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## ***In vivo* study of novel nanocomposite for prostate cancer treatment**

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**Abstract.** Because of high occurrence of side effects of conventional anticancer drugs, new therapies have been widely studied, such as the use of non-usual molecules and the association with nanomaterials. In this context, sildenafil is a molecule that has been lately considered as a co-adjuvant in cancer therapy because it inhibits an enzyme with a potential role in tumor progression, phosphodiesterase-5. Taking into account the combination of nanoparticles and sildenafil, we produced systems based on mesoporous silica nanoparticles, Pluronic F-127 and sildenafil as the main therapy for prostate cancer.

### **1. Introduction**

A major problem in cancer treatments is the high incidence of side effects intrinsic to anticancer drugs, which end up affecting the patients' lives and the therapies themselves. In an attempt to overcome this issue, the association of these drugs with non-usual molecules that act through different mechanisms as well as the association with nanomaterials are promising alternatives. In this way, sildenafil (SIL) is a molecule that inhibits an enzyme which expression is increased in many carcinomas, phosphodiesterase-5 (PDE-5) [1]. Moreover, it activates nitric oxide expression and apoptosis [2,3]. Among nanomaterials, mesoporous silica nanoparticles (MSNs) are good candidates to be used as drug carriers once they are safe and biocompatible [4]. They have high superficial area and great pore volume, very appropriate to carry bioactives [4]. They provide a sustained release of the drug, preventing it of premature degradation and increasing the effectiveness of drug delivery [4,5].

Another strategy to ensure a better drug distribution is the association with materials that can provide prolonged release [6]. In this perspective, Pluronic F-127 (PF-127) is a FDA-approved biocompatible block copolymer 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], applying the liquid formulation inside the patient body for it to form a hydrogel in there.

In order to obtain preliminary results about the antitumor potential of the association MSNs + SIL + PF-127, the effect of SIL on prostate tumor cells 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 nanocarriers for SIL, to reach a sustained release of the drug and PF-127 was used as the matrix of the hydrogel, to achieve *in situ* gelation to reach a long-term release of the components.

## 2. Methods

### *Synthesis and characterization of mesoporous silica nanoparticles (MSNs)*

The MSNs were synthesized through on a sol-gel method based on the methods of Stöber [8] [9] and Bein [9] [10] following the protocol developed by Paula et al. (2012) [10] [8]. This approach allow to synthesize spherical and monodisperse nanoparticles with high colloidal stability in aqueous medium. So, to synthesize the MSNs, 0.75 g of cetyltrimethylammonium bromide (CTAB) were added to a 0.050 mol L<sup>-1</sup> 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. 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.

Transmission electron microscopy (TEM) in the bright field mode was used to analyze size distribution and morphology of MSNs (TEM-BF, Zeiss Libra 120, operating at 80 kV). Surface area, pore volume and pore diameter were obtained through nitrogen-sorption assays (Accelerated Surface Area and Porosimetry System ASAP 2020 micromeritics) using BET (Brunauer-Emmet-Teller) to calculate surface area and BJH (Barret-Hoyner-Halenda) to calculate pore diameter, both using N<sub>2</sub> adsorption branch. The pore volume was calculated from the single-point value adsorbed at P/P<sub>0</sub> = ~0.94.

### *Synthesis and characterization of hybrid systems*

To evaluate the antitumor potential of the hybrid systems, the effect of SIL in tumor cells and the role of MSNs in the association, we developed three systems. The only difference between them was the presence and concentration of the MSNs (Table 1). The systems were produced by adding SIL to the PF-127 solution in an ice bath under magnetic stirring, then adding the nanoparticles lastly to avoid aggregation. The mixture was kept under magnetic stirring at 4°C 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 SIL was calculated based on Das et al. (2010) [1] [2] to reach 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 according to the desired range of gelation temperature (T<sub>gel</sub>): the optimum T<sub>gel</sub> would allow easy manipulation of the systems, avoiding gelation inside the and quickly gelation of the systems after applied intraperitoneally in the animals, avoiding diffusion to unwanted locations and premature release. So, the systems were characterized regarding their T<sub>gel</sub> 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.

