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Low Pressure Plasma Processing of Collagen Membranes for Anti-Cancer Drug Delivery

Alibi Baitukha[1]*, Iman Al-Dybiat[2], Azadeh Valinataj-Omran[2], Jerome Pulpytel[1], Marc Pocard[2], Massoud Mirsha'shi[2] and Farzaneh Arefi-Khonsari[1]

[1]*Sorbonne Universités, UPMC Univ Paris 06, CNRS, Laboratoire Interfaces et Systèmes Electrochimiques, 4 Place Jussieu, 75005, Paris, France*
[2]*Sorbonne Paris Cité, UMR Université Paris 7, INSERM U965 Carcinose, Angiogenèse et Recherche Translationnelle, L'Hôpital Lariboisière, 41 Bd de la Chapelle 75010 Paris, France*

Abstract

For targeting the cancer cells, a low pressure ICP reactor was utilised to fabricate a drug delivery system. On the surface of biocompatible collagen membranes, thin plasma polymer coatings were deposited to encapsulate an anticancer drug carboplatin and impart different biologically active functionalities. The characteristics of the deposited films were analysed by FTIR and XPS and the overall functionalities were tested by *in vitro* and *in vivo* experiments on the cancer cells lines on the cancer nodules respectively. Preliminary results of fabricated drug delivery systems showed the inhibition of cancer cell proliferation in vitro as well as tumour growth in mice models by 2.8 folds in mass compared to the control case.

Keywords: Delivery vehicle; Plasma polymerization; Low pressure inductively coupled plasma; Cell adhesive/repellent coatings

Introduction

Standard chemotherapy has a slight or no specificity for cancer cells, normally resulting in low deposit and accumulation at the tumor region and severe side effects. This disadvantage has led to the development of several strategies for chemotherapy agents to recover their concentration at the cancer nodule site, for increasing simultaneously their anti-cancer efficacy and reducing undesirable systemic effects. There are various types of drug delivery vehicles, such as polymeric micelles, liposomes, lipoprotein drug carriers, nano-particle drug carriers as well as the biocompatible and biodegradable membrane.

Among these carriers, collagen membranes are widely used in post-surgery treatments to provide isolation of operated zone, preventing adhesion of internal organs and scar tissue and intended to promote tissue regeneration [1,2]. Such membranes represent an excellent base for drug delivery systems (DDS) [3,4]. By encapsulating drugs on the surface of biocompatible patches with thin barrier films, it is possible to prolong the release of the drugs from the membrane into the surrounding tissue once it is placed after surgery on the operated zones and which provide the therapeutic effect as a drug delivery system [5].

Carboplatin is an anticancer platinum drug widely administered in clinical practice to treat different types of cancer [6-9]. In conventional practices, drug is systematically injected intravenously and then by covalently binding to DNA strains, it results in the formation of various defects in DNA molecules, forcing the cell to undergo apoptotic death path [10]. Because of the non-selective treatment of normal and cancer cells, the conventional treatments lead to many secondary effects and forces to dose limitation and decrease in efficiency [11]. In order to improve the selective drug delivery for the cancer treatment, it is necessary to develop new strategies and methods of extended and controlled drug delivery systems. A wide range of technologies are developed and applied [12,13], and the implantation of biocompatible polymer membranes loaded with drug is a trendy direction in this field and biodegradable membranes offer a great advantage of not requiring a surgical removal after depletion of the loaded drug [14,15]. The choice of the materials is limited to biocompatible ones. However to achieve required physico-chemical properties the surface of the materials should be tailored by processes such as grafting [16,17], or copolymerization [18-20].

Plasma polymerization is a universal technique to deposit films with a wide variety of properties ranging from dense barrier films to soft cell repellent coatings where the film properties can be controlled and changed *in situ* [21-23]. By plasma depositing thin coatings it is possible to add additional functions to the material and to control the biological activity on their surfaces [24,25]. In comparison to conventional wet chemical deposition processes, plasma enhanced CVD is a catalyst free, dry and solvent free process. Recent works showed that plasma co-polymerization of different organic precursors can be used to tailor specific physico-chemical properties on the surface of the films to control cell interactions on the interface with implanted membrane [26-29]. Polycaprolactone (PCL) and polyethylene glycol (PEG) polymers are FDA approved materials and excellent candidates for drug delivery systems in the class of biodegradable polymers and were the choice of materials in our previous works with low pressure [30,31] and atmospheric pressure plasma systems [32].

