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Oral keratinocyte stem cells behavior on diamond like carbon films

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Abstract

Diamond like carbon (DLC) is an amorphous carbon material suitable for use in a variety of in vivo biomedical applications, which may constitute a step forward in biotechnology of dental or orthopedic implants. Previous in vitro studies focusing on DLC films demonstrate that cellular behavior on DLC is cell type specific. The aim of the present study was to evaluate the interaction between DLC substrates and keratinocyte stem cells isolated from oral epithelia. Oral keratinocyte stem cells were isolated using a magnetic technique. Various DLC films were synthesized using Thermoionic Vacuum Arc plasma (TVA) by adjusting the distance between substrate and anode. We assessed important cell characteristics such as proliferation rate, colony forming efficiency of oral keratinocyte stem cells placed on various DLC films. Our results demonstrate that DLC substrates are biocompatible with the analyzed cell type, and do not affect cell size or colony forming efficiency, in specific conditions being able to stimulate their proliferative potential. The data demonstrates for the first time that oral keratinocyte stem cells are biocompatible with DLC films. Moreover the proliferative potential of cells may be enhanced by adjusting the deposition parameters of DLC films.

Keywords: oral keratinocytes, stem cells, diamond like carbon

1. Introduction

In their continuous quest to improve the patient's life, scientists have investigated various materials suitable for use in the biomedical field. Diamond-like carbon (DLC) is a promising versatile biomaterial with various atomic bond structures and compositions [1, 2]. Biotechnology and biomedical research have highlighted the high wear and corrosion resistance, low friction coefficient, high hardness, bioinertness, nontoxicity and biocompatibility of DLC films [1, 3, 4], suggesting their potential use in manufacturing of blood-contacting devices, contact lenses, implantable biosensors, biomedical implants, dental prostheses [5-20]. The biocompatibility of DLC films was assessed for several cell types and the reported results were successful [1, 4].

DLC coating of biomedical implants significantly reduces the release of metal ions into the surrounding tissues [21-23]. Thin amorphous carbon layers also showed a very good resistance to the oral cavity environment [24], suggesting that DLC films could be used to increase the biocompatibility of implanted dental devices [23]. *In vitro* studies revealed that

DLC coated surfaces cause no adverse effects on murine and human macrophages, fibroblasts or osteoblasts [25, 26]. *In vitro* exposure of macrophages to DLC surfaces has led to divergent results. If some experimental results have emphasized that DLC coating induces a decreased macrophage response [9], other studies have revealed no effect on macrophage morphology or activation [20]. Overall, macrophage releasing of proinflammatory signals, induced by contact with DLC films is not expected. Also, DLC induces no inflammatory response in bone marrow cell cultures [27]. Other studies also investigated the effects of DLC coating on glial cells and neurons and revealed the non-toxic nature of DLC films [26, 28]. There are also several successful *in vivo* tests regarding the biocompatibility of DLC coatings [22]. Experimental studies in rat and sheep showed that chronic implantation of DLC-coated CoCr or stainless steel rods is well tolerated by the surrounding tissues [5]. DLC coating of bioimplantable devices may reduce the risk of infection [18]. In an *in vivo* study investigating the effect of different coatings on skin and bone reaction in contact with an external fixator pin, DLC coated surface showed no remains of a bacterial colonization in contrast to uncoated steel and hydroxyapatite coating [29].DLC coating showed very promising results in studies of haemocompatibility. It reduced hemolysis, platelet adhesion, aggregation and activation. DLC films also inhibited fibrin network formation and protein absorption, demonstrating a high level of hemocompatibility [13, 30, 31]. On the other hand, various applications, such as biomedical implants request an increased cell adhesion and biocompatibility. Extensive research on various surfaces have shown that DLC coating can promote and stabilize attachment of different cell types, such as human micro-vascular endothelial cells [32], insulin-secreting INS-1 cells, chromaffin cells [33]. DLC coating also promote the attachment and proliferation of mouse and human fibroblasts and osteoblasts [2, 18, 34], suggesting a potential use in bone or cartilage replacements and implantology. However, only a few studies have investigated the effect of DLC on keratinocytes biological responses. DLC coating increased the *in vitro* viability of human epidermal keratinocytes on poly(dimethylsiloxane) (PDMS) surfaces [19]. In another recent study on primary cultures of human keratinocytes, DLC films allowed cell attachment and proliferation [35]. Nevertheless, it was emphasized an important variability regarding the differentiation process of keratinocytes, depending on the type of DLC coating and the energy densities used for their preparation [35]. This is in line with observations from other several studies which highlighted that the properties of DLC films and their impact on attachment and growing of various types of cells are highly related to the preparation method, atomic bond structure and composition [4]. To the best of our knowledge there are currently no reports on the interaction between DLC surfaces and different oral keratinocyte populations such as: oral keratinocyte stem cells, transit amplifying cells or post-mitotic differentiation keratinocytes. This data would represent an important step into understanding the healing process taking place at the interface between the oral epithelial and dental implants. Our group has successfully isolated, characterized and expanded *in vitro* a sub-population of oral keratinocyte stem cells [36-40]. The specific aim of the present study is to assess the behavior of oral keratinocyte stem cells on diamond-like carbon (DLC) films synthesized using Thermionic Vacuum Arc plasma (TVA). Cellular attributes such as biocompatibility colony forming efficiency, proliferation will be analyzed in relation to physical or chemical properties of different DLC films.

