Production and Evaluation of Biocompatibility of Polyacrylonitrile and Carbon Fibers

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THESIS

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DC

TABLE OF CONTENTS

CHAPTER PAGE

LIST OF TABLES

LIST OF FIGURES

Figure Page

SUMMARY

The main purpose of the work presented in the thesis is the preparation of Carbon fibers and evaluation of their toxicity as a preliminary screening for possible biomedical applications which have a really wide range; from example: reinforcement for prosthetic components, biosensors for detection of specific compounds or support for the synthesis of nanoparticles.

Actually, the main applications regards prothesis because carbon, thanks to its own strength and light weight, represents an ideal reinforcement material even if it reports brittle behavior under stress. Lot of promising researches are under way because even if allotropic form of carbons are not a recent discovery its own biocompatibility is debated and must be investigated. More over, the systematic investigation into biomedical applications of carbon are focused on nanotubes which possess unique features that make them extremely attractive for biosensing applications due to their combination of several exceptional chemical, electrical, physical, optical characteristics properties and many others. More over, it is described the synthesis technique of fibers with special attention of electro spinning technique which is a quite new, promising and really versatile technique to obtain fibers with different properties.

For what concern the biomedical testing, ISO 10993-5:2009, which describes the methods to evaluate in vitro cytotoxicity of medical devices, is used a s model for determine the biological response of cells during in vitro screening using appropriate biological parameters and methods. In details, the attention is focused only on the characterization of the material after the synthesis of it.

Standard mice fibroblast 3T3 cell line is used in the experiments due to the basics screening test of the materials used. In addition, the attention is focused is on cellular behavior in a limited amount of time.

CHAPTER 1

INTRODUCTION

The ultimate goal of this research is the preparation of fibers and the evaluation of their biocompatibility; in details, Polyacrylonitrile fibers obtained through electrospinning technique and Carbon Fibers.

Carbon based materials have a really huge and wide application range in biomedical field. Currently, carbon based products are part of assistive prosthetic devices for the disabled including above all components for orthopedic prostheses which are currently on the market having been certified according to the EC 93/42 standard, concerning medical devices, and ISO 10328. The study of medical devices requires knowledge of synthetic and biological materials which, of course, are characterized by significantly different physical, chemical and mechanical properties. For example, while fabrics contain cells, metallic, ceramic and polymeric materials do not. While the fabrics have a total or partial repair capacity, the metallic, ceramic and polymeric materials do not. (49)

In addition, Carbon fibers, which are considered a biomaterial, can be used as support in synthesis of metallic nanoparticles which can possibly enhance cellular functions or for the production of carbon nanotubes which have really promising applications in the purely biomedical field where experiments are underway, for example, for the production of artificial bones, for the reconstruction of nerve circuits damaged by trauma, to produce prosthetic components insensitive to wear and to construct "small ships" of nanonotubes to transport drugs directly into a cancer cell without damaging surrounding tissues and cells; both the previous three uses represent the frontier in the biomedical field due to the most futuristic uses of these revolutionary nanometric materials very light and much more resistant than steel. Anyway, even

if allotropic forms of carbon are not a recent technique, the first compounds have been synthesized in 1980, the biocompatibility has not been in depth analyzed and it still debated. (50) Due to still not well understood and well know biocompatibility of Carbon, is this thesis is showed the toxicity of polymer precursors of carbon sheets and the toxicity of Carbon fibers as possible support for synthesis of metallic nanoparticles.

In the end the fibers has been obtained through a quite recent and innovative technique called electrosspining which enable to obtain electrospun fibers that have demonstrated lot of applications in filtration, biomaterials and so on thanks to their nanoscale and/or microscale fiber diameters and through their property as high specific surface area-volume ratio and controllable morphologies.

1.1 Carbon Fibers and applications

Carbon fibers were first discovered by Edison towards the end of the nineteenth century. He noted that rayon could be converted into filamentous carbonaceous material through the use of incandescent lamps. Following this discovery, carbon fibers were used for the first time by the Union Carbide Corportation (UCC) for commercial purposes in the early 1960s. (36)

These fibers, although presenting an excellent resistance, especially in the direction of orientation of the filaments, have a rather low modulus of elasticity. The graphitic structure is, in effect, the fundamental characteristic of these fibers and the main reason for their use in the development of composite materials. The crystalline structure of graphite gives the carbon fibers particular mechanical properties and although the crystals tend to have an anisotropic behavior, it is possible to orient the crystalline structure in the desired direction through the fiber processing carbon. (35)

In details, the graphitic form of carbon has a specific structure which can crate fibers with a diameter between 5 and 15 μm. (37)

For the previous reasons, carbon fiber is a high quality composite material and it is generally used in the specific production of composite materials in rather high quantities being a very resistant material, but at the same time light. These materials acquire the denomination of composites since they consist of two or more materials and the carbon fiber only acts as a reinforcement and not as a carrier material.

A field of application where carbon fiber has found ample space is related to the transport industry, both the daily ones like the bicycle and the more sophisticated ones: in many aircraft and helicopter models, carbon composite materials represent up to 50% of the total construction materials used because, compared to other elements, it is much lighter and therefore makes the means of transport safer and reduces the amount of fuel that must be used. (44)

Anyway, the fields of application are very heterogeneous and range from sensors to the

biomedical sector where carbon is used as composite material for prothesis in order to reduce the weight and increase the strength and it is used for technopolymers which are thermoplastic materials that offer superior performance to those of traditional polymers; they can be supplied in granules and / or slab and can be processed using the most common technologies such as injection molding, thermoforming, calendering, etc; moreover, these materials can be transformed starting from a full with the use of traditional mechanical processing with tools; among the technopolymers used today in the biomedical sector, thanks also to its biocompatibility, PEEK stands out. (47)

Another application is suing carbon to create carbon nanotubes. The applications of nano-tubes range from the production of high-strength materials, to the construction of accumulators, to sensors, from a point of view of the possible problems related to their toxicity their biomedical applications are of particular importance. In particular their capacity to crossing biological membranes (both directly and through phagocytosis phenomena) thanks to the high length / diameter ratio, has made the carbon nanotubes a particularly promising system to convey pharmacologically active molecules directly to their biological receptor. (45)

The possible applications are the most disparate. They can be used as a support to create nanoparticles ore one of the most science fiction companies sees them used as building material for nano "intelligent" machines for transporting and releasing drugs directly into diseased cells, so as not to damage surrounding tissues. This would be a very important goal in the fight against cancer, for example, and in all those cases where the drugs used can be toxic even for healthy tissues. (46)

The main technique with which nanofibres are made is electrospinning, a technique that uses an electrostatic field to induce fiber extrusion. of production.

1.2 Electrospinning

The first studies concerning electrospinning date back to 1914 and were carried out by Zeleny, who first described this process in the technical literature. The first patent concerning a process for the production of fibers, micrometric fibers of cellulose acetate, starting from a solution through electrostatic means dates back instead to 1934 thanks to Formhal. From then on, several studies have been conducted on the topic, which laid the foundations for electrostatic spinning.

Among these are noteworthy those of Baumgarten in 1971, who was the first to obtain fibers with diameters less than a micrometer through electrostatic methods, and those of Larrondo and Manley, which in 1981 produced fibers from a polyethylene solution and from melted polypropylene . However it is above all thanks to the work of the last 20 years that it has been possible to acquire a thorough knowledge and a full understanding of the technique.

In fact, electrospinning has been recognized as new and efficient technique for the production of fibers with range of diameter from micrometer to nanometer scale in fact the initial low success of the electrospinning is due to the limited yield of the process, the low orientation at the molecular level, with consequent unsatisfactory mechanical properties and the high distribution of the diameters of the fibers produced. Consequently, even today, scientific information and technologies related to the characterization of the product are limited. However, in recent years, special requests for innovative applications in various fields (from military to medical) have stimulated new studies and renewed interest in this process, giving rise to a substantial resumption of research activities. (3)

Fibers are usually made by polymer solutions or melts. Due to the low mechanical characteristics of polymers the small dimension of fibers cannot be achieved with mechanical drawing processes; in fact, using electrostatic forces, a polymer melt is extruded through a die with a hole of specific size in a traditional fiber spinning process and significantly smaller diameters fibers can be obtained. (2)

The electrospinning process involves material science, applied physics, fluid mechanics, electrical, mechanical, chemical(material, especially polymer) engineering and rheology. The fiber morphology is controlled by the experimental parameters and is dependent upon solution conductivity, concentration, viscosity, polymer molecular weight, applied voltage, etc (4)

Furthermore, can be achieved fibers with either hollow or solid interiors; it is even possible to create fibers with special properties by adding different compounds as metallic, polymer, ceramic or even biological elements such as amino acids or DNA to the electro spun blend. (1)

1.3 Carbon Toxicity

Carbon, called also Carbon Black itself is the one produced industrially under controlled conditions, even if a vast spectrum of environmental emissions (eg vehicular traffic, domestic heating, industrial emissions, etc.) consists of nano-carbon particles (individually equivalent to those constituting Carbon Black) .

This particulate component, not being produced under controlled conditions, is much more heterogeneous than the industrial Carbon Black in terms of dimensions, morphology and chemical substances adsorbed on the surface.

Both the Carbon Black itself and the environmental particulate, despite their differences, converge towards a general inflammatory pathogenetic mechanism. More over, Carbon black is the base material of Carbon Fibers .

In cultured cells, Carbon Black has been observed to cause oxidative stress, increased transcription of pro-inflammatory genes, phagocytosis stimulation at low doses and inhibition at high doses (note that the effects on the immune system must always be understood in terms of the balance, see the sheet on carbon nanotubes), geno-toxicity and other potentially harmful behaviours. Epidemiological studies of workers in the Carbon Black industry have shown

evidence of morphological abnormalities of the lungs on chest radiographs and increased respiratory morbidity, whereas dubious results regarding lung cancer (which is however very difficult to obtain due to the many confounding effects). (48)

Direct toxicity of carbon is evaluated in this study due to possible application in direct contact with tissue of carbon in biomedical field. In details, standard procedure of test is performed following ISO 10993-5 of 2009 which describes test methods to assess the in vitro cytotoxicity of medical devices.

The first part of the norm, the one one which describes only the evaluation of biocompatibility of materials fro medical devices, is used as a standard for the development of the tests to be carried out for the evaluation of the biocompatibility of the materials under examination.

CHAPTER 2
PREPARATION OF FIBERS

2.0 Electrospinning of Polyacrylonitrile Fibers
2.1 PAN Nanofibers

Nanofibers fall into the field of nanotechnology, defined as the science that works with materials, structures or devices that have at least a size equal to or less than 100 nm. Although conventionally 100 nm represent the threshold value to be included in the field of nanotechnologies, structures, materials or devices with a size of a few hundred nanometers (even up to 500 nm) are also considered "nano". By breaking down the term nanofiber into two parts (nano and fiber), the meaning of the term is evident. In the technical language the prefix "nano" indicates a physical quantity one billion times smaller than the reference unit of measurement (for example a nanometer corresponds to one billionth of a meter, ie 1 nm = 10 9 m). With the term "fiber" instead yes means a long, thin and filiform material, with considerable resistance to extension and stretching in the direction of the fiber itself. At the molecular level, the fibers are made of long and linear polymer chains, which lie aligned in the direction of the fiber. Therefore the resistance to stretching is given by the strength of the covalent bonds of the polymer chain and the stability of the structure depends on the intermolecular attraction forces (hydrogen bonds, dipole-dipole forces, Van der Waals forces) that prevent the extended chains from folding .

In this study, therefore, the term nanofibra indicates a one-dimensional structure, in which the length is far greater than the diameter, which is in the order of a few hundred nanometers.

2.2 Electrospinning process

In a a conventional electrospinning process (Figure 1), a droplet of polymer solution or melt which held at the end of a capillary is attracted to a grounded collector due to the application of a high electrical potential between the nozzle and the collector. A constant flow of fluid through the needle is provided by an infusion pump. A charged jet of polymer solution or melt is ejected from the charged nozzle when the applied electric field overcomes the surface tension of the droplet; usually the applied voltage is between 5 and 50 kV. The jet grows thinner and longer thanks to bending instability or separating until it solidifies and then collects on a grounded collector which is placed at a certain distance. (1)

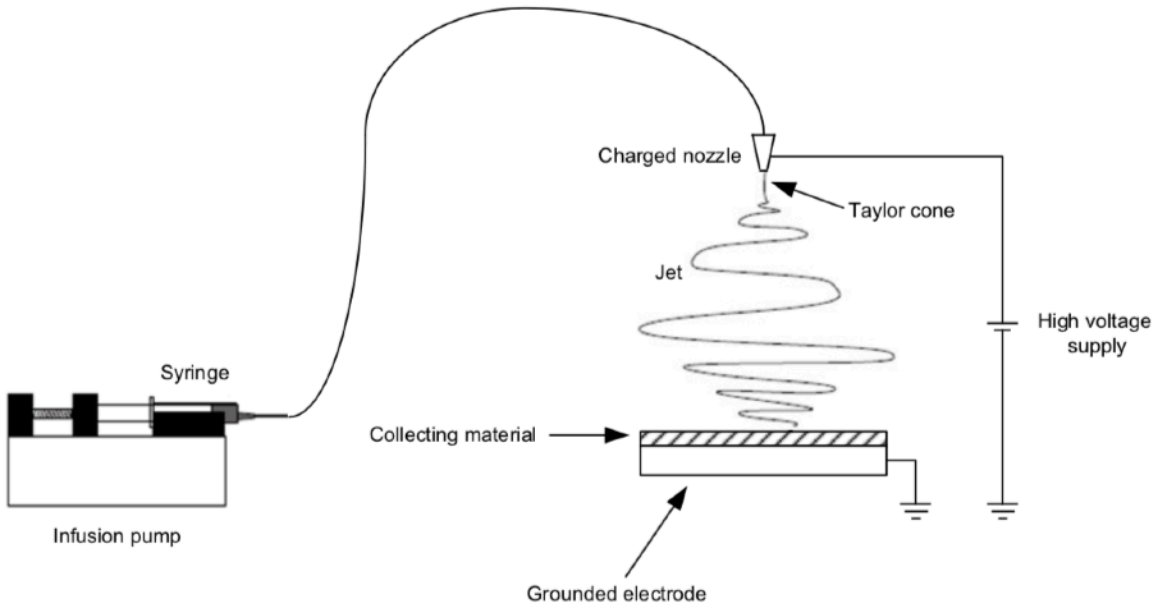


Figure 1: A characteristic electrospinning setup

When the electrostatic forces overcome the surface tension of the drop with a specific shape, which called Taylor's cone, a thin viscoelastic drop is released from the nozzle because the electric field is sufficiently high. While the charged jet is moving to the grounded electrode collector the polymer is affected by the electrostatic forces and it is continuously stretched.

During this short time the solvent evaporates and the combination of stretching forces and evaporation of solution perform a significant reduction of the fire diameter. (1)

The fiber's diameter and morphology are controlled by the experimental parameters and, for example, they differ dependent upon solution properties as: concentration, conductivity, viscosity of polymer and its molecular weight; more over, the fiber's diameter depends on other physical specification of the electrospinning-machine -set up like: applied voltage, distance between nozzle and collector, etc. (3)

Many researches have been done on the concatenate in an univocal and inescapable way morphology and specific parameters of fibers on the electrospinning process. The first and cleared results are shown below:

- The diameter size increases with polymer concentration according to a power law relationship.
- Fibers spun from higher concentration solution follow a bimodal distribution.
- Electrospun poly(glycolic acid) (PGA) fibers have linear relationship between diameter and concentration of polymer.
- Evidences are found between electrostatic effects influence and macroscale morphology of electrospun textiles. (5)

For what concern the applied voltage, its effect it is still debated:

- Electric field does not change much the diameter of polyethylene oxide fibers. (6)
- Increasing electrospinning voltage tends to decrease the fiber diameter although polymer concentration has a greater influence. (7)

The fiber diameter is decreased by roughly half doubling the applied electric field.

