On the Interaction of Doxorubicin with Oleate Ions: Fluorescence Spectroscopy and Liquid–Liquid Extraction Study

Emilie MUNNIER,[a,b,c] Frédéric TEWES,[a,b] Simone COHEN-JONATHAN,[a,b] Claude LINASSIER,[c] Laurence DOUZIECH-EYROLLES,[a,b] Hervé MARCHAIS,[a,b] Martin SOUCÉ,[a,b] Katel HERVÉ,[a,b] Pierre DUBOIS,[a,b] and Igor CHOURPA*[,a,b]

[a] *Université François-Rabelais, Faculté de Pharmacie, "Focalisation magnétique d'agents anticancéreux"; Tours, F–37200 France:* [b] *IFR 135 "Imagerie Fonctionnelle"; Tours, F–37000 France: and* [c] *CHRU Bretonneau, Service d'Oncologie Médicale; Tours, F–37000 France.* Received January 15, 2007; accepted March 31, 2007

Increase of lipophilicity of cationic doxorubicin (DOX) by its association with a fatty acid ion is of interest for pharmaceutical formulations and could have an impact on the drug delivery into cancer cells. On the basis of spectroscopic analysis of intrinsic DOX fluorescence, this study provides an experimental evidence of DOX–oleate interactions as function of ion/drug molar ratio (*R*) and pH. An electrostatic attraction to oleates is dominant for the cationic form of DOX (pH 6.5) and a hydrophobic interaction is characteristic of the molecular form of DOX (pH 8.6). A high content of sodium oleate vesicles ([oleate]≥0.2 mM, *R*≥20) limits the electrostatic and hydrophobic interactions at pH 6.5 while favoring the hydrophobic interactions at pH 8.6. The influence of these interactions on the lipophilicity of the cationic form of DOX is analyzed by measuring the apparent partition coefficient (aqueous buffer pH 6.5/methylene chloride). The results show a lipophilicity gain for the cationic form of DOX in presence of 10 : 1 ion/drug molar ratio, while no lipophilicity increase is observed at 50 : 1 molar ratio.

Key words doxorubicin; oleate; fluorescence spectroscopy

Doxorubicin (DOX) is an antineoplastic agent of the anthracycline family used as a first-line treatment in numerous neoplastic diseases, particularly in breast cancer.[1)] The progression of tumor cell resistance, in particular multidrug resistance (MDR), is one of the main factors which limit its employment.[2)] MDR is generally associated with a decreased intracellular accumulation of drug related to a membrane glycoprotein, the P-gp that contributes to a rapid expelling of the drug from the cell.[3)] An interesting way to overcome this active efflux would be to support the drug accumulation into the cell, in particular by increasing its capacity to cross lipidic membranes. One can act upon the membrane fluidity or act upon the hydrophilic/lipophilic properties of the drug.

DOX molecule (see structure on Fig. 1) is considered hydrophilic at physiological pH because it presents a positive charge on the sugar moiety. The more lipophilic molecular form of DOX is dominant at moderately alkaline pHs, between 8.2 and 9.5, the pK_a values of the amine and the phenol in position 11 respectively.[4)] The hydrophilicity of DOX is also depending on the drug concentration, since beyond a concentration of 30 μM DOX molecules are prone to form less hydrophilic dimmers (stacking effect) which precipitate in aqueous solution. The increase of the pH, *i.e.* of the neutral form percentage, is in favor of the stacking phenomenon.[5,6)]

It has been recently shown that lipophilicity of cationic drugs[7,8)] can be increased by association with an anion, namely with a long chain ion of a fatty acid.[9,10)] Nevertheless, no study is devoted to the molecular interaction between the drug and the ion. On the other hand, combined use of an anticancer drug, paclitaxel, with different fatty acids, has been shown to enhance mortality of cancer cells *in vitro*.[11)] It has not yet been established whether this gain in efficacy is dependent on an assembly between the drug and the fatty acid ion.

