

In vitro enhancement of anticancer activity of paclitaxel by a Cremophor free cyclodextrin-based nanosponge formulation

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Abstract A severe limitation for cancer therapy is the poor water solubility of many important therapeutic anticancer drugs. The development of novel delivery systems is therefore currently ongoing. We propose the use of β -cyclodextrin based nanosponges to deliver paclitaxel as an alternative to classical formulation in Cremophor EL. They are solid nanoparticles with mean diameter lower than 500 nm and spherical shape. Nanosponges show a safe profile being non-hemolytic and non cytotoxic. Nanosponges dissolved and encapsulated paclitaxel up to 2 mg/ml. The paclitaxel-loaded nanosponges formed a water stable colloidal system avoiding the recrystallization of paclitaxel. The in vitro release studies showed an almost complete release in 2 h without initial burst effect. Our study demonstrates that delivery of paclitaxel via nanosponges increased the amount of paclitaxel entering cancer cells and lowers paclitaxel IC₅₀, therefore enhancing its pharmacological effect. β -Cyclodextrin based nanosponges can therefore be considered an alternative system to solubilize and deliver the paclitaxel.

Keywords Anticancer agent · Drug delivery · Nanosponges · Paclitaxel · β -Cyclodextrin

Introduction

Chemotherapy is a complicated procedure whose success or failure is determined by many factors. Among others, the non-specificity of antineoplastic drugs is the cause of important toxicity, the most effective drugs being often highly toxic. Along with side effects properly due to anticancer molecule, often patients have to tolerate severe side effects due to formulation, since many important anticancer agents have poor water solubility and need to be dissolved in surfactants or in organic solvents. One significant example is represented by paclitaxel, a mitotic inhibitor used, among other, for the treatment of head and neck cancer, small and non-small cell lung cancer, and bladder cancer. Water solubility of paclitaxel is as low as 0.5 mg/l; the dosage form available for clinical administration requests the use of excipients consisting of Cremophor EL (polyethoxylated castor oil) and dehydrated alcohol. Besides extremely frequent severe anaphylactoid hypersensitivity reactions, it has been demonstrated that Cremophor EL causes side effects such as neurotoxicity, nephrotoxicity and cardiotoxicity [1]. Moreover, this formulation vehicle is associated with a number of pharmacological, pharmacokinetic and pharmaceutical concerns. Great efforts have been done in developing better formulations of paclitaxel by various approaches such as co-solvency, emulsification, micellization, liposome formation, non-liposomal lipid carriers (microspheres, nanocapsules, etc.) and many others [2–4]. A new formulation approved by the FDA by January 7, 2005 for the chemotherapy of recurrent metastatic breast cancer [5] is albumin-bound paclitaxel, with the commercial name of Abraxane [6, 7]. A very promising investigational new chemical entity is paclitaxel poliglumex, linking paclitaxel to a biodegradable polyglutamate polymer [8]. Currently,

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several clinical studies (Phase I and II) are ongoing mainly on ovarian carcinoma and non-small cell lung cancer [9–12]. In the meanwhile, research of new vehicles is still going on.

It has been reported that, by reacting cyclodextrins (cyclic oligosaccharides) [13] with suitable cross-linking reagents, a novel nanostructured material consisting of hyper-cross-linked cyclodextrins can be obtained, known as nanosponges [14, 15]. This new material shows interesting characteristics; in particular, cyclodextrin-based nanosponges are characterized by their marked capacity to encapsulate a great variety of substances that can be transported through aqueous media or from the opposite perspective, removed from contaminated water. Because of these properties, they could be a very efficient tool either to remove pollutants from water or to carry substances for biomedical applications. Thanks to their ability to complex molecules, nanosponges have been proposed as drug delivery systems [16]. The encapsulation of paclitaxel in nanosponges obtained by reacting cyclodextrins with diphenylcarbonate as cross-linker was studied [17]. Paclitaxel-loaded nanosponges showed a mean diameter of about 350 nm with a drug payload of 500 mg of paclitaxel/g of nanosponges. The pharmacokinetics of paclitaxel in nanosponges was evaluated after oral administration of the paclitaxel-loaded nanosponges in rats in comparison to commercial Taxol showing a three-fold increase of the drug oral bioavailability.

The objective of this study was to prepare and to compare, *in vitro*, the efficacy of a novel formulation consisting of paclitaxel carried in nanosponges, as an alternative to classical paclitaxel formulation in Cremophor EL, in terms of cytotoxicity against AT-84 [18], murine cells derived from a spontaneous oral squamous cell carcinoma, and the amount of drug entering those same cells. According to our results, β -cyclodextrin based nanosponges can be considered an alternative system without surfactant to solubilize and deliver the paclitaxel.

Materials and methods

Synthesis of nanosponges

β -Cyclodextrin nanosponges were synthesized following the procedure reported elsewhere [15]. Briefly, anhydrous β -cyclodextrin was dissolved in dry dimethyl sulfoxide (DMSO) and allowed to react with carbonyldiimidazole at 90 °C for 3 h. Once the reaction was complete, the solid was ground in a mortar, extensively washed with deionized water and ethanol and finally, Soxhlet-extracted with ethanol to remove possible by-products.

Synthesis of fluorescent nanosponges

Two-hundred milligrams of nanosponges was dispersed in 10 mL of dry dimethyl formamide and 20 mg of fluorescein isothiocyanate were added. The mixture was heated at 80 °C and allowed to react for 8 h. Once the reaction was over, the solid was filtered under vacuum and the filtrate was thoroughly washed with ethanol to completely remove unreacted fluorescein isothiocyanate. Finally, the obtained yellow powder was placed in a vacuum oven at 60 °C for 2 h.

