Enhanced in vitro antitumor activity of a titanocene complex encapsulated into Polycaprolactone (PCL) electrospun fibers

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"Enhanced *in vitro* antitumor activity of a titanocene complex encapsulated into Polycaprolactone (PCL) electrospun fibers"

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Abstract

Purpose: We aimed to achieve detailed biomaterials characterization of a drug delivery system for local cancer treatment based on electrospun titanocene trichloride-loaded resorbable polycaprolactone (PCL) fibers. **Methods:** The PCL fibers, were characterized for their structural, morphological and physical properties. The drug release kinetics of the titanocene complex was investigated at different concentrations, to obtain a set of correlations between structure and tuneable release. After exposing cancer cells directly onto the surface of PCL fibers, the anti-proliferative effects of titanocene-loaded PCL were evaluated by: (i) counting viable cells via live/dead staining methods, and (ii) analyzing cell apoptosis. **Results and Conclusion**: Titanocene concentration influenced fiber diameters that were reduced for PCL filled with the titanocene. X-rays analysis suggested that the titanocene, encapsulated into the PCL fibers, is not allowed to crystallize and exists as amorphous aggregates into the fibers. The titanocene release curves presented two stages not related to PCL degradation: an initial burst release followed by a release linear with time, extending for very long time. All of the titanocene-loaded fibers showed sustained drug release properties suggesting their potential clinical applicability for the treatment of local cancer diseases.

Running title: Antitumor activity of titanocene loaded PCL electrospun fibers.

Keywords: PCL electrospun fibers, titanocene complex, antitumor release, *in vitro* antitumor activity.

Introduction

In the last decades the successes reached by drug delivery systems based on nano-structerd biomaterials have been particularly relevant for cancer treatments.

In particular, the use of electrospun micro- and nano-fibres as antitumor drug carriers has attracted a great deal of attention as targeting delivery system of the antitumor drugs in postoperative local chemotherapy (1-15). Recent researches have shown that the fibers have many advantages, such as reduced toxicity and optimal drug release profile (16-28). Xie et al. encapsulated paclitaxel into PLGA and report that electrospun microfibers and nanofibers are efficient for sustained delivery of paclitaxel to treat C6 Glioma *in vitro*. They showed that the efficacy of the encapsulated molecule was comparable to the commercial paclitaxel formulation (29). Jing et al. developed implantable poly (ethylene glycol)-poly (L-lactic acid) diblock copolymer fibers for the controlled release of 1,3-bis (2-chloroethyl)-1-nitrosourea (30).

Recently, electrospun fibers as well as silica and non-silica-based materials have been used to incorporate organo-metallic compounds, such as titanocene dichloride, in order to improve both their safety and efficacy. Indeed, the titanocene complexes show low solubility and short half life in the human body, characteristics that have negatively affected their efficacy in phase II clinical trials in patients with metastatic breast cancer and metastatic renal cell carcinoma. Although titanocene dichloride, incorporated into PLLA fibers by electrospinning method, was released in an active form from the system, it is also evident that titanocene dichloride release mainly depended on PLLA degradation rather than simple diffusion from the fibers (31). Central to the further development of these titanocene-loaded PLLA fibers for application in local anti-cancer treatment is the prospective ability to control their degradation rate and the delivery of titanocene complexes. A problem not easy to solve since the inflammatory response associated with PLLA fibers implantation induces the production of “reactive oxygen species” (ROS) and enzymes, such as serine proteases and lipases, that might dramatically increase the PLLA degradation rate (32, 33)

Several studies have demonstrated that the transport of titanocene complexes into the cell, their binding to DNA and to biologically important molecules, and their citotoxicity may change on the basis of the titanocene derivative used.

These results moved our interest to the study of the dependence both of the titanocene complex type and of the biomaterial used for fiber synthesis on the final anticancer activity.

Poly(ε-caprolactone) (PCL) is selected as the candidate synthetic polymer because it is electrospinnable (34), biocompatible, biodegradable, economical and widely used for various biomedical applications including drug delivery system. PCL is a semicrystalline polymer with a melting point of about 60°C and a glass transition temperature of -60°C, which gives the polymer a rubbery behavior at room temperature and, as a consequence, good permeability to low molecular weight drugs in delivery systems. The homopolymer has a degradation time of the order of two years due to its hydrophobic and semicrystalline nature (35).

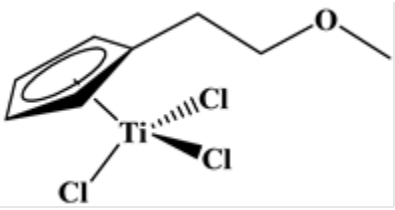
In addition, PCL nanofibers prepared by simply electrospinning blends of drug and polymer carriers for drug releases for pharmaceutical applications has been reported. However, the major disadvantage of this blend-electrospinning procedure is the severe burst release phenomenon (36). Burst releases lead to higher initial drug delivery, reducing the effective lifetime of the device (37). Many investigations using nanofibers for release applications have demonstrated the weakness of blend-electrospinning method and highlighted the significant role of drug-carrier interaction in controlled releases (38).

Between the titanocene complexes, our previous researches have demonstrated that titanocene trichloride showed the best performance in terms of hydrolytic stability .

In addition, titanocene trichloride, which has only one cyclopentadienyl ring coordinated, so less steric encumbrance, with more electronic unsaturation, and having three leaving groups, showed the best dispersion into the PCL fibers.

Finally, titanocene trichloride has shown a significant cytotoxic activity, similar to the cisplatin's on human embryonic kidney cells (1).

In this paper we report a study of the parameters for incorporation of a titanocene three chloride complex, shown below:



in biocompatible polymeric Polycaprolactone (PCL) nano-fibrous mats, obtained by electrospinning technology. The non woven membranes, obtained by optimizing the process parameters, were characterized for their structural, morphological and physical properties. The drug release kinetics of the titanocene complex was investigated at different concentrations, to obtain a set of correlations between structure and tuneable release. The anti-proliferative effects of complex and titanocene complex-loaded PCL was evaluated on glioblastoma cell line.