In this research work we used a low pressure inductively coupled plasma assisted chemical vapour deposition system to evaluate the effectiveness of the fabricated drug delivery system to deliver carboplatin drug *in vitro* and *in vivo* experiments. The objective of application of such drug loaded membranes in clinical practice is their placement at the area where tumour was removed to provide drug delivery locally and to prevent the risk of metastasis. In the framework of this research the goal was to investigate the kinetics and duration of the drug release, to study in *in vitro* the interaction of the cancer cells

***Corresponding author:** Alibi Baitukha, Sorbonne Universités, UPMC Univ Paris 06, CNRS, Laboratoire Interfaces et Systèmes Electrochimiques, 4 Place Jussieu, 75005, Paris, France, Tel: 02 38 49 43 31; E-mail: sad.neva@gmail.com

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with the drug loaded membranes and finally to investigate in *in vivo* the interaction with the tumour nodules in mice model.

Experimental Equipment

Materials

Diethylene glycol dimethyl ether (diglyme, 134.17 g/Mol, $C_6H_{14}O_3$, Sigma-Aldrich) and ε-caprolactone (Sigma-Aldrich) were used as the precursor materials. As substrates 0.1 mm thick collagen membranes (Biom'UP COVA+) of biological origin were used. Carboplatin (CBDCA, 150 mg/15 mL, Hospira Inc) was used as antineoplastic (anticancer) drug in this study.

Inductively coupled plasma (ICP) setup

The low pressure ICP reactor is schematically illustrated in Figure 1a. Precursor materials delivered to the reactor through bubbling in argon gas. Prior to the deposition process, the vacuum chamber (approx. 3L) was evacuated down to a base pressure of 1 mTorr and kept for 20 min to degas. The operating pressure was set at 375 mTorr and controlled through adjusting argon gas flow rate and gate valve of turbomolecular pump. A 13.56 MHz RF power supply was controlled and programmed to deposit a sequence of layers with varying density and thicknesses. This allowed us to automate multi-step deposition procedures to deposit composite films with different deposition parameters and fabricate films with alternating or gradually changing density, chemical composition and therefore mechanical properties.

FTIR spectroscopy

FTIR spectra were taken using VERTEX 70 (Bruker) in attenuated total reflection mode with germanium crystal as internal reflection element. Infrared absorption spectra were recorded in the range 700-4000 cm^{-1}, with a resolution of 8 cm^{-1} and 100 repetitions. ATR correction of the baseline, CO_2/H_2O lines and the effect of the depth of penetration in ATR mode was performed in OPUS 6.5 software.

FE-SEM

Deposited films were studied by Field Emission Scanning Electron Microscopy (FE-SEM). FE-SEM images were taken using Zeiss Ultra 55 FEG SEM with GEMINI Column on gold coated surfaces by sputter coating (Cressinton sputter coater-108).

Ellipsometry

The refractive index and the thickness of films deposited on Si substrates were measured by spectroscopic ellipsometry performed on a UV-IR (193-1690 nm) variable angle spectroscopic ellipsometer (VASE) M2000DI from Woollam at an incidence angle of 70°, and the data analysis was performed with the CompleteEASE software.

XPS spectroscopy

High resolution X-ray photoelectron spectroscopy (XPS) (Thermo Scientific ESCALAB 250) was used to analyse retention of C-O-C groups from precursor on the deposited film surfaces. Spectrometer equipped with a micro spot monochromatized Al Ka source and a flood gun combined with an argon gun for compensation of sample electrostatic charging. The Al Ka line (1486.6 eV) was used throughout the work. Survey scans (pass energy 100 eV) and detailed spectra (pass energy 20 eV) were recorded for each sample. A photoelectron take-off angle of 90° relative to the surface was used, which corresponds to a sampling depth of approximately 5-8 nm for the C1s peak. The values of binding energies (BE, eV) were taken relative to that of C1s electrons of hydrocarbon present on the sample surface which is equal to 285.0 eV. XPS peaks were fitted using the Avantage software package, which consists of a non-linear least square fitting program. The surface composition was determined using the instrument manufacturer's sensitivity factors. The curve fitting process was done by imposing the peak full width at half maximum (FWHM) of the most resolved peak (C1s at 285.0 eV) to the other peaks.