Preparation of paclitaxel-loaded nanosponges

The preparation of paclitaxel-loaded nanosponges was carried out by the freeze drying method. Briefly, nanosponges and paclitaxel as powders were mixed in the ratio 4:1 (w/w) and then resultant mixture was suspended in distilled water. The suspension was stirred for 24 h and then filtered to remove any undissolved drug particles. The filtrate was then lyophilized to obtain the paclitaxel-loaded nanosponges as a powder. An amount of the product was stored in the solid state while a part was suspended in water or in DMSO for the biological experiments.

Characterization of nanosponges

Fourier transform infrared spectroscopy (FTIR) analyses of nanosponges were carried out on a Perkin-Elmer system 2000 spectrophotometer (Perkin-Elmer, CT, USA). The spectra were obtained using KBr pellets in the region from 4000 to 400 cm^{-1} .

Differential scanning calorimetry (DSC) was carried out by means of a Perkin Elmer DSC/7 differential scanning calorimeter (Perkin-Elmer, CT-USA) equipped with a TAC 7/DX instrument controller. The instrument was calibrated with indium for melting point and heat of fusion. A heating rate of 10°C/min was employed in the 25–300 °C temperature range. Standard aluminium sample pans (Perkin-Elmer) were used; an empty pan was used as reference standard. Analyses were performed in triplicate on 5 mg samples under nitrogen purge.

Ultraviolet analysis was carried out using a Du 730 spectrophotometer (Beckman Coulter) to confirm the interaction of paclitaxel with nanosponges. The spectra of paclitaxel and paclitaxel-loaded nanosponges were recorded in the range between 210 and 255 nm.

Morphology and particle size analyses

Nanosponges sizes and size distribution were determined by dynamic light scattering, using a 90 plus particle sizer instrument (Brookhaven Instruments Corporation,

Holtville, NY, USA), with MAS OPTION particle sizing software at the fixed angle of 90° and at a temperature of 25 °C. Nanosponges were diluted in physiological phosphate buffer (PBS, pH 7.4) prior to measurements. Each sample was analysed in triplicate.

Transmission electron microscopy (TEM) was employed to evaluate the particle shape and size. A Philips CM 10 transmission electron microscope was used and the particle size was measured using the NIH image software. Diluted nanosponge aqueous suspensions were sprayed on formvar-coated copper grid and air-dried before observation.

In vitro release of paclitaxel from nanosponge formulation

The in vitro release was carried out using a multi-compartment rotating cell with a dialysis membrane (cut off 12,000 Da). The donor phase consisted of 1 ml of water dispersion of paclitaxel-loaded nanosponges (10 mg/ml). The receiving phase was phosphate buffer at pH 7.4 containing 0.1% of SDS to mimic sink conditions. The experiment lasted 2 h. At fixed time the receiving phase was completely withdrawn and replaced with fresh phosphate buffer. The amount of released drug was determined by HPLC as described later. The experiments were carried out in triplicate.

Physical stability of paclitaxel-loaded nanosponges over time

The stability of paclitaxel-loaded nanosponges, as aqueous suspension and as freeze-dried solid, was determined over time storing samples at 25° and 4 °C for six months. For stability studies paclitaxel content, crystallinity (by DSC analysis), sizes and particle size distribution of nanosponges were evaluated periodically as previously described.

Hemolytic activity

Paclitaxel-loaded nanosponges were incubated at 37 °C for 90 min with 1 ml of diluted human blood obtained from the blood bank (Molinette Hospital, Turin, Italy). Freshly prepared PBS was used for all dilution purposes.

After incubation, blood containing suspensions were centrifuged at 2,000 rpm for 10 min to separate plasma. The amount of hemoglobin released due to hemolysis was measured spectrophotometrically at 543 nm using a DU 730 Beckman Coulter (Beckman Coulter, Brea, CA, USA). The hemolytic activity was calculated with reference to blank and complete hemolysed samples (induced by addition of ammonium sulfate 20% w/v).

Quantitative determination of paclitaxel

Weighed amount of paclitaxel-loaded nanosponges were dispersed in methanol, centrifuged, the supernatant withdrawn, suitably diluted in methanol and were analysed by RP-HPLC. Briefly, a Shimadzu instrument model no. LC-9A (Shimadzu, Kyoto, Japan), equipped with C R5A chromatopac integrator and UV detector was used. Drug content analysis was performed using a C18 column (Lichrosorb 18 column, 5 μ , spherical, 4.6 mm \times 250 mm, Merck KGaA Germany) equipped with a C18 guard column at 25 °C with a mobile phase containing a mixture of methanol, acetonitrile, acetate buffer pH 5 (10/40/50, v/v/v) at a flow rate of 1 ml/min. Sample injection volumes were 20 μ l and paclitaxel detection was carried out with the UV detector at a wavelength of 227 nm.

Culture cells

AT84 cells (kindly provided by Dr. Shillitoe, State University of New York College of Medicine, Syracuse) were grown in RPMI supplemented with amphotericin B 2.5 μ g/ml, penicillin 100 U/ml, streptomycin 100 μ g/ml and 10% FCS (all reagents from Sigma, Italy) at 37 °C in a humidified 5% CO₂ atmosphere.

