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***In vivo* nanotoxicology of hybrid systems based on copolymer/silica/anticancer drug**

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Abstract. One of the major problems in cancer therapies is the high occurrence of side effects intrinsic of anticancer drugs. Doxorubicin is a conventional anticancer molecule used to treat a wide range of cancer, such as breast, ovarian and prostate. However, its use is associated with a number of side effects like multidrug resistance and cardiotoxicity. The association with nanomaterials has been considered in the past decade to overcome the high toxicity of these drugs. In this context, mesoporous silica nanoparticles are great candidates to be used as carriers once they are very biocompatible. Taking into account the combination of nanoparticles and doxorubicin, we treated rats with chemically induced prostate cancer with systems based on mesoporous silica nanoparticles and a thermoreversible block copolymer (Pluronic F-127) containing doxorubicin. Preliminary results show a possible improvement in tumor conditions proportional to the concentration of the nanoparticles, opening a perspective to use mesoporous silica nanoparticles as carrier for doxorubicin in prostate cancer treatment.

1. Introduction

It is known that one of the main problems related to cancer treatments is the high incidence of side effects caused by anticancer drugs. Doxorubicin (DOX), for instance, is an antibiotic and anticancer that is commonly used to treat many types of cancers, such as breast, ovarian and prostate [1,2]. However, its efficacy is yet very limited for the occurrence of aggressive side effects, including mielosuppression, alopecia, multidrug resistance and cardiotoxicity [2,3].

In the last years, many alternatives to minimize this issue have been studied. Among them, the association with nanoparticles is promising. Their use as drug carriers is advantageous once they can provide a sustained release and increase the effectiveness of drug delivery [4,5]. Among



nanomaterials, mesoporous silica nanoparticles (MSNs) have a good perspective to be used as drug carriers because of their biocompatibility and safety profile [4]. These particles have high superficial area and great pore volume, which is very suitable to carry actives [4].

Another strategy to overcome the intrinsic toxicity of anticancer drugs is the association with materials that can provide a long-term release of the drug. This ensures a better drug distribution and avoids a premature degradation of the drug [6]. In this context, Pluronic F-127 (PF-127) is a biocompatible block copolymer, approved by the FDA, that has a transition sol-gel temperature near room temperature [7]. That is, it is liquid at low temperatures (e.g. refrigerator temperatures) and it gels at room temperature, which makes it very suitable to reach *in situ* gelation [6].

So, to obtain preliminary results about the antitumor potential of the association MSNs + DOX + PF-127 and the role of the nanoparticles in this process, we developed three systems containing these components and tested them in rats with chemically induced prostate cancer. The MSNs were used as carriers for DOX, to reach a sustained release of the drug. The polymer, in turn, was used as the matrix of the hydrogel, to achieve *in situ* gelation (i.e. inside the animal body after inoculation) and thus reach a prolonged release of the components.

2. Methods

Synthesis and characterization of mesoporous silica nanoparticles (MSNs)

MSNs were synthesized following the protocol developed by Paula et al. (2012) [8], which consists of a sol-gel synthesis based on the methods of Stöber [9] and Bein [10]. This approach allows to produce spherical and monodisperse nanoparticles with high colloidal stability in aqueous medium. In this context, 0.75 g of cetyltrimethylammonium bromide (CTAB) were added to a 0.050 mol L⁻¹ ammonium hydroxide solution of pH 11. Then, 3.2 mL of absolute ethanol were added and after 15 minutes under magnetic stirring, 2.5 mL of tetraethyl orthosilicate (TEOS) were added. The reaction was maintained under reflux at 60°C for two hours. After completing the reaction, a centrifugation step was carried out to separate the products (60 minutes at 18,400 rcf). To extract the CTAB, the products were resuspended in 90 mL of absolute ethanol and 10 mL of hydrochloric acid were added (1:9 HCL:Ethanol volume ratio), sonicating the mixture for 10 minutes. Finally, to obtain ethanolic suspensions of the nanoparticles, the mixture was centrifuged (60 minutes at 18,400 rcf) following two washing steps with absolute ethanol and resuspended in absolute ethanol.