ICP-MS

The kinetics of carboplatin release into Dulbecco's Modified Eagle Medium (DMEM) culture cell medium (Thermo Fischer scientific, France) was measured by detecting Pt concentration using ICP-MS (Inductively Coupled Plasma Mass Spectrometry, Elan DRCe, Perkin Elmer®). The mass bias was corrected with a certified reference material (Common Lead Isotopic Standard, SRM 981, NIST) using the standard bracketing technique [33,34].

Fabrication protocol

The fabrication of DDS takes several steps (Figure1b), starting with 100 µm thick collagen patch covered with 1 µm plasma polymerized barrier layer (this is done to prevent leaching of drug through the collagen patch). After placement of the drug on the barrier layer, it was encapsulated with the second plasma polymerized barrier layer of the same thickness of 1µm. On top of DDS antifouling PEG layer is deposited in soft plasma conditions to prevent adhesion of cells on its surface.

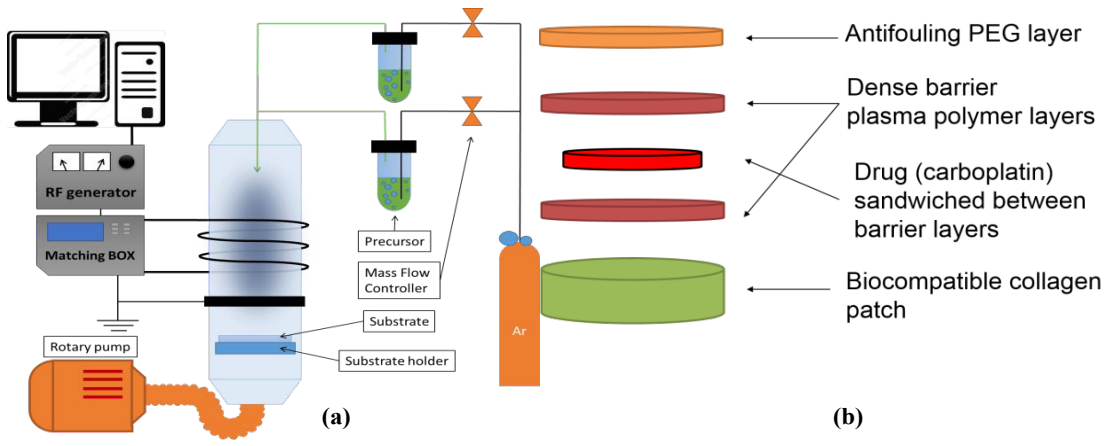

Figure 1: (a) Low pressure inductively coupled plasma enhanced chemical vapour deposition (ICP PECVD) reactor and (b) DDS structure.

In vitro and *in vivo* experiments

The ovarian cancer cell line OVCAR-3-NIH and the colon cancer cell lines CT26-luc were used (ATTC, Manassas, VA USA). In *in vivo* experiments were carried out on 8 to 10 week old BALB/c mice. 10^5 CT26 cancer cells in 100 µl of DMEM medium were injected into inguinal lymph nodes and incubated for 3-4 weeks (development of tumour). DDS membranes were positioned next to the developed tumour, and after 10 days, mice were sacrificed to analyse the interaction of tumour with the membrane. For anatomo pathologic analysis, the cancer nodules in all conditions were extracted and fixed by formaldehyde (4%) for paraffin embedding. The samples of 4 µm thickness were stained by Haematoxylin eosin coloration and analysed by photonic microscopy. Collected data were compared with the control which was the membrane with polymer coatings but with no encapsulated drug in between. The size of the developed tumour was estimated from photo analysis using ImageJ software in control case and with membrane loaded with 300 µg carboplatin before implanting membranes and after sacrificing the mice.