Cytotoxicity of blank cyclodextrin nanosponges

Cells were seeded into flat-bottomed 96-well microplates at a density of 500 cells/0.1 ml per well. After seeding, cells were allowed to attach over night in complete medium before treatment. Normal culture medium was replaced with medium containing increasing concentrations (0–20 μ g/ml) of nanosponges resuspended in milliQ water or in DMSO (0–1.2 M) (Sigma).

Plates were incubated at 37 °C with 5% CO₂ up to 72 h. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was then processed according to routine protocols. Briefly, 10 μ l of MTT, prepared at a concentration of 5 mg/ml in PBS, were added to each well. Cell culture were continued for another 3 h at 37 °C; after discarding supernatant, 100 μ l of DMSO were then added to each well and the absorbance was measured using a microculture plate reader (Microplate 450, Bio-Rad, Hercules, CA, USA) with a wavelength of 570 nm. Cell viability was expressed as percentage of living cells relative to controls.

Cytotoxicity of paclitaxel-loaded nanosponges versus paclitaxel

Cells were plated as previously described. The paclitaxel-loaded nanosponges and plain paclitaxel were resuspended in DMSO or water so that to reach the same concentration

(range 0–1 μM) of the antineoplastic drug. The amount of nanosponges added ranged between 0 and 2.36 $\mu\text{g}/\text{ml}$. Solutions were then added to the cells. MTT assay was performed as described in the previous section at 24, 48 and 72 h after drug addiction.

Uptake of fluorescent nanosponges by cancer cells

Cells were plated in 8-well permanox slides (Nunc, Rochester, USA) at the concentration of 5×10^3 cells/well. After adhesion, 2 $\mu\text{g}/\text{ml}$ fluorescent nanosponges were added. Negative controls were incubated with FITC unconjugated nanosponges under the same conditions. After 48 h, slides were thoroughly washed with PBS to eliminate unbound nanosponges, fixed in 4% paraformaldehyde pH 7.4, mounted with Dako Fluorescent Mounting Medium (Dako, Milan, Italy), observed under UV light and photographed by using a confocal Axiovert 100 M Zeiss microscope.

Internalization of paclitaxel-loaded nanosponges versus plain paclitaxel

Cells were grown for 24 h in presence of 62.5 nM paclitaxel or corresponding amount of paclitaxel-loaded in nanosponges. Cells were then harvested and counted. Pellet cell samples were sonicated by application of ultrasounds, then resuspended in 2 ml of HPLC grade water produced with Milli-DI system coupled with a Synergy 185 system by Millipore (Milan, Italy). Plain and loaded paclitaxel were extracted. Extraction was performed on 300 μl sample aliquots; after addition of 150 μl of methanol and 1 ml of diethyl ether, samples were centrifuged for 10 min at $3,000 \times g$ and superior phase was transferred into a new tube for evaporation to dryness under nitrogen. After evaporation, each dried sample was successively resuspended in 500 μl of a reconstitution solution (water:methanol:acetonitrile/50:10:40). All procedure steps were carried out at room temperature. Standard drugs solutions were prepared by dissolving in methanol 1 mg/ml of paclitaxel complex preparation and docetaxel, which has chromatographic properties similar to paclitaxel and was used as internal standard (IS). Docetaxel pure powder, diethyl ether and ammonium acetate were purchased from Sigma (Milan, Italy). Acetonitrile and methanol HPLC grade were purchased from VWR International (Milan, Italy). Untreated cell samples, spiked with determined volumes of paclitaxel-loaded nanosponges preparation, were used as calibrators. Calibration ranged from 6.25 to 100 $\mu\text{g}/\text{ml}$; blank and blank plus IS samples were also evaluated, in order to exclude potential interference peaks. Working solutions of IS was made at 100 $\mu\text{g}/\text{ml}$ in methanol; 50 μl of IS solution was added to each samples. The HPLC–UV

method, adapted from that of Alexander et al. [19] with modification, was used. The quantification of paclitaxel was normalized to the cell number of the relative sample.

Statistical analysis

One way analysis of variance followed by Bonferroni test was applied using statistical software (Prism).

Results

Characterization of blank nanosponges and paclitaxel-loaded nanosponges

Nanosponge formation was confirmed by FTIR analysis. The blank nanosponge spectrum showed the presence of the carbonate bond as evidenced by a peak at around 1700 to 1750 cm^{-1} corresponding to the stretching of carbonyl which is not present in the parent β -cyclodextrin spectrum (Fig. 1).

The paclitaxel complexation with nanosponges was evaluated using DSC and FTIR analyses

FTIR spectra are reported in Fig. 2. The reduction of O–H stretching vibration band at 3300 cm^{-1} and the change in the peak at about 1700 cm^{-1} corresponding to aryl and saturated ketone groups proved the interaction between paclitaxel and nanosponges. Significant changes was also evidenced in the 1500–1200 cm^{-1} region corresponding to the aromatic ring stretching.

The DSC thermograms of the paclitaxel-loaded nanosponges, blank nanosponges and plain paclitaxel are reported in Fig. 3.

The disappearance in the paclitaxel-loaded nanosponge thermogram of the endothermic peak at about 216 $^\circ\text{C}$, corresponding to the melting of the drug, evidenced that paclitaxel interacted with the nanosponges. Probably it remains molecularly dispersed in the nanosponge structure without the capacity of crystallization.

Ultraviolet spectra of free paclitaxel and paclitaxel-loaded in nanosponges showed a different profile (Fig. 4), confirming that paclitaxel was not merely on nanosponge surface but it is loaded in the structure.