2. Materials and methods

2.1. Deposition of Diamond Like Carbon films

A series of DLC films were synthesized using Thermoionic Vacuum Arc (TVA) plasma with a graphite anode, in vacuum of 10^{-6}torr [41-43]. The ignition principle of this high voltage anodic plasma source is based on obtaining carbon vapors and ionizing them using

accelerated electrons emitted from a tungsten filament. The electrons are progressively accelerated by increasing the voltage at the graphite anode, up to a point where a stable carbon plasma is obtained. The ions escape from the plasma and are accelerated in vacuum by the electric field created between the plasma and the chamber walls. As no process gas is necessary, carbon atoms and very energetic ions produced in the plasma are the only precursors of the DLC film. This unique combination of film precursors allows synthesis of films with particular composition and properties. Square microscope glass samples of 18x18x0.2 mm were used as substrates in this experiment. They were placed face down at different distances from the anode, as follows: 15, 20, 23, 31 and 36 cm.The sample holder contained five substrates at every such location, which were used for statistical analysis. The electrical parameters of the plasma used were: 90 A filament current, 2.9 A plasma current and 350 V plasma voltage.

2.2. DLC film composition

XPS spectra were recorded using SPECS XPS spectrometer equipped with a Phoibos 150 MCD electron energy analyzer operated in constant energy mode, an X-ray source using Al Kα anode and an electron flood gun charge compensation system (FG 15/40). Curve fitting was undertaken using XI-SDP32 version 7.0 software. A Shirley background was extracted and a 9/1 Gaussian/Lorenzian ratio was used. No constraints were used for finding the best fit.

2.3. AFM measurements

Topographical information regarding the physical structure of the coatings was obtained using a ThermoMicroscope AFM operated in contact mode. Image enhancement was restricted to the use of shading and leveling algorithms. A scan rate of 1 Hz was used.

2.4. Isolation and separation of oral keratinocyte sub-populations

Following a previously established cell culture protocol [36-38] human oral mucosa was collected from patients undergoing tooth extraction. Tissue samples were processed within 2 hours upon collection. Tissues were further rinsed with PBS and dissociated using 4 mg/mL Dispase II (Sigma, St. Louis, MO) and 3mg/mL Collagenase from *C. histolyticum* (Sigma, St. Louis, MO) at 4^{o}C overnight. Next day both the dermal side and the epidermal side were treated with trypsin 0.25% for 30 min at room temperature to obtain single cell suspensions. Primary keratinocytes were cultured in EpiLife® medium (Cascade Biologics, Portland, OR) supplemented with 1.2 mM calcium, EpiLife® Defined Growth Supplements (EDGS) (Cascade Biologics, Portland, OR) together with Fungizone and Kanamycin in an incubator set at 36.5^{o}C. A MACS Separator (Biotec Inc., CA, USA) was used to magnetically separate primary keratinocytes into three populations. The protocol employed two surface markers: integrin α6β4 and CD71 using the following antibodies: MicroBeads conjugated to monoclonal anti-human CD71 (isotype mouse IgG2a) (MiltenyiBiotec, Inc., Auburn, CA, USA); Mouse monoclonal [450-30A] antibody to integrin α6β4 (Abcam, Germany) together with Anti-mouse IgG MicroBeads (MiltenyiBiotec Inc.). In the first step the cells were incubated with integrin α6β4 antibody together with IgG Microbeads and placed in a magnetic field. After a washing step the positive cell fraction was incubated with CD71 antibody and placed in the same magnetic field. Following the two separations steps three distinct cell sub-populations were obtained:$\alpha6\beta4^{neg}$; $\alpha6\beta4^{pos}CD71^{neg}$ cells and $\alpha6\beta4^{pos}$ $CD71^{pos}$. As previously proven by our group $\alpha6\beta4^{pos}CD71^{neg}$ represents the oral keratinocyte stem cell sub-population. This fraction was routinely grown in 5% CO_2 at 36.5^{o}C and used in further experiments at different time points at 3, 7 and 14 days.