However, it is proved that the diameter of the jet reached a minimum after an initial increase in

field strength and then became much larger due to the increasing of the field. This effect shows one of the complexities of the electrospinning process and is is supposed that caused by the feed rate of the polymer solution through the nozzle. In fact, increasing the field not only increases the electrostatic stress and creates smaller diameter fibers, but it also draws more materials out of the syringe. (8)

By increasing the voltage again, a charged jet exits the tip of the cone and the electrospinning process begins (the direction of the jet is determined by the direction of the electric field). The charges carried by the jet arrive at the collector connected to the ground thus completing the electrical circuit. If the fluid has a low molecular weight the jet becomes unstable before reaching the collector and turns into a spray of small drops laden with static electricity. This process is called electrospraying. By using fluid polymers instead, the viscoelastic forces stabilize the casting, allowing the formation of very fine filaments. The electro spun fiber follows a devious path to the electrode collector, in fact, the polymer material which is detaching from the Taylor's cone oscillates chaotically because of the repulsion between material elements and an aerodynamically instability. (14)

The high charge density on the surface of the jet, causes a strong load instability and causes the electrospun fiber to oscillate quickly (the movement is comparable to that of a whip).

The lashes are so fast that it gives the impression that many nanofibers branch off from the single drop; in reality, photographs of the jet show that the fiber is only one and the speed with which the fiber moves creates an optical effect whereby the fibers look like many. This oscillation is called "Whipping Phenomenon" which produces a random deposition of the material creating a nonwoven mesh with properties hard to predict or characterize. The network of nanofibers deposited on the manifold is composed of a single nanofiber that is disposed on it in a completely random manner. A non-woven fiber is thus formed with diameters of the order of

nanometers which are well below the diameter of the extruded fibers with mechanical forces.

Although, a chaotic structure is not only sufficient but also fundamental for some applications as, in this case, the carbonization of PAN fibers. On the other hand some electro spinning applications prefer or even require a better oriented distribution of nano or micro fibers. The solvent (usually present in a solution above 80%) instead evaporates in the journey from the needle to the collector. The choice of the solvent, the distance between the needle and the collector and the temperature should be such as to ensure that the spun fiber is completely dry when it reaches the collector and that the solvent has evaporated completely. (14)

Worldwide, many research teams have studied and brevetted methods to control the orientation of fibers during electrospinning with various levels of success; anyway, lot of researches are run in order to obtain techniques and reproducible procedures the allow control of depositions, namely to obtain the fiber spacing in the mesh constant instead of chaotic. (1)

With Alignment is defined as the control on how parallel fibers can deposit to each other while Controllable spacing is defined as whether is possible to deposit fliers with a controllable space between each other.

There are three main groups of methods on how the orientation is achieved:

1. Mechanically: rotation or translation movements that accomplish alignment alignment of fibers.
2. Through electrostatic means: alignment is achieved solely through the shape of the setup.
3. Both mechanically and through electrostatic means: both previous techniques are used.

TABLE 1

Orientation control methods in literature	Controllable Spacing	Alignment	Autor	Reference
Mechanical:				
Rotating wooden or aluminum frame as collector	☐	✓	Huang 15	
Rotating disk collector V	☐	✓	Subramanian 12	
Aluminium foil on rotating cilinder	☐	☐	Bhattarai [13]	
Liquid bath-as-collector	☐	✓	Smit [16]	
Electrostatic:				
Ring auxiliary electrode	☐	☐		
Electrostatic lens as focusing element	☐	☐	Deitzel [18]	
Cylindrical auxiliary electrode	☐	☐	Kim [19]	
Auxiliary electric field	☐	✓	Huang [15]	
Electrode-gap spinning	☐	✓	Li/Xia [20]	
Metal frame electrode	☐	✓	Dersch [21	
Knife-edge electrode in needleless spinning	☐	☐	Yarin [22]	
Metal grid collector	☐	✓	Gibson 23	
Both:	☐	☐		
Copper wire drum collector	☐	✓	Katta 24	
Bobbin collector	☐	✓	Theron/Zussman	25
Cylinderspinner	☐	✓	Sundaray	26
X,Y,Z-positioned collector	✓	✓	Mitchell	27
Scanning tip spinning	☐	✓	Kameoka	28
Near-field electrospinning	✓	✓	Sun	29
Parallel grid of aluminium strips as electrode	☐	✓	Teo	30
Knife-edged blade electrodes	☐	✓	Teo	30
Aluminium foil on rotating cilinder /w copper electrodes	☐	✓	Bhattarai	31

The results from literature are divided in the following table on two qualitative fronts: controllable spacing and alignment can be seen in the table 1, controllale spacing is not well understood while alignment is a consolidated issue.

As can be seen, at an instrumental level a laboratory plant for electrospinning consists mainly of:

• glass or plastic syringe in which the polymer solution is contained;

• volumetric pump (syringe pump);

• electrode in contact with the solution;

• high voltage generator connected to the electrode;

• collector electrically discharged to the ground where the fibers are collected.

In Figure 2 the manifold shown is a static-plane collector, but in addition to this there are also other possible configurations.

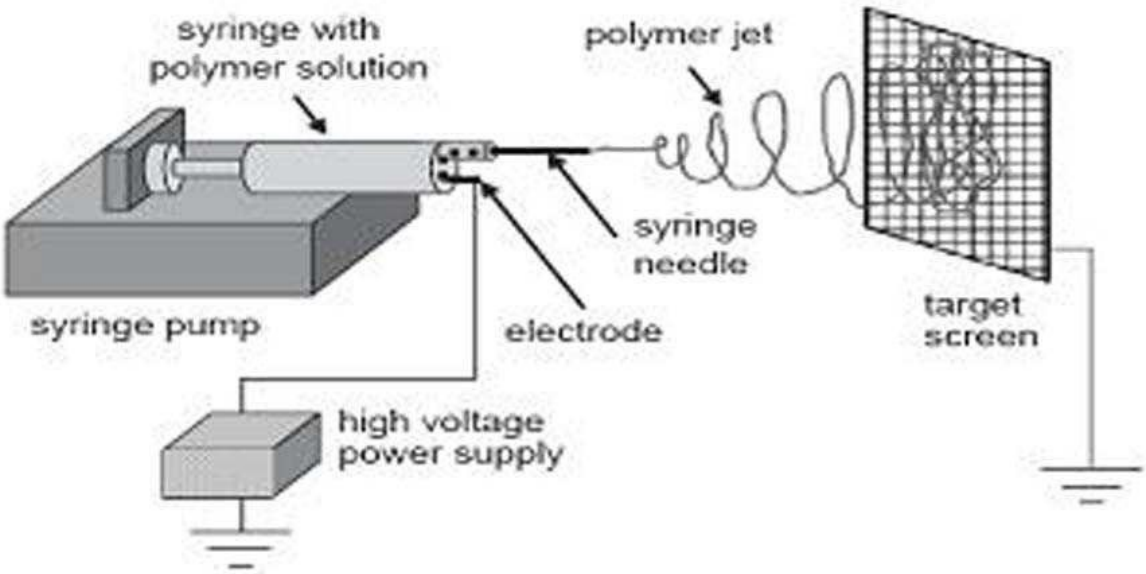


Figure 2: Schematic representation of the electrospinning process

2.3 Mechanism of formation of nano fibrils

As we have seen, the mechanism that leads to the formation of nanofibers is very complex. This mechanism can be divided into several key steps: launching the jet; elongation of the segment; region of instability (whiplash movement); solidification (18) with consequent formation of the fiber (solvent evaporation). In the following subparagraphs are illustrated one by one the steps just listed.

2.4 Generation of the drop

This first step can in turn be broken down into two intermediate stages: the generation of the drop and the formation of the Taylor cone.

The polymer solution is pumped through the syringe needle at very low flow rates. In the absence of an electric field, droplets form at the tip of the needle and fall due to gravity. The surface tension of the liquid (indicated with y) and the gravity force (FG) are the only forces acting on the surface of the drop; the radius of the droplet (r0) produced at the exit of the internal radius needle R is:

$$\sqrt[3]{\frac{3Ry}{2r0g}} \tag{1}$$

Where r0 is is the density of the liquid and g is the gravity acceleration.

The droplets can fall even in the presence of an electric field, if the generated voltage is low. Once a sufficiently high voltage has been reached, the electrical force (FE) and the force of gravity are opposed to the surface tension (Fγ = FE + FG) and the maximum diameter which allows the drop to be kept in equilibrium at the needle tip decreases r value, with r <r0. The electric force of the field that is created between the needle and the collector, placed at distance

L from each other, is:

$$Fe = \sqrt[3]{\frac{4\pi epV^2}{ln(4L/R)}} \quad (2)$$

Where ep is it is the perceptibility of the medium (generally air) and V is the applied voltage. By increasing the applied voltage, the value of the radius decreases until the critical voltage VC is reached, at which the instability condition of the drop is reached. Due to the electric field, within the drop of solution (capable of conducting electricity) there is separation of charges.

Since the needle is positively charged, positive charges accumulate on the surface of the droplet, while the negative charges migrate inwards, until reaching an equilibrium situation in which the electric field inside the drop is zero. The separation of charges generates a force that is opposed to surface tension. The speed of the charges depends both on the applied voltage and on the mobility of the ionic species present. The stability of an electrically charged drop on the tip of the needle is guaranteed until the surface tension (which acts inward) prevails with respect to the repulsion forces of the charges accumulated on the surface (facing outwards); the stability condition of the drop in the presence of an electric field is therefore:

$$Fe \leq gr0(\frac{r^2}{B} - V) \quad (3)$$

Where B is the factor of shape of the drop.

2.5 Taylor's cone formation

The drop, under the effect of the electric field, undergoes a deformation. The drop so stretched (19) takes the form of a cone, from which a thin stream of liquid starts. This cone takes the name

of the person who discovered it and was called Taylor's cone; it is formed at the critical voltage VC, expressed by:

$$V2 = (2L)\ 2\ [ln\ (2h)\ -1.5]\ (0.117\pi RT) \quad (4)$$

where h is the length of the needle and T is the temperature of the environment in which it is working.

It is precisely the transition from a spherical to an ellipsoidal shape that determines the onset of stretching forces that can then lead to the formation of drops (electrospraying) or fibers (electrospinning). From the considerations made up to now it can be deduced that liquids with high surface tension require higher values than the critical voltage; the same applies to liquids with high viscosity and low conductivity. Important for the purposes of this discussion is to understand what the mobility of ions depends on. An ionic species that is inside the drop of solution loaded, is subject to two forces: the electrostatic force FE, equal to the product between the ionic charge and the force of the electric field, and the viscous drag force FD = 6 π η r 'μ E (η is the viscosity of the solution and μ is the mobility of ions). The two forces are in contrast with each other and act in opposite fashion to each other; while the electrostatic force tends to move the charges in the direction of the electric field (towards the collector), the viscous drag forces oppose resistance and tend to retain the charges, thus acting in the opposite direction (towards the syringe).

In Figure 3 the geometry of the Taylor cone is shown. More over, is described the behavior of the electrospun fiber during deposition on the collector.

2.6 Elongation of the segment

As soon as the value of the critical voltage is exceeded, the formation of the jet is almost instantaneous. The repulsion force of the surface charges accumulated in the jet, since the electric field has a specific direction, has itself a direction. In particular, it has an axial

component that causes the elongation of the jet in its transit towards the collector (the direction of the electric field is in fact from the syringe needle towards the collector).

In a study conducted by Buer (20), he demonstrates that the velocity of the jet increases as one moves away from the Taylor cone; as a consequence, the diameter of the casting decreases, both due to the stretching of the casting and due to the evaporation of the solvent. The jet, therefore, accelerating more and more towards the collector, is becoming increasingly thinner. In this first phase, the jet is stable, and its stability is due to the chaining between the polymer chains: the motion regime is of laminar type.

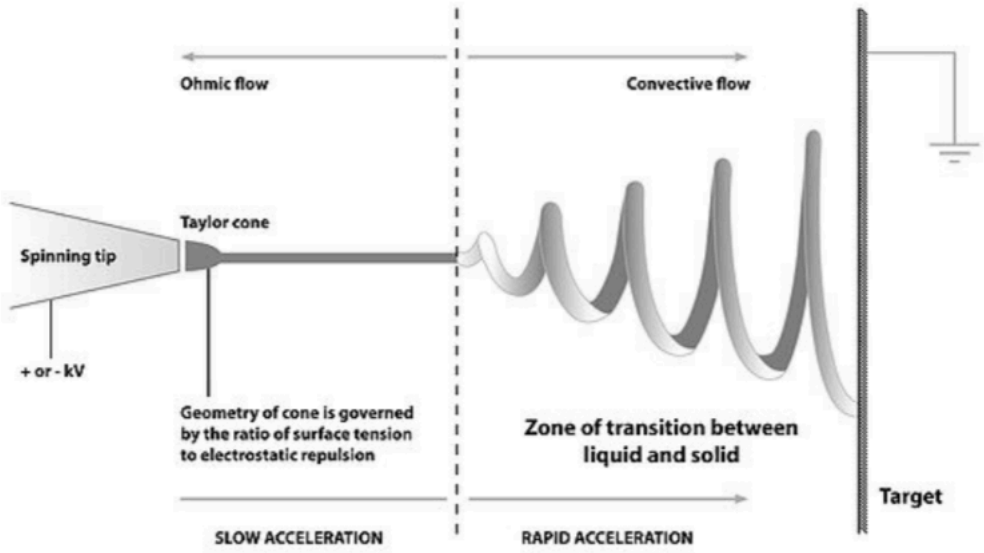


Figure 3: Representation of the jet. Note in particular the Taylor cone and the instability region (whippinginstability)

2.7 Region of instability

The jet, which for the initial stretch is straight, becomes unstable and in its transit towards the collector it curves and manifests an undulating and oscillating pattern. The bending

of the casting causes the surface area to increase: the density of the charges therefore decreases. Several studies are aimed at modeling the jet under the effect of the electric field: movement at the stroke of a whip it is given by the competition between different forms of instability, such as instability of Raleigh, instability symmetric with respect to the axis, instability with curvature. In particular, the type of instability that is obtained depends on the electric field: intense electric fields favor whiplash instability (whipping mode). (20) (21) (22) (23)

In this case the preponderant direction of the repulsive forces that are established inside the jet is not axial. The movements of the jet, comparable to flicks, are enclosed within a space of conical shape, and are arranged symmetrically with respect to the axis along which lies the straight line of the jet. In this phase, both the speed of increase in the surface area and the evaporation rate of the solvent are quite high; the diameter of the jet in this way is further reduced. Therefore, the whip-shake-up phase is the one most responsible for reducing the size of the nanofibers during the electrospinning process. In this stage, the forces acting on the jet are: the force of gravity, the electrostatic force (it lengthens the jet and pushes it towards the collector), the repulsive forces of Coulomb (introduce instability and "whip movements"), the viscoelastic forces (opposing the fragmentation of the jet in the electric field), the surface tension forces (working in opposition to the jet stretching), the friction forces between the jet surface and the surrounding environment (air or gas that are). The combination of all these forces determines the diameter of the jet. The quantitative description of the process is particularly difficult, since most of these forces vary very quickly over time due to the evaporation of the solvent and the dissipation of the fillers. As a consequence, the mathematical models available to the instability phase are not fully satisfactory. In Figure 3 you can see the shape of the jet with the Taylor cone, the rectilinear section and the area of instability.

2.8 Evaporation of the solvent

During the transit of the jet towards the collector, the solvent evaporates. What remains are polymer nanofibers. Ideally no trace of solvent should remain in the moment when the jet touches the collector, otherwise the newly formed polymeric nanofibres are dissolved again in the residual solvent. The evaporation rate of the solvent depends on a number of factors, among which for example the distance from the collector and the vapor pressure of the solvent. This phase is very important for the value of the diameters of the fibers: in fact the size of the obtained nanofibers depends also on the evaporation speed of the solvent and on the time it takes to evaporate.