The average diameter and polydispersity index of blank nanosponges were about 450 nm and 0.15, respectively. TEM analysis confirmed the nanosponge sizes and evidenced a quite spherical morphology (Fig. 5a). The sizes and shape of nanosponges were unaffected after drug incorporation. The FITC-labeled nanosponges are reported in Fig. 5b showing uniform fluorescence; sizes and spherical morphology were similar to those of unlabelled nanosponges.

These nanosponges obtained using carbonyldiimidazole as cross-linker showed a good ability of paclitaxel

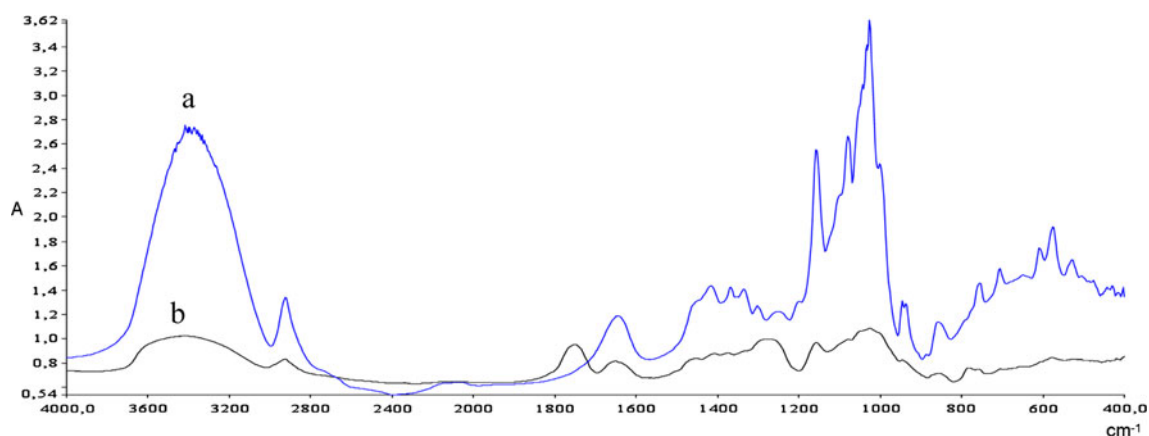


Fig. 1 FTIR spectra of β -cyclodextrin nanosponges (a) and β -cyclodextrin (b)

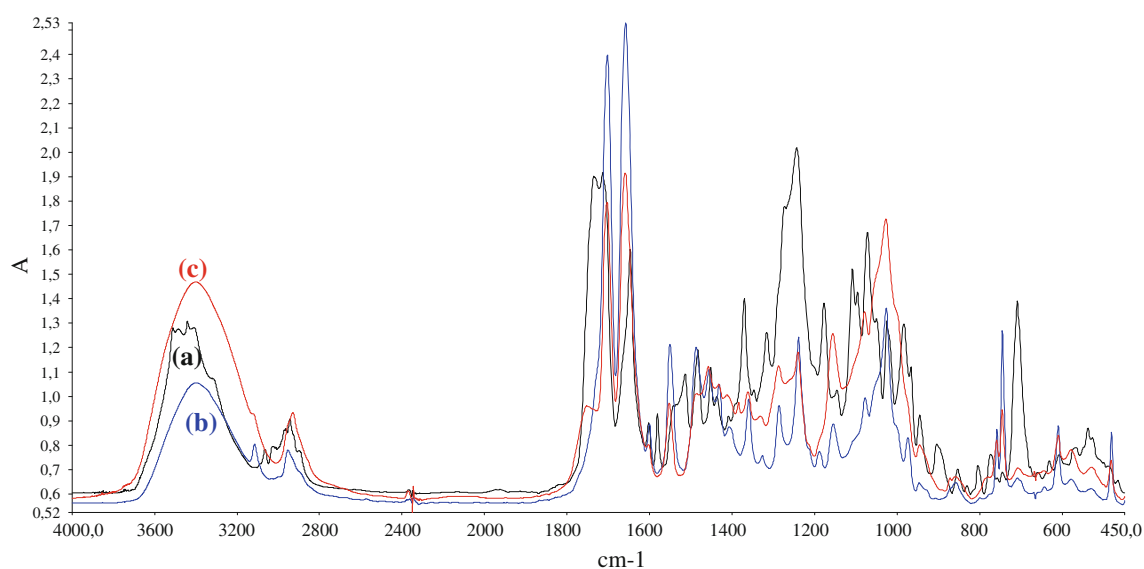


Fig. 2 FTIR spectra of paclitaxel (a), paclitaxel-loaded nanosponges (b) and blank nanosponges (c)

complexation as previously reported for other types of nanosponges synthesized in our laboratory [17]. One milliliter of a 1.5% nanosponge aqueous suspension was able to solubilize about 2 mg of paclitaxel. This result confirmed the nanosponge potential of wetting and solubilizing paclitaxel without the use of surfactants.

The stability studies showed that the paclitaxel-loaded nanosponges in aqueous suspension have a good physical stability for six months. The nanosuspension did not aggregate over time but maintained about the same sizes and size distribution. Moreover, paclitaxel formulated with nanosponges did not recrystallize or form less soluble hydrates which can separate and precipitate over time or upon dilution. The DSC analysis of paclitaxel-loaded nanosponges, either in suspension or in the solid state, confirmed that the drug did not crystallize over time.

The *in vitro* release kinetics of paclitaxel from nanosponges is reported in Fig. 6.

