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Tamoxifen encapsulation within polyethylene glycol-coated nanospheres. A new antiestrogen formulation

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Abstract

When dealing with solid tumors in vivo, pegylated long-circulating carrier systems show, after intravenous administration, an attractive extravasation profile with an enhanced localization in the tumoral interstitium. These systems could be of help for the delivery of cancer fighting drugs, such as Tamoxifen, a well known antiestrogen used in breast cancer therapy that possesses an extended biodistribution in vivo. This work aimed at encapsulating Tamoxifen in long-circulating poly(MePEGcyanoacrylate-co-hexadecylcyanoacrylate) 1:4 nanospheres. Tamoxifen-loaded poly(MePEGcyanoacrylate-co-hexadecylcyanoacrylate) nanospheres were successfully synthesized and characterized in terms of hydrophilicity/hydrophobicity by a model made up from near infrared spectra using principal component analysis. Zeta potential, drug loading, encapsulation efficiency, as well as biological effect, in vitro release and nanospheres integrity were also investigated. Even though near infrared spectroscopy could not detect Tamoxifen, it revealed that Pluronic F68 was associated with the pegylated nanospheres. HPLC measurements demonstrated that Tamoxifen was encapsulated in the pegylated nanospheres following a partition equilibrium between the polymeric and the aqueous phases. The Tamoxifen encapsulated in the nanospheres still showed a transcription inhibitory activity in ex vivo experiments. However, zeta potential and in vitro release suggested that Tamoxifen was essentially localized at the nanoparticles surface, resulting in an important and immediate drug release.

Keywords: Nanospheres; Near infrared hydrophilic/hydrophobic model; poly(MePEGcyanoacrylate-co-hexadecylcyanoacrylate); Tamoxifen; Transcription inhibitory activity

1. Introduction

Oral administration of the nonsteroidal antiestrogen Tamoxifen (partial agonist/antagonist) is today the endocrine treatment of choice for se-

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lected patients regardless of the stage of their breast cancer (MacGregor and Jordan, 1998). Despite being quite effective, Tamoxifen can have harmful long term side effects such as the development of endometrial cancer, or an acquired Tamoxifen resistance leading to further tumor progression (MacGregor and Jordan, 1998). To overcome these undesirable side effects, one could encapsulate Tamoxifen in stealth® pegylated nanoparticles: once in the blood stream, such a sterically stabilized carrier shows a reduced uptake by the reticuloendothelial system (Storm et al., 1995). This results in a prolonged circulation time, which enhances the probability for the pegylated nanoparticles to diffuse across the hyperpermeable, leaky tumoral endothelium in the interstitium (Seymour, 1992; Gabizon, 1995). Such a delivery method could improve the selectivity of the treatment by increasing the ratio of Tamoxifen absorbed by the tumor to the Tamoxifen absorbed by other tissues, such as the endometrium.

In aqueous solution, the copolymer poly(-MePEGcyanoacrylate-co-hexadecylcyanoacrylate) 1:4 (poly(PEGCA-co-HDCA) was found to form stealth® nanoparticles with a covalent MePEG grafting (Peracchia et al., 1998; Brigger et al., 2000). This eliminates the possibility of a rapid MePEG coating desorption/displacement by blood components in vivo and a consecutive blood clearance of the carrier by the reticuloendothelial system (Storm et al., 1995). Moreover, this copolymer is characterized by a rapid biodegradation profile, which suggests that after extravasation of the poly(PEGCA-co-HDCA) nanoparticles in solid tumors, the leakage of any encapsulated drug in the tumoral interstitium should be faster than in the case of nanoparticles with a slower biodegradation, like PEG-poly(lactic acid) nanoparticles for example (Peracchia et al., 1999).

Thus the aim of this work was to encapsulate Tamoxifen within poly(PEGCA-co-HDCA) 1:4 nanoparticles, to determine the physico-chemical characteristics and to evaluate this drug-loaded carrier system ex-vivo.