2.9 Parameters

The electrospinning technique is in itself simple: the principles on which it is based and the instrumentation necessary to make it are simple. However, the system of variables and factors that take part in it is very complex. The parameters involved are different and can be classified into three distinct groups, depending on their origin and their nature: parameters of the polymer solution, process conditions and environmental conditions. In the three paragraphs that follow will be examined in detail the groups just listed. (24)

The parameters related to the properties of the polymer solution are those that have a greater weight in the electrospinning process. The molecular weight of the polymer and the nature of the solvent, the viscosity, the surface tension and the conductivity of the solution, are decisive for the success of the process and for the production of fibers with the desired characteristics. The individual solution parameters are briefly described below and an explanation is given of why they are so important in electrospinning.

2.9.1 Molecular weight and viscosity

The viscosity of a polymeric solution is closely related to the molecular weight of the polymer to be dissolved; the higher the molecular weight of the polymer, the greater the viscosity of the solution. One of the conditions necessary for fiber formation is that the solution has sufficient viscosity. Only in this way is it possible to avoid the formation of drops; they are the entanglements that are established between the polymer chains to keep the jet cohesive and to prevent it from breaking, giving rise to drops of solution. The number of entanglements is closely related to the length of the polymer chains, which is directly proportional to the molecular weight of the polymer in solution. The use of a high molecular weight polymer is not the only way to obtain a sufficiently viscous solution: another parameter that can play in favor of viscosity is the concentration of the polymer in solution. Similarly, an increase in concentration means a greater number of polymer chains and therefore a greater number of entanglements. A solution that is not sufficiently viscous can lead to both the breaking of the casting and the formation of drops, and the formation of fibers sprinkled with beads (there are fibers with small drops arranged along them); in the latter case, the viscosity of the solution is not such as to overcome the surface tension, and results in a structure of this type (called a necklace).

2.9.2 Surface tension

In order for the electrospinning process to start, it is necessary for the solution charged by the effect of the electric field to be able to overcome its surface tension. However, even once the casting is formed, surface tension can play a negative role. First of all remember that the surface tension has the effect of reducing the surface area per unit of mass. In the case of a less viscous solution, the interactions between the solvent molecules and the polymer chains are scarce; therefore there are a large quantity of free solvent molecules, which tend to agglomerate with each other due to the surface tension. This does not happen in the case of more viscous solutions, where the interactions between solvent and polymer are more significant. When the solution is

stretched under the effect of the electric field, the solvent molecules are distributed on the polymer chains (in turn interacting through the entanglements): in this way the tendency of the solvent molecules to interact with each other to form agglomerates is reduced. , effect of surface tension. Starting from solutions characterized by low surface tension, it favors the formation of smooth fibers. To lower the surface tension you can intervene by choosing low surface tension solvents, or by introducing surfactants in solution.

2.9.3 Conductivity

As previously mentioned, the electrospinning provides for the stretching of the solution due to the repulsion of the charges on its surface. Consequently, a higher conductivity corresponds to a greater number of charges in the jet: the stretching effect will therefore be more relevant, there will be a reduction in the diameter of the fibers, as well as a lower tendency to form beads. To increase the conductivity it is sufficient to add a small amount of salt to the solution. The salt must be chosen bearing in mind that the mobility of the ions depends on their size (smaller ions have greater mobility); introducing smaller ions into solution therefore means being able to count on greater mobility of the same and obtain a greater stretching of the jet. An excessive increase in conductivity, however, causes the instability of the jet. The conductivity therefore favors the formation of smooth and thin fibers, but only within a certain range, beyond which flows into the instability and breaking of the jet. The accepted conductivity range depends on system-based system (polymer-solvent system).

2.9.4 Nature of the solvent

The nature of the solvent is a relevant factor for electrospinning. In particular, the dielectric constant directly affects the morphology of the fibers. The use of solvents with high constant results in a greater deposition area, demonstrating the fact that the instability zone is amplified; in this way smooth and small-diameter fibers are obtained (in fact, the flow of the jet from the

needle to the manifold increases, and the jet is then stretched more). However, the choice of solvent should not be made exclusively on the basis of its dielectric constant: we must not penalize the interactions that can be created with the polymer, with negative consequences on the solubility. In the choice of the solvent the solubility of the polymer should therefore be preferred.

2.9.5 Process conditions

Less important from the point of view of the consequences they have on the morphology of the fibers, but not negligible, are the process parameters. These include: the applied voltage, the flow rate, the temperature of the solution, the type of collector, the diameter of the needle and the distance between the tip of the needle and the collector.

2.9.6 Voltage

The voltage induces the charges inside the solution, generating a certain electrostatic force. By increasing the voltage value to exceed a certain critical value, the electrostatic force is such as to overcome the surface tension and allow the development of the Taylor cone and the formation of the jet. Depending on the flow rate, it will be necessary to set a more or less high voltage to ensure the stability of the Taylor cone. Due to the repulsive forces present in the jet and the electric field generated by the potential difference between the needle and the collector, the solution is stretched. The applied voltage and the electric field that results directly affect the stretching and acceleration of the jet, thus have a significant impact on the morphology of the fibers. In general, higher voltages result in a stronger electric field and more important coulomb forces: the stretching of the casting increases, the diameters of the fibers decrease and the evaporation rate of the solvent increases. Voltages too high, however, cause an acceleration of

the jet such as to drastically reduce the transit time of the jet towards the manifold; the fibers do not have the necessary time for stretching and elongation: there are fibers with higher diameters.

The applied voltage affects the morphology of the fibers also relative to the formation of beads and to the crystallinity of the fibers. Since the shape of the electric field (the direction and geometry of the field lines) has a considerable influence on the morphology of the fibers, additional electrodes and particular collectors are often used to obtain fibers with certain characteristics.

2.9.7 Flow rate

The flow rate determines the amount of solution available for electrospinning in the unit of time. Voltage and flow are closely related: depending on the applied voltage, it is necessary to set a specific flow value if the Taylor cone is to be kept constant and stable. As the flow rate increases, it is expected that the diameter of the fibers will increase, since the greater the volume of solution to spin in the unit of time. But this is not always true; if the flow rate is equal to the speed at which the solution is 'pulled' from the jet (determined by the strength of the electric field and therefore by the voltage), as the capacity increases, the number of charges increases. In this case, the stretching of the jet is greater and the fibers produced have smaller diameters, in contrast with what has been said previously. If you work with higher flow rates, because of the greater volume of solution to be treated, the time necessary for the solvent to evaporate completely is higher. If the speed at which the jet moves towards the collector is very high, and therefore the time taken to reach the collector is very low, the solvent does not have sufficient time to evaporate. Therefore, when the fibers are deposited on the manifold and come into contact with the already deposited fibers, they melt together and melt, precisely because of the residual solvent. For this reason it is therefore more appropriate to work with lower flow rates, so as to allow complete evaporation of the solvent.

2.9.8 Type of collector

The collector is generally a plate of conductive material, which is grounded to ensure that the potential difference between the needle and the collector itself remains stable and constant. If a non-conductive material was chosen, the charges present on the surface of the jet would tend to accumulate very quickly on the collector, causing the potential difference and therefore the number of fibers deposited to collapse. Moreover, due to the accumulation of charge on the manifold, repulsion forces are created which impede the deposition of new fibers. The result is a fiber mat very sparse compared to what is obtained in the collector placed on the ground, where the charges that are deposited are dissipated as soon as they touch the collector. Also in the case of a conductor manifold one can verify what happens in the case of a non-conducting manifold: when the deposited nanofiber layer becomes consistent, the same phenomenon of accumulation of charges occurs and the onset of repulsive forces that prevent the deposition of new fibers. Collectors are often chosen characterized by a design (pattern): the lines of the pattern present on the manifold become the preferential lines along which the fibers are deposited. The deposited fiber network then reproduces the collector pattern. Then there is the possibility to choose between static and moving collectors. The rotating collectors allow to obtain more aligned fibers. They also give more time to the solvent to evaporate, as well as speeding up the evaporation itself.

2.9.9 Needle diameter

Needles with lower internal diameter reduce the tendency to form droplets along the fibers and allow to obtain fibers with reduced diameters. This happens because in the case of needles with small internal diameter the surface tension of the solution on the needle tip increases and a larger Coulomb force is therefore needed to break the drop and form the jet (at the same applied voltage). Therefore, the acceleration of the jet decreases and the solution has more time to be stretched before reaching the manifold. However, with too small needles it is sometimes not

possible to get a drop of solution out of the tip.

2.9.10 Distance between the needle tip and the manifold

Changing the distance between the tip of the needle and the collector changes both the time and the jet it takes to reach the collector, and the strength of the electric field. When the distance between the needle and the manifold is reduced, the jet will have less space to travel before reaching the manifold; furthermore, by increasing the electric field, the acceleration of the jet will be greater. Consequently the solvent will not have enough time to evaporate. According to the characteristics of the solution, the distance can also affect the morphology of the obtained fibers. If, for example, the distance is too low, the strength of the electric field increases to such an extent that it greatly increases the instability of the jet: in this way the formation of droplets along the fibers is encouraged. By increasing the distance, the jet has more time to be ironed and therefore smaller diameter fibers are obtained. However, if the distance exceeds a certain value, related to the set potential difference, the electric field is not strong enough and the stretching of the fibers is less. For excessive distances there is no deposition of fibers. Once the electric field is fixed, it is necessary to find the optimal distance that allows to maximize the stretching of the fibers and the evaporation of the solvent and to minimize the diameters of the fibers.

2.10 Environmental parameters

The effect of the external environment on the electrospinning process is not currently a field studied in depth. Any interaction between the surrounding environment and the polymer solution can have effects on the morphology of the obtained fibers. Moreover, any variation that occurs in the environment in which the process is conducted can influence the electric field and therefore the process itself. The main environmental parameters investigated are: humidity, type of environment, temperature and pressure.

2.10.1 Humidity

If the humidity of the environment is high, pores may form on the fibers, the size of which depends on the same degree of humidity. This happens because the jet, due to the evaporation of the solvent, cools and when it comes into contact with its surface, the water vapor present in the air can condense. The pores are due to the evaporation of water and solvent. The humidity also affects the evaporation rate of the solvent: when the humidity is very low, the solvent evaporates quickly; evaporation of the solvent may also be greater than the rate at which the polymer solution escapes from the needle. If this occurs, the needle will be fired within a few minutes. Finally, humidity also affects the distribution of charges, but this is a problem still to be studied.

2.10.2 Type of environment

The composition of the air that constitutes the electrospinning environment has an impact on the process. Different gases in the presence of an electric field behave differently.

2.10.3 Temperature

The increase in temperature plays a double role: on the one hand it involves a higher evaporation rate of the solvent, on the other it reduces the viscosity of the polymer solution and favors the solubility of the polymer in the solvent. At high temperatures, therefore, the coulomb forces are able to exert a greater stretching on the jet since the viscosity is lower (remember that viscosity is the resistance that a fluid offers when subjected to a force). The electrospinning therefore benefits from the increase in temperature. However, care must be taken when working with biological substances, such as enzymes and proteins, that are extremely thermosensitive.

2.10.4 Pressure

In general, reducing the pressure of the external environment does not improve the electrospinning process. When working at pressures lower than atmospheric pressure, the polymeric solution is sucked out, causing great instability in the starting phase of the jet. As the pressure drops, the solution starts to boil on the tip of the needle. At very low pressures it is not possible to conduct the process as the fillers are instantly dissipated.

2.11 Theoretical model

As mentioned above, Taylor introduced the concept of critical voltage and determined its formulation. The critical voltage is therefore that voltage value at which, keeping all the other parameters constant, the drop of polymer solution present on the tip of the needle is deformed to form a cone (Taylor cone) and remains in equilibrium under the action of the electric field and surface tension. The critical voltage is given by:

$$Vc^2=(\frac{2L}{h})^2[\ln(\frac{2h}{R})-1.5](0.117\pi RT) \quad (5)$$

where Vc is the critical volume, h is the length of the needle, R the inner diameter of the needle, L la

distance between the tip of the needle and the collector and T the temperature.

Notice how the formula just reported does not take into account either the conductivity or the

viscosity; however it can serve as a reference in the case of solutions with medium-low viscosity and little conductivity.

In 1971 Baumgarten observed that the increase in viscosity causes an increase in the diameter of the fibers, according to the following law:

$$d = \eta^{0.5} \tag{6}$$

where d is the diameter of the nanofibres and η is the viscosity of the solution.

Other studies report quantitative methods that can evaluate the optimal conditions because the electrospinning process is carried out, trying to analyze the various aspects involved, such as shape and charge density of the jet coming out of the capillary. These methods are based on the equations of conservation of mass, momentum and charge. (25)

2.11.1 Conservation of the mass

Consider an infinitesimal portion of jet of length dz; the mass contained in it is equal to:

$$m = \rho\pi D^2 (dz) \tag{7}$$

where ρ is the density of the solution and D the diameter of the jet. After an infinitesimal time interval we have:

$$\rho\pi D^2 v(dt)\,|_z - \rho\pi D^2 v(dt)\,|_{z+z} = 0 \tag{8}$$

where v is the velocity of the liquid.

2.11.2 Conservation of momentum

For the momentum we have:

$$\rho\pi D^2 v^2 (dt)|_z - \rho\pi D^2 v^2 (dt)|_{z\,z+dz} + p\pi D^2 (dt)|_z - p\pi D^2 (dt)|_{z+dz} = 0 \tag{9}$$

where p is the liquid pressure. From this equation it results:

$$\frac{\partial v}{\partial t} + \frac{\partial v}{\partial z} = -\frac{1}{\rho D^2}\frac{\partial pD2}{\partial z} \tag{10}$$

Below is the balance equation obtained from Feng(25), more precise and detailed from moment that takes into account a greater number of phenomena and factors that can affect the behavior of the jet:

$$\frac{d}{dz}(\pi D^2 \rho v) = \pi D^2 \rho g + \frac{d}{dz}([\pi D^2(-p+\tau)] + \frac{\gamma}{D} 2\pi D D' + 2\pi D(t_t - t_n D') \tag{11}$$

where g is the acceleration of gravity, γ is the surface tension, D 'is the distance between the surface of the jet and the axis of the jet, tt and tn are the tensile forces respectively tangential and normal to the surface of the jet, due to the electric field.

2.11.3 Charge retention

Feng(25) proposed the charge retention report:

$$I = \pi D^2 KE + 2\pi D v \sigma \tag{12}$$

with I electric current, K liquid conductivity, and vertical component of the electric field, σ charge surface density.

The forces acting on the jet must satisfy Newton's second law. A model (26) suggested, however, that it does not take into account the instability of the jet:

$$m\frac{d^2P}{dt^2} = f_C+f_E+f_V+f_s+f_A+f_G \tag{13}$$

The following describes the expressions for the forces that fall into the above equation

$$\text{Coulomb force: } f_C = \frac{e^2}{l^2} \tag{14}$$

$$\text{Electric force: } f_e = -\frac{eV}{L} \tag{15}$$

$$\text{Viscoelastic force: } f_v = \frac{d\sigma v}{\partial t} = \frac{G}{l}\frac{dl}{dt} - \frac{G}{\eta}\sigma v \tag{16}$$

$$\text{Surface tension: } f_s = \frac{\alpha\pi D^2 k}{\sqrt{x^2+y^2}} \tag{17}$$

$$\text{Friction force with air: } f = 0.65\pi D\rho_{aria}v^2\left(\frac{2\nu D}{air\nu}\right)\text{^(-0.81)} \tag{18}$$

$$\text{Gravitational force: } f_G = \rho g\pi D^2 \tag{19}$$

where e is the electric charge, l the length of the ideal straight jet, V the difference in potential, L the distance between the drop and the collector, σV the viscoelastic tension, G the elastic modulus, η the viscosity, α the coefficient of tension surface, k the curvature of the jet, ρ the density, ν the kinematic viscosity and air ν the kinematic viscosity of air.