Association DOX–fatty acid could be more lipophilic than DOX alone, and enter more efficiently into the cell. It could also facilitate drug encapsulation into pharmaceutical formulations based on hydrophobic polymers or liposomes.[7,9)] Oleic acid is a good candidate for such an association within a feasibility study: it is the simplest of the ω-9 fatty acid family (Fig. 1), and possesses a long hydrophobic chain that could contribute to a gain of lipophilicity of the association. Moreover, oleates are non-toxic and the behavior of oleic acid and its ion (oleate, OA^-) in aqueous solution is rather well studied.[12—15)]

Fluorescence spectroscopy has been successfully used to study interactions between DOX and its surrounding, for instance when the drug intercalates DNA[16,17)] or penetrates within membrane models or liposomal drug carriers.[18,19)] Indeed, the dihydroanthraquinone moiety of DOX is a well known fluorophore excitable with visible light (absorption maxima at 480—500 nm). Fluorescence spectroscopy

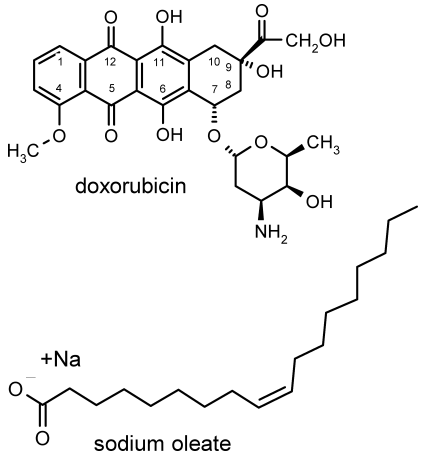

Fig. 1. Structural Formulae of DOX Molecule and Oleate Ion

* To whom correspondence should be addressed. e-mail: chourpa@univ-tours.fr

is particularly appropriate to our experimental conditions: i) in view of the experimental reasons described above, DOX–oleate molecular interactions should be studied at relatively low concentrations of both molecules; ii) much sensibility is required to highlight even weak interactions between DOX and OA^-.

The present study is an attempt to assess the ability of DOX to interact with OA^- in aqueous solution. We describe a fluorescence approach for quantitative and qualitative analysis of two forms of DOX, molecular and cationic, in presence of increasing concentrations of sodium oleate in buffered aqueous medium. Furthermore, we investigate the influence of such interactions on the lipophilicity of the more hydrophilic form of the drug, the cationic one: we measure its apparent partition coefficient between a pH 6.5 buffered aqueous solution and an organic solvent in presence and in absence of sodium oleate.

Experimental

Chemical Reagents DOX hydrochloride salt was purchased from DAKOTA Pharm (France). Methylene chloride (MC) of reagent grade and oleic acid were purchased from Sigma-Aldrich (France). Di-sodium tetraborate decahydrate and sodium citrate were purchased from Merck (France). NaOH and HCl commercial solutions were obtained from Carlo Erba (France).

Solutions All solutions were prepared with fresh de-ionized water. Sodium oleate (OA^-) solutions were prepared by adding oleic acid to a NaOH solution, resulting in concentrations of sodium oleate of 2 mM and 20 mM. We realized solutions of 20 mM of doxorubicin in 2 different buffers: borate buffer pH 8.6 and citrate buffer pH 6.5.

Titration of Doxorubicin by Sodium Oleate Increasing quantities of sodium oleate were added to a constant 10 μM DOX concentration in buffer solution. Sodium oleate concentrations went from 0 to 5 mM, knowing that the known critical micellar concentration is 0.9—2.4 mM in a 0.1 M NaCl water solution at pH 7.5 at 25 °C.[20] We added respectively the appropriate volumes of DOX solution, buffer and sodium oleate solutions to obtain the desired concentrations in a 1 ml sample. The samples were prepared progressively, homogenized 10 s by vortex and immediately analyzed by fluorescence spectrometry. We checked the adding of the larger of alkaline sodium oleate solution did not change the pH of the final sample. Each experiment was realized at least twice. The samples are described referring to the OA^-/DOX molar ratio (R).

Liquid–Liquid Extraction of Doxorubicin Three different extractions were performed at pH 6.5. The aqueous phase was constituted of 10 ml of buffer in which DOX and sodium oleate were dissolved. DOX concentration was 10 μM and sodium oleate concentration varied to reach molar ratios [OA^-]/[DOX] of 0, 10 and 50. An extraction was performed at pH 8.6 without any addition of oleate ions. The organic phase was constituted of 20 ml of MC. Phases have been agitated together for 4 h in a 25 °C water bath. DOX concentration was measured in the aqueous phase by spectrofluorimetry before and after extraction. Each experiment was realized at least twice. All results will be expressed as apparent partition coefficient (P), defined as the ratio of DOX concentration in the organic phase divided by DOX concentration remaining in the aqueous phase after extraction.