2. Methods

Poly(PEGCA-co-HDCA) nanoparticles were prepared by the nanoprecipitation technique: 40.0 mg of poly(PEGCA-co-HDCA) 1:4 and 200 μg of Tamoxifen (corresponding to a concentration of 25 μg/ml in the final suspension, unless otherwise specified) were dissolved in acetone (4.0 ml). Nanosphere formation was obtained by pouring this organic phase in an aqueous phase (8.0 ml) containing 0.5% w/w of Pluronic F68 (BASF, Germany) (unless otherwise specified), a non-toxic tensio-active agent used to reduce the particle size to 110 nm. The acetone was then removed under reduced pressure. The resulting colloidal suspension was filtered through a sintered glass membrane (Millex® AP 20, Millipore) and finally washed by ultracentrifugation (145 000 × g, 1 h, 4°C) to remove the nonencapsulated Tamoxifen, the Pluronic F68 and the fraction of the copolymer still soluble in the aqueous solution (about 50% w/w) (Brigger et al., 2000).

Tamoxifen-loaded and unloaded nanospheres, with or without Pluronic F68, were first analyzed by our hydrophilic/hydrophobic model computed by principal component analysis (PCA) of near infrared (NIR) spectra, described elsewhere in detail (Brigger et al., 2000). Briefly, PCA is a data treatment used to extract the chemical and physical information contained in NIR spectra (Chaminade et al., 1998). The objective of PCA is to model the variability of each class of compounds and to represent each class as a unique cluster made up of the spectral data (Chaminade et al., 1998). The principle of PCA calculation is to find new variables (the loadings) replacing wavelengths to describe the spectra. The loadings are thus independent variables, not correlated between each other, describing the wavelength space on the basis of the main cause of variance in spectra. In our model, based on the analysis of various compounds involved in the preparation of nanoparticles, it appeared that loading two separated the different products according to their hydrophilic/hydrophobic balance (Brigger et al., 2000).

The different nanospheres formulations were then characterized by zeta potential, in KCl 1 mM (Zeta Sizer, Malvern, France).

The encapsulation efficiency was evaluated by HPLC, after filtration of nanospheres samples (Waters corporation, 1996). Practically, the amount of Tamoxifen encapsulated into the nanospheres was determined by subtracting the amount of Tamoxifen left in the dispersion medium after ultracentrifugation (145 000 × *g*, 1 h, 4°C) of the nanospheres, from the total amount measured in the colloidal suspension after dissolving the nanospheres with acetonitrile (1:1). The drug loading was also determined by HPLC (Waters corporation, 1996), after extraction of Tamoxifen contained in the freeze-dried nanospheres.

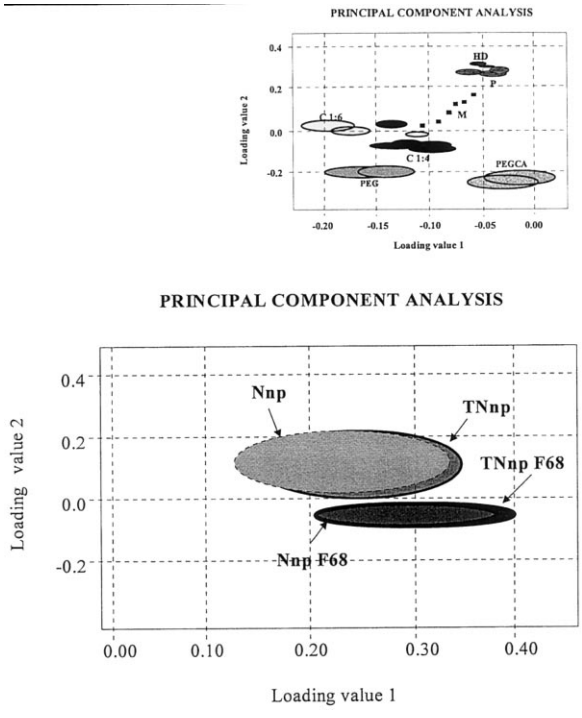

Fig. 1. Hydrophilic/hydrophobic model (top) (Brigger et al., 2000) with principal component analysis performed on near infrared spectra of the following compounds (bottom): Nnp: poly(PEGCA-co-HDCA) nanospheres without Pluronic F68 (data taken from Brigger et al., 2000), Nnp F68: poly(PEGCA-co-HDCA) nanospheres with Pluronic F68, TNnp: Tamoxifen-loaded poly(PEGCA-co-HDCA) nanospheres without Pluronic F68, and TNnp F68: Tamoxifen-loaded poly(PEGCA-co-HDCA) nanospheres with Pluronic F68.

Ex vivo experiments were carried out on cultured MELN-cells (human MCF-7 breast cancer cells stably transfected with an ERE-tk-LUC construct) as described by Demirpence et al., 1993. The transcription inhibitory activity (luciferase activity) was measured in the presence of estradiol 10^{-10} M, for intact and dissolved Tamoxifen-loaded nanospheres, after a 20 h cell exposure.