2.12 Possible nanofiber structures

By varying the electrospinning parameters, different nanofibers can be obtained from the morphological point of view. In particular, several studies describe the production of porous, banded, branched, helical and hollow fibers. Depending on the application for which the fibers are intended, it is convenient to choose one type of nanofiber rather than another. (25)

For example, when high surfaces are required, porous nanofibers become useful. The humidity of the environment plays an important role in the production of porous fibers but it is not the only parameter that comes into play: in fact, the type of polymer and solvent used are also significant. During the evaporation phase of the solvent the solution becomes unstable from the thermodynamic point of view and there is the formation of two distinct phases, one rich in polymer, the other poor in polymer. The rich phase solidifies very quickly and forms the matrix within which the pores created by the polymer-poor phase (whose solidification is slower) are created.

The mechanism by which ribbon-shaped (flat) nanofibres are produced is closely related to solvent evaporation. In particular, if the solvent does not evaporate completely, the fibers are still wet when they reach the collector and then yes (27) flatten by the impact.

The branched fibers are obtained when smaller secondary jets develop from the main jet, or when the jet breaks into more jets. This happens because during the phase of stretching and evaporation of the solvent, the shape and the amount of charge per surface unit present in the casting are modified; it follows that the balance between the electric forces and the surface tension is no longer respected and the jet becomes unstable. This instability leads to the branching of one or more jets smaller than those of the main jet.

The helical fibers are produced starting from the mixture of two different polymers, of which one of them is conductor. When the fiber is deposited on the collector, the charges of the

conducting polymer rise on the surface of the fiber: in this way an imbalance is created between the repulsive electrostatic forces and the viscoelastic forces. What happens is that the fiber structure is modified in an attempt to return to a situation of (28) balance between forces, helical fibers are formed in this way.

Hollow fibers can be obtained using the coaxial electrospinning technique. They are used a polymer and a mineral oil. The central part is removed with a bathroom in a (29) solvent that is able to dissolve the oil but not the polymer. The hollow fibers allow to functionalize the external and internal surface in a different way: this makes the more effective nanofibers in view of their use, as well as more flexible from the point of view application, since the type of functionalization will be specific depending on of employment. (30)

Thanks again to the coaxial electrospinning technique, fibers can be obtained with different composition, using two different polymers, one on the inner side (heart or core) e the other on the outside (shell or shell). The coaxial technique consists of using two capillaries one inside the other, inside which pass the polymer that will make from shell and the polymer that will be the heart. Another technique that allows to obtain fibers of different composition is that of the electrospinning side – by – side, where the two capillaries come positioned side by side. (31)

2.13 Applications

Regarding the fields of application of nanofibres, four can be identified large areas of interest: Bioengineering, Environmental Engineering and Biotechnology, Energy and (25) Electronics and, finally, Defense and Security.

2.13.1 Bioengineering

In the field of Bioengineering, nanofibres are used for tissue engineering (tissue reconstruction), wound dressing, affinity membranes and drug release. In the case of tissue reconstruction and wound dressing, the nanofibers act as scaffolds, which, when properly immersed in cell culture, promote cell growth and proliferation. The cells themselves are implanted inside the support matrices, which are subsequently inserted into the patient's body in order to repair and reconstruct the damaged tissue. Most tissues in the human body have a fibrous structure; this allowed us to focus attention on nanostructured scaffolds as supports for this purpose. Several studies report the production of scaffolds through electrospinning for the reconstruction of vascular, cartilage, nervous and bone tissues.

Wound dressing (wounddressing) is a therapy that aims to repair damaged skin due to burns and wounds. The use of nanofiber membranes in this sector allows us to meet requirements such as high gas permeation and protection from infection and dehydration. The ultimate goal is to be able to create a structure characterized by high porosity, as well as excellent barrier properties.

As for the release of drugs, the nanofibers are used as a "vehicle" to carry and release the drug precisely. For example, polymeric nanofiber membranes have been made in which drugs are incorporated. nanofibers are produced by electrospinning, it is important for this type of application to study and monitor the release rate of the drug by the membranes, and consequently the release time. The release rate depends on several factors, including the total amount of active ingredient present, the diameter of the fibers and the distribution of the active principle within the nanofibres themselves. Therefore, depending on therapeutic needs, you can play with these parameters to obtain a product with the desired characteristics.

Affinity membranes (affinity membranes) have the function of separating the target molecules using ligands chemically bound to the surface of the nanofibers. The separation is based on the

selectivity of the membranes and their ability to trap specific molecules, specific according to the ligands present on the surface of the membranes themselves. These membranes, placed in comparison with the traditional filtration columns, have a much greater separation efficiency, thanks to the high surface area that the fibers themselves offer.

2.13.2 Environmental engineering and biotechnology

In reference to these sectors, nanofibres are found to be excellent for the production of membranes for filtration and neutralization of bacteria (antibacterial membranes).

Filtration allows to purify the air both from solid particles, such as viruses, dust of various origins, etc., and from liquid particles, such as smog, humidity, chemical solvents and others. In both cases these are very small particles; therefore, to filter ultrafine, liquid or solid particles, it is necessary to have a porous structure characterized by very small pores or a significant thickness. The pressure losses for a gas that passes through a half of this type are quite high; it is therefore necessary to have a high pressure. It is desirable, on the basis of these considerations, to be able to create filter media with low load losses and it is precisely here that nanofibres come into play. In fact, nanofiber membranes exhibit high filtration efficiency, while maintaining high gas permeability and therefore low load losses.

If the surface of the nanofibers is chemically modified with substances capable of killing bacteria, then these filters also become useful as antibacterial filters.

By inserting catalyst particles inside the nanofibers, nanofiber membranes can also be used in the field of photocatalysis. Moreover, purely for this purpose, purely ceramic membranes can be made.

2.13.3 Energy and Electronics

As is known, great efforts have been made in research in the field of clean energy and renewable energy. Examples are wind and solar generators, the hydrogen battery and polymer batteries. Recent studies have turned to creating batteries made up of polymeric nanofiber membranes as a new type of energy generation. Nanofibers become useful for this purpose since they enjoy a porous structure due to the network of fibers themselves and a high specific area (little bulk for high available surfaces). Therefore several scholars have worked on the design and manufacture of polymer batteries using conductive polymer nanofibers.

2.13.4 Defense and security

In the defense and security sector, nanofibers are used in protective clothing for chemical and biological agents and in sensors (biosensors, chemical sensors, optical sensors and gas sensors). In recent years there has often been talk of means of mass destruction and terrorist attacks related to the use of toxic substances. Several researches have therefore been carried out in the field of protective clothing. The nanofibres become part of a filtering fabric, capable of trapping substances and particles that can be harmful to the human body. By way of example, masks with filters for nerve gas are mentioned.

The second application in the field of defense and security is that of sensors. Sensors are devices that can transform physical or chemical responses into electrical signals. Sensors characterized by a high sensitivity can be produced starting from nanofibres with a high specific area. The principle on which these sensors are based is that of take advantage of the chemical or physical reaction between the substance to be detected and that capable of detecting it.

Subsequently, the sensor converts the result of the physical or chemical phenomenon that occurs into an electrical output; from this we finally find a quantitative measure of the substance to be detected.

2.14 Polyacrylonitrile Fibers

Polyacrylonitrile (PAN), whose formula is shown in Figure 5 is an important polymer which has many properties such as resistance to solvents, abrasion resistance, thermal and mechanical stability and high tensile strength. It also finds many applications in different fields such as composite materials, protective clothing, nanosensors, separation of gas, purification of biochemical products and biomedical applications. (15)

Polyacrylonitrile, often referred to by the acronym PAN, is the polymer obtained from the polymerization of acrylonitrile. It is produced in a watery medium, at a temperature of 40-55 ° C, through the use of catalysts (potassium persulphate, potassium hydroxylamine disulfonate dihydrate and ferrous sulfate) (11)

There is also another method that exploits the use of peroxide as a radical initiator and requires higher temperatures or polymerization reaction by precipitation in aqueous solution starting from acrylonitrile. (16)

The nitrile groups present in the polyacrylonitrile allow the introduction of new functional groups through specific reactions. Some reactions that have been studied are, for example, hydrolysis and reduction to produce carboxylic or aminic functionalities useful for medical applications. (16) (17)

The amine-like functions can be introduced into the polyacrylonitrile by various mechanisms, illustrated below, which lead to polymers with characteristics, functions and properties that are very different from one another.

The first obtained acrylic fiber, known under the trade name Orlon, was produced by DuPont in 1950 and consisted of polyacrylonitrile. The use of this polymer is mainly aimed at the production of synthetic fibers resistant to aging, moths, physical and chemical agents and characterized by considerable mechanical properties. The PAN is also used as a precursor to obtain high-quality carbon fiber. (12)

The polyacrylonitrile decomposes before melting (315-320 ° C) and is quenched by solubilization in aprotic solvents such as dimethylformamide, dimethylacetamide and dimethyl sulfoxide. (13)

Polyacrylonitrile is a polymer that is used to obtain synthetic fibers that look like silk but have thermal insulation characteristics similar to wool or as a precursor of carbon fibers.

Polyacrylonitrile homopolymers are used as fibers in gas filtration systems, outdoor tents, boat sails but also in reinforced concrete fiber. It is often used in copolymers to make fabrics consisting of acrylonitrile and methacrylate or methyl methacrylate monomers.

The starting monomer is acrylonitrile having the formula C3H3N consisting of a vinyl group bound to a nitrile - C≡N.

Acrylonitrile is obtained by catalytic ammonialation of propylene according to the process known as the Sohio process according to the reaction:

$$2CH3 - CH = CH2 + 2NH3 + 3O2 \rightarrow 2CH2 = CH - CN + 6H2O \qquad (20)$$

The polymer is obtained by radical polymerization using as a starter a peroxide or a mixture of potassium peroxidisulphate K2S2O8 and a reducing agent such as potassium hydrogen sulphite KHSO3.

The polymerization reaction can be schematized as follows:

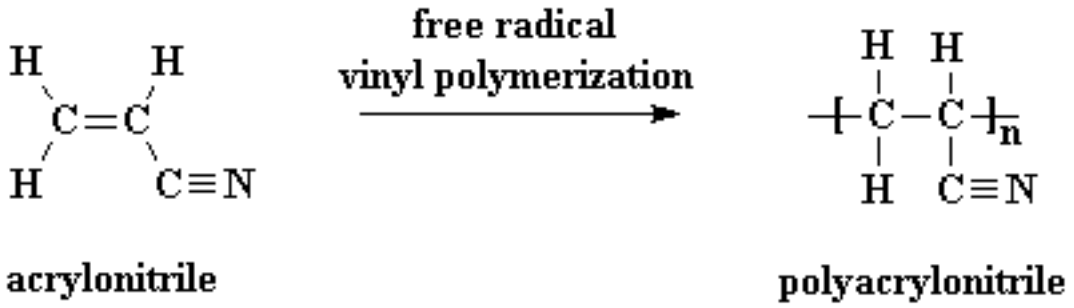


Figure 4: Polymerization reaction

Polyacrylonitrile fibers have a density of 1.17 g / cm3 and are lighter than wool which has a density of 1.32 g / cm3 but have an insulating power, compared to the wool of about 20% and have a 15% elongation at break . They show a good thermal stability and they only decolorate for exposure to temperatures above 175 ° C for long times. Polyacrylonitrile fibers shrink by about 1.5% when treated with boiling water for 30 minutes and have good resistance to mineral acids and diluted alkaline solutions while they are attacked by strong hot alkali. These fibers are not affected by molds and moths and show exceptional stability towards bleaching agents. (10)

This polymer is obtained thanks to a polymerization reaction by precipitation in (14) aqueous solution starting from acrylonitrile.

The nitrile groups present in the polyacrylonitrile allow the introduction of new functional groups through specific reactions.

Some reactions that have been studied are, for example, hydrolysis and reduction to produce carboxylic or aminic functionalities useful for medical applications. (9) (15)

The amine-like functions can be introduced into the polyacrylonitrile by various mechanisms, illustrated below, which lead to polymers with characteristics, functions and properties that are very different from one another.

2.15 Preparation of Fibers

The materials and the description of the electrospinning laboratory system for the preparation of fibers are shown in the following pages.

2.15.1 Materials

The materials used in the experimental phase are the powdered polymer to be functionalized and electrophilated, the solvents used to prepare and purify the products.

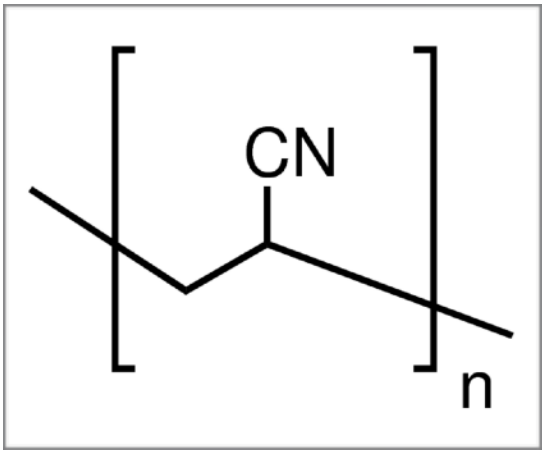


Figure 5: PAN chemical formula

Characteristics:

- Boiling point: Degrades

- Formula: C3H3N

- Density: 1184 kg / m³

- Molar mass: 53.06 g / mol

- Melting point: 572 °F (300 °C)

- ID IUPAC: Poly(1-acrylonitrile)

<u>Polymer</u>

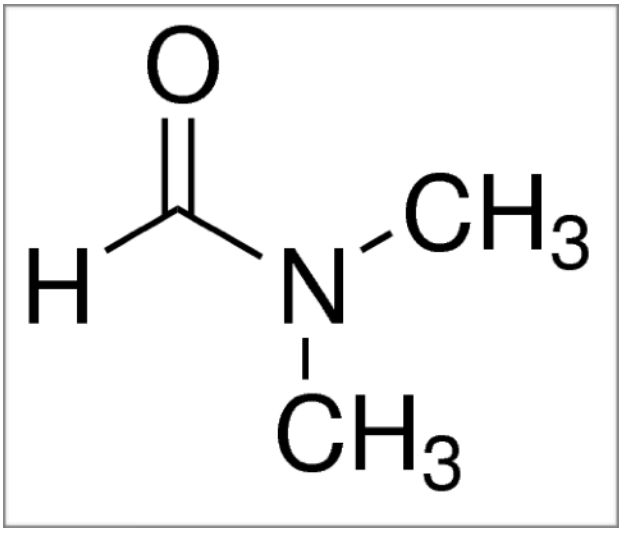
The polymer used for the functionalization reaction and to produce the fibers through "electrospinning" is the polyacrylonitrile (PAN) homopolymer, whose structural formula is shown in Figure 5.

Polyacrylonitrile(PAN with average molecular weight of 150 000) is purchased from Aldrich and used without further purification. PAN is generally used to produce synthetic fibers with remarkable mechanical properties and resistant to aging and to physical and chemical agents.

Solvents

Figure 6: DMF chemical formula

Characteristics:
- Boiling point: 307.4 ° F (153 ° C)
- Formula: C3H7NO
- Density: 944 kg / m³
- Molar mass: 73.09 g / mol
- Melting point: -77.8 ° F (-61 ° C)
- ID IUPAC: N, N-Dimethylmethanamide



The solvent used for the electrospinning process is N, N dimethylformamide (DMF), whose structural formula is shown in Figure 6.

The 99,8% N,N-Dimethylformamid (DMF) is obtained too from Sigma-Aldrich.

The choice of solvent is of fundamental importance because it must allow conditions optimal electrical conductivity, viscosity and surface tension of the solution to be subjected to electrospinning.

Tools for electrospinning

The main components that make up the plant for the electrospinning and electrospraying process are:

• a high voltage generator;

• a volumetric pump;

• a plastic syringe containing the polymeric fluid;

• a rotating metal manifold.

