

Reversion of multidrug resistance using nanoparticles in vitro: influence of the nature of the polymer

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Abstract

Previous studies have already shown that polyalkylcyanoacrylate nanoparticles were able to overcome multidrug resistance in vitro. The study described in this paper investigated other types of nanoparticles, made of poly (lactic acid) PLA, poly (lactic-co-glycolic acid) PLGA and alginate, in order to determine if overcoming of resistance could be achieved with any nanoparticles independently of the nature of the polymer. In a first step, PLA and PLGA nanoparticles loaded with doxorubicin have been formulated, and the cytotoxicity of each preparation has been evaluated against P388 sensitive and resistant cells. Only polyalkylcyanoacrylate nanoparticles were effective: doxorubicin release experiments allowed to incriminate too rapid doxorubicin release in the case of PLA, PLGA and alginate nanoparticles to explain their lack of efficacy to overcome MDR.

Keywords: Nanoparticles; Doxorubicin; Polyalkylcyanoacrylate; Alginate; Polylactide; P388; Multidrug resistance; Reversion

1. Introduction

Chemoresistance of cancer cells, either primary or secondary to treatment, is a frequent cause of treatment failure. The simultaneous resistance to multiple, structurally dissimilar drugs, poses a particularly difficult problem to the oncologist. Such resistance called 'multidrug resistance'

(MDR) is explained by a decreased accumulation of the drug in tumor cells (Bradley et al., 1988). A membrane glycoprotein which acts as an energy-dependent pump, designed as P-glycoprotein (P-gp), may be responsible for accelerated drug efflux (Roninson, 1987). Many strategies have been developed to overcome multidrug resistance. For example, much work has been done using chemosensitizing compounds such as verapamil (Tsumo et al., 1982) or cyclosporin A (Twenty-

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man, 1992) which allow restoration of the sensitivity of tumor cells *in vitro*. However, clinical applications of these agents have met important limitations, especially because of toxicity and side effects observed at concentrations needed to overcome multidrug resistance (Ozals et al., 1987). Instead of a direct inhibition of Pgp, another way of bypassing resistance is to protect the drug against the pumping action of Pgp, by means of chemical modifications of drug structure (Tapiero et al., 1986), or by association with colloidal carriers. Liposomes have been widely used for this purpose, with varying results. When a significant effect was observed, overcoming resistance was explained by phospholipids (either acting as direct Pgp inhibitors (Thierry et al., 1992) or modifying cell membrane fluidity (Warren et al., 1992)) rather than by a modified intracellular trafficking of the drug. Polyalkylcyanoacrylate nanoparticles have also been used successfully on a wide range of tumor cell lines (Cuvier et al., 1992; Bennis et al., 1993; Némati et al., 1994). However, one has no idea if the nature of the polymer constituting the nanoparticles is an important factor or not leading to a bypass of the resistance. For this purpose, we investigated the potential of two other types of polymer, differing from polyalkylcyanoacrylate (PACA) by their biodegradation rate or by their hydrophobicity. Hence, polylactic acid and a copolymer with glycolic acid were first chosen because they degrade at two different rates, both much more slowly than PACA. Secondly, we investigated alginate nanoparticles, recently developed as a hydrophilic carrier supposed to avoid opsonisation *in vivo* (Rajaonarivony et al., 1993). This paper concerns the growth inhibition studies performed on the P388 cell line with these nanoparticles loaded with doxorubicin. The results obtained are discussed in terms of doxorubicin release.

2. Materials and methods

2.1. Materials

2.1.1. Cell lines and culture

P388 (sensitive cell line) and P388/ADR (resistant subline) were kindly supplied by the

Institut de Recherche sur le Cancer (IRSC, France). The resistant subline overexpresses the P-glycoprotein. Suspension cultures were grown in RPMI 1640 medium (Gibco, France) supplemented with 10% foetal calf serum (Gibco, France), penicillin-streptomycin 1‰ (Eurobio, France) and 2-mercaptoethanol 3 nM (Sigma, USA).