Fluorescence Spectroscopy Analysis A Hitachi F-4500 fluorescence spectrophotometer was used for fluorescence measurements. The excitation wavelength was 500 nm, with a 5 nm slit. The field of emission wavelengths went from 530 to 700 nm, with a 10 nm slit, to be able to visualize entire spectrum even if a shift in emission wavelengths occurred. Obtained spectra were fitted (least square method) with either Gaussian profiles or characteristic experimental spectra using Labspec 4.0 software (Jobin Yvon Horiba, Villeneuve d'Ascq, France). The Gaussian positions were established from derivative spectra and kept constant upon the fitting.

Results and Discussions

Fluorescence Spectroscopy Data The two forms of DOX, cationic and molecular, were generated in aqueous solutions at pH 6.5 and 8.6, *i.e.* both sides of pK_a 8.2 of the amine moiety (described above). At these two pHs, the carboxylic moiety of oleic acid (pK_a=4.5) is deprotonated and carries a negative charge (oleate ion, OA^-). Further lowering of pH would decrease the hydrosolubility of oleic acid, whereas further increase of the pH would favor the deprotonation of the phenol group in position 11 (pK_a=9.5) of DOX thus diminishing neutral drug fraction.

In presence of OA^-, both intensity and shape of DOX fluorescence spectra are affected (detailed below), and the nature of the changes is dependent on the ion/drug molar ratio (R). The spectra shape indicates an interference of a light scattering that becomes significant over certain critical R value: R=20 at pH 6.5 and R=100 at pH 8.6 (oleate concentrations of 0.2 and 1 mM, respectively). Hereafter, the diffusion signal is subtracted prior to the fluorescence analysis. Nevertheless, the light scattering renders impossible to interpret the fluorescence shape for the samples with R>30 at pH 6.5 while at pH 8.6 the spectra are still interpretable up to R=200.

At both pHs, the increase of oleate excess up to R~25—30 is accompanied by a progressive decrease in DOX fluorescence intensity (data not shown). The intensity decrease is particularly strong at pH 6.5: with R close to 25, the intensity goes down to *ca.* 50% of the initial value and then remains nearly constant. At pH 8.6, the intensity decreases moderately (down to 90% when R=30), then it is increased to 100% with R=50 and to 130% with R=200.

Analysis of the shape of DOX fluorescence spectra indicates that at each pH, two fluorescence forms, significantly different from the free drug spectrum appear successively during titration. These spectra, characteristic of different kinds of DOX–oleate interaction, are represented on Fig. 2 and will be analyzed in details below.

Two types of interaction between DOX and oleates are observable at pH 6.5 (their fluorescence spectra are hereafter

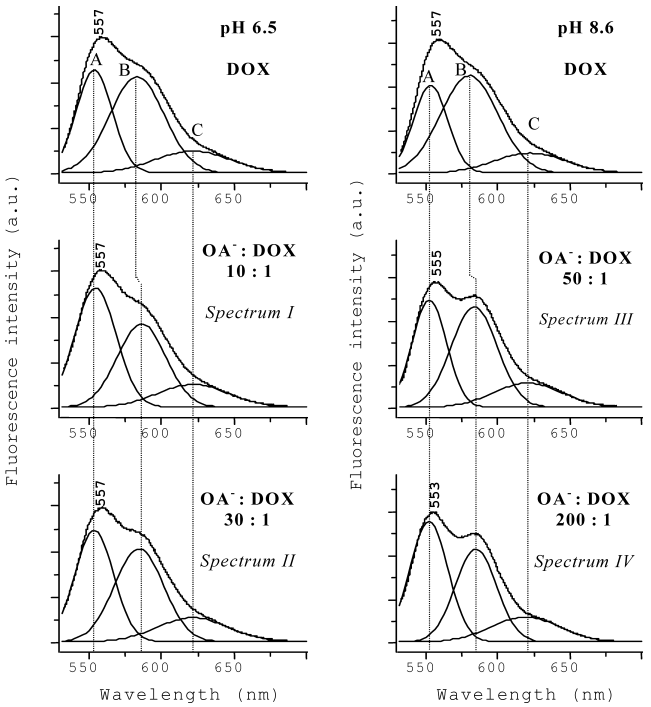

Fig. 2. Characteristic Fluorescence Spectra of Doxorubicin Free and When in Presence of Different Excess of Sodium Oleate

Numeric data of the Gaussian profiles are provided in Table 1.