Figure 7 shows a diagram of a typical electrospinning plant. For these experiments the working environment was delimited in an ordinary chemical hood chamber in order to avoid external disturbances.

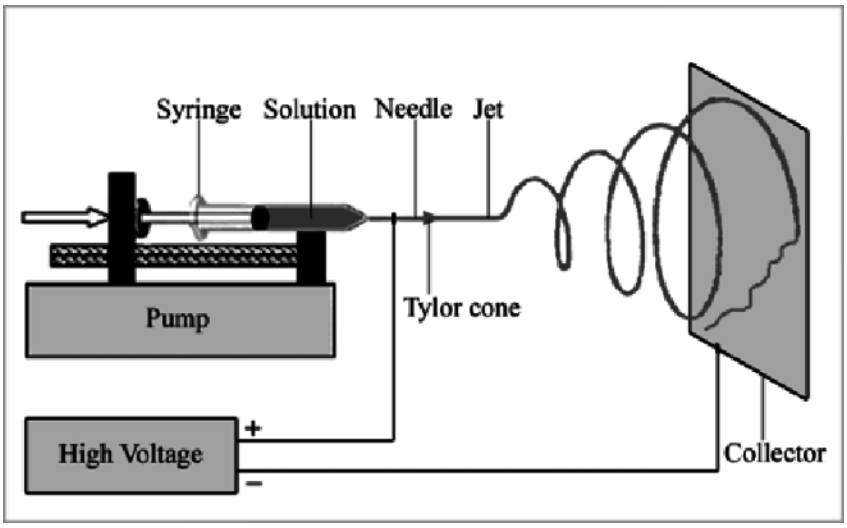


Figure 7: Horizontal set-up of electro spinning machine.

2.15.2 Procedure

The solution with concentration of 10% PAN/DMF (ratio of volume/volume) is prepared by stirring at room temperature for 2 hours at 60° Celsius at a speed up to 1000 rpm(the exact value is 1220) in order to obtain homogenous solution. The previous operation has been performed on Digital magnetic stirrer; in details, the model of the stirrer is Thermo Scientific Cimarec Multiposition Electromagnetic Stirrers 62125.

Fibers are electrospun as reported in literature (2) but changing some mechanical experimental parameters; in fact, tiers are electro-spun horizontally and not vertically as can be observed in the schematic set- up of figure 8.

In figure 8 is shown the real set-up through fibers has been obtained.

In details, after two hours of stirring on Digital magnetic stirrer Thermo Scientific Cimarec Multiposition Electromagnetic Stirrers 62125, 3 ml of the polymer solution is collected through

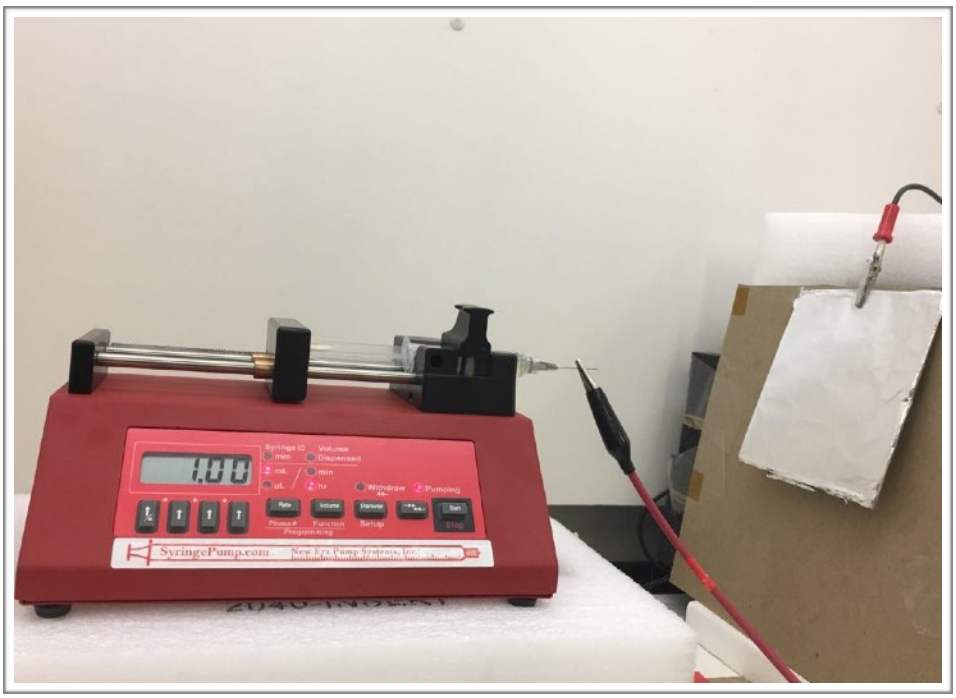


Figure 8: Electrospinning set-up

5 ml syringe equipped with a 22 gauge needle.

The syringe is put in NE-1010 Higher Pressure Programmable Single Syringe Pump

Then the parameters of the pump are selected:

- Flow rate: 1.5 ml/hour
- Distance between machine and collector: 15 cm

The collector of the syringe-pump machine(NE-1010 Higher Pressure Programmable Single Syringe Pump) is covered with a square non sticky aluminum sheet which dimensions are 12*12 cm.

The collector is connected to Bertan Spellman 230-20R High Voltage Power Supply DEL HVPS

INST 230 20KV .600mA battery and the voltage selected is 18KVolt.

Figure 9: Formation of fibers on collector

After one hour the solution is pumped and electro-spun and so the aluminum foil with the PAN fibers is removed.

In figure 9 it can e possible to see the formation of the fiber on the collector.

In fact, after few seconds after the activation of the pump a white spot on the luminescence aluminum foil can be observed.

The spot is the deposition of electrospinned fibers in random order. After more than one hour(the rate can be changed modifying the parameters of the pump) of electro spinning all the surface of the collector is covered and depending on the thickness of the PAN layer on the collector it can be possible to detach easily the fibers without breaking them.

In Figure 10 in reported the layer of PAN fiber obtained after one hour and half of electro spinning with the rate of 1.5 ml/hour. The fibers randomly deposit on the collector following the field lines of the electric field formed between the gauge and the collector but at the end of the process the layer formed is enough homogenous to be carbonized.

Figure 10: PAN fibers layer on the collector

2.16 Carbon nano-fibers synthesis

2.16.1 Introduction

The raw materials used for the production of carbon fibers are essentially two: the polyacrylonitrile fiber, PAN; obtained from the polymerization of acrylonitrile, and pitch, also called pitch, a residue obtained from the distillation of tar or oil; in detail fibers were obtained from 10% polyacrylonitrile (PAN)/*N*,*N*-dimethyl formamide (DMF) solution (35)

The precursor that actually started the era of carbon fibers, is the polyacrylonitrile fiber, PAN, characterized by an adequate chemical composition, a particular molecular orientation and a certain morphology.

The chemical composition is important to moderate the exothermicity of the cyclization reaction of the Carbon nanofibers; for example the property of 18kcal / mole is obtained when cyclization is conducted at 220 - 260 ° C for a few hours. (32)

In the following pages, are reported the main steps to obtain carbon nanofibers by the process of carbonization.

2.16.2 Carbon fibers

As reported in the Introduction, carbon fibers were first discovered by Edison towards the end of the nineteenth century. He noted that rayon could be converted into filamentous carbonaceous material through the use of incandescent lamps. Following this discovery, carbon fibers were used for the first time by the Union Carbide Corportation (UCC) for commercial purposes in the early 1960s (36).

The definition of carbon fiber is quite strict in fact by carbon fiber we mean a polymer of carbon atoms structured in graphitic form with a diameter between 5 and 15 μm (37).

These fibers, although presenting an excellent resistance, especially in the direction of orientation of the filaments, have a rather low modulus of elasticity. The graphitic structure is, in effect, the fundamental characteristic of these fibers and the main reason for their use in the development of composite materials. The crystalline structure of graphite gives the carbon fibers particular mechanical properties and although the crystals tend to have an anisotropic behavior, it is possible to orient the crystalline structure in the desired direction through the fiber processing carbon(35).

2.16.3 Process of carbonization

The synthesis of PAN fibers as precursor of carbon fibers involves different steps common to all fibers with polymeric precursors:

1) Polymer in chemical form which does not melt at high temperatures/treatments.

2) Spinning of the polymer into fine fibers.

3) Carbonization process at high temperature(~1000°C)

The final product is made up predominantly of hexagonal honeycomb networks of carbon. PAN fibers achieve a well-ordered crystalline structure(such as materials made of mesophase pitch) that is why the further heat treatment at temperatures up to 3000 °C is not used, this step called graphitization is not needed because for tour purpose to create Carbon fibers a sufficient degree of order is still reached. The high degree of orders of fibers for specific reasons is achieved only through further heat treatments around 3000 °C . (31)

Carbonization allows the formation of carbon fibers that have an almost pure, continuous and regular graphitic structure along the entire fiber.

More over, the cyclization reaction leads to a fireproof material of black color, oxidized PAN, but with modest mechanical properties.

The subsequent carbonization process (400-1000 ° C) is generally carried out in an inert or vacuum atmosphere and leads to the removal of atoms from the structure and to the development of the graphitic structure. (32)

In the end, the possible graphitization process takes place at temperatures between 2500 ° C and 3000 ° C and contributes to improving the mechanical characteristics of carbon fibers. (35)

The PAN fibers, as it is reported in the previous chapeter, are spun at room temperature from a solution with controlled ratio of PAN in Dimethyl formamide(DMF), in fact, because of the strong intermolecular interaction of the chains, PAN can only dissolve in strong polar solvents.

Water can be added to the solution depending on the purpose of fibers ut in the production of fibers described is not used. After, fibers are heated in an oxygen-containing atmosphere at 260 °C for 2 hours in air in order to stabilize them and prepare for the following carbonization step; in fact, PAN precursor fibers usually are preoxidized under low temperature

in order to pass from the linear PAN molecular chains into aromatic ladder structure which is suitable for further carbonization. (33)

In Figure 11 can is shown the main passages to obtain carbon fibers from PAN.

The main reaction can be divided generally in three main steps:

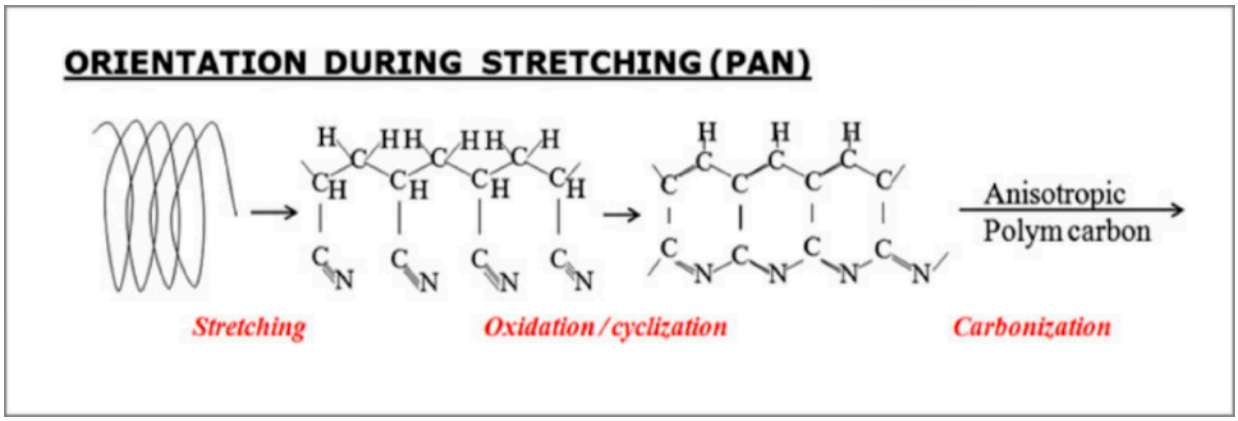


Figure 11: Main passages of PAN carbonization.

1. <u>Initial stage: Stretching</u>

   During the initial stage, at. 195°C, PAN precursor fibers undergo morphological structure rearrangements. Moderate stretching can induce additional orientation and order in the fibers that is why it is usually imposed in this period.

   The contents of C, H and N decrease slowly, while, due to oxidation, the one of O increases.

   In the first stage, the trivalent bond existing in the elementary cell of the polymer, between nitrogen and carbon, is broken. At the end of the heating, a cyclic ring structure is formed called

   tetrahydropyridine.

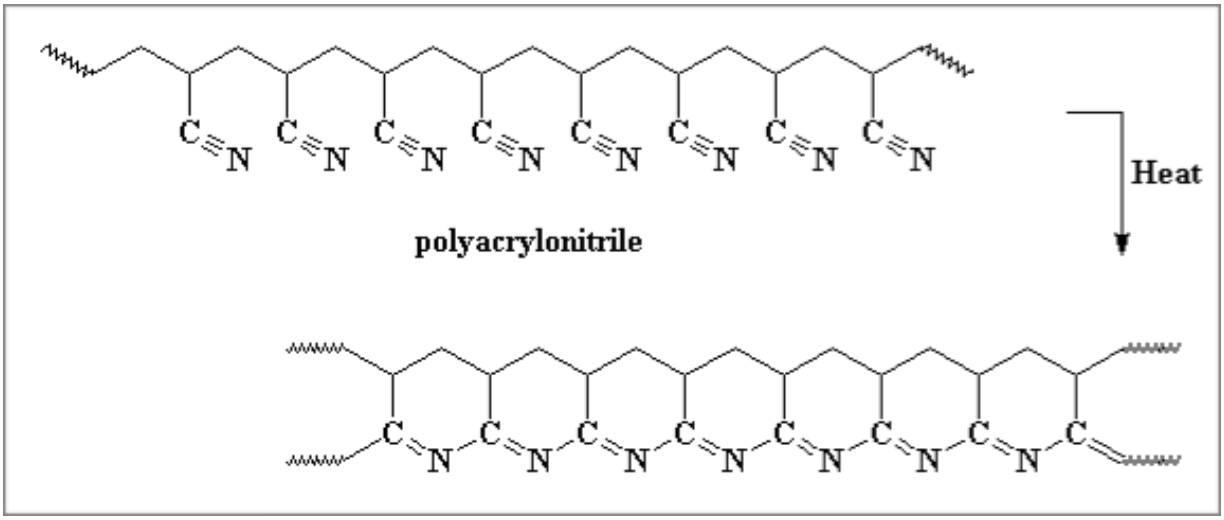


Figure 12: Heating and Stretching- Cyclic ring structure is formed called tetrahydropyridine.

2. <u>Middle stage: Oxidation</u>

The content of C, H and N decreases along with the gradual decrease of O and the density of the compound increases rapidly, which is the result of three reactions of: the cyclization reaction, dehydrogenation reaction and oxidation reaction.

Oxydation during heat up: O2 breaks the triple end of N and cyclization starts; it is favored in amorphous domain. In fact, in this phase the bonds between carbon and hydrogen are broken. The previously formed rings become aromatic, and hydrogen is released in gaseous form.

In the presence of oxygen, the triple bond between C and N is converted into a double bond with the formation of ladder structure. The cohesive energy between the relative chains drops significantly due to the absence of this bond, which can usually induce the decrease in tensile strength of preoxidized fibers. The carbonization process results in cross-linking between adjacent chains ending with turbostratic graphite structure. AN example of this step is reported in figure 13.