2.1.2. Chemicals

Isohexylcyanoacrylate (IHCA) was kindly supplied by SOPAR (Sopar, Belgium). Free doxorubicin (Adriblastin®) (Dox) was obtained from Farmitalia (Farmitalia, Carlo Erba, Italy). Isobutylcyanoacrylate (IBCA) was obtained from Sigma (Sigma, USA). Sodium alginate was obtained from Sigma (France). Poly (lactic acid) 50 and poly (lactic-co-glycolic acid) 25/50 were supplied by Birmingham (USA) and Boehringer Ingelheim (Germany), respectively. All other chemicals were obtained commercially and were of analytical grade.

2.2. Methods

2.2.1. Preparation of polyalkylcyanoacrylate (PACA) nanoparticles

Nanoparticles were obtained by emulsion polymerization of a cyanoacrylic monomer as previously described by Couvreur et al. (1979). Typically, 66.5 mg of monomer (IBCA or IHCA) were dropped under mechanical stirring into 6.5 ml of a medium containing 5 mg of doxorubicin, 5% glucose, 1% dextran 70, and 0.5% citric acid. After 6 h (IBCA) or 20 h (IHCA) polymerization, nanoparticles were obtained, and lyophilised (Couvreur et al., 1979). Unloaded nanoparticles of IBCA or IHCA (NS PIBCA and NS PIHCA, respectively), were prepared in the same way, no drug being present in the polymerization medium.

2.2.2. Preparation of alginate (ALG) nanoparticles

Alginate nanoparticles were prepared according to the method of Rajaonarivony et al. (1993). Briefly, 4.5 ml distilled water were added to 5 ml

of a solution of sodium alginate (0.12%). In the case of doxorubicin loaded nanoparticles, 25 μ l of a solution of doxorubicin (10 mg/ml) were also added. Then, 0.5 ml of calcium chloride 0.2% were introduced dropwise. Two minutes later, 2 ml of a solution of polylysine 0.05% were added dropwise. The suspension was then energetically stirred for 5 min, then the agitation was maintained gently for 24 h.

2.2.3. Preparation of poly(lactic acid) (PLA) and poly (lactic-co-glycolic acid) (PLGA) nanoparticles

PLA and PLGA nanoparticles were prepared according to the method of Fessi et al. (1988); 10 ml of an organic solution containing 100 mg of polymer were mixed with 20 ml of an aqueous phase containing Pluronic F68. The nature of the organic solvent, the nature of the aqueous phase (water or buffer) and the concentration of the surfactant have been varied. The only solvent we found able to dissolve both PLA and PLGA was acetone. Nanoparticles formed instantaneously. The organic solvent was then removed by evaporation under vacuum at 40°C, until a final volume of 10 ml was reached. When doxorubicin-loaded nanoparticles were prepared, doxorubicin was added in the aqueous phase (preliminary experiments had shown that the encapsulation rate did not vary when doxorubicin was introduced in the organic phase). The effect of the concentration of the drug has been studied.

2.2.4. Assay of the doxorubicin associated to nanoparticles

The nanoparticles have been assayed for the amount of non-adsorbed doxorubicin, following ultracentrifugation (30 000 rev./min for 1 h, L7-75 Ultracentrifuge Beckman, type 70,1 Rotor Beckman). The supernatants were analysed by HPLC. Briefly, samples were injected automatically (Waters 712 Wisp, Waters, France) onto a C18 column (SFCC, France). The mobile phase was composed of methanol/sodium acetate 0.01 M/acetic acid (70:30:1.3), and used at a flow rate of 1.5 ml/min (Waters 600E, Waters, France). Doxorubicin, and daunorubicin, used as internal standard, were detected by fluorimetry (Waters 470,

Waters, France), with an excitation wavelength of 470 nm and an emission wavelength of 550 nm. Peak areas were integrated using a Waters 412 data module (Waters, France).