Materials and Methods

Materials

All manipulations were carried out under oxygen- and moisture-free atmosphere in a MBraun MB 200 glove-box. All the solvents for the synthesis were thoroughly deoxygenated and dehydrated under argon by refluxing over suitable drying agents, while NMR deuterated solvents (Euriso-Top products) were kept in the dark over molecular sieves. The anhydrous compound TiCl4, (Strem, Aldrich) was used as received. Potassium hydride and lithium buthyl, PCL (M_n 80 000), acetone and phosphate buffer were purchased from Sigma-Aldrich. A mechanical mixture of the PCL and PCL + 5% [C_5H_4-$CH_2CH_2OCH_3$]$TiCl_3$ powders was obtained by moulding both components in a hot press (Carver Inc.) at 90 °C, forming a 50 ± 5 μm thick film, which was rapidly quenched in a bath at 25°C. Glioblastoma human cancer cell line (A-172) used in the present investigation was obtained from ATCC (American Type Culture Collection), grown in Dulbecco's Eagle medium, supplemented with 10% fetal bovine serum, penicillin (100U/mL) and streptomycin (100mg/ml) (all purchased from Hyclone), at 37°C in a 5% CO_2 and 95% air atmosphere.

Synthesis of [C_5H_4-$CH_2CH_2OCH_3$]$TiCl_3$

The half-titanocene [C_5H_4-$CH_2CH_2OCH_3$]$TiCl_3$, was synthesized following the procedure previously reported in the literature (13, 39).

To a solution of neutral ligand [C_5H_5-$CH_2CH_2OCH_3$], prepared following the procedure reported in the ref. (45) (1.0 g, 8.1 mmol) in THF dry (40 ml), a stoichiometric amount of *n*BuLi (2.5 M solution in hexane, 3.5 ml) was slowly added at -78°C. The solution was warmed up to room temperature and left stirred overnight, obtaining a yellow lithium intermediate. Afterward the solution was treated at -78°C with 0.85 ml (8.1 mmol) of $TiCl_4$ and stirred overnight and then it was filtered to remove LiCl. The solvent was evaporated at reduced pressure and the red-brown solid dried in vacuum. The yield was quantitative. Elemental analysis (C, H, N) agreed with the proposed formulation and ^1H and ^{13}C NMR experiments were used for the full characterization of the complex.

Electrospinning Procedure

Electrospinning of PCL fiber was carried out at room temperature at a high voltage of 20-30 kV (HV Power Supply, Gamma High Voltage Research, Ormond, FL). The spinneret used in the experiments had an inner diameter of 0.8 mm. A copper wire was mounted in the spinneret and used as the positive electrode. Grounded aluminium foil was used as the counter electrode and mounted at a distance of 20, 25 and 30 cm from the spinneret.

Voltage, distance of the counter electrode, flow rate, polymer concentration were optimized to produce PCL nano-fibrous mats composed of individual fibrils without bead formation. PCL with 1%, 3% and 5% (w_t/w_t) of the Titanocene complex, respect to the polymer, were stirred vigorously for 1 h in acetone until complete dissolution of both components, and then spun utilizing the same conditions of pristine PCL.

Material characterization

X-ray Diffraction (XRD) Measurements. XRD measurements were carried out on the spun samples with a Brucker diffractometer (equipped with a continuous scan attachment and a proportional counter with Ni-filtered Cu KR radiation (λ) 1.54050 Å).

Scanning Electron Microscopy (SEM). The morphology and diameter of the electrospun nanofibers were determined with a scanning electron microscope (SEM; JEOL JSM-T300). A small section of the fiber mat was placed on the SEM sample holder and sputter-coated with gold prior to the analysis.

The fiber diameter distribution was determined by Sigma SacnPro 5. About 200 fibers were considered, comparing their dimensions respect to the reference bar of SEM image. Samples were taken from many places of the membranes to control the homogeneity of the fibers distribution.

Energy Dispersive X-ray Spectroscopy (EDX). Some samples were also characterized by microanalysis to investigate their chemical structure. Elemental analysis and element mapping were conducted with a field-emission scanning electron microscope (FESEM, model LEO 1525, Carl Zeiss SMT AG, Germany) equipped with an EDX spectroscope (INCA Energy 350, Oxford Instruments, Witney, UK).

Elemental analysis. The elemental analyses for C, H, N, Cl were recorded on a ThermoFinnigan Flash EA 1112 series and were performed according to standard microanalytical procedures.

NMR analysis. 10 mg of complex was dissolved in $CDCl_3$ and ^1H and ^{13}C $\{^1H\}$ NMR

spectra were recorded at 298 K on a Bruker Avance 300 spectrometer operating at 300 MHz (^{1}H) and 75 MHz (^{13}C) and referred to internal tetramethylsilane.

Release kinetics. The *in vitro* release kinetics of the Titanocene molecules in a fixed volume of a physiological saline solution or in phosphate buffer solution were obtained by ultraviolet spectrometric measurement at ambient temperature, using a Spectrometer UV-2401 PC SHIMADZU.

Solutions were prepared at a known concentration of titanocene and a linearity between the absorbance and concentration was found. The tests were performed using rectangular specimen of 8 cm^2, with thickness of 30-60 micron and weight of 20 mg, placed into 25 mL of physiological saline solution (0.9 %) at room temperature and 100 rpm in an orbital shaker (VDRL MOD. 711+, Asal S.r.l.). After specific intervals, the solution was removed and the absorbance measured, and the same volume was replaced with fresh one. The concentration value at specified time intervals was derived from the linear dependence of concentration on absorbance.

Determination of cell viability and apoptosis.

In vitro antitumor activities of titanocenes loaded-PCL fiber mats were analyzed by Apotox-GloTriplex Assay (Promega, Milan, Italy). Exponentially growing A-172 cells were seeded in quadruplicate into 96-well flat-bottomed plates in 200 µl of complete medium at a concentration of 5×10^3 cells/well and allowed to attach overnight. . After 24 h, complex and PCL+complex (5%) fiber mat were added to cell cultures and culturing for 96 h. Then, 20 µl of Viability/Cytotoxicity reagent containing both GF-AFC substrate and bis-AAF-R110 substrate was added to all wells, and briefly mixed. After incubation for 30 min at 37°C, fluorescence (400Ex/505Em) was measured by a Fluorescence Multi-well Plate Reader. For analysis of apoptosis, 100 µl of Caspase-Glo 3/7 reagent was added to all wells and incubated for 1 h at room temperature. Luminescence was measured and recorded. Control wells (100% viability), in which the test compound was absent, were included in all experiments. All data points represent an average of at least four assays.