The paclitaxel release profile did not show an initial burst effect suggesting the drug complexation and the absence of weakly adsorbed or uncomplexed drug on the nanosponge surface. The loaded paclitaxel was completely released in 2 h from nanosponges after dilution. This fast release proved a good solubilization of paclitaxel without crystallization also in the absence of surfactants or solubilizers.

Hemolytic activity

The paclitaxel-loaded nanosponge aqueous suspension showed a negligible hemolytic activity. Blank nanosponges did not affect red blood cells morphology and did not show hemolytic effects.

Fig. 3 DSC thermograms of blank nanosponges, paclitaxel and paclitaxel-loaded nanosponges

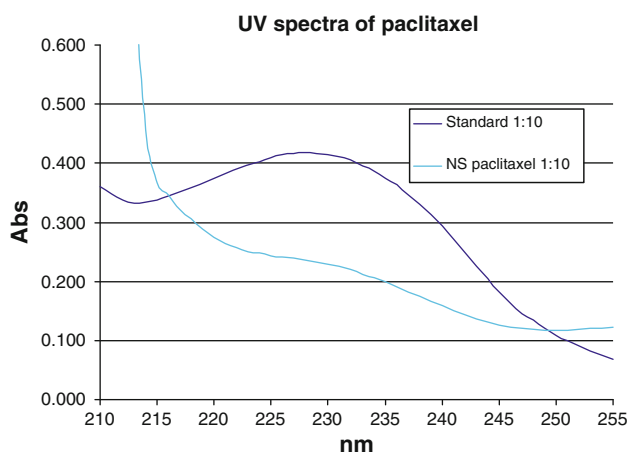
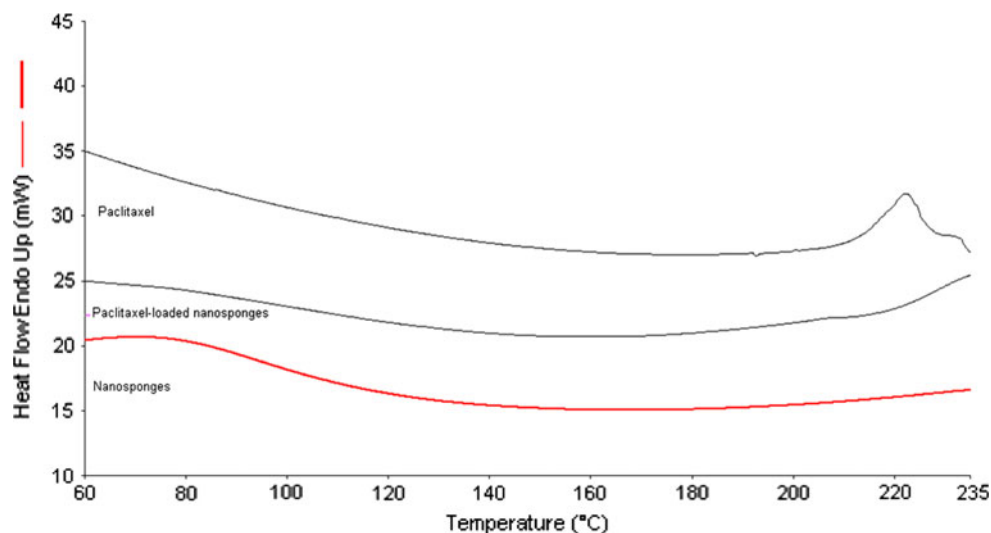


Fig. 4 UV spectra of paclitaxel and paclitaxel-loaded nanosponges

Cytotoxicity of blank cyclodextrin nanosponges

No cytotoxicity could be ascribed to cyclodextrin nanosponges at any of the considered time-point (24 and 48 h data not shown, 72 h see Fig. 7). Cytotoxicity at 10 and 20 $\mu\text{g mL}^{-1}$ is due to DMSO (41.5 $\mu\text{L mL}^{-1}$ and 83 $\mu\text{L mL}^{-1}$ respectively), as demonstrated by the absence of significance toward solvent at both concentrations (P always > 0.05).

Cytotoxicity of paclitaxel-loaded nanosponges versus plain paclitaxel

After 24 h incubation (Fig. 8a) neither plain nor complexed paclitaxel significantly inhibited cell proliferation at any of the considered concentrations. After 48 h (Fig. 8b), vehiculated paclitaxel significantly inhibited cell proliferation already at 31.5 nM ($P < 0.01$), while plain paclitaxel inhibition was significant only at 125 nM ($P < 0.001$).

After 72 h incubation (Fig. 8c) nanosponge-vehiculated paclitaxel significantly inhibited cell proliferation already at 1.9 nM ($P < 0.001$). Toxicity of plain paclitaxel was significant against untreated cells at 31.5 nM ($P < 0.05$), and even more strikingly at 62.5 nM ($P < 0.001$). At this same time point, nanosponge-vehiculated paclitaxel inhibition was significantly higher than that obtained with plain paclitaxel already at 1.9 nM. The IC_{50} values were 32 and 73 nM respectively.

No differences were evidenced between cytotoxicity of paclitaxel-loaded nanosponges dissolved in DMSO or in water (data not shown).

Uptake of nanosponges by cancer cells

As shown in Fig. 9, after 48 h incubation, AT84 clearly internalize fluorescent nanosponges. Incorporation appears cytoplasmatic with a sporadic perinuclear accumulation. In order to confirm the specificity of the experiment, no fluorescence signal was seen in cells treated with nanosponges non FITC-conjugated (data not shown).