2.2.5. In vitro release of doxorubicin associated with nanoparticles

Drug loaded nanoparticles were incubated in the cell culture medium at a doxorubicin concentration varying from 540 to 2000 ng/ml at 37°C in a humidified atmosphere containing 5% CO₂. Free doxorubicin was incubated under the same conditions as a control. Samples were taken after various incubation periods and nanoparticles were separated from the released drug by ultrafiltration on a polysulfone membrane (300 000 Da molecular weight cut off; Millipore, France) at 2000 \times g for 5 min in the case of PACA and PLA, PLGA nanoparticles. Free doxorubicin were separated from nanoparticles by ultracentrifugation at 100 000 \times g for 1 h in the case of alginate nanoparticles.

The filtrates or the supernatants were assayed by HPLC. Samples were injected automatically (Waters 712 Wisp; Waters, France) onto a C18 column (SFCC, France). The mobile phase was composed of methanol/0.01 M sodium acetate/acetic acid (65:35:1.3, by vol.) and used at a flow rate of 1.5 ml/min. Doxorubicin was detected spectrofluorimetrically (Waters 470; Waters, France) at an excitation wavelength of 470 nm and an emission wavelength of 550 nm. Peak areas were integrated using a Waters 412 data module (Waters, France).

2.2.6. Drug treatment and cytotoxicity

Cytotoxicity of free doxorubicin and nanoparticles with doxorubicin was determined by measuring the inhibition of cell growth using the tetrazolium dye (MTT) assay. Samples were free doxorubicin (Dox), doxorubicin-loaded nanoparticles (NS-Dox), or unloaded nanoparticles (NS). All treatments were performed on cells in log-phase growth seeded in 96-well plates at a cellular density of 5×10^3 cells/well and in a humidified atmosphere (5% CO₂). Typically, cells were incubated for 48 h with various concentrations of drug. At the end of the experiment, the MTT

assay was performed. The survival rate of the treated cells was calculated in regard to untreated cultures. The IC_{50} (inhibition concentration) values were evaluated as the drug concentration providing a decrease of 50% of cell survival.

3. Results

Doxorubicin loaded PACA and ALG nanoparticles were already described, and the operative conditions well defined. On the other hand, the encapsulation of doxorubicin in PLA50 or PLGA25/50 nanoparticles needed to be optimized. Hence, two main parameters were investigated: the pH of the aqueous phase and the doxorubicin concentration in the aqueous phase.

3.1. Preparation of doxorubicin loaded PLA and PLGA nanoparticles

3.1.1. Influence of the pH of the aqueous phase on the encapsulation rate

As can be observed in Fig. 1, the amount of doxorubicin encapsulated in the PLGA25/50 nanoparticles increased when the pH of the aqueous phase increased. The slight discontinuity of the curve around pH 5.5 could be attributed to the buffer change (acetate from pH 4 to 5.5 and citrate from 6 to 7). No effect of the concentration of the stabilizer (Pluronic®) was seen for the

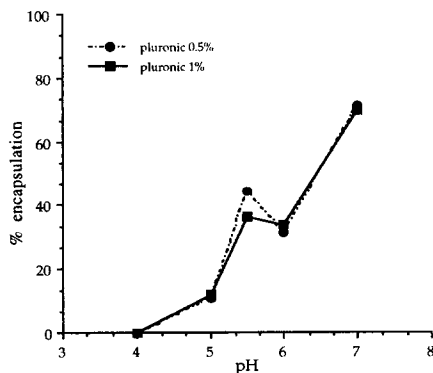


Fig. 1. Encapsulation ratio of doxorubicin in PLGA25/50 nanoparticles versus pH, and for two different concentrations of Pluronic® in the aqueous phase.