Measurement of cell density

The cell density was evaluated at different time points by phase-contrast microscopy after being exposed to control PCL or PCL+complex (5%) fiber mats. A-172 cells (5×10^4) were seeded into 24-well culture plate, incubated with PCL or PCL+complex (5%) up 96 h, then stained with trypan blue and photographed post-plating under a phase-contrast

microscope with the use of an Olympus camera and Kodak T-Max 400 film, at the same magnification for all cultures. Five random fields were selected containing minimally 200 cells/field. Data from three to seven assays were analyzed for statistical significance by analysis of variance (ANOVA), and are shown graphically as mean ± SEM (p values <0.05 were defined as significant).

Results and discussion

Morphology and structure of the pure and filled samples

Many internal as well as external parameters can influence the electrospinning process and, as a consequence, the fiber morphology. Indeed it is difficult to isolate the effect of each parameter since they all are interrelated. For this reason, typically a trial-and-error approach has been employed by varying the solution properties and spinning parameters until uniform defect-free fibers are obtained.

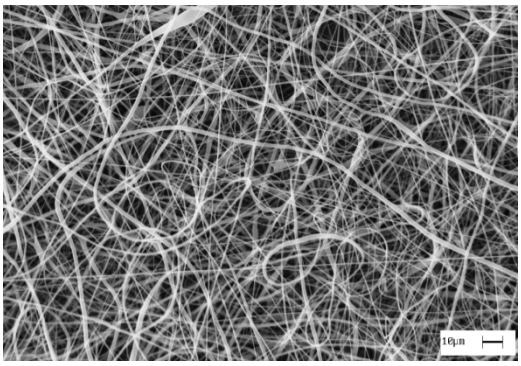
According to the results of a previous paper (40), we chose acetone, as solvent for PCL and PCL loaded with different concentrations of titanocene, also considering that titanocene is soluble in this solvent. Dissolving PCL in acetone, we tried different polymer concentrations, with a distance of the needle from the screen of 20 cm, 25 cm or 30 cm, alternatively. The concentration of the PCL solutions influences the spinning of fibers and also controls the morphology. As reported in literature (13-23), also in our case the formation of beads along the fibers took place at low PCL concentrations, and at high flux (mL/h).

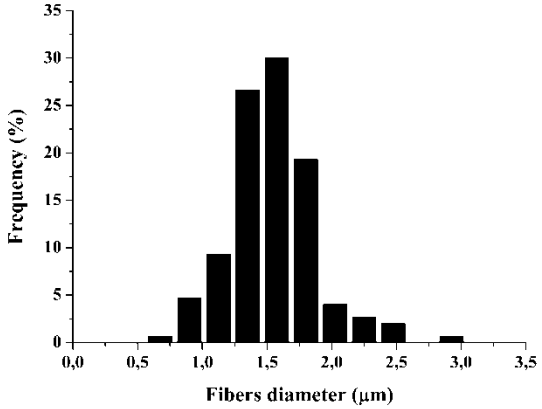
As the concentration of PCL was increased and the flux decreased, continuous nanofibers without bead formation were obtained by electrospinning from 15 and 17.5 wt % solutions.

Analyzing the experimental results, we chose a concentration of 17.5 %, with a distance of 30 cm, to spin either the pure PCL or the samples filled with titanocene, and in Figure 1 we show the SEM of the obtained fibers of PCL (a), and PCL charged with titanocene at 1% (b), 3% (c), and 5% (d). The fibers diameter distribution is shown for each sample, too.

Fig. 1

(a)


10µm


Frequency (%)
0
5
10
15
20
25
30
35
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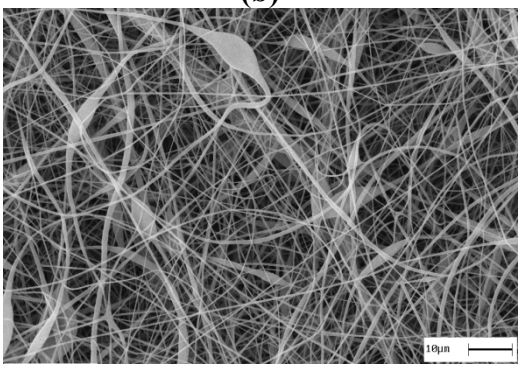
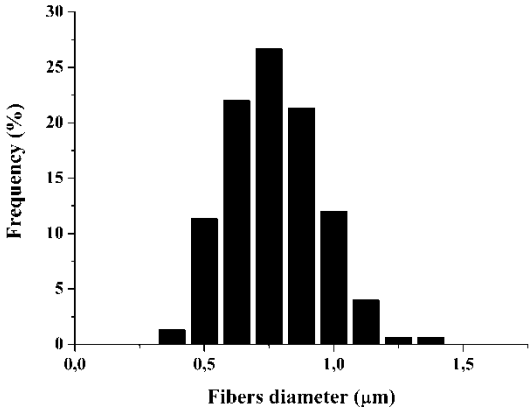
2,0
2,5
3,0
3,5
Fibers diameter (µm)

(b)