The size distribution and morphology of MSNs were analyzed by transmission electron microscopy (TEM) in the bright field mode (TEM-BF, Zeiss Libra 120, operating at 80 kV). Nitrogen-sorption assays were run to obtain information on nanoparticles surface area, pore volume and pore diameter (Accelerated Surface Area and Porosimetry System ASAP 2020 micromeritics). To calculate surface area and pore diameter, the BET (Brunauer-Emmet-Teller) and BJH (Barret-Hoyner-Halenda) methods were used, respectively, both using N₂ adsorption branch. The pore volume was calculated from the single-point value adsorbed at P/P₀ = ~0.94.

Synthesis and characterization of hybrid systems

To evaluate the antitumor potential of the hybrid systems as well as the role of MSNs in the association, we developed three systems. The only difference between them was the presence and concentration of the nanoparticles (Table 1). The systems were produced by firstly adding DOX to the copolymer solution in an ice bath under magnetic stirring and adding the nanoparticles lastly to avoid aggregation. The mixture was kept under these conditions overnight. All systems were produced in physiological saline solution (0.9% w/v NaCl) to keep the same osmotic pressure than the animals' cells.

The concentration of DOX was calculated based on Das et al. (2010) [2] to reach 1.5 mg of drug per kilogram of animal body weight, considering a dose application of 0.3 mL and an animal body

weight of 150 g (average). The PF-127 concentration was chosen regarding its gelation temperature (T_{gel}): T_{gel} had to fit within a temperature range that would allow easy manipulation of the systems, avoiding gelation inside the syringe and on the needles' walls and quickly gelation of the systems once applied inside the animals bodies, avoiding the diffusion of the systems to unwanted locations and premature release. So, the systems were characterized regarding their T_{gel} by adding 5 mL of the liquid mixture in a beacker in an ice bath with a thermometer. The mixture was heated in a constant rate and the temperature in which the bar stopped moving was considered the gelation temperature.

In order to corroborate the potential for prolonged release of the systems, the release profile was studied *in vitro* through a membraneless dissolution method. To do so, 0.5 mL of ht hybrid systems were added to a weighed vial and incubated at 35°C (which is the body temperature of mice used in the *in vivo* assays), using a dry bath, to reach thermal equilibrium. So, the vials were weighed and 0.5 mL of the release medium (NaCl 0.9% w/v) were put carefully in order to avoid mixture. At pre-determined intervals, the release medium was removed, the vial was weighed and a new release medium was put to avoid saturation. The dissolution rate was calculated in terms of weight loss against time.

In vivo model

To accomplish the *in vivo* study, seventeen 7-week-old male Fischer 344 rats were used and they were obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) at University of Campinas (UNICAMP). The prostate cancer induction was performed in 13 animals and followed a new protocol based on Fávaro et al. (2014) [11] in which the animals are ready to be treated in approximately four months. Firstly, the animals received a daily subcutaneous injection of 100 mg kg⁻¹ testosterone cypionate diluted in 0.5 mL of peanut oil for three days. Then, the animals were anesthetized with 5 mg kg⁻¹ of 2% xylazine hydrochloride and 60 mg kg⁻¹ of 10% ketamylene hydrochloride in order to perform 0.5 cm suprapubic incision and inoculation of 0.2 mL of 15 mg kg⁻¹ of *n*-methyl-*n*-nitrosourea (MNU) dissolved in 0.3 mL of 1 M sodium citrate (pH 6.0) and 25% PF-127, which allows *in situ* gelation of the solution. After one week, the animals received subcutaneous injections of 5 mg kg⁻¹ testosterone cyprionate diluted in 5 mL of peanut oil on alternate days for 120 days.

Of the total number of animals, 4 animals comprised the healthy control group (Control, n=4, received no cancer induction) and 4 comprised the cancer group (Cancer, n=4, received no treatment), both groups received physiological saline (NaCl 0.9% w/v) for treatment. The rest of the animals were divided in 3 groups (n=3): Group S1 was treated with S1 system, Group S2 was treated with S2 system and Group S3 was treated with S3 system. The treatment consisted of a weekly-dose of intraperitoneal inoculation of 0.3 mL of the respective treatment for 30 days. To prevent gelation inside the needles, the systems were kept in ice bath before each application. The animals received water and the same solid diet *ad libitum* (Nuvilab) and were allocated in single solid-bottom boxes lined with wood shavings in a room with controlled light and temperature (12 hours light and 12 hours dark, 20-25°C). The experimental protocol followed ethical principles in animal research. After treatment, the animals were euthanized and the occurrence of macroscopic changes were observed.