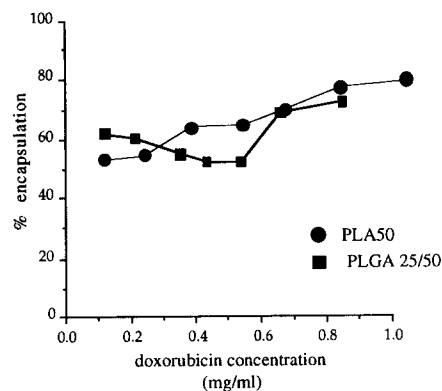


Fig. 2. Encapsulation ratio of doxorubicin in PLA50 and PLGA25/50 nanoparticles versus doxorubicin concentration in the aqueous medium.

two concentrations used. The experiment was not performed at pH above 7.4, because of doxorubicin degradation. Very similar results were obtained in the case of PLA50 nanoparticles.

3.1.2. Influence of the drug concentration in the aqueous medium

Nanoparticles were prepared using a Pluronic® concentration of 1% and a pH of 7.0. For both PLA50 and PLGA25/50, the percentage of drug encapsulation could be slightly increased when the initial concentration of doxorubicin was increased (Fig. 2), up to an optimal concentration of 0.8 mg/ml.

Thus, for the growth inhibition studies described below, nanoparticles were prepared with a Pluronic® concentration of 1% at a pH of 7.0 and with a doxorubicin concentration of 0.8 mg/ml, which corresponded to a polymer/drug ratio of 12, quite close to the polymer/drug ratio used in the case of PACA nanoparticles (13.3).

The main characteristics of the different nanoparticles investigated on P388 cells are reported in Table 1.

3.2. Growth inhibition studies

3.2.1. PACA nanoparticles

As reported elsewhere with slightly different experimental conditions (Nemati et al., 1994), doxorubicin loaded PIHCA nanoparticles showed

Table 1

Physico-chemical characteristics of PACA, ALG and PLA or PLGA nanoparticles investigated in vitro on tumor cell line

		Size ^a (nm)	Polymer/dox ratio	% Encapsulation
PIBCA	Non-loaded	236	—	—
	Dox-loaded	230	13.3	87
PIHCA	Non-loaded	150	—	—
	Dox-loaded	300	13.3	85
ALG	Non-loaded	292	—	—
	Dox-loaded	425	24	87
PLA50	Non-loaded	205	—	—
	Dox-loaded	270	12	80
PLGA25/50	Non-loaded	246	—	—
	Dox-loaded	274	12	72

^aSize measured by laser scattering (Nanosizer N4MD®, Coultronics, France).

the same cytotoxic effect as free doxorubicin in the case of the P388 sensitive cell line (Fig. 3a). On the other hand, in the case of resistant cells (Fig. 3b), doxorubicin loaded nanoparticles greatly increased the cytotoxicity of the drug: the IC₅₀ decreased from a value of around 1500 ng/ml in the case of the free drug, to a value of 100 ng/ml in the case of nanoparticles.

Non-loaded PIHCA nanoparticles themselves had an IC₅₀ of 600 ng/ml, expressed in the equivalent dose of doxorubicin (7800 ng/ml polymer), and this value was the same for sensitive and resistant cells.

Very similar results could be obtained for PIBCA nanoparticles (data not shown).

3.2.2. ALG nanoparticles

As shown on Fig. 4, doxorubicin loaded onto alginate nanoparticles were not more cytotoxic than the free drug, whether the cell line was sensitive or resistant. Drug-unloaded nanoparticles were not cytotoxic at the investigated doses.

3.2.3. PLA50 and PLGA25/50 nanoparticles

In the case of sensitive P388 cells (Fig. 5) as well as resistant P388/ADR cells (Fig. 6), both doxorubicin-loaded PLA50 and doxorubicin-loaded PLGA25/50 nanoparticles exerted the same cytotoxic effect as the free drug. Drug-unloaded nanoparticles were not cytotoxic at the investigated doses.

3.3. In vitro release studies

3.3.1. PACA nanoparticles

As shown in Fig. 7, doxorubicin release was much faster in the case of PIBCA nanoparticles than in the case of PIHCA nanoparticles. Release from PIBCA started immediately and was complete within 1 h. In the case of PIHCA, release displayed a biphasic character. No significant release occurred within the first hour, and complete release was only observed after 400 min.