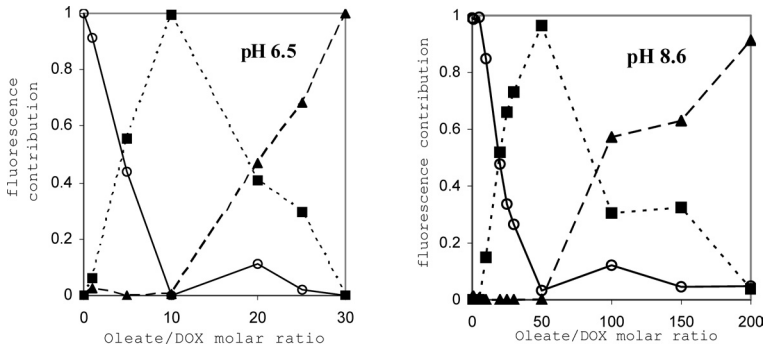

Fig. 3. Evolution of Doxorubicin Fluorescence Spectra as Function of Oleate/Drug Molar Ratio

For each pH, the experimental spectra are described through their deconvolution into three characteristic spectra (shown in Fig. 2): free drug (○) and two types of drug–oleate associations observed at lower (■, spectrum I or III) and higher (▲, spectrum II or IV) excess of sodium oleate.

Table 1. Numeric Data of Gaussian Profiles from Fig. 2

	pH 6.5			pH 8.6		
	DOX	OA^- : DOX 10 : 1	OA^- : DOX 30 : 1	DOX	OA^- : DOX 50 : 1	OA^- : DOX 200 : 1
Band A						
Position, nm	552	553	552	552	551	551
Width, nm	25	29	27	24	24	27
Area, %[a)]	34.3	45.3	39.7	29.4	36.0	43.8
Band B						
Position, nm	582	585	584	579	583	584
Width, nm	38	34	34	40	32	29
Area, %	50.0	39.0	44.0	56.2	47.0	38.8
Band C						
Position, nm	621	621	621	622	620	618
Width, nm	52	50	50	50	48	48
Area, %	15.7	15.7	16.3	14.4	17.0	17.4

a) Relative band contribution to the fluorescence spectrum normalized to 100%.

named spectrum I and spectrum II) and two other ones are noticeable for pH 8.6 (spectrum III and spectrum IV). Figure 3 shows how the relative fluorescence contribution of the characteristic spectra I—IV to the experimental spectra evolves as function of oleate/drug molar ratio. As seen from the profiles in Fig. 3, the spectrum of free DOX is completely converted into spectrum I (pH 6.5) at $R_I=10$ and into spectrum III (pH 8.6), at $R_{III}=50$. Further R increase leads to the spectra conversion into spectrum II (pH 6.5) at $R_{II}=30$ or into spectrum IV (pH 8.6) at $R_{IV}=200$. It is noteworthy that, simultaneously with the appearance of spectrum II or spectrum IV, we observe a rise of light scattering. Interestingly, at low content of spectrum II ($R=20$) or spectrum IV ($R=100$), there is a punctual reappearance of a moderate fraction of free DOX fluorescence.

The spectral shapes of the fluorescence characteristic of free DOX, as well as spectra I—IV, are shown in Fig. 2. At pH 6.5, the differences between free DOX fluorescence and spectra I and II are minor: only small variation of the shoulder intensity can be noticed and the position of the main emission maximum remains at 557 nm. In contrast, at pH 8.6 the spectral changes in presence of oleates are more obvious: in addition to the shoulder changes, there is a slight hypsochrom shift of the emission maximum, from 557 to 553 nm.

To analyze these spectral changes in more details, we performed the curve fitting with theoretical Gaussian profiles, intended to accentuate the vibrational fine structure of the emission band.[18] As seen in Fig. 2, all the spectra are well fitted with 3 Gaussian components situated at *ca.* 552 nm (band A), 582 nm (band B) and 620 nm (band C). The graphs in Fig. 2 and the numeric fitting results in Table 1 indicate that the most significant changes concern bands A and B whereas band C is not significantly affected by the adding of OA^-.

At both pHs, oleate ions induce a redistribution of the emission intensity in favor of shorter wavelengths that is traduced by the increase of the relative contribution of band A over that of band B. Band A undergoes some widening for spectra I, II and IV, but not for spectrum III. In presence of oleates, band B is reduced in both intensity and width, the narrowing being particularly pronounced at pH 8.6. Furthermore, in all the spectra of DOX–OA^-, band B is red-shifted of 3—5 nm, this is also more clearly observed at pH 8.6 because of the increased band resolution.