Results and Discussion

In this work and for the development of the DDS, we used the pulsed and continuous plasma polymerization of diglyme precursor only to obtain either cell repellent coatings with the retention of the ether functions of the precursor or highly cross-linked polymers respectively. To deposit cell adherent coatings, ε-caprolactone precursor was used in the pulsed plasma mode.

By applying an effective power of 25 W at 100% of duty cycle on mixture of 1.5 sccm of diglyme precursor in 30 sccm of working argon gas at 375 mTorr on 10 cm^2 substrates placed downstream of ICP plasma, it is possible to reach a high level of fragmentation of the introduced precursor and to deposit highly cross-linked dense films with a high refractive index equal to 1.83 (at 600 nm) similar to diamond like carbon films [35]. W/FM or also known as Yasuda parameter [36] was calculated for our system and the energy input per monomer flow was 64 MJ × kg^{-1} and according to the literature [37] the deposition process takes place in a monomer deficient zone. The FTIR spectra of such films (Figure 2, black line with 100% of duty cycle) show no retention of C-O-C bonds (1100 cm^{-1}) present in the precursor molecule.

Such coatings are excellent candidates for barrier coatings in drug delivery systems. In Figure 3 the drug concentration increase

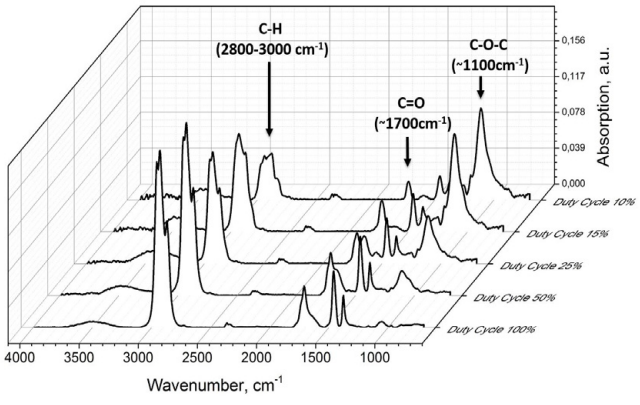

Figure 2: FTIR spectra of films deposited from diglyme precursor at different duty cycle: 10%, 15%, 20%, 25% and 100% (continuous).

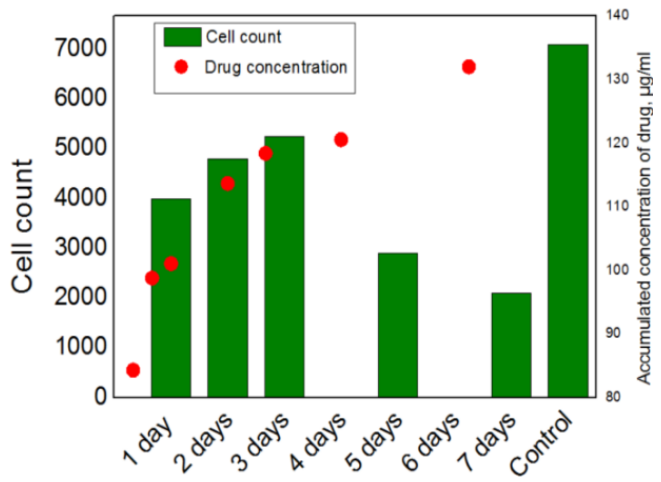

Figure 3: Left axis, green bars: OVCAR cancer cells count after incubation for 48h in the media with different amount of carboplatin released from DDS (1d, 2d, 3d, 5d, 7d). Right axis, red dots: Carboplatin release kinetics and accumulation in DMEM medium from 1 µm thick barrier layers detected by ICP-MS.

in the medium over the time and cell viability in different mediums with different amount of drug are represented. The carboplatin (300 µg) release and accumulation in DMEM medium (2 mL) measured by ICP-MS, sandwiched with barrier coatings from both sides with thickness of each 1 µm (red dots on the right axis). 1 µm thick barrier coatings enabled to extend drug release time to 7 days. The kinetics of carboplatin release was measured *in vitro* as well in the following experiments: prepared DDS membranes with encapsulated carboplatin (300 µg) kept in different wells filled with the medium (2 ml) for various times (24 h, 48 h, 72 h, 120 h, 168 h) and the accumulated amount of carboplatin in each well was different for each sample. Later incubation with OVCAR cancer cells for 48 h led to different levels of inhibition of cell proliferation depending on carboplatin concentration present in the medium (Figure 3, green bars).