The in vitro release study was performed using ^{3}H-Tamoxifen (85 Ci/mmol). Briefly, the Tamoxifen-loaded nanospheres suspension (1 μCi/ml) was diluted at 1/10 in a DMEM cell culture medium supplemented with 10% fetal calf serum (FCS) and maintained at 37°C, similar to the ex vivo experiment. At predetermined time intervals, the medium was ultracentrifuged (145 000 × *g*, 1 h, 4°C) and the ^{3}H-Tamoxifen released in the supernatant was determined by liquid scintillation counting.

Finally, turbidity measurements were also performed (Perkin Elmer UV/VIS spectrophotometer), using the same ratio of nanospheres suspension to FCS as in the in vitro release study.

3. Results and discussion

Fig. 1 presents the PCA performed on NIR spectra of unloaded poly(PEGCA-co-HDCA) nanospheres prepared in the presence (Nnp F68) or in the absence (Nnp) of Pluronic F68. With this hydrophilic/hydrophobic model, nanoparticles scored between -0.1 and 0.2 on loading two, in the amphiphilic region. Moreover, we obtained different clusters and a more hydrophilic character for poly(PEGCA-co-HDCA) nanospheres with Pluronic F68 in the formulation. This clearly shows chemical and/or structural differences between these nanospheres, probably because Pluronic F68 remained in the colloids by entanglement with poly(PEGCA-co-HDCA) chains. Our model obtained by NIR followed by PCA is thus an efficient technique for surface characterization of nanoparticles. Unfortunately, Tamoxifen-loaded nanospheres, with or without Pluronic F68 (TNnp F68 and TNnp, respectively), scored

Table 1
Zeta potential measurements for Tamoxifen-loaded and unloaded nanospheres formulations

Formulations			
Nanoparticles	Pluronic F68 (% w/w in aqueous phase)	Tamoxifen (initial concentration; μg/ml)	Zeta potential (standard deviation) [mV]
Poly(PEGCA-co-HDCA)	0.0	0	−27 (±0.5)
Poly(PEGCA-co-HDCA)	0.5	0	−12 (±0.9)
Poly(PEGCA-co-HDCA)	0.5	10	−6 (±0.0)
Poly(PEGCA-co-HDCA)	0.5	25	−4 (±0.5)
Poly(PEGCA-co-HDCA)	0.5	37.5	+2 (±0.2)
Poly(PEGCA-co-HDCA)	0.5	50	+3 (±0.2)

at the same values as the corresponding unloaded nanospheres. This shows that NIR spectroscopy is not sensitive enough to differentiate between unloaded nanoparticles and nanoparticles loaded with small quantities of drug (0.46% w/w in our case) (Fig. 1).

The results of the zeta potential measurements are presented in Table 1. Poly(PEGCA-co-HDCA) nanospheres displayed a surface charge of −27 mV, which is consistent with previously published data (Brigger et al., 2000). Nanospheres prepared in the presence of Pluronic F68 displayed an increased zeta potential, compared to those obtained without Pluronic (−12 vs. −27 mV). This result confirms the association of Pluronic F68 at the surface of the already pegylated particles, as evidenced by our NIR–PCA investigations.

On the other hand, the surface charge of poly(PEGCA-co-HDCA) nanospheres with Pluronic F68 increased linearly with Tamoxifen loading and reached +3 mV for a Tamoxifen concentration of 50 μg/ml (Table 1). This was presumably due to both the positive charge borne by the Tamoxifen amino group and the localization of Tamoxifen at the surface of the nanoparticles. Moreover, when the concentration of Tamoxifen was higher than 25 μg/ml, the lack of electrostatic repulsion between the particles resulted in the floculation of the suspensions followed by the crystallization of Tamoxifen in the aqueous phase. Consequently, the highest possible Tamoxifen concentration in the formulation was set to 25 μg/ml.

