ATMOSPHERIC PLASMA SURFACE MODIFICATION OF ELECTROSPUN POLY(L-LACTIC ACID): EFFECT ON MAT PROPERTIES AND CELL CULTURING

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AIM OF THE WORK

Investigation on the effectiveness of atmospheric pressure non-thermal plasmas as a tool for biocompatibilization of PLLA nanofibers

OUTLINE

Tissue engineering & electrospun scaffolds

Surface modification

Low pressure plasmas vs atmospheric pressure plasmas

Plasma sources

Plasma treatment: definition of optimal operative conditions

Chemical functionalization

Electrospun scaffold characterization & cell culturing

Conclusions
**Pioneer in plasma science**

Doctoral dissertation on glowing platinum wire and hot gases at low pressure

Studies on thermoionic emission

Studies on electricity effects on gases -> coined the term **PLASMA**

Invented the electric probe (Langmuir’s probe)

Discovered electron density waves in plasmas (Langmuir waves)

Inventor of first plasma welding device

**IRVING LANGMUIR**

**1932 Nobel prize for chemistry**

(studies on surface adsorption)

**Pioneer in surface chemistry**

Studies on surface catalysts

Introduced the concept of monolayer

Co-invented the **Langmuir-Blodgett film**
(technique to produce organic monolayers)

Invented **Langmuir films**
(organic monolayer immersed in liquid environment)

**FIRST INDUSTRIAL RESEARCHER TO ATTAIN NOBEL PRIZE**
TISSUE ENGINEERING

Tissue engineering
Implantation of temporary artificial scaffolds containing both cells and bioactive molecules

Scaffold
Temporary 3D environment for cells to produce new biological tissue

SCAFFOLD REQUIREMENTS

- biocompatibility (no cell toxicity before and after degradation of the scaffold material)
- promotion of cellular activities (cell adhesion, proliferation....)
- biodegradability with a controlled rate of degradation, matching that of new tissue growth
- 3D highly porous structure with interconnected pore network to allow tissue growth and permeation of nutrient medium
- mechanical properties consistent with those of the tissue to be regenerated
- highly reproducible and versatile fabrication process

http://biomed.brown.edu

HIGHLY BIOMIMETIC

EXTRA CELLULAR MATRIX (ECM)
SCAFFOLD ARCHITECTURE: WHY NANOFIBERS

MORPHOLOGICALLY BIOMIMETIC

Self assembly

Phase separation

Electrospinning

Science, 2005, 310, 1135-1138
**ELECTROSPINNING**

**Electrospinning**
Liquid polymer solution fed through a capillary tube into a region of high electric field

- Polymer nanofibers

**Advantages**
- Cost-effective process
- Long continuous nanofibers
- Broad range of polymers
- Tailoring of mechanical properties
- Variety of architectures and morphologies

**Disadvantages**
- Sub-micrometric fibers
- Use of organic solvents
- Poor control over 3D structure

LAB & INDUSTRIAL SCALE PROCESS
SURFACE MODIFICATION OF ELECTROSPUN NANOFIBER

TARGET: SURFACE FUNCTIONALIZATION AND MODIFICATION OF SURFACE PROPERTIES (HYDROPHILICITY)

SURFACE MODIFICATION
Introduction of functional groups on the surface
Can be followed by functionalization

- **Plasma treatment**
  Superficial treatment, does not affect bulk properties

- **Chemical treatment**
  Adapt for modification of thick scaffolds
  May lead to polymer degradation

SURFACE GRAFT POLYMERIZATION
Introduction of functional groups through grafting of polymers.
Plasma and UV radiation as free radical sources to introduce polymerization

CO-ELECTROSPINNING
Nanoparticles/polymeric fragments added to the solution
Additives exposed and oriented on fiber surface
LOW TEMPERATURE NON-EQUILIBRIUM PLASMAS

\[ T_b << T_e \]

- Electrons
- Heavy particles
LOW PRESSURE vs ATMOSPHERIC PRESSURE PLASMAS

PLASMA ASSISTED SURFACE MODIFICATION
- Plasma treatment (introduction of reactive species or functional groups)
- Plasma grafting (creation of a cross-linked polymer top-layer)
- Plasma polymerization (polymeric coating from vapor phase monomers)