3.3.2. ALG nanoparticles

The release of doxorubicin was investigated with two different drug concentrations: 540 and 1900 ng/ml. The higher the concentration, the lower the percentage of doxorubicin released (Fig. 8): 65% released in the case of a concentration of 1900 ng/ml versus 90% in the case of 540 ng/ml. However, in both cases, the maximum level of doxorubicin released was already reached within a few minutes (< 15 min) and did not change over 6 h.

3.3.3. PLA50 and PLGA25/50 nanoparticles

The results reported in Fig. 9 clearly show that the whole doxorubicin content released immediately from both PLA50 and PLGA25/50 nanoparticles as soon as they were dispersed in the cell culture medium. Doxorubicin could even be released by a simple effect of dilution of the samples in water (Fig. 10). For a dilution ratio of 10, 80%

of the drug has already been released into the suspension medium. It must be noticed that for the purpose of growth inhibition studies, the samples needed to be diluted at least 100 times.

4. Discussion

The goal of the present study was to investigate different polymeric nanoparticles as potential drug carriers able to bypass multidrug resistance. The preparation of polyalkylcyanoacrylate nanoparticles (Couvreux et al., 1979) and alginate nanoparticles (Rajaonarivony et al., 1993) were already described with doxorubicin. In both cases, doxorubicin could be encapsulated with a high efficiency (85% and more). On the other hand, the

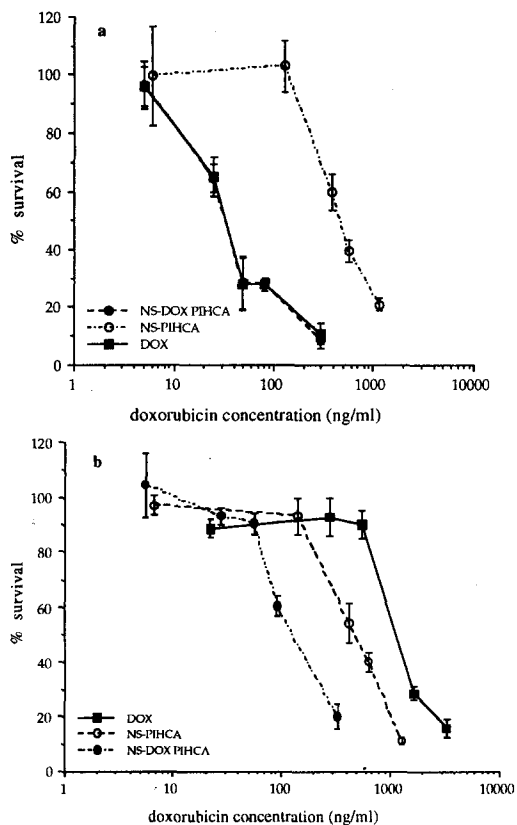


Fig. 3. Cytotoxic effect of doxorubicin and PIHCA nanoparticles, loaded with doxorubicin (NS-Dox PIHCA) or not (NS PIHCA) incubated with P388 sensitive cells (a) and P388/ADR resistant cells (b).