10µm


Frequency (%)
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25
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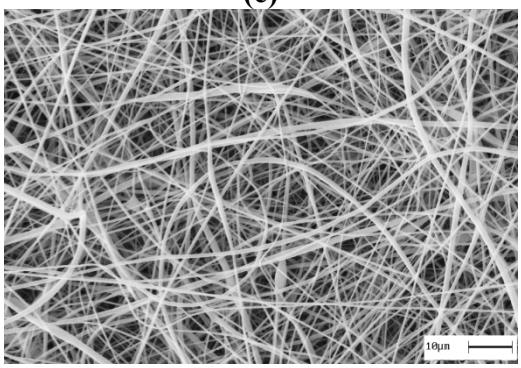
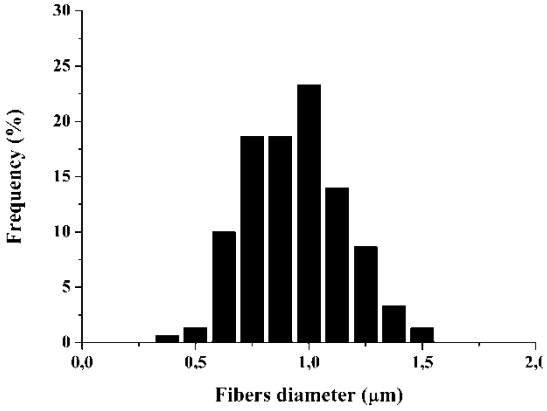
0,0
0,5
1,0
1,5
Fibers diameter (µm)

(c)


10µm


Frequency (%)
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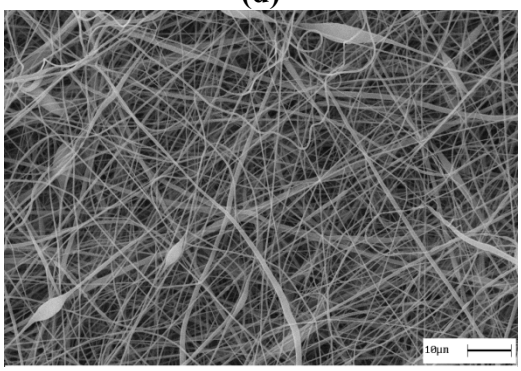
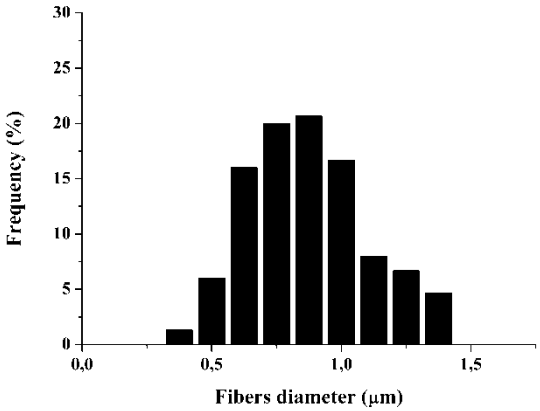
30
0,0
0,5
1,0
1,5
Fibers diameter (µm)

(d)



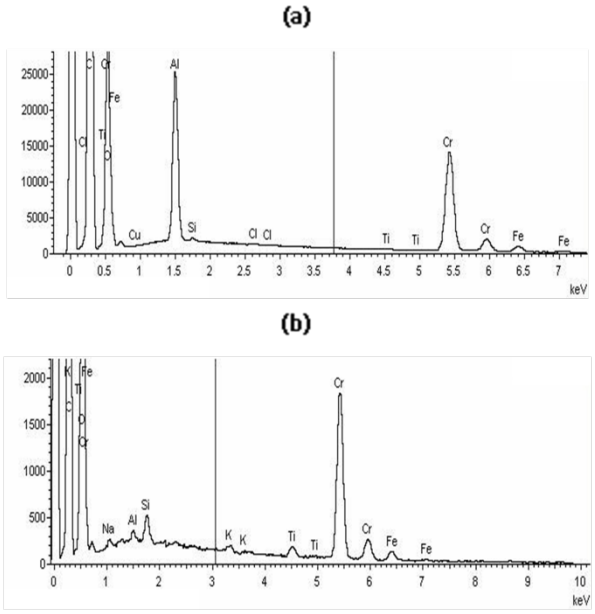


The nano-fibrous structure of pristine PCL sample is evident, composed of individual, uniform, and randomly oriented fibers with an average diameter at 1.5 μm.

The addition of the titanocene complex at different concentrations of 1%, 3%, and 5% caused no noticeable change in the morphology. However it resulted in electrospun nanofibers with significantly lower average diameter, in comparison with pure PCL. The fiber diameters are centred at 0.75 μm (1%), 1.0 μm (3%) and 0.85 μm (5%) for the three concentrations, respectively. Thus, it is most probable that the dispersion of titanocene complex structures improved the electrospinnability of the mixture, as denoted from the lower average diameter of nanofibers obtained. Since increase in conductivity and charge density generally is reported in literature as responsible of the smaller fibre diameters, we can hypothesize this effect by adding the titanocene complex into the PCL solution in acetone. Some rare beads appear, possibly due to clusters of the titanocene complex molecules on the surface of the microfibers.

To determine the structural organization of the titanocene complex in the PCL fibers, we determined the chemical structure on the surface of the composite fibers with FESEM-EDX. Figure 2 shows the EDX analysis of electrospun fibers PCL + 5% Titanocene on the surface (a) and after thermal degradation at 450°C (b).

Fig. 2



On the surface (2a) besides the elements always appearing in the micrographs (such as Fe and Cr due to the iron support of the samples and Al, on which the membrane was electrospun) we observe that the peaks of Titanium are very small, almost in the noise. However, if we submit the sample to a thermal oxidation up to 450°C, followed by an EDX analysis (2b) we observe the peaks of Titanium much higher respect to the very small ones on the surface. This is an indication that the titanocene complex is located inside the fibers, probably with a very small fraction on the surface.

A confirm of the titanocene presence into the electrospun membranes was obtained by analysing a part of the membrane treated with the complex via ^{1}H NMR in acetone-d_6, and shown in Figure 3, in which ^{1}H-NMR spectra of: PCL (3a), $[C_5H_4\text{-}CH_2CH_2OCH_3]TiCl_3$ (3b) and PCL+ 5% $[C_5H_4\text{-}CH_2CH_2OCH_3]$ $TiCl_3$ (3c) are reported.

We observe the presence of the peaks ascribable to the complex well developed and evident. However some of the peaks of the complex changed significantly their chemical shift. In fact, for example, by examining the zone relative to the hydrogens of cyclopentadienyl we noticed some signals shifted to lower fields (see Fig. 3). This fact can be tentatively attributed to a strong interaction between the complex, in neutral or cationic form, and the membrane. The cationization could occur because of strong electric field used for electrospinning.