To obtain information on 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*

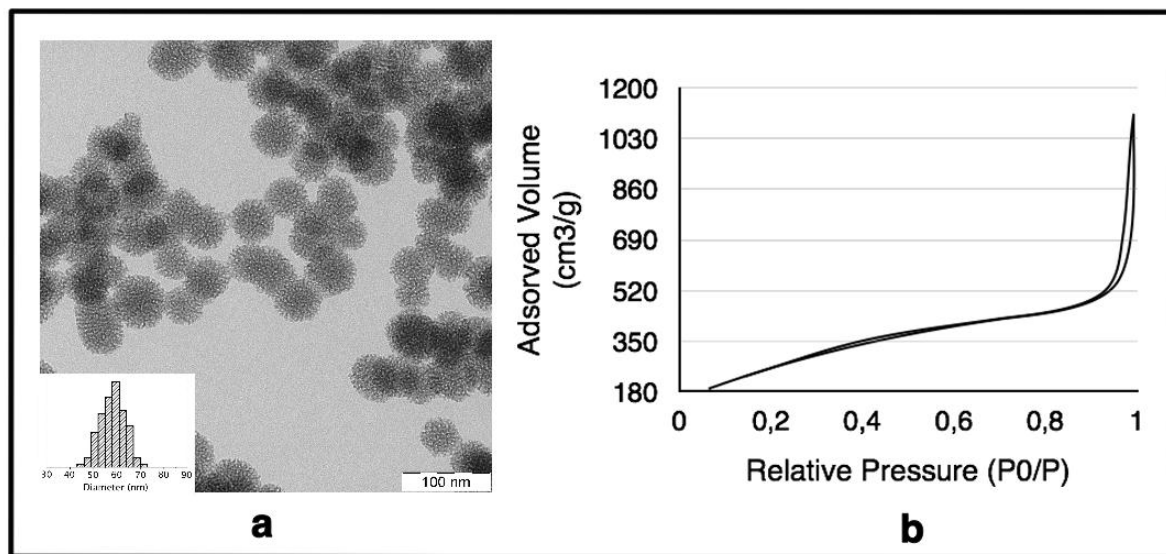
The *in vivo* study was accomplished using seventeen 7-week-old male Fischer 344 rats, obtained from the Multidisciplinary Center for Biological Investigation (CEMIB) at University of Campinas (UNICAMP). The prostate cancer induction followed a new protocol based on Fávaro et al. (2014) [11 - 11] and was performed in 13 animals. This new approach allows the animals to be ready for treatment in approximately four months. The animals received a daily subcutaneous injection of 100 mg kg<sup>-1</sup> testosterone cypionate diluted in 0.5 mL of peanut oil for three days. Then, the animals were anesthetized with 5 mg kg<sup>-1</sup> of 2% xylazine hydrochloride and 60 mg kg<sup>-1</sup> of 10% ketamine hydrochloride in order to perform 0.5 cm suprapubic incision and inoculation of 0.2 mL of 15 mg kg<sup>-1</sup> 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<sup>-1</sup> testosterone cypionate 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*

It is possible to see through TEM images (Fig. 1a) that MSNs have 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 cm<sup>2</sup> g<sup>-1</sup> and the pore volume was 1.6 cm<sup>3</sup> g<sup>-1</sup>, 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<sub>0</sub>, 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

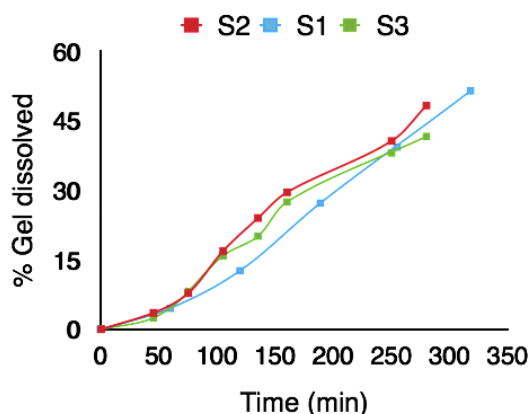
#### *Synthesis and characterization of hybrid systems*

The hybrid systems were characterized regarding their  $T_{\text{gel}}$  (Table 1). All systems have a  $T_{\text{gel}}$  that fits the temperature range preferred, 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.