The supporting membrane substrate originates from porcine collagen and once introduced into the aqueous medium tends to swell. The collagen membranes increase 5 times of its original thickness during the swelling process. The swelling in the surface area is more crucial as it induces additional stress on the barrier layer and strongly affects its integrity and ability to control kinetics of release. The surface area of the collagen membrane was measured by taking time-lapse photos (every 2 seconds for 20 hours) and subsequently area of the collagen surface was estimated by ImageJ software for each photo. The swelling of the collagen membrane was about 14 % of its original surface area. Also the internal stress in plasma polymers is induced due to the mechanism of growth [38,39]. In combination with swelling of the collagen as a substrate, accumulated stresses in barrier coatings leads to the formation of microcracks which give rise to a burst release of the encapsulated drug. It is difficult to control the kinetics of release from case to case and in order to reduce the formation of microcracks and the burst of the drug, we used the approach of depositing a multi-layer coating [40] with altering "soft" and "hard" layers, by varying the duty cycle of the pulse mode, during the deposition process. Such films showed good results and SEM an image of such multi-layer barrier coating is illustrated in Figure 4.

Pulsing and thus lowering the effective input power lead to retention of the functional groups of the precursor molecules and impart to

the deposited films a better retention of the functional groups of the precursor molecules. PEGylation is the process where PEG blocks are introduced onto the material to impart antifouling properties [41-43]. In Figure 2 FTIR spectra presented for films deposited at various pulsing duty cycles has been shown. Indeed, with a decrease of the duty cycle one can observe an increase of the ether functions C-O-C bonds (1100 cm^{-1}) from diglyme precursor molecule in the deposited film. At 10% duty cycle of 25 W effective input power is 2.5 W with Yasuda parameter equal to 6.4 $MJ \times kg^{-1}$, thus making it an energy deficient deposition process, and resulting in "soft" films with a low density and refractive index equal to 1.55 (at 600 nm).

To deposit coatings with more complex biological functionalities, plasma copolymerization of ε-caprolactone (PCL) and diethylene glycol dimethyl ether also known as diglyme (PEG), a mixture of the two different precursors could be fed into the reactor. In Figure 5, FTIR spectra are shown for films deposited in the pulsing mode from two precursors with different feed rate into plasma reactor. Ratio of PCL to PEG was calculated referring to the feed rate of the respective precursor into the reactor, which was varied in the range of 0-1.5 sccm. Both precursors result in biocompatible materials, PCL coatings are more hydrophobic compared to PEG ones, and thus by combining these two materials it is possible to control the hydrophilic properties of the resulting film. By controlling the flow rate of each precursor it is possible to control the chemical composition of the deposited film, and thus to control the functionality of the film.

Pulsed plasma deposited PCL film result in cell adhesive coatings, whereas PEG films are cell repellent [44,45]. As the plasma deposited layers will be in contact with tumours, it is important to observe the behaviour of cancer cells with the coatings. In Figure 6 SEM image with two different coatings on glass is shown. The sample was prepared in two steps, top part on the picture is cell adhesive PCL film, and lower part is PEG cell repellent film deposited under soft pulsed plasma conditions. The edge between cell adhesive and repellent is very distinct and defined by the sharp edge of the mask.

The high resolution C1s and survey XPS spectra of pulsed plasma polymerized diglyme films are shown in Figure 7, a) and b), respectively. Four component fitting was carried out of C1s spectra corresponding to: C-C/C-H (C1, 285.1 eV), C-O (C2, 286.6 eV), C=O (C3, 288 eV) and O=C-O (C4, 289.4 eV). The presence of an intense peak at 286.6 eV attributed to ether peak (C2/total C1s=68%) on the surface of deposited film confirms high retention of precursor moieties, in pulsed plasma deposition process [30].