Let us compare now the DOX–OA^- associations at the two pH conditions. At pH 6.5, the ratio of the A/B bands area is higher in the fluorescence spectrum I ($R=10$) than in spectrum II ($R=30$). Between these two spectra, the difference is mainly due to the relative intensity and not to the width of bands B and A (Table 1). At pH 8.6, on going from the spectrum III ($R=50$) to spectrum IV ($R=200$), the ratio of the

A/B bands area increases. With $R=200$, band B becomes not only less intense but also more narrow, than with $R=50$.

Liquid–Liquid Extraction of DOX in Presence of Oleate Ions The effect of the DOX–OA^- association on the drug lipophilicity can be evaluated by measuring DOX partition coefficient between aqueous phase and organic phase.

We proceeded to the extraction of DOX from a 10 μM aqueous solution pH 6.5 to an organic phase constituted of MC. We performed these extractions for free DOX and for two oleate/drug molar ratios that correspond to two characteristic situations distinguished by the spectral analysis: spectrum I ($R=10$) and spectrum II ($R=30$).

At pH 6.5 and in the absence of oleate ions, the partition coefficient of DOX between MC and aqueous buffer is very low: *ca.* 0.5. The adding of the concentration of OA^- characteristic of spectrum I ($R=10$) permits to increase significantly the partition coefficient as it is multiplied by two and reaches 1. When a larger quantity of OA^- is added (spectrum II), the lipophilicity benefits are cancelled and the partition coefficient is equivalent to the one without oleates (0.3). For comparison, we realized free DOX extraction at pH 8.6 in absence of OA^-. The obtained apparent coefficient of DOX between aqueous phase pH 8.6 and MC is 3.4.

The results described above show a different behavior of doxorubicin in presence of oleate ions in function of two determining parameters: the pH and the ion/drug molar ratio. We compare DOX–oleate interactions in aqueous solutions at pH 6.5 and 8.6 that favor respectively cationic and molecular forms of DOX. These pHs below and above the pK_a of the amine group of DOX allow us to determine the influence of the charge on the interaction between DOX and OA^-.

Fluorescence Interpretation At pH 6.5, the interaction is mainly indicated by an important quenching of the drug fluorescence in presence of OA^- (static quenching). At this pH, DOX carries a positively charged NH_3^+ group that favors electrostatic interaction with the negative charge on the hydrophilic head of an oleate molecule[6] (Fig. 4a). This electrostatic interaction has almost no effect on the fluorescence spectral shape because it implies a group distant from the fluorophore (aromatic moiety) of DOX.

In addition to electrostatic attraction, DOX molecules are exposed to certain hydrophobic interaction with oleate chains, which is responsible for the minor but objectively present changes in the spectral shape. This kind of interaction is namely indicated by the increase of the A/B band area ratio and moderate narrowing of band B in both spectra I and II. It is noticeable that oleate-induced spectral changes are more moderate for spectrum II ($R=30$) than for spectrum I ($R=10$). We suggest that the oleate–doxorubicin interactions could be hindered by the formation of oleate-consuming aggregates or vesicles[15] that we observe through the light scattering. According to Edwards *et al.*, the apparent pK_a of fatty acid is shifted to considerably higher values when it is incorporated into a micelle or an aggregate.[12] Thus, the number of sites available for the DOX–oleate electrostatic interaction should be additionally decreased due to a partial reprotonation of assembled oleates. This is confirmed by the free drug reappearance at $R=20$ (Fig. 3), when the light diffusion becomes significant relatively to the DOX fluorescence. At $R=30$, there is no free DOX fluorescence and the spectrum II should correspond to a situation when DOX is electrostatically bound to a surface of oleate/oleic acid aggregates or vesicles (Fig. 4b). In this case, the drug fluorophore should remain in the aqueous phase,[21] minimizing the hydrophobic interaction.