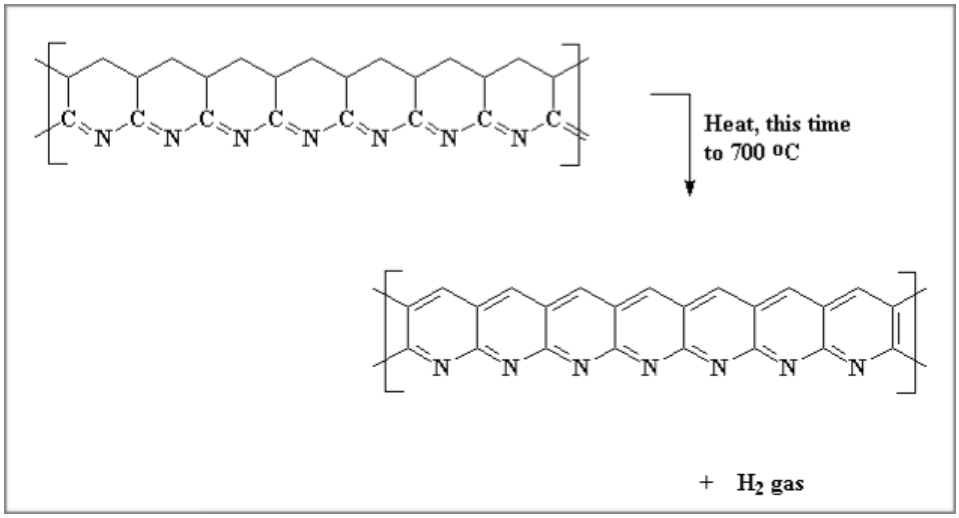


Figure 13: Oxidation Process - The carbonization process results in cross-linking between adjacent chains ending with turbostratic graphite structure.

3. <u>Last step: Cyclization</u>

The nitrile groups condence and form closed ring structures which represents a more stable conformation called turbostratic graphite structure (33).

During carbonization reactions, there is a huge loss in weight which produce a large quantity of contaminants as volatile gases and some tarry substances, and the amount O, H and N decreases because they are released as gasses while the amount of O increases.

The carbonization phase takes place in the absence of air. In a first stage the temperature is raised to values between 400 and 600 ° C. Previously formed aromatic chains literally merge by expelling hydrogen atoms, which is released in the form of gas. At the end of this process, a strip polymer is obtained, consisting of three chains of aromatic rings which have nitrogen atoms at their lateral ends.

The increase of C indicates that more basal planes are formed duringcarbonization, which could lead to the rearrangement and compactness of structures along the fiber axis.

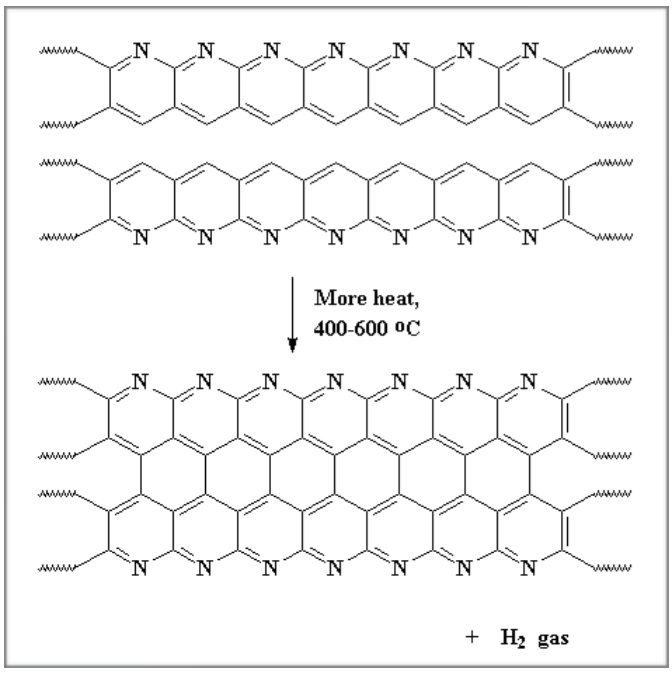


Figure 14: Carbonization - turbostratic graphite structure is achieved.

Carbon fibers with about 95% of C and 1.76g/cm^3 of density can be obtained as a result thanks to this process.

In this step, the oxidized fibers were subjected to low-temperature carbonization in a pure nitrogen atmosphere from 350°C to 600°C for 5 hours using tube furnace: "Compact 1100ºC Tube Furnace with Solid Precursor Sublimator for CVD Growth of 2D Layers of TMDs - OTF-1200X-4-NW-UL" and the cyclization pattern of the fiber is obtained as shown in

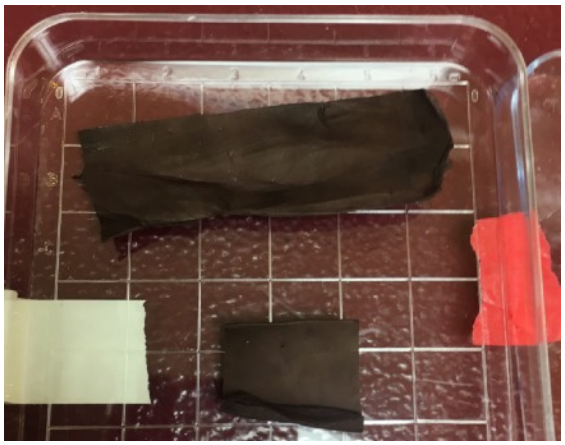


Figure 15: Carbonization - Fibers made by turbostratic graphite layers.

Figure 16: Turbostratic graphite fiber.

figure 14.

From 400 to 1000 ° C HCN, NH3 and N2 develop; CO, CO2 and H2O can also develop as a function of the amount of O2 that the oxidized precursor has bound during the treatment at 220-260 ° C in air.

The final aspect of fibers after Cyclization step is shown in Figure 15 where are shown two fibers layers and Figure 16 where the attention is focused only on one fiber layer..

2.16.4 General considerations

After treatment at 1000 ° C the fiber contains more than 90% of carbon and about 5% of nitrogen.

It is very important to check the fiber retraction during the cycling phase at 220-260 ° C, since in this phase the alignment of the molecular segments along the fiber axis is determined, the orientation on which the final elastic modulus depends. The molecular orientation imparted to the original acrylic fiber influences the toughness and the elastic modulus of the final fiber. An excessive orientation is negative because it introduces superficial and inside defects of the fiber. In any case, fibers obtained through thin PAN fibers layers have a really fragile behavior and are really delicate to handle. (32)

2.16.5 Carbonization pattern scheme

In Figure 17 it is shown the pattern that the tube furnace (the model is Compact 1100ºC Tube Furnace with Solid Precursor Sublimator for CVD Growth of 2D Layers of TMDs - OTF-1200X-4-NW-UL”) usually follows.

1) First step: n this case the temperature inside the tube arrives at 200 °C in 30 minutes and it is hold at 200 °C for five hours in air. This one is the first step, fibers are heated in an oxygen-containing atmosphere at 260 °C for 2 hours in air in order to stabilize them and prepare for the following carbonization step.

2) Second step: then, the tube is filled with Argon and the temperature increases until 800 °C and it is hold at that temperature for 2 hours.

3) Third step: then the machine is turned off and it takes 3 hours to arrive at room temperature.

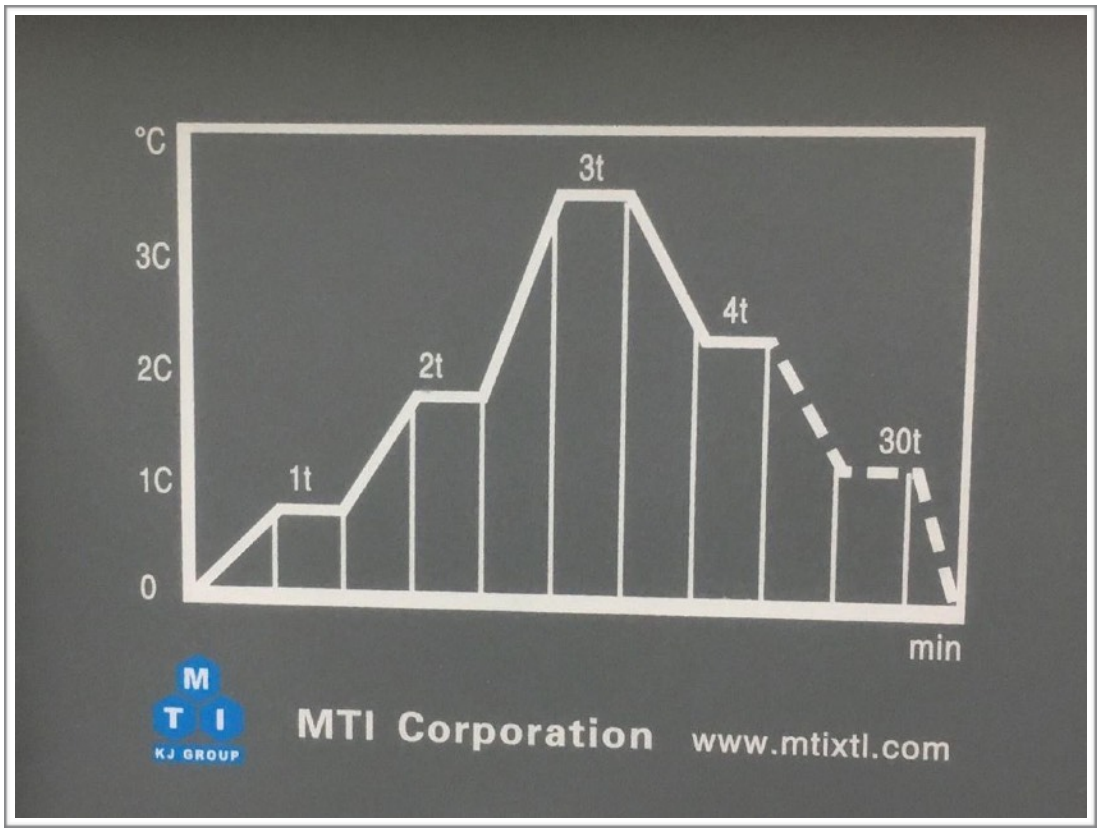


Figure 17: Heating scheme

2.16.6 Check of the reaction

When the fibers are heated to higher temperatures, using the X-ray diffraction (XRD), can be proved that the linear molecular chains change into aromatic ladder structure.

It is reported the analysis thanks to Xray diffraction of the procedure of carbonization that can be a possible way to verify the structure of layers obtained.

FTIR spectra of fibers are analyzed at various stages. At the starting stage of preoxidation (below 235°C), the intensity of absorption peaks are:

- C≡N —> 2939 cm^{-1},
- C–H —> 1454 cm^{-1}, and 1358
- C–CN —> 1070cm^{-1}

There is no change of 1620 cm-1 while the peaks of precious bands gradually became more weak. Due to the increase of temperature and due to dehydrogenation, the absorption peak at 1620 cm-1 begins to intensify. In the meantime, thanks to of oxidation reaction the peak at 1772 cm-1 attributed to C=O gradually increases [5].

The other bands, in details: the bands at 2243 cm-1, 2939 cm-1, 1454 cm-1, and 1358 cm-1, and 1070cm-1 basically disappear at 280°C.

FTIR could not provide information about chemical structure related to carbonization progress due to the highly absorbing nature of black carbon fibers, In fact, the FTIR spectra of carbon fiber appear like a line.

2.16.7 Honeycomb crystal lattice

The graphene crystal lattice has a hexagonal honeycomb structure in which each atom is able to bind to three adjacent atoms, placed at 120 ° one another, with interatomic distance = 1.42 Å and center-to-center distance of 2.46 Å. To illustrate the crystalline lattice of graphene I need some mathematical-geometric tools to help us describe the spatial arrangement of atoms in crystalline solids. I then introduce the director concept of Bravais, an infinite set of points having a fixed, regular geometric arrangement and periodic in space. If I place the originecartesian on any of these points, every other point is identified by a vector. We therefore create a base of vectors

that, through a linear combination, allow to identify each point of the lattice. These vectors are primitive primitives. In the case of a two-dimensional crystal the primitive vectors are two and we call them a_1 and a_2 as can be seen in figure 18. In this way we mathematically define the Bravais lattice simply as the set of vectors R = $n_1a_1 + n_2a_2$, with n_1 and n_2 integers, and we are also able to identify a primitive cell, a volume of space which, translated through all the Bravais vectors, fills it completely without overlapping and without leaving space. Depending on how primitive they are we have different possible crystals with different geometries and symmetries; in two dimensions there are five: square, rectangular, rectangular centered, hexagonal and oblique. The honeycomb lattice cannot be traced to the simple cases, since it does not satisfy the definition of any of the possible Bravais lattices. The lattice of graphene belongs to a different class of crystalline lattice lattices, which can be constructed from a Bravais lattice, with the addition of a "base", which in this case is represented by two carbon atoms C1 and C2e from the primitivebe vectors. In the case of graphene between the two primitives there is an angle of 120 °. The Bravais lattice which is more similar to the graphene lattice is the hexagonal one, but it is not identical, presenting an additional node in the center of the hexagon.

The honeycomb lattice is therefore not a Bravais lattice; rather it can be seen as the interpenetration of two hexagonal Bravais lattices. Otherwise we can consider an extension to the Bravais lattice definition, introducing what is called the ethics.

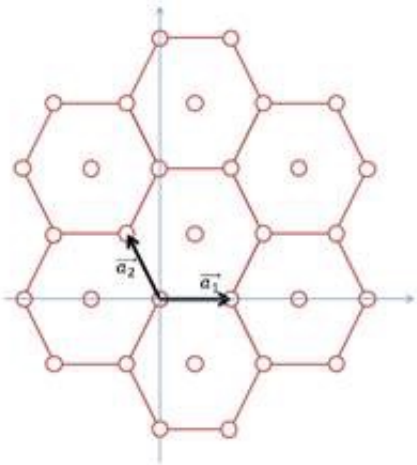


Figure 18: Exagonal bravais lattice

3 SEM Characterization of images

Scanning electron microscope, commonly referred to S.E.M. from the English Scanning Electron Microscope, is a type of electron microscope.

The microscope does not use light as a source of radiation. The beam is generated by an electronic source, typically a Tungsten filament, which emits a stream of primary electrons concentrated by a series of electromagnetic lenses and deflected by an objective lens. The latter, in addition to refocusing the beam further, imposes a controlled deflection on the same, so as to allow the scanning of areas of the sample.

The scanning electron microscope can obtain images that appear almost three-dimensional even of relatively large objects.

SEM must operate in a high vacuum (with pressures lower than 10-3 Pa) to allow production and the sample must be conductive and grounded, so as to be able to remove from the analysis area any possible accumulation of charge that would make it impossible observation. Non-conductive samples can however be observed at the SEM by making metallic coatings, dehydrating the wet samples or performing the operation quickly so as to prevent the accumulation of charges from overheating the object in question. (51)

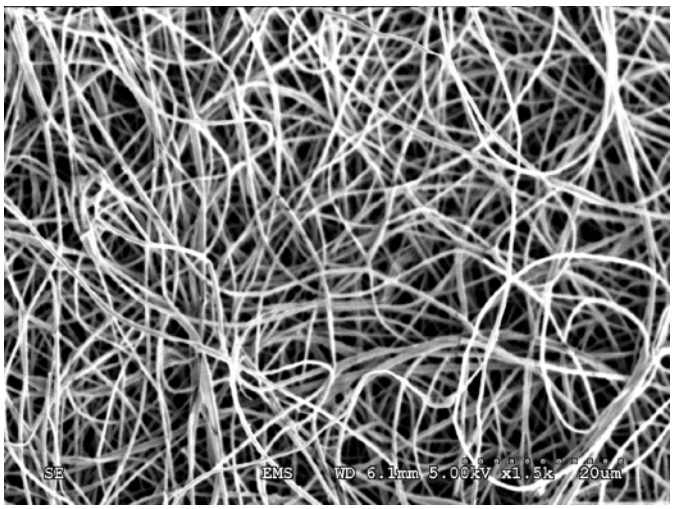


Figure 19: Fibers of PAN

In image 19 it is shown how PAN fibers appear through SEM. The average diameter of the fiber is calculated through the open source software FIJI, acronym for Fiji Is Just Imagej and the value its value is 1,2 µm.