2.5. Cell viability

Cell viability was assessed for each time point and substrate using Trypan blue dye exclusion. Following Trypan blue staining the percentage of dead cells, colored in blue was calculated from the total number of cells. For statistical calculus the experiment was performed five independent times.

2.6. Cell proliferation

Each DLC substrate and control was seeded with OKSCs (Oral Keratinocyte Stem Cells) at 1 x 10^4 density and grown for 3, 7 and 14 days. For each time point cells were removed from the substrate using trypsin and counted using a cell counting chamber under an optical microscope.

2.7. Colony forming efficiency

Colony forming ability was assayed by seeding 1 x 10^4 OKSCs cells on different DLC substrates as well as controls. After 3, 7, and 14 days in culture colonies were fixed with paraformaldehyde followed by crystal violet staining. Each assay was performed five times using Cell Analyst software (AssaySoft, Inc., Fountain Valley, CA, USA) ; each time only colonies with more than 20 cells were taken into consideration.

2.8. Statistical analysis

Each experiment was performed five independent times; results are presented as means ± SD. Statistical analysis was performed using StataIC 11 statistical software (StataCorp LP, USA). Statistical significance was accepted at $p<0.05$.

3. Results

3.1. Characteristics of the DLC films

Topography

It is already known that the DLC films synthesized using TVA are generally very smooth but some contain particles whose size and population density is directly correlated to distance from anode [44]. A typical AFM image of such films is given in Figure 1.

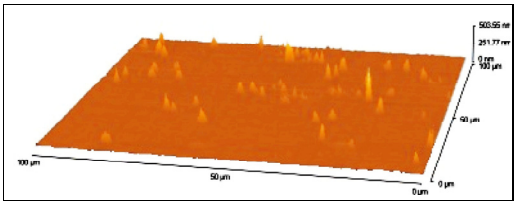

Figure 1. Topography assessment of diamond like carbon films using AFM.

Pulsed laser deposition (PLD) and filtered cathodic vacuum arc (FCVA) are H-free DLC deposition methods that use graphite precursor, like TVA. The deposited film surface topography is mainly influenced by the presence of particulates, deposition rate and ion energy. The TVA films also contain particulates formed during deposition. In PLD, the size of particulates is of the order of microns. Very low surface roughness (lower than 1 nm) is obtained by FCVA only due to the filtering systems which are usually employed in the cathodic arc set-up. Ion energies below 200 eV were reported to give roughness lower than 1 nm in both PLD and FCVA, with a minimum at 100 eV and a continuous increase thereafter. As ion energy in TVA is given by the applied voltage, a value of 350 eV was used here for DLC deposition. Although no reported data was found in current literature for DLC film roughness at ion energies around this value for PLD and FCVA, an extrapolation above 100 eV of the reported curves suggests higher rms values than in the TVA films.

Composition

The H-free DLC films obtained by TVA contain sp^3 and sp^2 bonded carbon. Their sp^3 content was found to increase with distance from the anode, as can be observed in Figure 2.

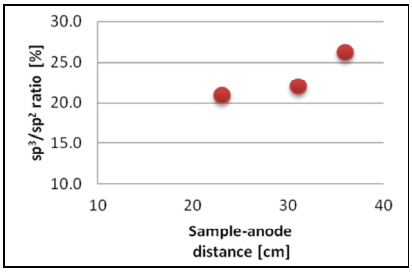

Figure 2. sp^3/sp^2 ratio for the DLC films obtained by TVA.