Fig. 2 shows the drug loading and encapsulation efficiency as a function of the total Tamoxifen concentration measured by HPLC. Encapsulation efficiency was constant and equal to 80% (±10%) regardless of the Tamoxifen concentration, whereas drug loading increased linearly with increasing Tamoxifen concentration. This suggests that the association of Tamoxifen with poly(PEGCA-co-HDCA) nanospheres fol-

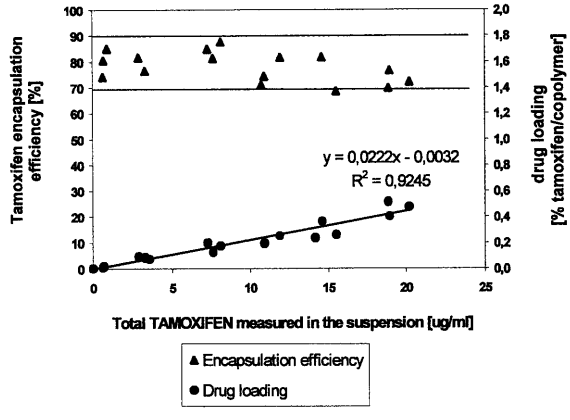

Fig. 2. Tamoxifen encapsulation efficiency and drug loading in poly(PEGCA-co-HDCA) nanospheres.

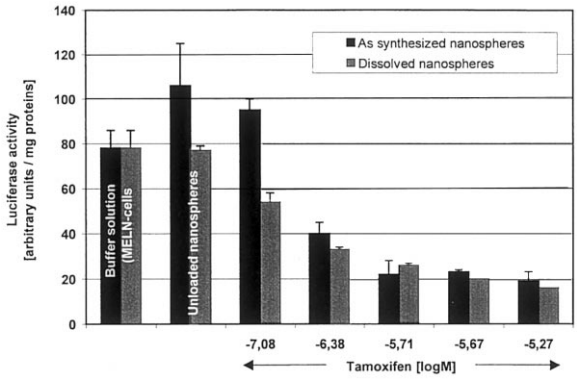

Fig. 3. Transcription inhibitory activity on MELN-cells for intact and dissolved Tamoxifen-loaded nanospheres (and the controls), in the presence of 10^{-10} M estradiol, as a function of their total Tamoxifen concentration (determined by HPLC).

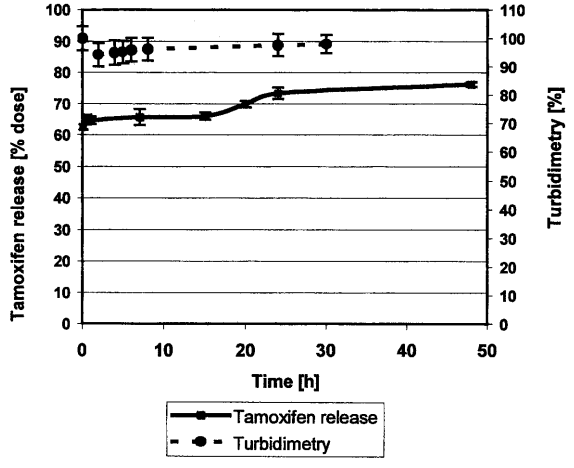

Fig. 4. Cumulative ^{3}H-Tamoxifen release and turbidity measurements of poly(PEGCA-co-HDCA) nanospheres in a DMEM cell culture medium supplemented with 10% FCS.

lowed a simple partition between the copolymer phase and the dispersion medium phase. At the highest possible Tamoxifen concentration, we obtained a drug loading of 0.46% ($\pm$0.04) and an encapsulation efficiency of 73% ($\pm$4), corresponding to a final drug concentration of 38 μM.

As shown in Fig. 3, representing the ex vivo experiment, the dissolved unloaded nanospheres solution did not modify the estradiol-induced transcription level ($\approx$LUC activity: 78 units/mg of proteins). On the other hand, the activity of the unloaded nanospheres was surprisingly about 36% higher ($\approx$106 LUC/mg of proteins). Furthermore, whether dissolved or not, the Tamoxifen-loaded nanospheres displayed inhibition of the estradiol-mediated Luciferase gene expression. However, their Luciferase activity were not significantly different, except for low Tamoxifen concentrations, where the drug is known to have an estrogen agonistic activity (MacGregor and Jordan, 1998). A quick release of Tamoxifen or an early degradation of the nanospheres could result in the equivalent activities witnessed at higher concentrations.

Finally, Tamoxifen was released in the cell culture medium with an important burst effect (release of 62% within a few minutes), as shown in Fig. 4. A complementary turbidimetry analysis (Fig. 4) revealed that this burst effect did not result from a rapid biodegradation of the nanospheres. This result confirms that most of the active compound was located on the surface of the nanospheres.

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