In image 20 it is reported an explanatory example of attachment on PAN fibers. In the center of the image there are cells at different steps of attachment procedure and they are highlighted with a green color. Cells 1, 4 and 5 are still in the reversible phase of attachment and they do not adhere to the substrate as can be evaluated due to their round shape. While cells 2 and 3 have already release proteins to attach to the fibers and have already started the procedure as can be evaluated through their shape non anymore round but spread.

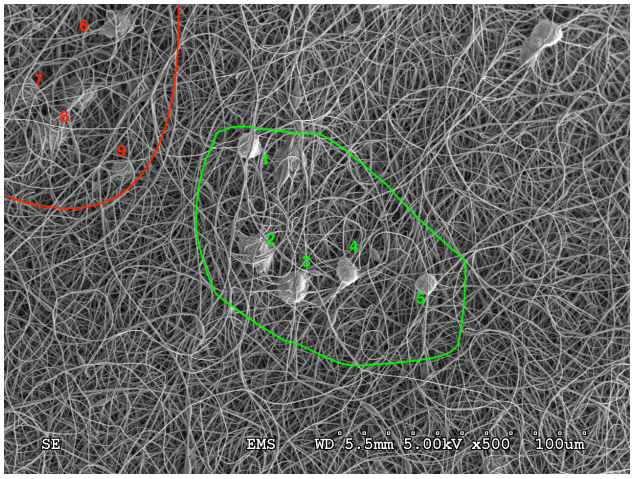


Figure 20: Cells anchored on Pan Fibers

Cells on the top left corner of the image are highlight with red color. In this part of the figure it is possible to see cells that not only have attached to fibers but that even started to penetrate in the substrate. Their shape is stretched because cells have already secreted protein and other elements to used to attach irreversibly to the substrate.

Carbon fibers are reported in figure 21; the average value of the diameter is evaluated similarly to PAN fibers and its value is 1.6 µm.

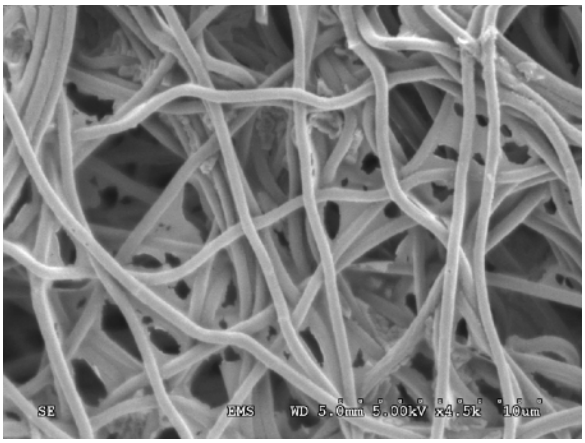


Figure 21: Carbon Fibers

The average diameter is 4 µm bigger than average PAN Fibers diameter.

In figure 22 it is reported the attachment of cells on carbon fibers. Lot of proteins and other material is released by the cells in order to recreate a biological network useful for attachment procedure and for signaling procedure between cells and between cells and extra cellar matrix. More over, as it can be observed in all the image cells stick together near to each other which is fundamental for communication using cell-cell junctions, a kind of tight or gap junctions though which cells can communicate or exchange natural products as proteins.

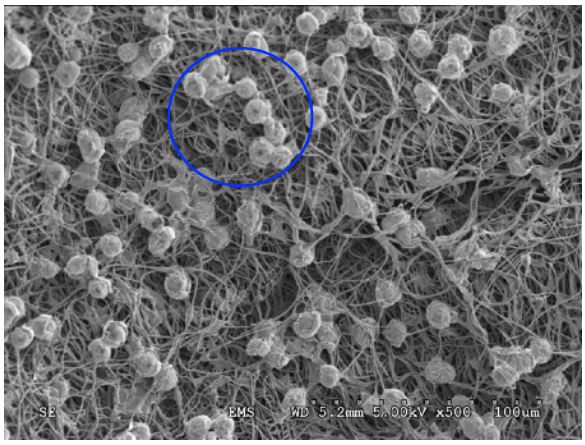


Figure 22: Cells anchored on Carbon Fibers

Figure 22 shows this behavior in the blue circlet in the top centre of the image.

It is hypothesized that the other cells present in the image were migrating on the surface of fibers layers to recreate the natural network present in animal natural tissues.

3 BIOLOGICAL ACTIVITY

Fibroblast cells interaction with nanofibers at various weight percentages

4.1 Fibroblast

Fibroblasts (or fibrocytes) are the most numerous cellular elements of the loose connective tissue. They are deputed to elaborate the constitutive elements of the collagen and elastic fibers as well as the proteoglycans and the glycoproteins of the fundamental amorphous substance. (1)

In the body there are specialized cells responsible for the synthesis and maintenance of extracellular material. More precisely, we are talking about fibroblasts, which derive from precursor cells of the primitive connective tissue. (2)

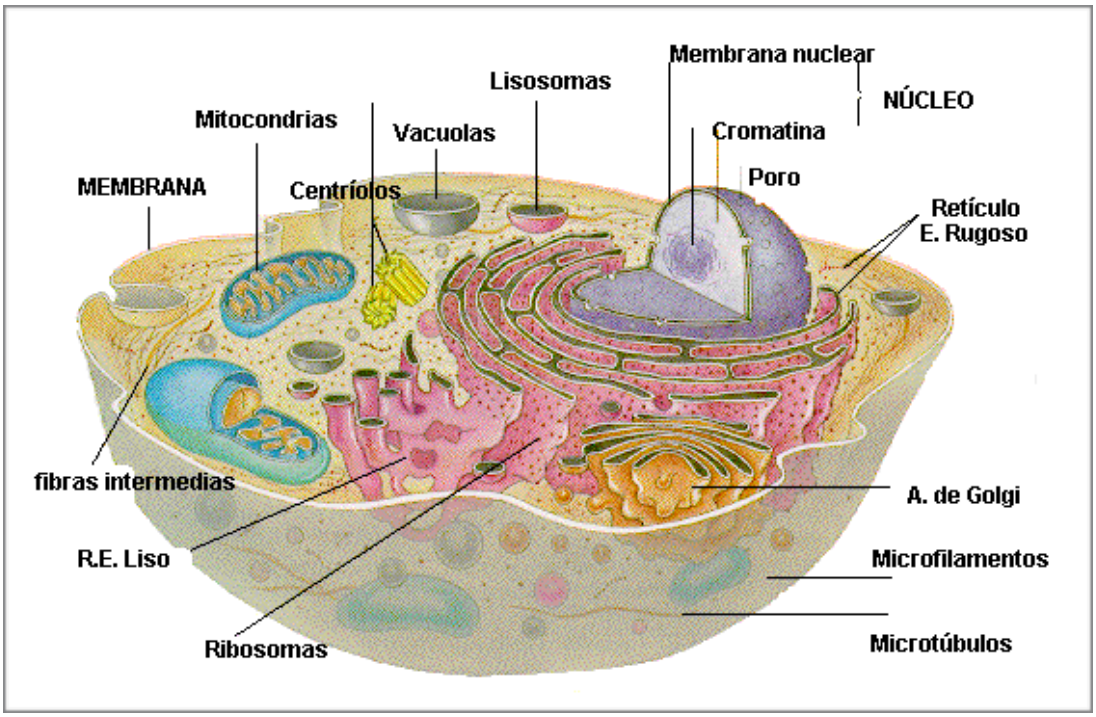


Figure 23: Cell section

They also intervene in the synthesis of all the macromolecular components of the elastic fibers which, in the locations where fibroblasts are lacking (like the elastic tunic of the arteries) are produced by smooth muscle cells. (1)

The fibroblasts are young connective cells that synthesize the pro collagen, a molecule

that will give rise, outside the cell, to the trope collagen with a process of exocytosis. Procollagen is produced not only by fibroblasts, but also by odontoblasts, chondroblasts and osteoblasts. (2)

Fibroblasts do not have a characteristic shape and it is difficult to distinguish them from other cell types. They are often arranged along the bundles of collagen fibers and appear as elements cast with an elongated core; in other locations they have a starry shape with numerous extensions. Near the nucleus are the diplomas and the Golgi apparatus; there are long and thin mitochondria and flattened cisterns of endoplasmic reticulum. In the Golgi apparatus the synthesis of the macromolecular components constituting the fundamental amorphous substance and their sulphation, as well as the link with the protein component produced on the ribosomes takes place. Fibroblasts do not possess a basal lamina. As in other cells, there are microtubules (20-25 nm thick), intermediate filaments containing vimentin (10 nm thick) and actin-like and myosin-like microfilaments (5 nm thick) dispersed throughout the cell but more concentrated in a thin area immediately below the plasma membrane. All of these filaments can be detected under light microscopy using anti-tubulin, anti-desmin, anti-actin and anti-fluorine anti-myosin antibodies. (1)

Fibroblasts are fixed cells but in some surroundings they may have modest migrant properties.

They are devoid of phagocytic activity and therefore do not ingest appreciable quantities of dye at least that the latter is not in high quantities. (1)

A common method used to distinguish these two cell types is that of granulopexy or vital staining with colloidal dyes which exploits the phagocytic properties of macrophages. If an animal is injected with a vital dye, such as the blue tripan or the lyocarmin, the injected material

is phagocytosed by macrophages and concentrated in large granules in their cytoplasm, while the fibroblasts and other cellular types of the connective tissue do not take the substance or they take on little, appearing therefore colorless. When fibroblasts propagate in their substrates they adhere to the surfaces through two particular sites.

A site, called focal adhesion, is characterized by a finely dotted area due to the close contact of the ventral cell surface to the substrate. Focal adhesions are responsible for the strong adhesion of cells to their substrate.

The second type of adhesion, called close contact, is characterized by a more focal area, often surrounding or adjacent to the focal adhesion, where the cell surface is separated from the substrate (30 nm space). The close contact site contributes only partially to cell adhesion. Fibronectin, a protein of the extracellular matrix, is located adjacent to the sites of focal adhesion. (1)

4.2 NIH 3T3

In details, NIH 3T3 are used. The NIH3T3 cell line, not only is one of the most commonly used fibroblast cell lines but it has become the standard fibroblast cell line to perform experiments.

NIH 3T3 cells are obtained from a NIH Swiss mouse embryo. These kind of cells is sensitive to sarcoma virus and leukaemia virus focus formation and it suffer from contact inhibition.
Two scientists at the Department of Pathology in the New York University School of Medicine, George Todaro and Howard Green cell line established this cell line from the tissue of a swiss albino mouse embryo in 1962.
The '3T3' name alludes to the abbreviation of "3-day transfer, inoculum 3×10^5 cells.

At first, the cell line was settled from the primary mouse embryonic fibroblast cells cultured by the officially assigned protocol, called '3T3 protocol'. The embryonic mouse primary cells were transferred (the "T") every 3 days (the first "3"), and then the fibroblast were

introduced into a culture medium at the fixed density of 3×10^{5} cells per 20 cm^{2} dish (the second "3") continually. (3)

Culture of cells with an indefinite and considered immortal life, called a cell line to distinguish it from the cell line that is not perennial. In order to clone individual cells or to study and modify their behavior, biologists need cell cultures capable of maintaining over one hundred replications. All animal cells, even if kept in the best environmental conditions and fed with everything they need, live and replicate only for a certain amount of time, after which they age and die. These cell cultures, called primary cultures, have a limited duration in time. Continuous cell lines, on the other hand, are 'transformed' cells, ie cells derived from tumors or cells that have undergone a genetic modification, an oncogen transformation, which has enabled them to grow indefinitely. These abilities of 'immortalization' and of indefinite growth depend on the species from which they derive: for example, normal chicken cells die after a few replicative cycles and only rarely do they transform. The first human cell line is the HeLa cell line, obtained in 1952 from a carcinoma of the uterine cervix: the name HeLa derives from the name and surname of the woman from whom the tumor was removed. This cell line has proved to be of immeasurable value for research on human cells and is still one of the most used cell models in the laboratory. In most cases, cell lines are undifferentiated although some may perform functions typical of the normal differentiated cells from which they derive. (6)

Cell cultures are simplified systems that allow the maintenance of cells in vitro; cells grown in vitro are used both for the study of biological phenomena such as growth and differentiation, and for the study of the structure and function of genes through genetic manipulations. Availability and relative cost-effectiveness, easy standardization and experimental reproducibility make the methodology advantageous, however, in the evaluation of the results, the remarkable

simplification of the in vitro experimental system must be considered compared to the in vivo one (for example exposure to exogenous substances takes place different from what occurs in vivo, moreover the concentrations chosen to work in vitro do not always correspond to the physiological values of the organism under examination). In an in vitro system it is possible to maintain cells of different origins, from bacteria to yeasts to primary cultures of animal cells and cell lines; naturally cells from different organisms require specific growth conditions. (4)

4.3 Primary Cultures

A primary culture is generated by isolating cells directly from tissues or organs, the cells isolated in this way better reflect the biochemical activities of cells in vivo, however they have a limited life and, for the realization of long-term projects, it is necessary to foresee different isolations.

To proceed with the isolation of a primary tissue culture it should be remembered that a tissue is composed of different cell types, held together by matrix; it is therefore necessary to break the matrix to separate the cells then proceeding to the selection of the cell types necessary for the experimental purposes. The procedure requires that the tissue sample be treated with trypsin and collagenase in order to digest the extracellular matrix, and with EDTA for the purpose of chelating Ca ++ on which cell-cell adhesion depends. A primary culture is obtained from the cell suspension obtained. The cells of the primary culture adhere to the plate and grow to cover the entire available surface. At this point they are removed from the culture plate and placed in new low density plates in order to obtain secondary cultures. (4)

4.4 Immortalization

After a certain number of duplications (variable by cell type), the primary cells stop dividing and die as a result of shortening of the telomeres. To obtain a cell line capable of proliferating without limit (continuous cell line), it is necessary to immortalize the culture; there are several methods to achieve this result, for example:

1) Introduction of the exogenous gene coding for the catalytic subunit of telomerase
2) Use of oncogenes derived from tumor viruses (SV40 antigen), which trigger transformation mechanisms similar to those that determine tumor proliferation.

Cells from tumor tissues are naturally immortalized cell lines, not by chance that the first continuous cell line was isolated by George Gey in 1951 from cervical tumor tissue (Hela).

The possibility of producing continuous cell lines allows to reduce the experimental variability linked to the use of primary cultures, ensuring greater reproducibility. However, when analyzing the results, it should be borne in mind that the immortalized culture is generally further from the physiological conditions of the starting organism. (4)

4.5 Culture conditions

Since within the body there are specific control systems that regulate vital functions such as nutrition, pH control, protection, temperature maintenance, the cultivation of cells in vitro must take place ensuring the maintenance of the same physical-chemical conditions, for this reason they are the functions performed by the culture medium and by the tools used for incubation are fundamental.

The first definition of culture medium capable of supporting the growth of eukaryotic cells is due to Harry Eagle - 1955. The culture media for animal cells normally contain inorganic salts that ensure cations and anions such as Na +, K +, Mg ++, Ca ++, Cl- , indispensable for cellular function, glucose for metabolic functions related to energy requirements, essential amino acids essential for protein synthesis, vitamins that support innumerable cellular functions and serum, which is a source of proteins and growth factors that are generally indispensable for proliferation.

The pH of the culture medium is buffered to pH ~ 7.4 by generally adding bicarbonate / carbonic acid. The presence of an indicator such as phenol red makes it possible to monitor the pH of the culture medium, while the yellowish color indicates an acid pH value whereas the violet-red corresponds to a basic pH. (4)

When the pH is around 7.3 (physiological value) the color of the soil is red-orange.

At acidic pH (6.8-7.0) the color turns to yellow: with the proliferation of cells the pH of the soil is acidified for the production of C02 from part of the cellular metabolism: the soil must be changed.

