiments were not performed above pH 9, because of high DOX degradation rate at pH exceeding this value [4]. As can be observed in Fig. 1a, DOX affinity to the organic phases increased when pH of the aqueous phase increased from 4 to 9, with a strong augmentation between 7.8 and 8.6. This observation is close to the results published by Raghunand et al. [4], who used octanol as organic phase. An increase in the DOX affinity to the organic phase has also been reported when partitioning between chloroform and aqueous phases buffered between pH 5 and 7 [5].

This pH dependence in partitioning is related to the ionic state of DOX in the aqueous phase. Literature values for the pK_a of the 3′ amine function in the sugar moiety of DOX (NH_3^+, Fig. 1) range from 7.2 to 8.6, depending on the DOX concentration, ionic strength and method of measure [4–8]. The pK_a of the more acidic phenol group of DOX (position 11) is given as 9.5 [8]. Using these pK_a values, and considering the system as a simple equilibrium between two consecutive dissociations, one can approximate the proportion between the molecular (HO–DOX–NH_2) and the ionic forms of DOX versus pH of the aqueous phase (Fig. 1b). At low pH (4–6), the amino sugar of DOX is protonated and therefore carries a positive charge (cationic DOX, Fig. 1b). Above pH 6, the fraction of pos-

itively charged amine groups decreases in favor of neutral molecular form of DOX. However, above pH 7.5 the fraction of neutral DOX is again reduced because of deprotonated phenol group (anionic DOX). Part of the neutral DOX molecules might exist in zwitterionic form, but they should revert to the molecular form before being extracted into the organic phase.

In the pH range investigated, the fraction of DOX extracted (Fig. 1a) seems to be well correlated with the fraction of uncharged DOX molecules (Fig. 1b). This confirms that the optimum pH for extraction to the organic phase is the one that favors the neutral form of DOX.

To favor DOX lipophilicity without further favoring drug instability, we used initial aqueous phase pH 8.6 (borate buffer) to extract the drug into organic solvents. At this pH, the apparent partition coefficient P of DOX was found to be about 8 with MC and about 1 with EA solvent. The difference in P obtained with the two organic solvents could be explained by the higher water solubility of EA compared to MC, 8.7 and 1.32 wt% at 25 °C, respectively [3]. Therefore, the aqueous phase is highly saturated in EA, increasing in DOX solubility in this phase and decreasing P. In presence of MC, the solubility of DOX in the water rich phase is slightly increased, allowing DOX extraction. Since DOX was more efficiently extracted to MC, we performed further formulation experiments with this organic solvent.

3.2. DOX loading in PLGA nanoparticles

Fig. 2 shows the shape of the particles obtained by SE and DE as observed by transmission electron microscopy (TEM). SE was used to encapsulate the neutral form of DOX extracted in MC from an aqueous borate buffer at pH 8.6. DE was used to encapsulate the cationic form of DOX (DOX hydrochloride salt dissolved in water). With the two methods, nanoparticles seem spherical. The mean hydrodynamic diameters in volume were close, 280 ± 17 and 316 ± 21 nm for particles formulated by single and double emulsification methods, respectively.

In order to quantify the amount of DOX encapsulated in PLGA nanoparticles, we dissolved them in DMSO and measured the absorption of the solution at 480 nm. The results of DOX encapsulation levels are shown in Table 1. These formulations are not optimized in terms of loading but performed to evaluate the difference in entrapment efficiency of the two methods of formulation. PLGA nanoparticles prepared by SE exhibited better entrapment efficiency than those formulated by DE. The EE values obtained for SE nanoparticles were similar to the EE values previously reported for nanoparticles formulated from DOX–PLGA conjugate [9] and higher than those obtained using nanoprecipitation method (Nemati et al. [10]). These results confirm the assumption that the increased affinity of the neutral DOX form to the organic phase should reduce the loss of the drug in the external aqueous phase during the emulsification step.