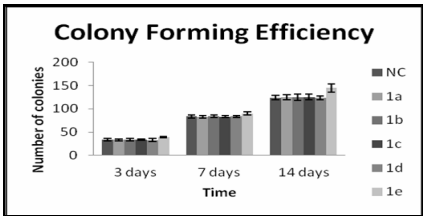

Figure 3. Colony forming efficiency – Oral keratinocyte stem cells were analyzed for their proliferative potential i.e. colony forming efficiency, following exposure to five different diamond like carbon substrates (synthesized at different distances from the plasma source). Data represents n=5 independent experiments, ANOVA test with a p value set at <0.05.

3.2. Cell viability

Trypan blue assay was performed in order to assess the percentage of viable cells after exposure to different DLC surface. Statistical analysis showed that the percentage of viable oral keratinocyte stem cells was over 90% for all five analyzed groups and no statistical difference could be observed between these groups and control. At the same time cell viability was the same at each analyzed time point (3, 7 and 14 days). These results demonstrate that DLC films do not affect cell viability (See Table 1).

Table 1. Oral keratinocyte stem cells viability (percentage of cells out of total cell population) following exposure to various diamond like carbon substrates: each film being placed at different distances from the anode, analyzed at different time points: 3, 7 and 14 days.

Distance from anode	3 days	7 days	14 days
Uncoated	95.96±1.79	93.86±1.17	93.82±1.28
15 cm -1a	94.72±1.68	94.26±1.85	93.74±1.66
20 cm - 1b	95.04±1.58	93.46±1.85	93.86±1.17
23 cm - 1c	96.92±1.49	95.04±0.98	94.76±2.83
31 cm - 1d	95.20±0.57	93.10±1.64	94.16±0.79
36 cm - 1e	94.00±1.67	95.08±1.05	93.52±0.90

3.3. Colony forming efficiency

Colony forming efficiency represents a good indicator of stem cell's proliferative potential. For the first time our data shows that the proliferative potential can be boosted by adjusting the distance between the substrate and the anode. Statistical significance could be observed between the control group and DLC substrates placed at 36 cm from the plasma source at 3, 7 and 14 days respectively: at 3 days - 33.42± 2.74 vs. 33.26± 2.53 vs. 33.4±2.91 vs. 33.3±1.46 vs. 32.96±3.12 vs. 39.02±1.76; at 7 days - 83.7±3.55 vs. 82.28±2.78 vs. 83.82±2.49 vs. 83.14±1.84 vs. 83.9±1.96 vs. 89.98±3.05; at 14 days 123.92±4.95 vs. 124.86±5.77 vs. 124.88±6.28 vs. 125.36±6.28 vs. 123.08±4.29 vs. 144.66± 8.53 for negative controls and substrates number 1a, 1b, 1c, 1d and 1e respectively (results represent average and standard deviation for 5 independent experiments, ANOVA test, $p<0.05$) (see Figure 3).

3.4. Cell proliferation

Cell proliferation was assessed for all DLC films at 3 days. Data shows that DLC substrates do not have a negative impact on cell number, the results being comparable with the control group. Moreover when grown on a DLC 1e, OKCS count was increased albeit without any statistical significance when compared to controls. More long term studies are needed in order to analyze the cell growth on all DLC films (Figure 4).

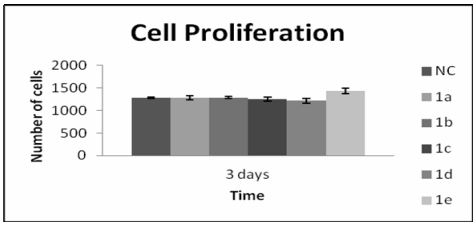

Figure 4. Cell proliferation – Oral keratinocyte stem cells were counted after 3 days of being grown on specific DLC substrates. The results show that DLC substrates can increase cell number. Data represents n=5 independent experiments, ANOVA test with a p value set at <0.05.