LOW PRESSURE PLASMAS
- Stable discharge
- Good control over plasma chemistry

ATMOSPHERIC PRESSURE PLASMAS
Advantages
- Lower investment costs
- In-line process
- Scalability

Disadvantages
- Discharge instabilities (local temperature increase)

CLASSICAL APPROACH

INDUSTRIAL DEMAND
- Cost-effictiveness
- Flexibility

INNOVATIVE APPROACH
PLASMA SOURCES

Floating Electrode - Dielectric Barrier Discharge (FE-DBD)

- Working gas: **Air**
- Dielectric layer on high voltage electrode to prevent transition to arc
- Homogeneous or filamentary discharge depending on operative conditions

Schematic of operative configuration

P&M application
PLASMA SOURCES

DBD roller

Working gas: **Air**

Dielectric layer to prevent transition to arc

**Large area** treatment

Easily **scaled-up**

Schematic of direct treatment configuration

Schematic of indirect treatment configuration

Nylon dielectric

Highly homogeneous discharge (fixed gap height = 2mm)

Treatment uniformity
PLASMA SOURCES

DBD roller

Working gas: **Air**

Dielectric layer on both electrodes to prevent transition to arc

**Large area** treatment

Easily **scaled-up**

Negligible surface modification

Schematic of direct treatment configuration

Schematic of indirect treatment configuration

Highly homogeneous discharge (fixed gap height = 2mm)

**Treatment uniformity**
PLASMA SOURCES
LINEAR CORONA

Working gas: **He, Ar, N₂**

**Large area** treatment

Easily **scaled-up**

- No dielectric layer
- Transition to arc is prevented through voltage waveform

P&M application

Schematic of operative configuration
PLASMA SOURCES
LINEAR CORONA

Working gas: He, Ar, N₂
Large area treatment
Easily scaled-up

No dielectric layer
Transition to arc is prevented through voltage waveform

P&M application
Schematic of operative configuration
PLASMA SOURCES
Waveform effect

ALTERNATE CURRENT

Function generator + Amplifier
Trek 30/20A
Peak voltage (PV): 0 – 60 kV
Frequency (F): 0 - 5000 Hz

MICROSECOND PULSES

Plasma Power LLC – PG100-3D
Peak voltage (PV): 15 – 25 kV
Repetition rate (RR): 50 - 3500 Hz
Pulse duration: 1 - 10 us
Rise time: ~5 us

NANOSECOND PULSES

FID GmbH - FPG 20-1NMK
Peak voltage (PV): 7 – 20 kV
Repetition rate (RR): 50 - 1000 Hz
Pulse duration: ~40 ns
Rise time: 3 ns

STRONGLY FILAMENTARY

PV: 40kV
F = 1000Hz

DIFFUSE

PV: 40kV
RR = 1000Hz

PV: 20kV
RR = 1000Hz
DEFINITION OF OPTIMAL OPERATIVE CONDITIONS
DISCHARGE & TEMPERATURE UNIFORMITY

- Z-type schlieren optics
- Dual view optics
- Plasma source
- 450 W Xe Lamp
- Nanosecond pulsed generator
- NAC Memrecam GX3 1000 fps
- NAC Memrecam K3 1000 fps
DEFINITION OF OPTIMAL OPERATIVE CONDITIONS

DISCHARGE & TEMPERATURE UNIFORMITY

Discharge spatial uniformity
Filaments motion

Side view

NAC Memrecam GX3 1000 fps

450 W Xe Lamp

Nanosecond pulsed generator

NAC Memrecam K3 1000 fps
DEFINITION OF OPTIMAL OPERATIVE CONDITIONS

DISCHARGE & TEMPERATURE UNIFORMITY

Discharge spatial uniformity

Filaments motion

Side view

Number of filaments

Filaments motion

Bottom view

450 W Xe Lamp

Nanosecond pulsed generator

NAC Memrecam K3 1000 fps
DEFINITION OF OPTIMAL OPERATIVE CONDITIONS

DISCHARGE & TEMPERATURE UNIFORMITY

Discharge spatial uniformity
Filaments motion

Side view

Temperature gradient (*main contribution*)
Ionization degree and gas composition play a role where filaments occur