In-vivo results

Intra-lymphatic injection with 10^5 cells in 100 µl of culture medium and afterwards incubation for 3-4 week period was carried out to

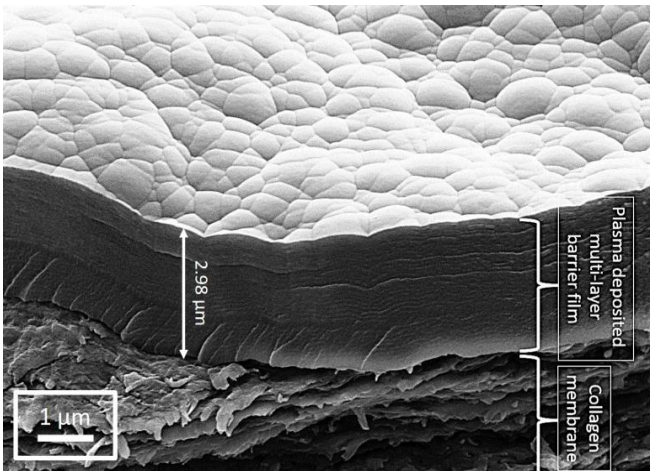

Figure 4: SEM cross-section image of multilayer barrier film with alternating 60 layers of 40 nm thick "soft" and 60 nm thick "hard" films on collagen membrane.

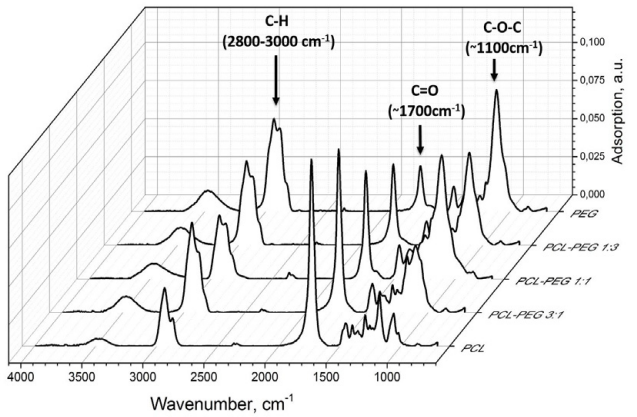

Figure 5: FTIR spectra of thin films deposited from two different precursors with different feed rate into plasma reactor. Ratio of introduced diglyme (PEG) to ε-caprolactone (PCL) in presented films spectra varied between 4:0, 3:1, 2:2, 1:3, 0:4.

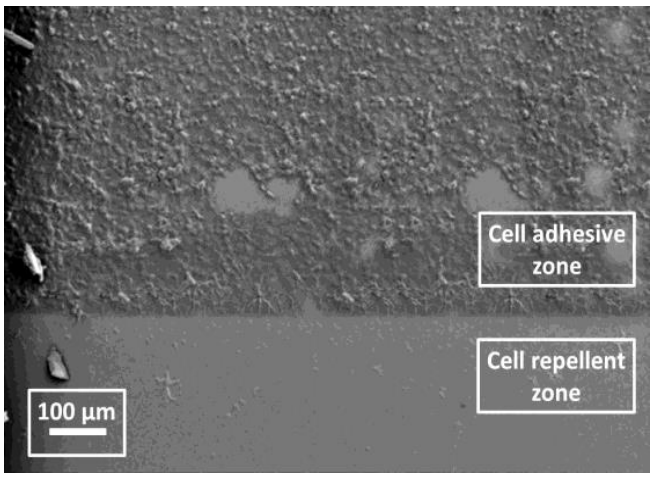

Figure 6: SEM images of OVCAR cells on glass surface coated with cell repellent PEG like film and cell adhesive PCL.