3. Results and Discussion

Synthesis and characterization of MSNs

Transmission electron microscopy images (Fig. 1a) show MSNs with spherical morphology and size distribution from 45 to 75 nm. Nitrogen-sorption experiments using BET and BJH methods revealed that the superficial area was 970 cm² g⁻¹ and the pore volume was 1.6 cm³ g⁻¹, with an average pore diameter of 4.8 nm. The nitrogen-sorption isotherm (Fig. 1b) shows an adsorption pattern that

resembles a type IV isotherm (IUPAC) with a subtle stepwise behavior around 0.4 P/P_0 , which is characteristic of well-ordered mesoporous materials [12].

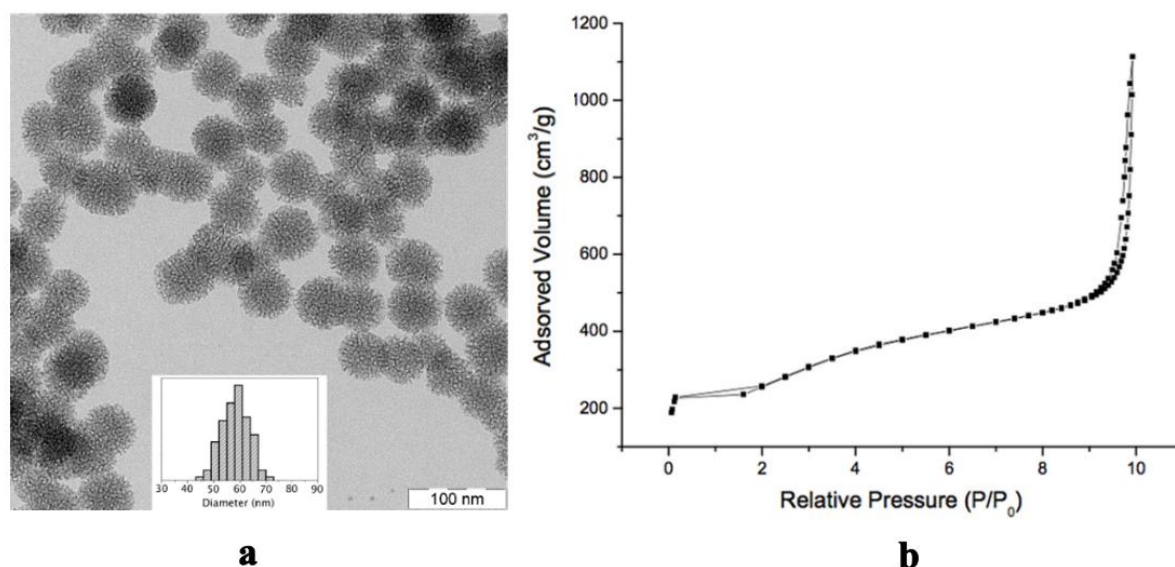


Figure 1. a) TEM images of MSNs with size distribution histogram (measuring at least 100 nanoparticles) **b)** Nitrogen sorption isotherm of MSNs

Synthesis and characterization of hybrid systems

The hybrid systems were synthesized and firstly characterized regarding their T_{gel} (Table 1). All systems have a T_{gel} around 20°C, which allows easy manipulation of them during dose applications and a rapid gelation inside the animal body (which temperature is around 35°C).

Table 1. Description of the hybrid systems.

System	Components and final concentration	Gelation temperature (T_{gel} , °C)
S1	PF-127 (18%) DOX (1.25 mg mL ⁻¹)	21.0 ± 0.5
S2	PF-127 (18%) DOX (1.25 mg mL ⁻¹) MSNs (1.0 mg mL ⁻¹)	21.0 ± 0.5
S3	PF-127 (18%) DOX (1.25 mg mL ⁻¹) MSNs (5.0 mg mL ⁻¹)	22.0 ± 0.5

The achievement of long-term release was confirmed during the *in vitro* dissolution experiment (Fig. 2). Taking into account the concentration gradient induced by cells permanently on the hydrogel,

it is indeed expected that the hybrid system will delay the release of the components (DOX and MSNs, when it is the case) after the application.