Bottom view

Schlieren

Number of filaments
Filaments motion

NAC Memrecam K3
1000 fps
DEFINITION OF OPTIMAL OPERATIVE CONDITIONS
DISCHARGE & TEMPERATURE UNIFORMITY

PV = 23 kV, RR = 1000 Hz, gap = 1 mm

PV = 23 kV, RR = 1000 Hz, gap = 6 mm
DEFINITION OF OPTIMAL OPERATIVE CONDITIONS
DISCHARGE UNIFORMITY – iCCD IMAGING

Exposure time = 3 ns, images not overlapped

Gap = 1 mm, PV = 9 kV

Gap = 3 mm, PV = 16 kV

PIMAX 3, Princeton Instruments
DEFINITION OF OPTIMAL OPERATIVE CONDITIONS
MORPHOLOGICAL ANALYSIS - SEM

<table>
<thead>
<tr>
<th>Pristine</th>
<th>FE-DBD</th>
<th>DBD roller</th>
<th>Linear corona</th>
</tr>
</thead>
</table>

- **Pristine**: no morphological alteration
- **FE-DBD**: thermal degradation due to high current density filaments
- **DBD roller & Linear corona**: no morphological alteration

**FIBER UNIFORMITY**

- Diameter
- Morphology
- Arrangement
DEFINITION OF OPTIMAL OPERATIVE CONDITIONS

SUBSTRATE TEMPERATURE MEASUREMENT

Temperature acquisition system:
Fiber optic temperature transducer
Model: OPSENS
Calibration range: 20°C-60°C
Sensitivity: 0.2°C

PLLA glass transition temperature around 60°C
Maximum substrate temperature: 25°C

PLASMA TREATMENT – OPTIMAL CONDITIONS

<table>
<thead>
<tr>
<th>Source</th>
<th>Abbreviation</th>
<th>Gap (mm)</th>
<th>Duration (s)</th>
<th>Gas (slpm)</th>
<th>PV (kV)</th>
<th>RR (Hz)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linear corona</td>
<td>LC He</td>
<td>1</td>
<td>10</td>
<td>3 (He)</td>
<td>17</td>
<td>500</td>
</tr>
<tr>
<td>Linear corona</td>
<td>LC N₂</td>
<td>2</td>
<td>20</td>
<td>3 (N₂)</td>
<td>20</td>
<td>1000</td>
</tr>
<tr>
<td>DBD roller</td>
<td>DBDR</td>
<td>2</td>
<td>10</td>
<td>Air</td>
<td>17</td>
<td>500</td>
</tr>
</tbody>
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DEFINITION OF OPTIMAL OPERATIVE CONDITIONS

SUBSTRATE TEMPERATURE MEASUREMENT

Temperature acquisition system:
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PLLA glass transition temperature around 60°C
Maximum substrate temperature: 25°C

Visible damages after non-optimized plasma treatment
No damages after optimized plasma treatment
CHEMICAL FUNCTIONALIZATION

Carboxyl (COOH) groups are the most used reactive group to covalently attach molecules using primary amine.
Treated scaffolds show a 2 to 5 times higher fluorescence

Higher fluorescence with N2 Linear corona corresponding to higher -COOH concentration

Negligible non-specific binding
CHEMICAL FUNCTIONALIZATION
AGING OF PPLA AFTER LC N$_2$ PLASMA TREATMENT

Functionalization at different times (0, 6, 24 and 48 hours) after plasma treatment

Mean of fluorescence intensity of non-treated PLLA scaffold obtained at the same time point

Data represent the mean intensity fluorescence ± SD of ten area acquisitions

Negligible variation of fluorescence intensity

A satisfactory reproducibility was obtained CV% = 15%
**MECHANICAL TENSILE MEASUREMENTS**