Uptake of loaded and plain paclitaxel

When cells were incubated with plain paclitaxel, $0.2 \times 10^{-8} \mu\text{g cell}^{-1}$ of paclitaxel was extracted, while, when the drug was nanosponges-vehiculated, we extracted $3.6 \times 10^{-8} \mu\text{g cell}^{-1}$.

Discussion

At present, about 40% of small-molecule drugs in the pipeline of pharmaceutical companies have low water

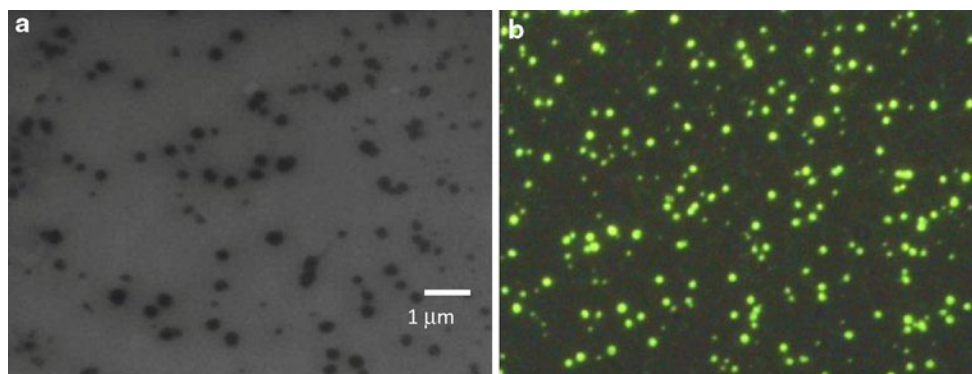


Fig. 5 Morphological analysis. TEM photomicrograph of paclitaxel-loaded nanosponges (a) and photograph of fluorescent nanosponges (b)

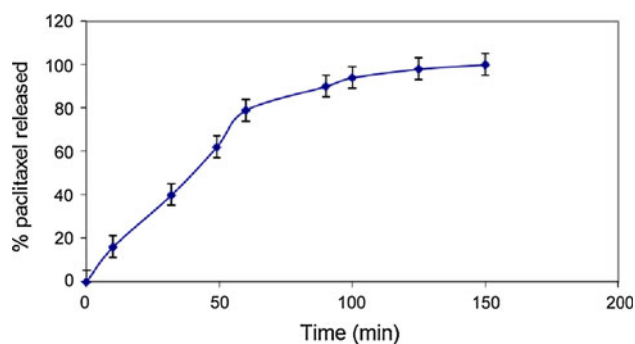


Fig. 6 In vitro release kinetics of paclitaxel from nanosponge formulation

solubility and therefore cannot be administered by the preferred route or, in some cases, at all [20].

Various nanomedicine approaches have been proposed to increase solubility and bioavailability of paclitaxel, including liposomes, parenteral emulsions and nanoparticulate systems [3].

However the development of a stable and suitable paclitaxel formulation for parenteral administration without surfactants remains a challenge. One formulative strategy for paclitaxel formulation consists of improving

its water solubility using solubilizing agents. Among these, the complexation with β and γ -cyclodextrins has been studied [21]. Cyclodextrins are polysaccharides with a toroidal hollow shape with a hydrophobic internal cavity and a hydrophilic external region. They are able to encapsulate molecules forming inclusion complexes or supramolecular aggregates in aqueous solutions. The cyclodextrin complexation can enhance the solubility and stability of drugs. Previously it was shown that different types of cyclodextrins were able to increase the solubility of paclitaxel [22]. Many paclitaxel-cyclodextrin formulations are stable in the solid state, but the cyclodextrin complex become weak in solutions producing a drug precipitation upon dilution. Moreover, the amount of cyclodextrins needed to formulate paclitaxel complex at the dose required for the parenteral administration is so high that it could induce toxic effects.

To overcome these limitations, various cyclodextrin derivatives have been proposed.

Amphiphilic cyclodextrins can self-aggregate in water forming solid nanoparticles [23]. Amphiphilic β -cyclodextrin nanoparticles have been prepared for paclitaxel delivery showing an increase either of the physical stability or of the anticancer activity on cells of the drug [24].

Fig. 7 Nanosponge cytotoxicity. Cytotoxicity of blank cyclodextrin nanosponges compared to vehicle (DMSO)