At pH 8.6, the cationic form of DOX is minor compared to molecular form (about 15—20% *versus* 75—80%, respectively[22]). Therefore, the hydrophobic interactions are mainly responsible for the major spectral change in the drug fluorescence, *i.e.* the blue shift of the overall emission maximum. The increase of the A/B band ratio and the narrowing and shift of band B are much more significant at pH 8.6 than at pH 6.5. In a more general manner, the changes observed in the spectra in presence of oleates are consistent with a decrease of the dielectric constant of the molecular environment.[18] On the other hand, this interaction is observed with much higher oleate excess: one must reach the ratio $R=50$ to observe a change in the fluorescence spectra. With $R>50$, the fluorescence quenching is rapidly supplanted by an intensity increase. Fluorescence increase has been reported upon embedding of the fluorescent molecule into lipidic vesicles or liposomes.[6] Taken together, all these spectral observations at pH 8.6 indicate that DOX molecular form could avoid the aqueous medium by partial insertion into aggregated oleates or by complete embedding into oleate vesicles (Figs. 4c, d, respectively).

Lipophilicity of DOX–Oleate Assemblies The electrostatic interactions DOX–oleate at pH 6.5 results in the formation of an uncharged association DOX–oleate (Fig. 4a), currently called ion pair.[7—9] This association should then be prone to quit the aqueous environment to rejoin the organic phase. This suggestion is confirmed by the increase of the

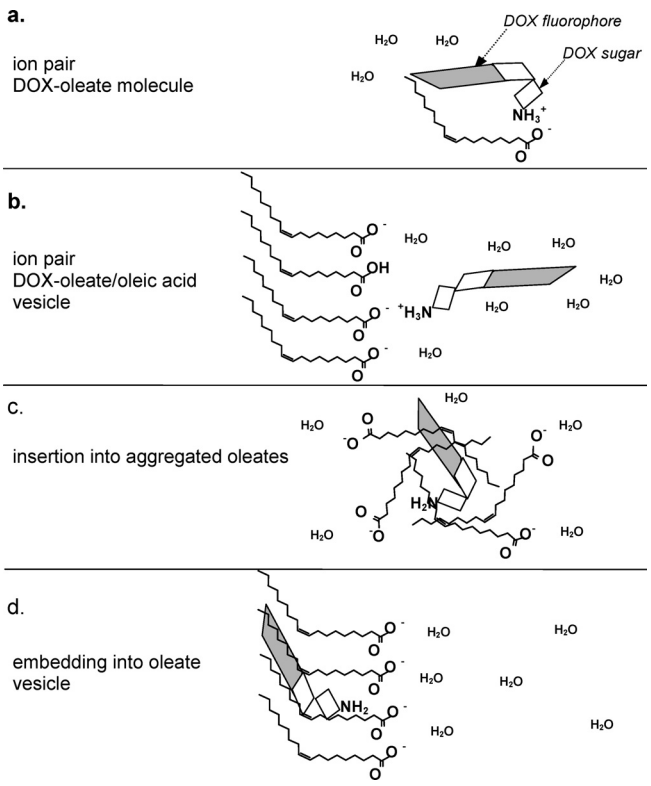

Fig. 4. Attempt of Representation of DOX–Oleate Interaction Modes Deduced from the Drug Fluorescence Spectra I—IV

DOX partition coefficient in presence of a small excess of OA^- ($R=10$). The model proposed for the higher oleate excess ($R=30$) is in agreement with the extraction data: the increased presence of vesicles (Fig. 4b) seems to hinder the DOX extraction from the aqueous to the organic phase.

The gain in lipophilicity of DOX described here is quite moderate and does not permit to achieve the partition coefficient of the molecular form of the drug. The gain could be improved by additional protocol optimization, namely by using other fatty acid ions. Nevertheless, this gain is obtained i) by association of the drug with non-toxic ion, ii) at acidic pH conditions that allows to prevent DOX from degradation, known to be a serious problem at basic pH.[23] In view of this, the DOX–oleate association could have interesting perspectives.

Conclusions and Perspectives

The results of the present study provide an experimental evidence of DOX–oleate interactions as function of pH and molar ratio and show that it is possible to act upon lipophilicity of DOX by associating it with fatty acid ions.

The improvement of doxorubicin lipophilicity is of interest for pharmaceutical formulations based on hydrophobic constituents, since it could help to increase the formulation efficiency and to reduce the drug loss.[22] Once released from the formulation, the lipophilic association of DOX with a non-toxic fatty acid ion could be advantageous in terms of drug transport into cancer cells. Furthermore, one can imagine to associate doxorubicin with one of ω-3 polyunsaturated fatty acids known to enhance the chemosensitivity of cancer cells *in vitro*.[24,25]

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