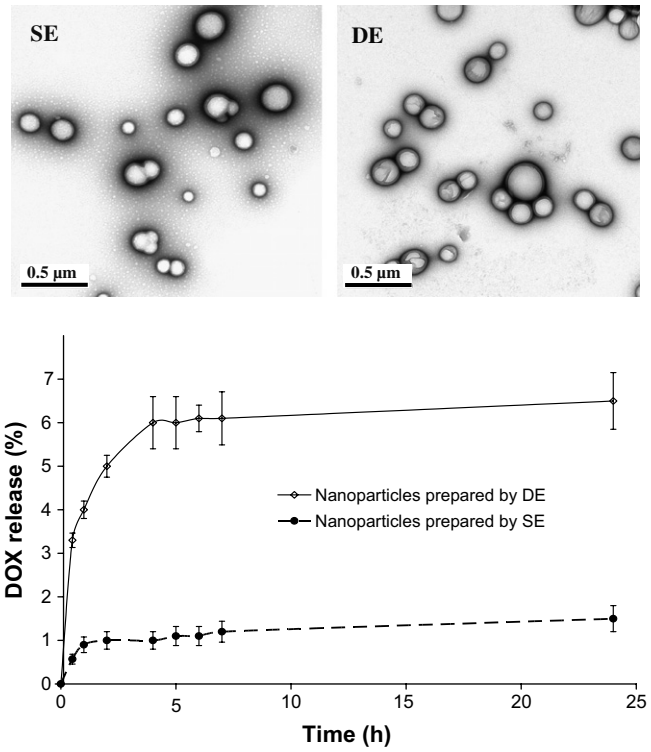

Fig. 2. Top: Transmission electron microscopic images of DOX–PLGA nanoparticles prepared according to single emulsion (SE) and double emulsion (DE) methods. Bottom: DOX release kinetics from the nanoparticles prepared by SE and DE methods (particle concentration 27 and 38.5 g/L, respectively, to introduce equivalent DOX concentration of 0.25 g/L). Release medium: phosphate buffer, pH 7.4 at 37 °C.

3.3. Kinetics of DOX release from the nanoparticles

Fig. 2 shows the release profile of DOX from nanoparticles prepared by DE and SE into phosphate buffer pH 7.4 at 37 °C. The nanoparticle amount employed was chosen to introduce 500 μg of DOX. For both kinds of nanoparticles, the drug release follows a two-phase kinetics. In the first phase, the amount of DOX released increases rapidly (burst effect). The second phase corresponds to a pseudo steady state for which the release rate is constant and much slower. One can interpret the first phase as characteristic of the DOX diffusion through the superficial layers of the polymeric particle. The second, much slower phase may correspond to the drug release due to DOX diffusion from the inner polymer matrix.

One can see that after 4 h, the DE particles released about 6 times more drug than SE particles. This could indicate that the superficial layers of nanoparticles formulated by SE are less enriched in DOX than those formulated by DE, or a difference in porosity between the two categories of particles. Over the following 20 h, both SE and DE particles released nearly the same additional quantity of DOX. Over a period of 24 h, only 1.5% or 6.5% were liberated by DE and SE particles, respectively. These release profiles seem similar to those obtained from nanoparticles formulated with DOX–PLGA conjugates (molecular weight of

Table 1
Characteristics of doxorubicin-loaded nanoparticles

Method of preparation	Initial amount of polymer (mg)	Initial amount of DOX (mg)	Theoretical DOX loading (%, w/w)	Experimental DOX loading (%, w/w)	Entrapment efficiency (%, w/w)
Simple emulsion	100	1.00	0.97	0.92 ± 0.06	95 ± 6
Double emulsion	100	1.00	0.97	0.65 ± 0.10	67 ± 10

10,000) [9]. After longer periods of time (several weeks), the expected hydrolysis of the polymer should contribute to the release of the remaining DOX. Thus, these SE and DE particles appear to be indicated for controlled release on an extended time scale. Once accumulated in the tumor by magnetic control of a shorter duration, the particles would have to remain immobilized at the site of the tumor by interaction with cancer cells. In this purpose, specific target molecules could be attached to the particle surface. A release of 100% of the DOX upon 24–48 h seems to be more compatible with application of magnetic targeting. Faster DOX release is not envisaged since this could be responsible for the lack of efficacy of nanoparticles to overcome multidrug resistance phenomenon as suggested by Némati et al. [10].

The release rate could be increased using shorter PLGA polymer (i.e. $\overline{M}_w$ below 40–75 kDa) or by decreasing the PLGA concentration in the MC solution. This would also lead to a higher DOX content if the DOX concentration in the MC solution is kept the same. In the literature, the usual i.v. administered dose of DOX in rats is 2–4 mg/kg/day. For a dose in nanoparticles of 100–200 mg/kg, the DOX loading must be at least of 2% (w/w). This might be reached by decreasing the PLGA concentration in the MC solution.

4. Conclusions

These results demonstrate that the control of doxorubicin polarity permits us to realize DOX–PLGA nanoparticle formulation according to SE protocol that implies both polymer and DOX to be in organic phase. We achieved an effective extraction of DOX into methylene chloride solvent by adjusting the pH of the initial aqueous phase to 8.6, where the neutral form of DOX predominates. In addition to being simpler and more economic, the SE protocol provides a nearly 1.4-fold higher loading efficiency than DE approach. On the other side, DOX release from PLGA nanoparticles was remarkably slower with SE than DE samples.

Further studies we continue in our group should allow additional optimization of these protocols with respect to DOX loading rates and kinetics, for instance, by using polymer of lower molecular weight and by varying the polymer/drug ratio. In addition to DOX, ultra-small iron oxide nanoparticles (~10 nm) are being incorporated into the PLGA particles for magnetic drug targeting. In near future, we will evaluate these DOX–PLGA nanoformulations on cancer cells in culture, namely in terms of intracellular drug concentration and cytotoxicity.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejpb.2007.02.016.

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