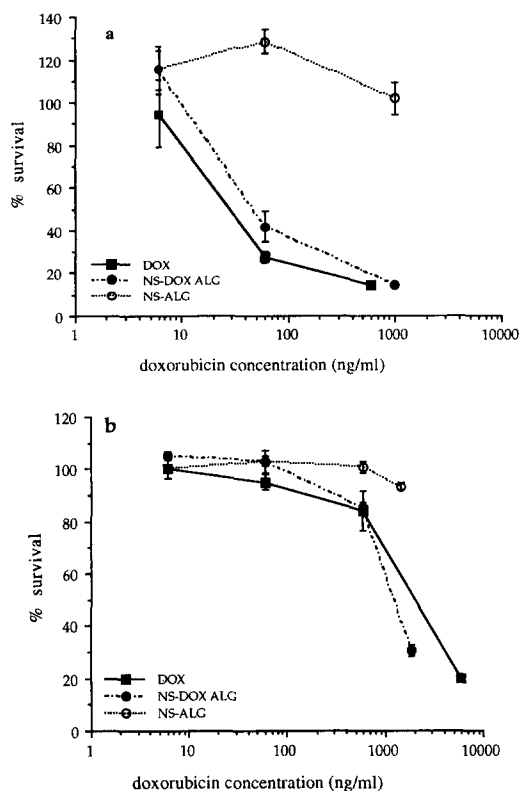


Fig. 4. Cytotoxic effect of doxorubicin and alginate nanoparticles, loaded with doxorubicin (NS-Dox ALG) or not (NS ALG) incubated with P388 sensitive cells (a) and P388/ADR resistant cells (b).

conditions for doxorubicin attachment to PLA or PLGA nanoparticles were never investigated. Thus, the aim of the first part of the work described here was to optimize this preparation.

It was found that the stabilizer had no effect on the encapsulation rate of doxorubicin, which indicated that Pluronic® did not participate in the partitioning of doxorubicin between the aqueous and the polymer phases. On the other hand, a marked effect of pH was noticed. This has to be related to the pK_a of the drug (8.2 according to Vigeveni and Williamson (1980)). Indeed, when the pH increased from 4 to 7.4, the proportion of the non-ionized form of doxorubicin increased from zero to 90%. In contrast to the ionized form which preferentially stayed in the aqueous medium, the non-ionized drug partitioned to a much greater extent in the hydrophobic polymer

phase, leading in turn to an increase in the encapsulation efficiency. Lastly, the slight improvement of the encapsulation observed when the drug concentration was increased suggested that the encapsulation did not respond to a single partitioning effect, nor to a simple monolayered adsorption. Indeed, the encapsulation efficiency would have been constant in the first case, and would have more likely decreased with increasing concentrations in the second. It is well known that doxorubicin has a tendency to aggregate (Martin, 1980). Our observations could then be interpreted as a multilayer adsorption process. The very fast release of doxorubicin from both PLA and PLGA nanoparticles, as simply resulting from the dilution of the solid phase, is in agreement with the multilayered adsorption model of possibly aggregated drug molecules onto the particle surfaces.

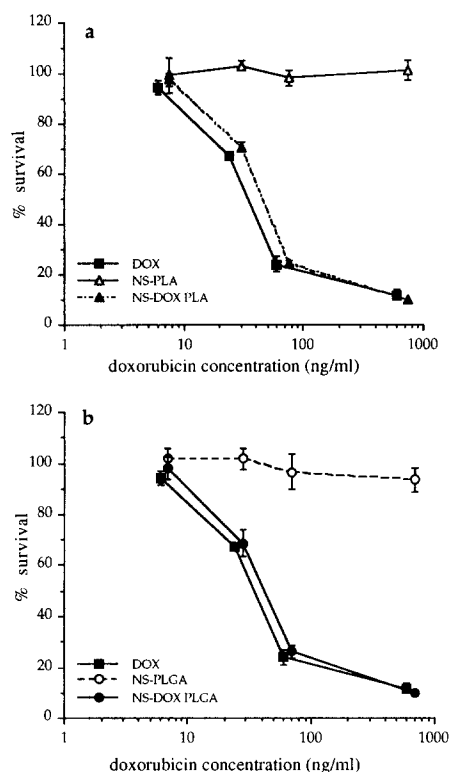


Fig. 5. Cytotoxic effect of doxorubicin and PLA nanoparticles (a) or PLGA nanoparticles (b) incubated with P388 sensitive cells.