**LINEAR CORONA**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Thickness (um)</th>
<th>Elastic modulus $E_{\text{AVERAGE}}$ (MPa)</th>
<th>Stress at break $\sigma_{b\text{AVERAGE}}$ (Mpa)</th>
<th>Deformation at break $\varepsilon_{b\text{AVERAGE}}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 – Pristine</td>
<td>35 - 70</td>
<td>96.9 ± 10.0</td>
<td>3.8 ± 0.7</td>
<td>59.3 ± 4.0</td>
</tr>
<tr>
<td>1 – LC He</td>
<td></td>
<td>78.5 ± 7.4</td>
<td>2.6 ± 0.3</td>
<td>76.3 ± 11.2</td>
</tr>
<tr>
<td>2 – Pristine</td>
<td>20 - 40</td>
<td>75.0 ± 15.5</td>
<td>3.0 ± 0.2</td>
<td>52.8 ± 6.6</td>
</tr>
<tr>
<td>2 – LC N$_2$</td>
<td></td>
<td>49.0 ± 8.4</td>
<td>2.0 ± 0.3</td>
<td>66.1 ± 7.0</td>
</tr>
</tbody>
</table>

**Test conditions** - Sample width: 5mm; Sample length: 20mm; Repetitions: 10

Slightly modification of mechanical properties  ➡️  **LESS RIGIDITY**

Commercial biodegradable thermoplastic materials typically employed in tissue engineering, such as poly(lactic acid) are often too rigid to be replacements for soft tissues

Materials with lower elastic moduli are required for soft tissue engineering applications

No differences were observed in thermal properties (from **TGA** and **DSC**) before and after plasma treatment, indicating that no significant reduction in PLLA molecular weight occurred.
CONTACT ANGLE MEASUREMENTS (WCA)
LINEAR CORONA - LC N₂

- WCA pristine: $123.3 \pm 4.0$ deg (stable); PLLA film: 93 deg
- After plasma treatment: **complete absorption** within 90s
- After **72h** from the treatment:
  - Complete absorption is preserved
  - WCA decreases at lower rate
  - The same value of the WCA is found at 60s
  - Scaffold hydrophilicity maintained after 72 h

<table>
<thead>
<tr>
<th>Temporal evolution (s)</th>
<th>After 3h (deg)</th>
<th>After 24h (deg)</th>
<th>After 48h (deg)</th>
<th>After 72h (deg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>117.5</td>
<td>121.0</td>
<td>119.5</td>
<td>121.7</td>
</tr>
<tr>
<td>5</td>
<td>82.1</td>
<td>102.4</td>
<td>106.1</td>
<td>119.4</td>
</tr>
<tr>
<td>10</td>
<td>64.4</td>
<td>80.0</td>
<td>88.9</td>
<td>109.1</td>
</tr>
<tr>
<td>20</td>
<td>45.4</td>
<td>53.8</td>
<td>70.4</td>
<td>88.0</td>
</tr>
<tr>
<td>30</td>
<td>36.0</td>
<td>44.2</td>
<td>54.0</td>
<td>68.2</td>
</tr>
<tr>
<td>60</td>
<td>22.5</td>
<td>25.6</td>
<td>19.7</td>
<td>24.5</td>
</tr>
</tbody>
</table>

Water absorption after plasma treatment
A. SEM micrograph of PLLA electrospun scaffold.
B. Representative picture of rat embryonic stem cells (RESCs) grew on PLLA scaffold; cells were visualized by cytoplasmatic actin staining in red and nuclear Hoechst33258 staining in blue; PLLA nanofibers were labelled with FITC (green). Scale bar: 20 μm.
C. Visualization of RESC-SC by Oct4 immunostaining (green); cells grew as a monolayer on es-PLLA scaffold and could be localized only on the surface of the scaffold thickness.

CONCLUSIONS

The introduction of COOH functional group on PLLA electrospun scaffold has been achieved using low temperature atmospheric pressure plasmas.

Plasma stable homogeneous condition for avoiding polymer damage can be obtained by properly setting operative conditions using DBD roller and Linear corona sources.

FE-DBD has been found to damage the polymer due to local heating.

Linear corona with N\textsubscript{2} produces the highest amount of COOH groups (4-5 times the amount of pristine scaffolds).

After 48h no COOH groups reduction is observed.

After 72h induced hyrophilicity is preserved.

Rat embryonic stem cells (RESCs) grown on pristine PLLA electrospun scaffolds showed higher proliferation rate compared to standard 2D conditions.
THANK YOU FOR YOUR ATTENTION

ANY (PLASMA RELATED) QUESTIONS?