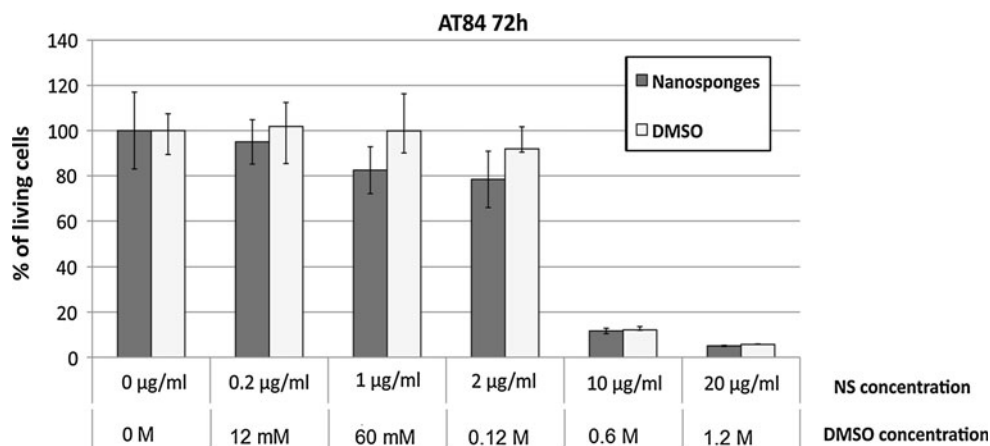
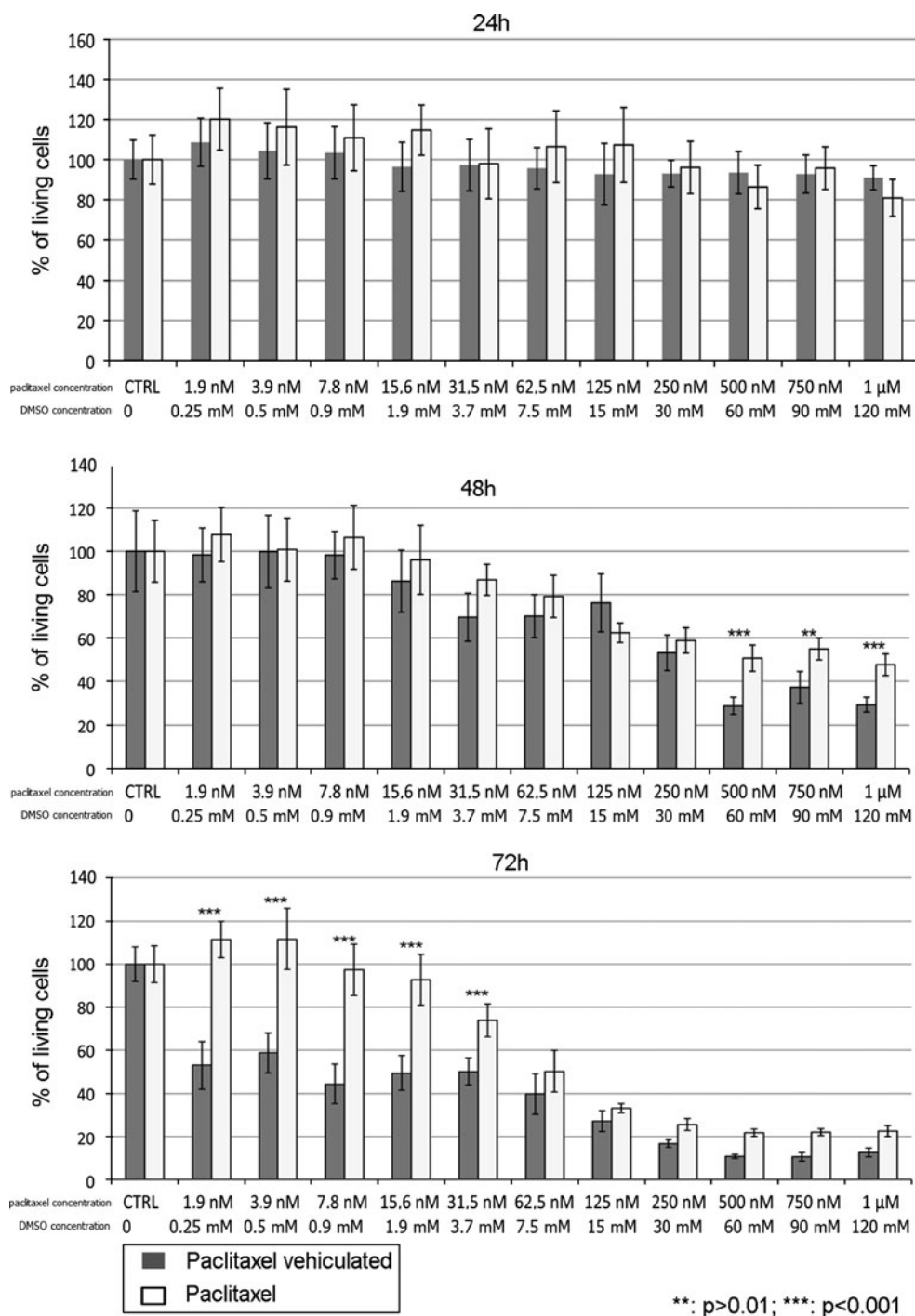


Fig. 8 Nanosponge-vehiculated paclitaxel. Toxicity of paclitaxel-loaded nanospheres dissolved in DMSO compared to plain paclitaxel at three different time points. ** $P > 0.01$; *** $P < 0.001$. Significance against internal control as described in the text



Another type of derivatives, synthesized to enhance the cyclodextrin binding ability, are bridged bis(β -cyclodextrin)s, which possess dual hydrophobic cavities. The cooperative binding of two adjacent cyclodextrin units in the structure can enhance the complexation ability and solubilization capacity. Liu et al. showed that oligo(ethylenediamine) bridged bis(β -cyclodextrin)s dramatically increased the paclitaxel solubility [25].

Nanosponges are solid nanoparticles obtained by the cross-linking of many cyclodextrin units [14]. They showed a huge capacity for the encapsulation of a great variety of molecules including gases.