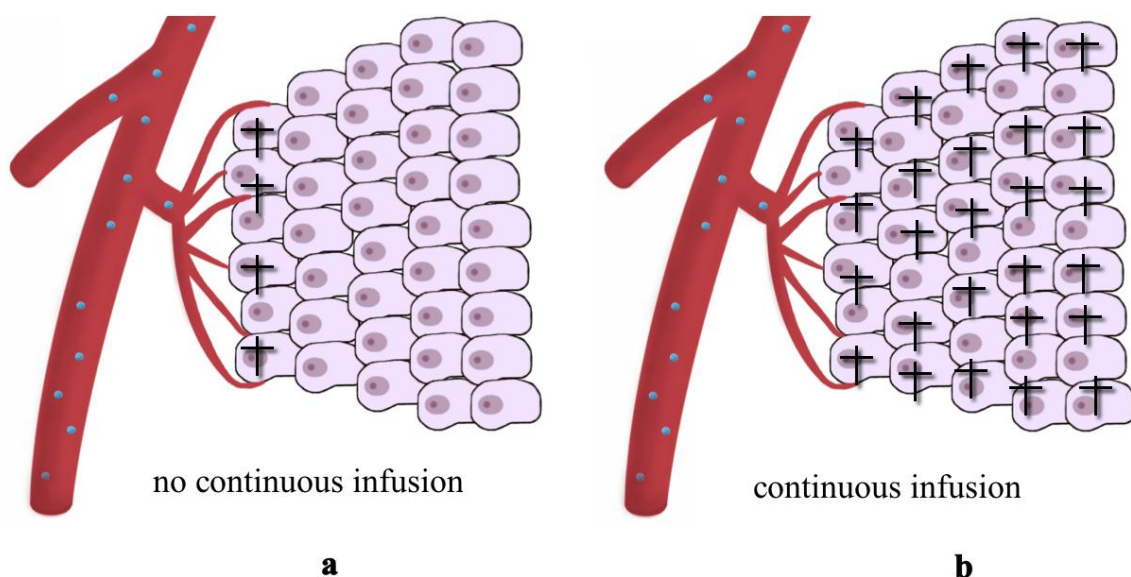
System	Components and final concentration	Gelation temperature ( $T_{\text{gel}}$ , °C)
S1	PF-127 (18%) SIL (4.16 mg mL <sup>-1</sup> )	21.0 ± 0.5
S2	PF-127 (18%) SIL (4.16 mg mL <sup>-1</sup> ) MSNs (1.0 mg mL <sup>-1</sup> )	20.0 ± 0.5
S3	PF-127 (18%) SIL (4.16 mg mL <sup>-1</sup> ) MSNs (5.0 mg mL <sup>-1</sup> )	19.0 ± 0.5

The potential for prolonged release was confirmed during the *in vitro* dissolution experiment (Fig. 2). Considering the concentration gradient induced by cells permanently on the hydrogel, it is expected that the hybrid system will delay the release of the components after the application.



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

The gelation of the system after its inoculation leads to a prolonged release of the components as the gel dissolves inside the animal body, which is a key factor to get a better drug distribution in tumors [13, 14]. It is known that the drug access to tumor cells is achieved via bloodstream [13, 14]. So, if there is no continuous infusion, only drugs that are near the blood vessels can be killed and cells that are more distant are able to survive and regenerate the tumor [13, 14]. Oppositely, a continuous infusion permits a longer residence time of the drug, ensuring a better distribution so a larger number of cells are reached (Fig. 3).



**Figure 3.** Schematic illustration of drug distribution in **a)** no continuous infusion and **b)** continuous infusion (prolonged-release)

#### *In vivo macroscopic analyses*

After treatment, macroscopic changes regarding organ conditions 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 apparent macroscopic changes	100%	-	-	-	66,6%
Lesion in prostate base	-	100%	100%	66,6%	33,3%
Metastasis	-	100%	100%	33,3%	-

It is possible to observe in Table 2 a decrease in frequency of metastasis and prostatic lesions that is clearly proportional to MSNs concentration. This calls the attention for the role of the nanoparticles in this process, suggesting that the MSNs might be determinant in a possible improvement of tumor conditions.

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 [15]. This may increase the delivery of SIL, leading to a combined effect from the drug and the nanoparticles on tumor cells.

#### 4. Conclusions

To get preliminary results of the antitumor potential of the association of SIL, MSNs and PF-127, we developed three systems that differed only by the MSNs concentration and treated rats with chemically induced prostate cancer. Preliminary macroscopic analyses showed an interesting decrease in frequency of metastasis and prostatic lesions that was proportional to MSNs concentration. The results suggest that the MSNs might be determinant in the treatment. Further studies are necessary to confirm the antitumor potential of the systems, but in a first moment, we can associate the results to a combined effect from SIL and MSNs, caused by EPR effect along with sustained and prolonged release of the drug. This avoids premature degradation of the drug and enables its cell internalization, achieving a better drug distribution and increasing its efficacy. Thus, we open a new perspective of using MSNs as carriers for SIL in a new platform for prostate cancer treatment, using SIL as the main therapy.

#### 5. Acknowledgements

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