Fig. 3

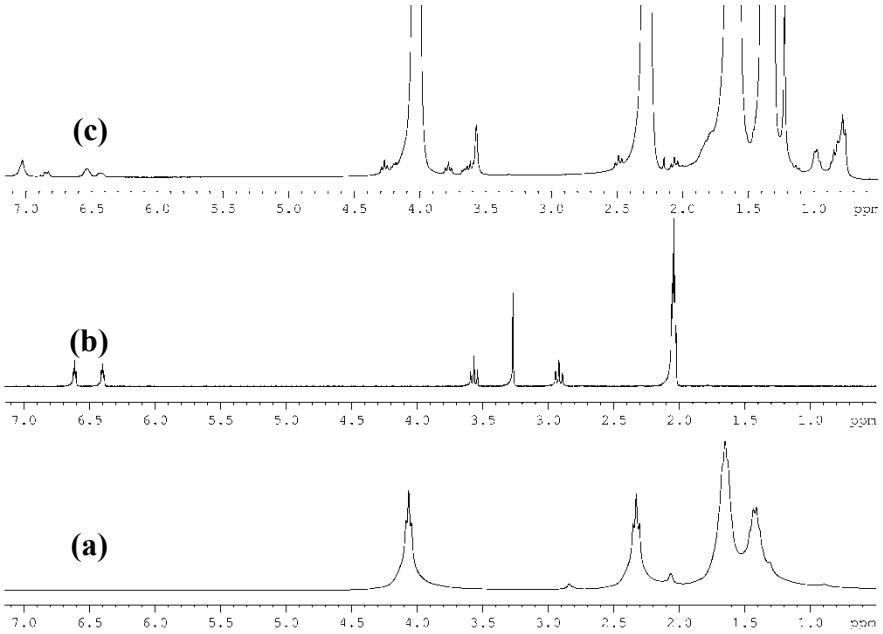
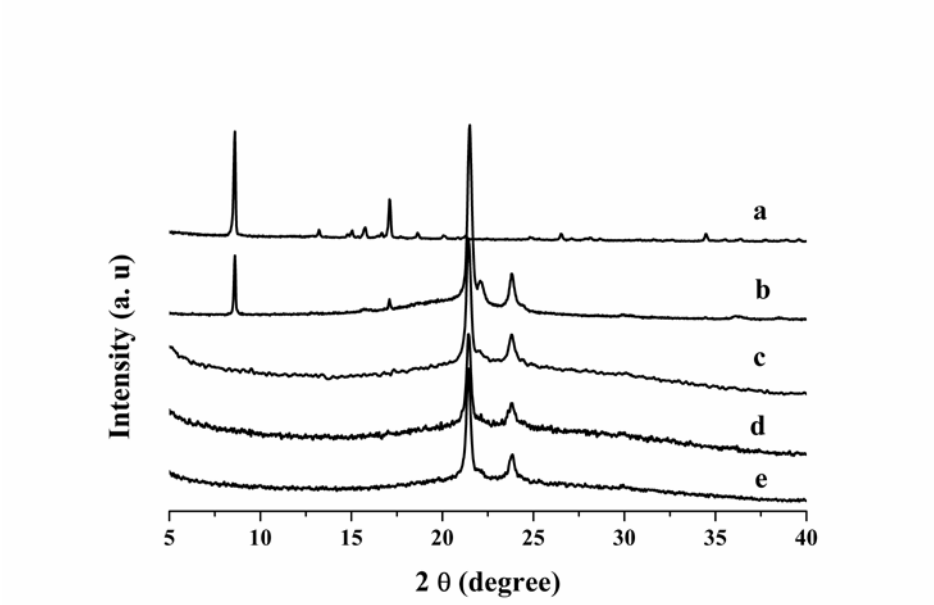


Figure 4 shows the XRD diffractograms of pure $[C_5H_4\text{-}CH_2CH_2OCH_3]TiCl_3$ (a); PCL+ 5% [C5H4-CH2CH2OCH$_3$]TiCl$_3$ mechanical mixture (b), electrospun PCL charged with titanocene at 1% (c), 3% (d), and 5% (e).

Fig. 4



The $[C_5H_4\text{-}CH_2CH_2OCH_3]TiCl_3$ complex powders (4a) are very crystalline, showing the most intense peaks at 8° and 17° of 2ϑ. The PCL, spun from the 17.5 % solution in acetone, with 1% (4c), 3% (4d) and 5% (4e) shows the PCL crystalline structure well developed with the main peaks appearing at 21.4° of 2ϑ and 23.8° of 2ϑ and a reduced amorphous fraction. Interestingly, observing the diffractograms of the filled electrospun membranes, we can notice that even in the most concentrated sample, that is the 5% $[C_5H_4\text{-}CH_2CH_2OCH_3]TiCl_3$, the crystalline peaks of the titanocene complex do not appear at all (4e). We suggest that the complex, encapsulated into the PCL fibers, is not allowed to crystallize and exists as amorphous molecular aggregates or solid solution into the fibers. This result was already reported for a titanocene-dichloride in polylactic acid (PLLA) electrospun from dichloromethane (25). To ascertain that the absence of crystalline peaks of $[C_5H_4\text{-}CH_2CH_2OCH_3]TiCl_3$ is not due to its low concentration, we prepared a mechanical mixture of the complex and PCL, at the same concentration (5% complex) as the electrospun membrane, and the diffractogram is shown in Figure 4 (b). We observe that both the most intense peaks of the titanocene complex are very well evident into the diffractogram, confirming that in the membranes, where the peaks do not appear, the complex is in the amorphous state.