Particularly nanospheres, due to the cooperation of the cyclodextrin cavities and the mesh of the cross-linker network present in their structure, show the capacity to increase the solubility of poorly water molecules without

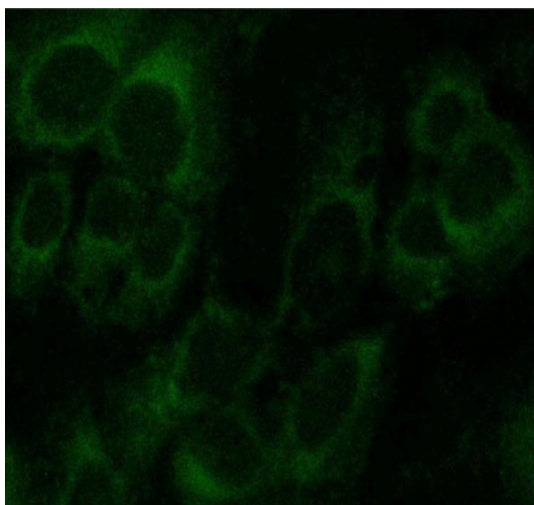


Fig. 9 Nanosponge uptake. AT84 incubated with fluorescent nanosponges

surfactants. Moreover, being a nanoparticulate solid, they can also act as a drug delivery system.

Our previous results with camptothecin [26] suggest that nanosponges may offer a solution for the solubilization and the stabilization of lipophilic drugs.

Based on these premises, we studied β -cyclodextrin nanosponges to develop a novel paclitaxel formulation.

For this purpose we synthesized novel β -cyclodextrin nanosponges using β -cyclodextrin as building block and carbonyldiimidazole as cross-linker in a 1:4 molar ratio. Solid cyclodextrin nanoparticles were obtained with spherical morphology and an average diameter of about 400 nm with a narrow size distribution. The nanosponges dispersed in water formed aqueous nanosuspensions with sizes suitable for parenteral administration.

The nanosponges showed a great capacity of wetting and solubilize paclitaxel forming a stable Cremophor free formulation. A nanosponge aqueous suspension (1.5% w/v) was able to solubilize paclitaxel up to about 2 mg/ml (about 2.3 mM) without the addition of surfactants or other co-solvents. On the contrary, a direct suspension of crystalline paclitaxel in water reaches just a concentration ranging between 0.2 and 0.9 μ M.

The sponge-like matrix of nanosponges with cyclodextrin cavities besides the cross-linked network might favour the paclitaxel complexation, either as inclusion or non inclusion complex, due to the presence of various binding sites. DSC and FTIR analyses confirmed the interaction of paclitaxel with the nanosponge structure.

The paclitaxel-loaded nanosponges suspended in water formed a stable system up to 6 months without recrystallization and precipitation phenomena of the drug. A physical stability of the Cremophor free paclitaxel nanosponge formulation was observed also upon dilution.

Dilution of the nanosponge suspension was also possible without the rapid precipitation of paclitaxel. Recrystallization of paclitaxel in solution is due to the hydrophobic interaction between the lipophilic region of paclitaxel molecules. In aqueous media paclitaxel undergoes easily to hydrophobic clustering and the multimeric structures can nucleate and precipitate. As previously observed with cyclodextrins, nanosponges can reduce the tendency of paclitaxel molecules to interact each other resulting in increased drug solubility and increased physical stability. This behavior confirms the strong interaction between nanosponge and paclitaxel.

Moreover paclitaxel-loaded nanosponges could be freeze-dried and easily resuspended in water by simple vortexing, forming the original colloidal system. The in vitro release profile of paclitaxel from the Cremophor free formulation showed a fast release kinetics. The release from the paclitaxel bulk powder was negligible after 3 h. The release data confirmed the capacity of nanosponge formulation to solubilize paclitaxel and to maintain the drug molecularly dispersed also without surfactants.

The absence of Cremophor can play an important role also in the biopharmaceutical properties of the nanosponge formulation. The presence of Cremophor forming micelles can sequester the drug reducing the free fraction of paclitaxel available for the pharmacological activity. Cremophor reduces the paclitaxel tissue penetration as previously shown.

Besides the physical stability, for the parenteral administration the biocompatibility and safety of the nanosponge formulation is mandatory. The blank nanosponges showed a safe profile being non-hemolytic as previously described [26] and non cytotoxic on cells.

As a matter of fact, the biological effect of paclitaxel in vitro is highly enhanced by nanosponges: not only its cytotoxicity is greatly increased after 72 h incubation, but even intracellular paclitaxel concentration is significantly enhanced when compared to plain paclitaxel. Fluorescent-labeled nanosponges have been prepared to study cell uptake. The presence of FITC on the surface of nanosponges did not change their sizes and morphology. Our results clearly show a cellular internalization of labeled nanosponges which is compatible with the higher amount of vehiculated paclitaxel recovered in treated cells and with the lower IC_{50} of paclitaxel-loaded nanosponges. These results showed that nanosponges, beside a solubilizing agent, could be also considered as delivery systems for paclitaxel.

Though the proper mechanism is still unclear, we might speculate that nanosponges increase paclitaxel concentration in cell vicinity, probably by adhering to/interacting with the cell membrane. This might facilitate the cells in internalizing higher amount of paclitaxel, thus explaining

the experimental data. This phenomenon might be enhanced by nanosponges entering the cells, as demonstrated by our own results and by other scientific works [26]. It remains to explore the fate of nanosponges after internalization, since they could be degraded, due to their glycosidic bond nature, or extruded as it happens for other nanoparticulate delivery system.

Therefore paclitaxel-loaded nanosponges could be proposed as a safe Cremophor free formulation with the capacity at the same time of solubilize and selectively deliver the drug to cancer cells. Considering the increased antitumor activity, besides the enhanced water solubility and prolonged physical stability in the absence of Cremophor of the paclitaxel encapsulated in β -cyclodextrin nanosponges this new formulation might be potentially useful for clinical application as innovative delivery system for the drug.

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