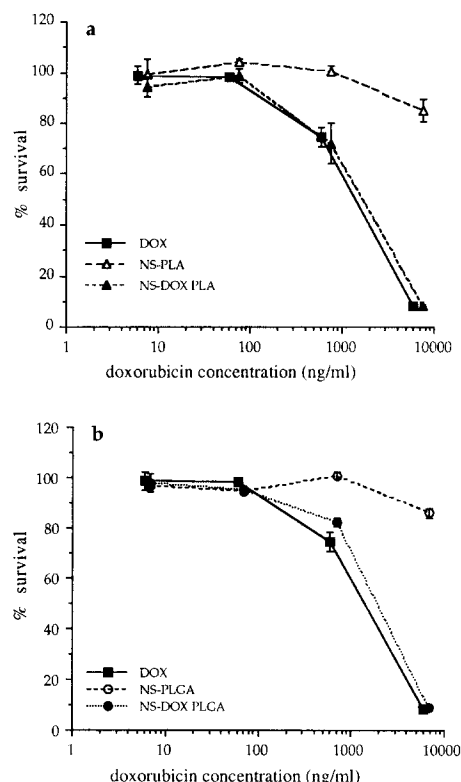


Fig. 6. Cytotoxic effect of doxorubicin and PLA nanoparticles (a) or PLGA nanoparticles (b) incubated with P388/ADR resistant cells.

Concerning the overcoming of the MDR resistance in vitro, neither alginate nanoparticles nor PLA or PLGA nanoparticles were able to increase doxorubicin cytotoxicity towards P388/ADR re-

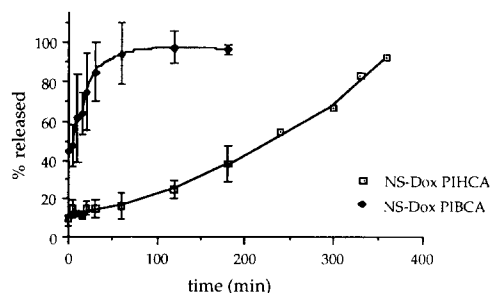


Fig. 7. In vitro release of doxorubicin in culture medium at 37°C from PIBCA and PIHCA nanoparticles (doxorubicin concentration = 2000 ng/ml).

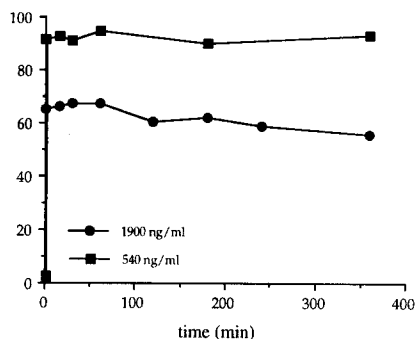


Fig. 8. In vitro release of doxorubicin in culture medium at 37°C from alginate nanoparticles (doxorubicin concentrations = 540 and 1900 ng/ml).

sistant cells. On the other hand, doxorubicin-loaded PACA nanoparticles (both PIBCA and PIHCA) allowed the IC_{50} to decrease to a value close to that measured with the sensitive cells. In a previous work, we showed that PACA nanoparticles were not endocytosed by P388/ADR cells (Colin de Verdière et al., 1994) which was quite relevant as regards the poor endocytic capacity of these tumor cells (Sehested et al., 1986). To explain the cytotoxic effect of PACA nanoparticles, it was hypothesized that nanoparticles adsorbed to cell surface and released doxorubicin close to the membrane, leading to a high local drug concentration gradient. A similar mechanism was proposed by Cavallaro et al. (1994) with antibi-

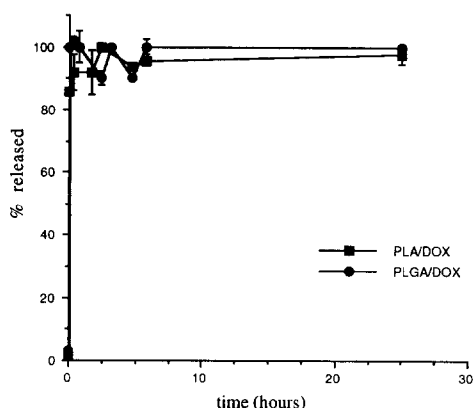


Fig. 9. In vitro release of doxorubicin in culture medium at 37°C from PLA50 and PLGA25/50 nanoparticles (doxorubicin concentration = 1000 ng/ml).