In vitro Release Properties

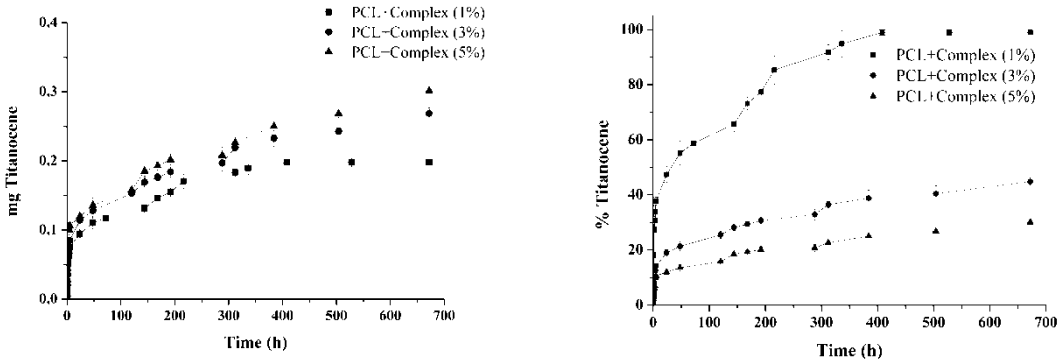
Because of the particular characteristics of the tumor microenvironment and tumor angiogenesis, it is necessary to design drug delivery systems that specifically target anti-cancer drugs to tumors. Most of the conventional chemotherapeutic agents have poor pharmacokinetics profiles and are non-specifically distributed in the body leading to systemic toxicity associated with serious side effects. Therefore, the development of drug delivery systems able to target the tumor site is becoming a real challenge that is currently addressed. To this aim it is of utmost importance to tailor the release profiles in different environments, depending on the many parameters that it is possible to modulate for the specific applications. For a local controlled release of elecrospun membranes it is possible to modulate: a) drug concentration; b) drug location (inside and/or outside the fibers); c) diameter of the fibers, which in turn determines the membrane porosity; d) biodegradation time of the polymeric matrix, that can be quicker than drug diffusion or can

simultaneously occur, contributing to the delivery. In the present case we chose a polymer, PCL, whose biodegradation is very slow, and therefore we can modulate other parameters without its influence. We investigated the effect of varying the concentration of the titanocene complex, whose location is mainly inside the PCL fibers and studied the release kinetics in physiological and phosphate buffer solution. In forthcoming papers we will investigate the other parameters (fiber diameter and polymeric matrix biodegradation time) with the aim to reach a complete correlation picture for the release of the titanocene complex from electrospun polymeric membranes.

In Figure 5 the absolute and pecentage release of titanocene at different concentrations, monitored up to 29 days, is shown. As explained in the experimental part, we took rectangular specimen of 8 cm^2 of same weight (20 mg), and therefore the maximum quantity of complex to be released is 0.2 mg (for 1% membrane); 0.6 mg (for 3% membrane); and 1 mg (for 5% membrane).

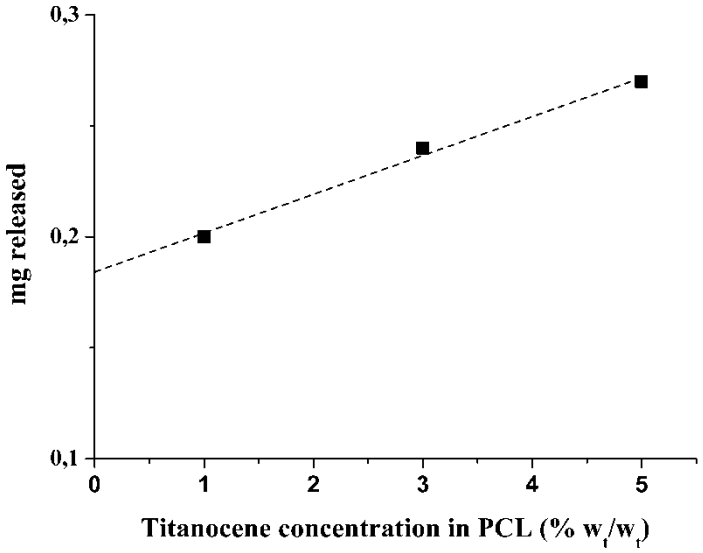
Since in the first stage the fraction of complex released for the membrane with 1% of it is about 50%, this membrane reaches the 100% release in a shorter time respect to the others, that is after 400 hours. The 3% and 5% membranes, at this time have released 38% and 21%, respectively. If the quantity of complex released in the burst is mainly dependent on the geometric factors, it is evident that it influences the less concentrated samples, and its influence decreases on increasing the complex concentration.

Fig. 5



The three curves present mainly two stages: a first stage, quick as a "burst" followed by a successive stage, in which the release of the complex is linear with time. It is worth noting that the absolute quantity (in mg) released in the first stage is almost the same for the three samples, and therefore it results independent of the initial concentration. This noticeable result is confirmed if we report the quantity of the complex delivered in the second stage (at 500 hours), as a function of the initial concentration, as shown in Figure 6:

Fig. 6

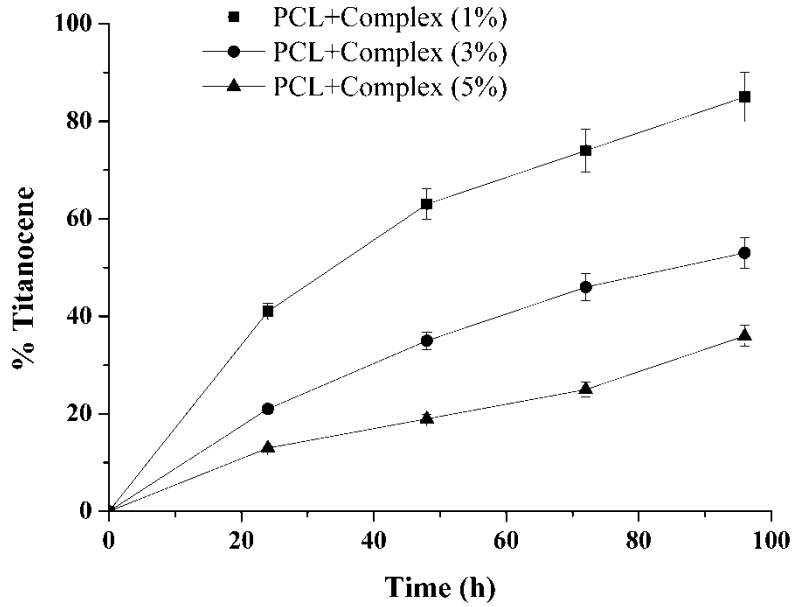


The points follow a straight line that extrapolates at zero loading to the same value (0.18 mg) observed in Figure 6, as first exit of the complex. The initial burst, found in many systems, has been generally associated to the drug on the surface of fibers, that is directly delivered, without being slowed down by the diffusion phenomena. The present result could indicate that the fraction of drug deposited on the surface, during the electrospinning process, mainly depends on geometrical factors and less on the drug concentration. This result would be very interesting, if confirmed, and needs further investigations to be accepted as a general behavior.