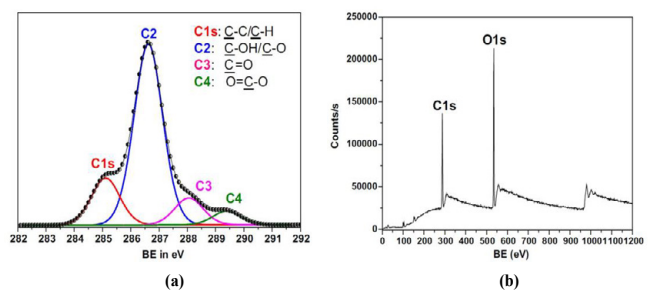

Figure 7: (a) High resolution C1s and (b) survey scan XPS spectra of pulsed plasma polymerized diglyme.

develop colon cancer tumour in inguinal lymph nodes of BALB/c mice. 1 × 1.5 cm^2 collagen membranes loaded with 300 µg of carboplatin were implanted and samples were collected after 10 days (Figure 8). Preliminary results from *in vivo* experiments on drug delivery systems showed the inhibition of growth of colon cancer tumour during the implantation of the membranes. In comparison to the control case, the size of the developed tumour was 2.8 times less in weight. In the control case, the collagen membrane was covered with plasma polymerized coatings but without any anticancer drug, loaded and sandwiched in between the polymer barrier films.

The estimation of the tumour size with ImageJ software from photos taken before and after implantation showed that in the control case, the tumour size increased 10 folds. While for the tumour loaded with carboplatin membranes, the increase of the size was 3.5 times the original size before implanting DDS. This makes 2.8 times difference between drug loaded and control cases at the final stage. The advantage of using ImageJ is that to estimate the tumour's size it is not necessary to remove the tumour, the comparison with weighing after tumour removal showed that results well correlated in these two methods.

Histopathologic analysis (Figure 9) showed necrosis zones not only on the surface of tumour, which was in direct contact with DDS membrane, but also 3 mm deep inside the tumour. Drug loaded membranes also affected the geometrical shape of the tumour, preventing it to develop and proliferate on the side where membranes were applied, forcing it to elongate in other direction. Drug loaded membranes were intended to be used as a patch applied to the area from where the tumour was removed, however, direct application of such membranes to the tumour shows its effectiveness in the inhibition of the tumour growth.

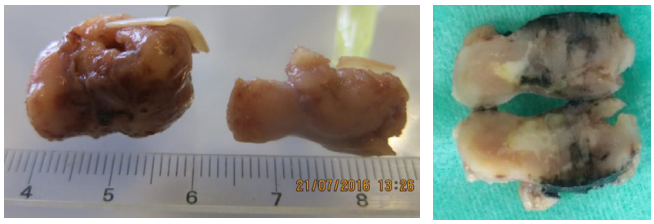

Figure 8: Tumour after 4 weeks of incubation and 10 days treatment with membranes: (a) on the left control case and on the right with membrane loaded with 300 µg carboplatin;(b) cross-section of the tumour treated with membrane loaded with drug.

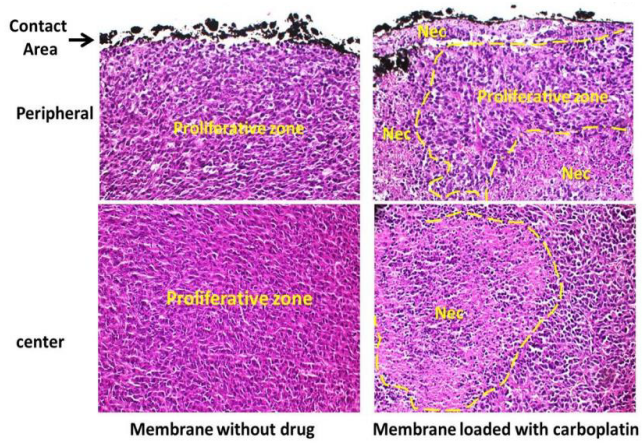

Figure 9: Hematoxylin eosin coloration of the peripheral and central zones of tumour nodules. Contact area with membrane shown by black coloration (arrow). Compared with the control sample (left), several necrotic zones were detected in the periphery and in the center of the tumour (right). The proliferative zones were not received the cytotoxic dose of drug and presented in inter-necrotic regions.

Conclusion

Low pressure PECVD approach to deposit functional coatings for biomedical devices could be used to process both cross-linked hard barrier coatings and soft coatings with retention of functional groups of the precursor. Both *in vitro* and *in vivo* tests showed good results i.e., prevention of cancer cells proliferation and inhibition of tumour growth by 2.8 times compared to the control case. For more effective implementation of the proposed method, careful choice of the drug dosage should be the future goals to reach for the optimized performance of such drug delivery system model.

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