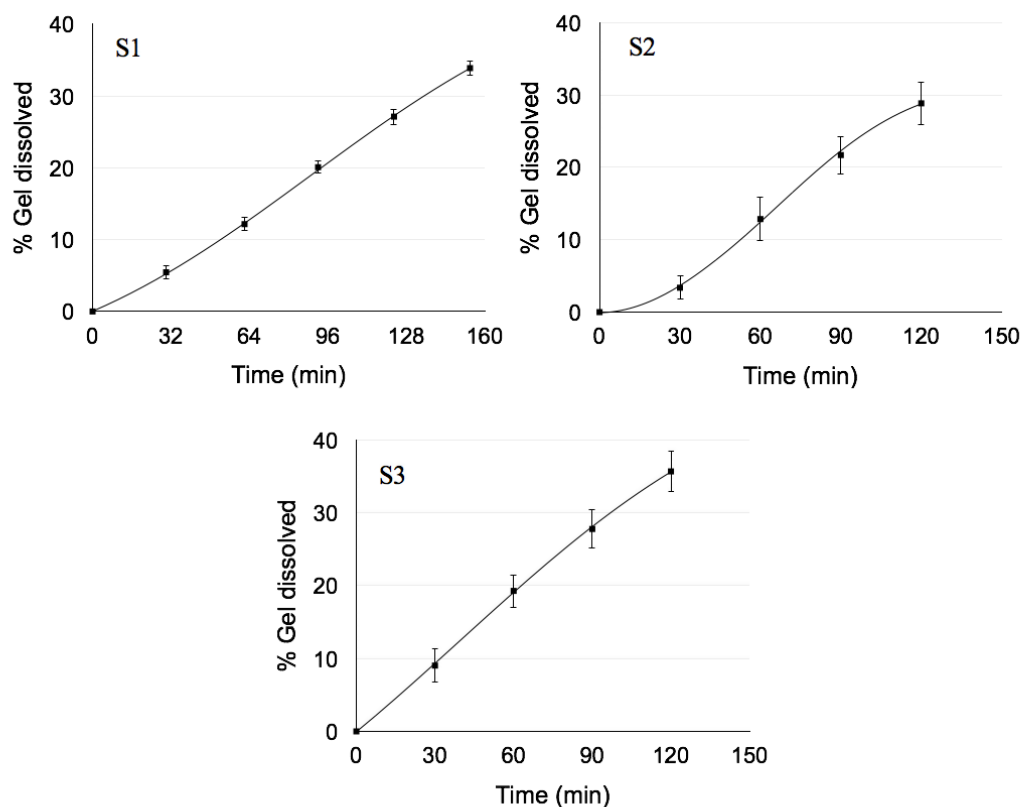


Figure 2. *In vitro* gel dissolution profiles of the systems.

In vivo macroscopic analyses

After treatment, the animals were euthanized and macroscopic changes were observed in order to get preliminary information on the antitumor potential of the systems (Table 2).

Table 2. Observed macroscopic changes of five experimental groups.

Macroscopic observation	Control	Cancer	S1	S2	S3
No macroscopic changes	100%	-	33,3%	100%	100%
Cardiomegaly	-	-	66,6%	-	-
Hernia	-	-	33,3%	-	-

Lesion in prostate base	-	100%	33,3%	-	-
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The results from Table 2 show a decrease in frequency of lesions that is proportional to MSNs concentration. It might be a possible improvement in tumor conditions as the MSNs are added in the formulations, calling the attention for the role of the nanoparticles in this process. Also, we can a high frequency of cardiotoxic effects in group S1 (the one treated with the system with no nanoparticles), probably caused by intrinsic toxicity of DOX, although these effects can not be seen in groups treated with systems containing nanoparticles (S2 and S3). This is particularly interesting once it suggests that the MSNs might lead to a decrease in drug toxicity.

It is known that nanoparticles are submitted to the enhanced permeability retention (EPR) effect, that allow macromolecules and particles up to 100 nm to accumulate in interstitial space of tumor cells [13]. This may increase the delivery of DOX. In turn, the prolonged and sustained release promoted by the hydrogel and the MSNs might protect the organisms from the intrinsic toxicity of the drug, increasing the efficacy of the treatment.

4. Conclusions

After treating rats with chemically induced prostate cancer, preliminary macroscopic analyses showed a possible improvement in tumor conditions that was proportional to MSNs concentration and a possibly cardioprotection induced by the MSNs. The results suggest that the nanoparticles might play an important role in the treatment and that they have good perspectives to minimize the side effects of DOX. It might be occurring a combined effect from DOX and MSNs, caused by EPR effect along with sustained and prolonged release of the drug. This avoids premature degradation of DOX and enables its cell internalization, achieving a better drug distribution, increasing the efficacy of the drug and possibly decreasing its toxicity.

5. Acknowledgements

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