In figure 7 the release in phosphate buffer solution as % released up to 96 hours is reported. We observe a very similar behaviour as in the previous phisiological solution. Also in this case the amount released in the burst is very similar for the three

concentrations, indicating a general behaviour for titanocene complex into polycaprolactone.

Fig. 7



Thus, our study indicates that the use of titanocene loaded-PCL devices can alleviate the initial burst release and improve the sustainability of nanofiber-based releasing devices.

In vitro antitumor activity of titanocene on Glioblastoma cells

Glioblastoma is the most common and most aggressive primary brain tumor, with a median survival time from diagnosis of up to 1 year. Since the blood brain barrier prevents many chemotherapeutic agents from reaching these tumors in adequate concentrations, one approach could be targeted drug delivery to a particular site near the tumor to increase drug accessibility. Titanocene dichloride previously encapsulated into electrospun fibers shows some drawbacks such as low solubility, instability and short half-life in the human body (41), at variance with Titanocene three-chloride is more stable and more active. An intracerebral delivery system loading of carmustine [1,3-bis-(2-chloroethyl)-1-nitrosourea] has been shown to have promising initial activity and limited toxicity (42). However, relatively minor achievements were obtained with this approach, due to the resistance of many brain tumors to carmustine, as well as the low stability of the drug and its tendency

to ionize at physiological pH. In addition, the major limitations of these implants are in attaining the required amount of drug for a given amount of time in a proper distribution of the antiproliferative drug.

To assess the anti-tumor potential of titanocene complex in human glioblastoma cells, A-172 cells were treated at various concentrations (0, 1, 5, 10, 25, 50, 100, and 150 mg/L) of the drug, and cell viability was evaluated by trypan blue dye exclusion assay up 96 h.

Titanocene complex significantly inhibited the growth of cancer cells in a dose- and time-dependent manner, showing the half maximal inhibitory concentration (IC50) value of 100 mg/L at 96 h culture. Surprisingly, the IC50 value of the complex after loading in the fibers was 11.60 mg/L after 96 h culture.

Next, we compared the anti-proliferative effect of the titanocene released from PCL with that of equimolar concentrations of titanocene complex, (here with control drug). In detail, using Apotox-Glo Triplex Assay we assess viability, cytotoxicity and apoptosis events in cells incubated in the presence of control drug (complex 5%) PCL and PCL+complex 5% fiber mats.

Figure 8a, shows that PCL+complex 5% induced a marked decrease in the vitality of A-172 cells relative to the control (60-65% decrease). Importantly, there was difference between the extent of activity of PCL+complex 5% and control drug ($p<0.05$; Fig. 8), indicating that titanocene's pharmacological activity was increased rather than decreased by the incorporation into PCL. Moreover, titanocene complex 5%-loaded PCL exerted cytotoxicity on 96 h, as indicated by decreased viability fluorescence and increased cytotoxicity fluorescence (Fig. 8b), while, complex 5% control drug, induced preferentially cell apoptosis with negligible level of cytotoxicity (Fig. 8c).

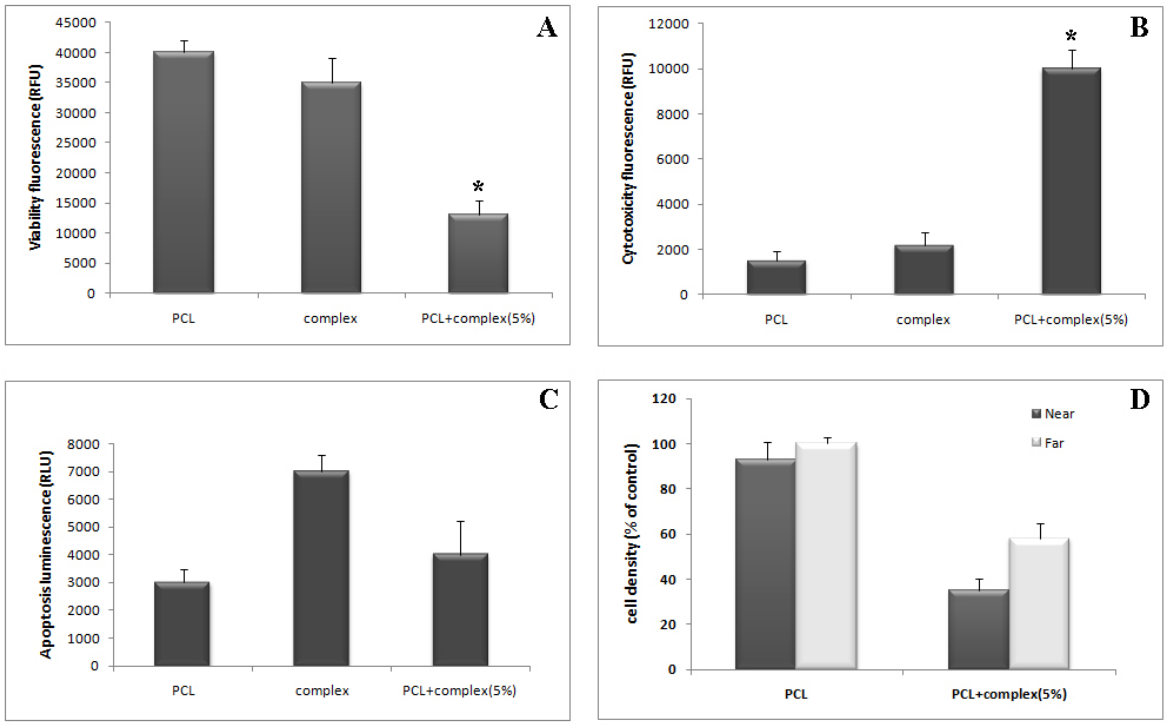
Considering that the concentration of free active complex in the medium was the same in all the cultures, these results seem to be apparently in contrast with the current opinion that the mechanism of titanocene-induced cell death is concentration dependent. Indeed, to maintain the sequence of events which lead to apoptosis, titanocene complex must remain under an overly toxic level, otherwise the cells will be driven into necrosis (41).