4. Discussions

DLC is a material synthesized from graphite or from carbon-containing gases such as methane or ethane. Depending on the deposition method and also on the precursors used, DLC films are composed of different types of carbon-carbon and/or carbon-hydrogen bonds. Due to this bio-friendly composition, DLC films are intensely studied for applications where biocompatibility is necessary. Apart from composition, DLC film characteristics such as roughness and hydrophilicity are additional parameters in fluencing their overall compatibility with living cells. DLC films were also employed as barrier for metal ion release. A recent review on applications of DLC films in medicine is presented by Hauert et al. [7]. Some applications of DLC films have already been implemented. Medical device companies are selling stents coated with DLC. The medical use of DLC at a large scale is hindered by the lack of ideal deposition technologies for very adherent and flexible films. Cracking of DLC films during *in vivo* use is of real concern. In this work, an original high voltage anodic plasma source with graphite anode was used to deposit hydrogen-free DLC films. It is the first time an assessment of biocompatibility of the DLC films obtained using this plasma source is made. Further studies on finding technologies offering highly adherent films on medical devices are envisaged. Previous studies evaluate the behavior of different tissues and cell types in relation with various DLC coatings. [45-47]. It was previously demonstrated that DLC films do not induce cytotoxicity or inflammation in skin fibroblasts, osteoblasts and macrophages and show minimum adherence for platelet cells, thus preventing thrombosis [13]. Reports involving cellular behavior on DLC films show that cell behavior in relation to DLC is cell-type specific: while some cell types show good adhesion and increased proliferation, i.e. monocytes, others such as glial cells or fibroblasts demonstrate low adhesion [26]. Fibronectin, a protein assembled in the fibrillar extracellular matrix, seems to be a key element in the adhesion and spreading of cells on a material surface. Surfaces which adsorb fibronectin, such as DLC-coated surfaces, determine a better cell proliferation [48]. DLC has also been shown to be effective for oral cavity implants, due to its ability to prevent the release of Nickel (Ni) ions from Nickel-Titanium alloys used in orthodontic archwires and their resistence to saliva [23]. However most reports are in consensus with the conclusion that DLC does not have cytotoxic effects, does not elicit an inflammatory response, does not alter the cytoskeletal architecture and is biocompatible with most studied cell types [1, 20]. To date

the knowledge on the interaction of oral keratinocyte cells, key players in the healing process following implant procedures, and DLC surface is very limited. The present work analyses important cellular attributes such as: cell viability, colony forming efficiency, cell proliferation and cell size in relation to DLC films synthesized at different distances from the anode. Viability assessment demonstrated that cells plated on DLC substrates retain the same viability percentage as the negative controls. Necrosis or late apoptosis was a rare event, occurring in less than 5% of the total cell number. Although the cell culture experiments cannot exactly reproduce the complexity of *in vivo* responses [49], this data suggests that DLC coating does not affect cell viability and can be used for medical applications. Colony forming efficiency is a key characteristic of stem cell behavior *in vitro*. Stem cells switch from a relative quiescent state in which they are found in *in vivo* conditions and became actively proliferative cells when placed in cell culture condition. One of their attributes is the potential to divide indefinitely and to form the highest number of colonies among the other cells types derived from the same tissue. Our previous studies show that oral keratinocyte stem cells have the highest colony forming efficiency when compared to transit amplifying cells and to post mitotic differentiated keratinocytes. The present study demonstrates that DLC substrates can enhance this potential when compared to negative control substrates. Thus following 14 days in culture 1e DLC substrate formed statistically significant more colonies than the other DLC substrates and negative control. This result suggests that DLC can be used in medical applications where a rapid colony forming process is needed, such as oral epithelial healing following dental implant placement. These data correlate with the cell proliferation assessment: short term *in vitro* growth shows that after 3 days in culture, OKSC placed on 1e DLC substrate have a higher proliferation rate when compared with the other samples.

5. Conclusions

The present work demonstrates for the first time that DLC films are biocompatible with oral keratinocyte stem cells. Moreover, cell proliferation, cell viability and colony forming efficiency are dependent on the physical attributes and chemical composition of DLC films. The results of this work come in line with findings in current literature where DLC is reported as being biocompatible, regardless of the synthesis technique used. The thermoionic vacuum arc plasma was proved to be part of this "community". The best combination of composition and roughness (from the point of view of biocompatibility with oral keratinocyte stem cells) will be further used to develop a technology for maximum adherence to medical materials and devices.

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All authors have equally contributed in the conception and preparation of the manuscript.

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