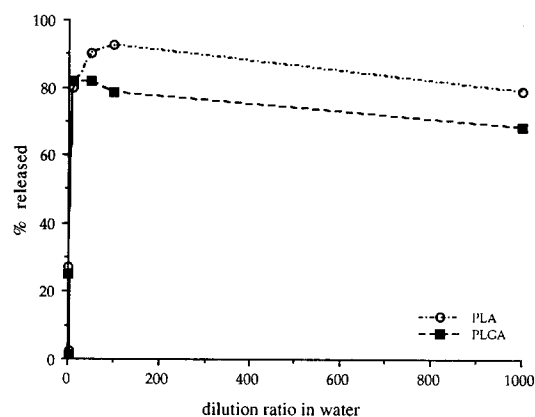


Fig. 10. In vitro release of doxorubicin from PLA50 and PLGA25/50 nanoparticles as a consequence of the dilution in water (dilution ratio = volume of water/volume of colloidal suspension).

otics loaded onto polyethylcyanoacrylate nanoparticles. The fact that PLA, PLGA and alginate nanoparticles, loaded with doxorubicin, exerted the same cytotoxicity as the free drug itself, suggested that (i) either these nanoparticles did not interact with cells, releasing the drug directly into the cell culture medium, or (ii) the nanoparticles were able to adsorb to the cells' surface, but released doxorubicin too fast, before the adsorption process could take place. The first hypothesis could have been particularly justified in the case of alginate nanoparticles. Indeed, these particles are hydrophilic (Rajaonarivony et al., 1993) and may have limited interaction with cells. Nevertheless, release experiments clearly demonstrated that, at the concentrations used in the culture medium, both PLA/PLGA and alginate nanoparticles released doxorubicin immediately. Therefore, doxorubicin became diluted in the culture medium just as the free drug, and could not, in that way, have a greater cytotoxic effect. In contrast, PACA nanoparticles released doxorubicin more slowly, especially in the case of PIHCA, within a period of time compatible with the adsorption of nanoparticles to cells.

The fast release of doxorubicin from PLA or PLGA nanoparticles could be explained by the fact that the drug was simply adsorbed onto the surface of the polymer which is not the case for

PACA nanoparticles for which most of the drug was found to be dispersed throughout the whole polymer matrix (Henry-Toulmé et al., 1994). In the case of alginate nanoparticles, it is likely that doxorubicin was encapsulated in the whole nanoparticle matrix too, but not through hydrophobic binding as with PACA nanoparticles: doxorubicin could interact with the negatively charged alginate carboxylic groups through electrostatic binding. Doxorubicin could then be released by a simple ion exchange mechanism. Release experiments suggested that cations present at a given concentration in the cell culture medium were effectively able to exchange with doxorubicin: the percentage of drug released then increased when drug initial concentration was lowered, since competition with cations of the culture medium occurred.

From all these experiments, the idea could emerge that overcoming MDR resistance in vitro may only be achieved using nanoparticles in which doxorubicin is deeply incorporated, which prevents an immediate drug release. Thus, it is supposed that doxorubicin-loaded PACA nanoparticles prepared in such a way that doxorubicin is only adsorbed onto the particles' surface (by simply mixing polymer particles and free drug), would not be able to bypass multidrug resistance. In fact, the situation is not so simple since we found in a previous work (Nemati et al., 1994) that the single mixture of PACA nanoparticles and free doxorubicin was more cytotoxic than the free drug (the IC_{50} was intermediate between that of non-loaded nanoparticles and that of doxorubicin-loaded nanoparticles). Consequently, PACA itself (or at least its degradation products) seems also to participate in the enhancement of the cytotoxicity of doxorubicin.

Our conclusion is that the kinetics of release of the encapsulated drug is an important parameter in overcoming the resistance in vitro, and other important parameters are the nature of the polymer itself and its ability to modify drug cytotoxicity.

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