To provide answers for these questions we designed a set of experiments in which the density of cells were imaged at various time points after being exposed to control PCL or PCL+complex 5% (Fig. 8d).

A relatively homogenous distribution of cells with approximately the same density was observed around the control PCL fiber mat. In contrast, cell density around the PCL+complex 5% was significantly reduced at 96 h, whereas their density was higher at a relatively far distance from the fiber mat. Thus, we could demonstrate a decrease in cell density as a function of increased distance from titanocene-loaded PCL mats. Cell density was relatively low near the fiber, (cell exposed to the highest drug concentration), and relatively high close to the edge of the well, (cell exposed to a lower drug concentration). This correlation is consistent with cell necrosis and apoptosis near and at a greater distance from the fiber attributable to high and low drug complex concentrations, respectively.

Accordingly, Fung et al. reported a similar gradient concentration effect using either a Carmustine, 4-hydroperoxycyclophosphamide (4-HC), or paclitaxel-loaded polyanhydride pellet implanted intracranially in cynomolgus monkeys as a new modality of chemotherapy delivery in primates (42).

Fig. 8



Conclusions

The technical parameters for electrospinning a solution of PCL, and PCL filled with a Titanocene complex at different concentrations in acetone, were defined and set up. A trial-and-error approach has been employed by varying the solution properties and spinning parameters until uniform defect-free fibers are obtained. The morphological analysis showed pure PCL fibers with an average diameter of 1.5 μm, whereas the fiber dimensions were reduced for PCL filled with the titanocene complex to a mean diameter between 0.75 and 1.0 μm, indicating an improved electrospinnability of the mixture respect to pure polymer. X-rays indirectly suggested that the complex, encapsulated into the PCL fibers, is not allowed to crystallize and exists as amorphous molecular aggregates or solid solution into the fibers. The release properties in physiological and phosphate buffer solutions show two stages for all the samples: after an initial burst release (first stage) released titanocene complex was approximately linearly increased with time, extending for very long time (stage II).

Titanocene complex significantly inhibited the growth of cancer cells in a dose- and time-dependent manner, showing an IC50 value of 100 mg/L at 96 h culture. Surprisingly, the IC50 value of the complex after loading in the fibers indicates that the titanocene pharmacological activity was increased rather than decreased by the incorporation into PCL.

Overall, we have described an electrospun PCL delivery platform for a new family of compounds that show promising in vitro anti-tumor activity and may provide improved treatment options for the management of glioblastoma achieving an adequate drug level near the tumor cell. This is of primary importance because an inadequate tumor cell drug-burden will lead to low cell killing and to a potential for early development of drug resistance.

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Figure legends

Fig. 1 - Fibers diameter distribution of PCL (**a**), and PCL charged with titanocene at 1% (**b**), 3% (**c**), and 5% (**d**).

Fig. 2 - EDX micrograph of PCL + 5% [C_5H_4-$CH_2CH_2OCH_3$] on the surface (**a**) and after thermal degradation of PCL at 450°C(**b**).

Fig. 3 - ^{1}H-NMR spectra of: (**a**) PCL, (**b**) [C_5H_4-$CH_2CH_2OCH_3$]$TiCl_3$ $TiCl_3$ and (**c**) PCL+ 5% [C_5H_4-$CH_2CH_2OCH_3$].

Fig. 4 - X-ray spectra of: (**a**) [C_5H_4-$CH_2CH_2OCH_3$]$TiCl_3$, (**b**) PCL+ 5% [C_5H_4-$CH_2CH_2OCH_3$]$TiCl_3$ mechanical mixture, electrospun PCL charged with titanocene at 1% (**c**), 3% (**d**), and 5% (**e**).

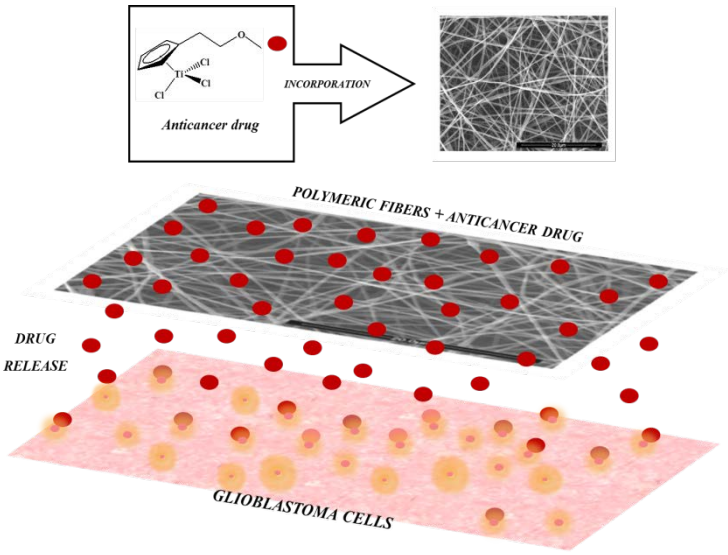
Fig. 5 - *In vitro* release of titanocene complex.

Fig. 6 - Titanocene mg released *vs* Titanocene concentration in the membrane.

Fig. 7.- Percentage of release of titanocene complex in phosphate buffer.

Fig. 8 - Antitumor activity of titanocene-loaded PCL on A-172 glioblastoma cells at 96 h. (a-c) Effect on cell viability, cytotoxicity and apoptosis; (d) effect on cell density around control PCL and PCL+complex (5%) fiber mats.* indicated $p < 0.05$.

TOC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