At a basic pH (> 7.5 -7.6) the color turns to red-violet: this coloring indicates that the cells are not active metabolic. (5)

4.6 Temperature and CO2 control

The physical environment necessary for in vitro growth is provided by incubators, instruments capable of maintaining controlled temperature and atmosphere. Cells grown in vitro require a strict temperature control at least as much as that of the living organism; mammalian cells are commonly grown at 37 ° C, while for other cell types, such as drosophila cells, the incubator must be able to be regulated at temperatures as different as 30 ° C or 34 ° C.

The CO2 partial pressure suitable for the in vitro culture of animal cells must be close to that measured inside the tissues (35-45 mmHg, equal to 4.6- 5.9% of CO2), for this reason the atmosphere inside incubators consists of 95% air and 5% CO2. (4)

4.7 Sterility control

Cell cultures are extremely sensitive systems and, to handle them, specific skills and instruments are needed to guarantee sterility. All the activities that require the manipulation of the culture are in fact carried out under a vertical laminar flow hood: the air coming from the outside is filtered, so as to retain contaminating particles, and driven by the laminar flow and a positive pressure to cross the work space vertically from top to bottom; a system made in this manner ensures that only sterile air is circulated in the working space dedicated to the handling of the crops.

All materials that come into contact with crops must be sterile; for materials such as glassware and metals dry stoves are used, at 150 ° C, for saline solutions autoclaving is used, while 0.22 μm pore filters are used for solutions containing macromolecules or thermolabile molecules such as culture media .

The contaminations that most commonly affect a cell culture are due to microorganisms, such as bacteria and mycoplasmas, but also to yeasts and molds. Bacterial contamination is an easy event to recognize because it causes a sudden acidification and cloudiness of the culture medium. It causes irreversible damage and the culture must be eliminated. Infections with intracellular organisms such as mycoplasma are much less evident and, at least in the early stages, do not interfere with cell viability, also for this reason it may be appropriate to intervene with antibiotics in an attempt to eliminate the contaminant. (4)

4.8 Types of cultures

Depending on whether the crop requires to adhere to a surface or grow in a liquid medium, crops grown from those in suspension are distinguished. Some cell types, characterized by adherent growth, due to contact inhibition, stop proliferating when they form a monolayer. The most common culture surfaces are the Petri dishes, available in various sizes and made of plastic material treated appropriately in order to expose negative charges that promote cell adhesion.

The growth curve of an in vitro culture, expressed as the logarithm of the cell number as a function of time, begins with a slow growth phase known as the latency phase, continues with an exponential growth phase and ends with the senescence phase and death. The duration of a crop can also vary widely: in fact, it is possible to distinguish short-term cultures, whose growth curve ends after a reduced number of replications, and long-term cultures, in which the cells divide many times before dying. We will see later that it is however possible to intervene in such a way as to lengthen the life of the crop indefinitely; to obtain this result it is necessary to proceed with the immortalization.

4.9 Biocompatibility study

Biocompatibility can be described as the measurement of how compatible an instrument is with a specific biological system and the main aim of performing biocompatibility testing is to determine the fitness of a device for human use and avoid potentially harmful physiological effects.

In details, in this thesis is analyzed the cytotoxicity of a material namely the ability of a material or substance to cause a toxic effect on the cells which leads to a change in their normal morphology and functionality.

The interaction between the material and the biological environment is divided in the evaluation of 4 main steps:

1) Interaction on the surface of element of specific proteins (nanometer scale).
2) Answer of cells to the element (micrometer scale).
3) Answer of the tissue considered to the external element(millimeter scale)
4) Answer of the guest organism

1) The proteic adsorbent is a dynamic equilibrium is influenced by lot of different elements like the primary structure, the charge, the concentration of the protein and others.
   Usually an alteration of the protein conformation occurs which can also means the lose of specific biological activity by the protein.
2) In this step specific membrane receptors interact with adsorbed proteins and there is a conformational modification of the transmembrane proteins which means the trigger of the biochemical response.
   After the protein attachment where the chemical-physical bond is unstable there is the cellular adhesion the stable binding is mediated by specific proteins and induce the cellular activation.

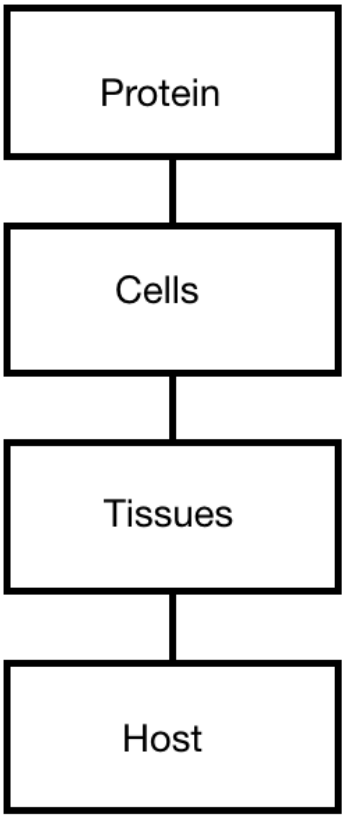


Figure 24: 4 Steps for biological evaluation

3) At this level, the superficial characteristics of the material plays a fundamental role as the dimensions, morphological irregularity, shape, presence of angles, etc.

4) The answer of the guest organism is due to all the previous step.

The interaction between the material and the biological ambient is studied in vitro through cellular cultures evaluating the cellular answer through a qualitative and a quantitative methods. This kind of evaluation is fundamental and basic for further investigation of biocompatibility. In fact, the characterization of materials is the phase one of the procedure for evaluation medical devices (ISO10993-5 of 2009 which describes *in vitro* test methods to assess the cytotoxicity of medical devices). The law is usually an example to follow in the evaluation of materials.

In vitro biocompatibility is divided in three phases:

1. In vitro screening:

   in this phase the cytotoxicity, cytocompatibility, mutagenicity, genotossicity and hemocompatibility of the material are investigated.

2. In vivo screening:

   in this phase the acute systemic toxicity, short-term chronic toxicity, sen sibilization and carcinogenity of the material are investigated.

3. Clinical monitoring:

   in this phase the preclinical evaluation, clinical investigation and retrospective survey are investigated.

The study is focused only on in vitro screening of cellular response in contact with the two different materials; in fact, cytotoxicity is defined as the ability of a material or substance to cause a toxic effect on the cells which leads to a change in their normal morphology and functionality;

In details, different elements have been evaluated as: a qualitative evaluation of adhesion, cell morphology, proliferation and metabolism of cells after contact with materials; the parameters that have been evaluated are:

- Cell morphology through optical microscope.
- Cellular vitality (DAPI test and MTT test).

In details two kind of tests have been performed.

1) Citotoxicity test: indirect contact with 5 days media

- Evaluation of cells through optical microscope after 1-3-7 days from sowing.
- Evaluation of cells vitality through MTT test after 1-3-7 days from sowing.

2)Biocompatibility test: direct contact

- Evaluation of cells through optical microscope after 1-3-7 days from sowing.

- Evaluation of cells vitality through MTT test after 1-3-7 days from sowing.
- Evaluation of cells through DAPI test after 1-3-7 days from sowing.

In the details, in the citotoxicity test where the cells come into indirect contact (they are in contact with eluates) with different elements are valuated: Morphology through optical microscope, Proliferation through optical microscope and metabolic activity through MTT test.

Both for PAN and Carbon fibers, three media with different concentration of compound have been prepared; in details:

- 1 media with 50 µg/ml of PAN or Carbon fibers which means that for every ml of media there are 50 µg of PAN or Carbon fibers.
- 2 media with 100 µg/ml of PAN or Carbon fibers.
- 3 media with 500 µg/ml of PAN or Carbon fibers.

The media is composed with the following ratio:

- 89% of DMEM acronym for Dulbecco's Modified Eagle Medium that is standard cell culture media that contain a stabilized form of L-glutamine, the dipeptide L-alanyl-L-glutamine, that prevents degradation and ammonia buildup in both adherent and suspension cultures.
- 10% of FBS acronym for Fetal ovine Serum which is a kind of Sera for standard research applications
- 1% of Pen Strep that is an antiseptic made by mixture of penicillin and streptomycin and it is commonly used in mammalian cell culture media to prevent bacterial contamination

The media has not been filtered because all the tests have been performed with fibroblast which are one kind of the strongest cells in mammals.

4.10 Opportune considerations on cells

A screening on the health of cells has been performed through the measure of proliferation rate.

The normal proliferation rate of Fibroblast is around 20- 26 hours for doubling and the cells used have a proliferation rate of 21.84 hours which is symptom of healthy cells without any contamination. The μ called growth rate of cells has been measure through the following formula: $X = X_0 e^{\mu t}$ where:

- X is the number of cells at a certain time t
- X_0 is the initial number of cells
- μ is the growth rate of cells

Both the initial number and the number at certain time t have been measured and so the μ value has been obtained. (38 and 39)

TABLE 2	PROLIFERATION RATE DATA:	CONTROL CELLS
Time (h)	cell density (cells/ml)	cells in 1 ml
0	70000	70000
24	72500	72500
48	92750	92750
72	275000	275000
96	687500	687500
Day 1-Day 4		
μ (growth rate)	doubling time (days)	hours
0,75	0,92	22,19
Day 0 to Day4		
0,7615221958	0,9102127086	21,84510501

More over, optical observation through optical microscope of cells has been used in oder to evaluate the morphology and the health ofd cells.

As can be seen in Figure 25 cells at 16 passage in culture have been seeded. The low number of cells and the shape are synonyms of healthy cells in fact, when fibroblasts and epithelial cells are grown in vitro on a plate the lower surface of the cell is not uniformly pressed against the substrate. The cell is anchored to the surface only at some scattered and well-defined sites: focal adhesions. Focal adhesions are structures dynamics that can be rapidly disassembled if the cell is stimulated to move or to enter into mitosis.In a focal adhesion the plasma membrane contains clusters of adhesion proteins called integrins, mostly αvβ3.

In the image are clear the protein complex focal adhesion useful for the cells to attach to the surface.

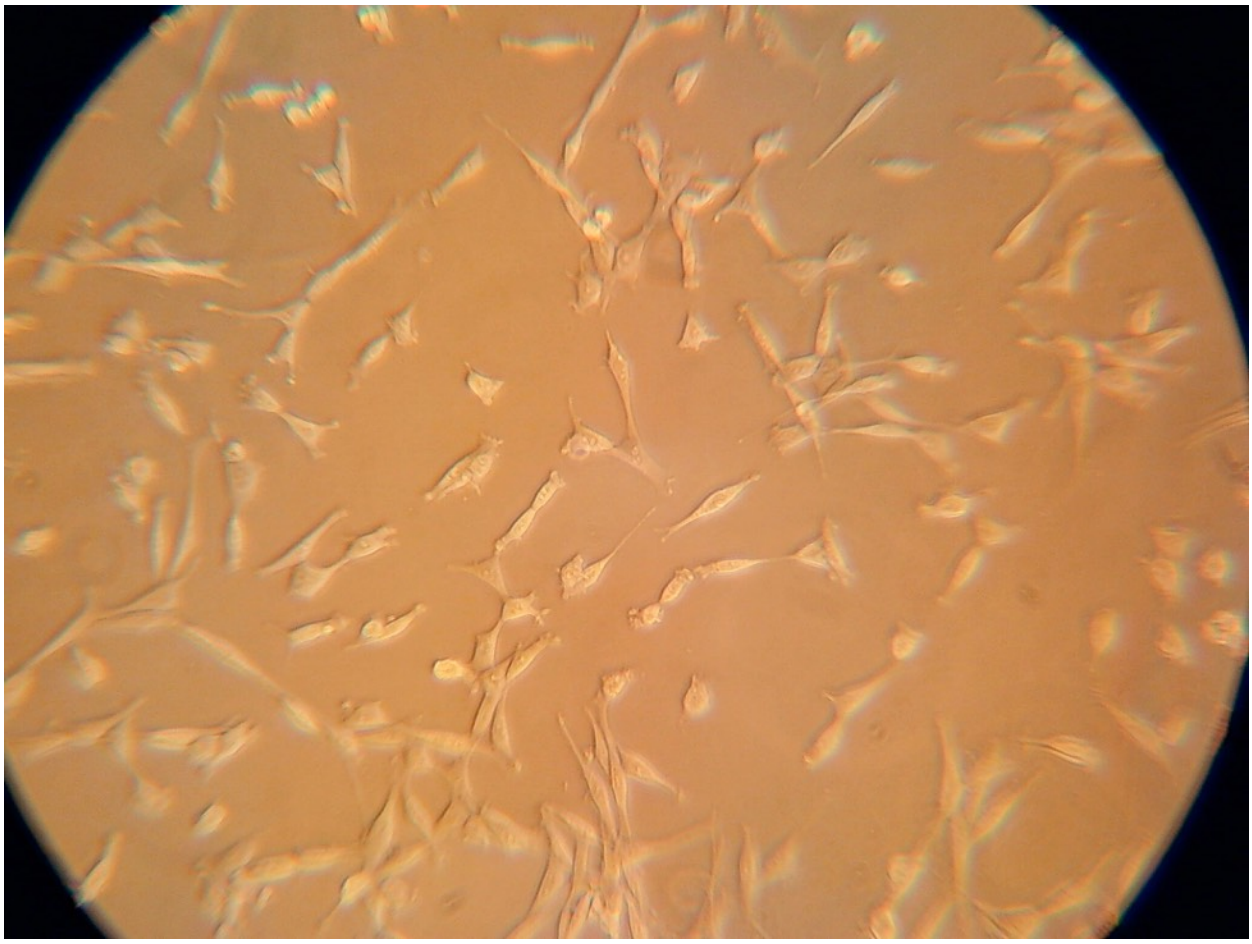


Figure 25: Behavior of Healthy Fibroblast

The proliferation rate of cells has been evaluated also for cells in contact with PAN fibers media and with Carbon fibers media. The results are reported in table 3 and table 4

Has can be seen in the tables the proliferation rate of cells in contact with carbon fibers is around two days, while usually, healthy cells have a proliferation rate around one day.

These data show the non healthy environment for cells where they have difficulties in proliferating due to the release of harmful particles by the fibers.

TABLE 3	PROLIFERATION RATE DATA	PAN FIBERS MEDIA
Time (h)	cell density (cells/ml)	cells in 1 ml
0	200000	200000
24	301000	301000
48	520000	520000
72	780000	780000
96	850000	850000
Day 1-Day 4		
μ (growth rate)	doubling time (days)	hours
0,346	2,00	48,079
Day 0 to Day4		
0,361	1,92	46,081

TABLE 4	PROLIFERATION RATE DATA	CARBON FIBERS MEDIA
Time (h)	cell density (cells/ml)	cells in 1 ml
0	70000	70000
24	420000	420000
48	92750	92750
72	275000	275000
96	687500	687500
Day 1-Day 4		
μ (growth rate)	doubling time (days)	hours
0,225	3,08	73,93
Day 0 to Day4		
0,243	2,85	68,46

4.11 Preparation of samples

4.11.1 Indirect contact

All the operations have been performed under biological hood opportunely sterilized before the usage.

The media has been in contact with fibers opportunely sterilized for 5 days, then the eluate has been removed through syringe with a 19 gauge needle equipped with a filter and subsequently cells have been seeded in different well plates with the proper concentration of media.

In details, the for the 24 hours test 48 well plate has been used; for the 72 hours test 24 well plates has been used and for the 7 days test 6 well plate has been used. As can be seen in Figure 26.

Figure 26: Well Plates used

Cells that have been grown in T flasks(usually T-50 or T-75) have been detached from the flask using trypsin and then counted through hemocytomer or Neubauer chamber which is a high precision automated cell counter when is coupled with an optical microscope.

In every well 2000 cells have been seeded; the number is due to the fact that for every experiment a control element has been used and the purpose is not to obtain 100% of confluence

of cells after 1,3 and 7 days in the control sample but a lower percentage of confluence.

More over, the media for of the 3 days test and the 7 days test has been changed every two days after seeding.

An example of seeded samples is reported if Figure 27 and Figure 28.

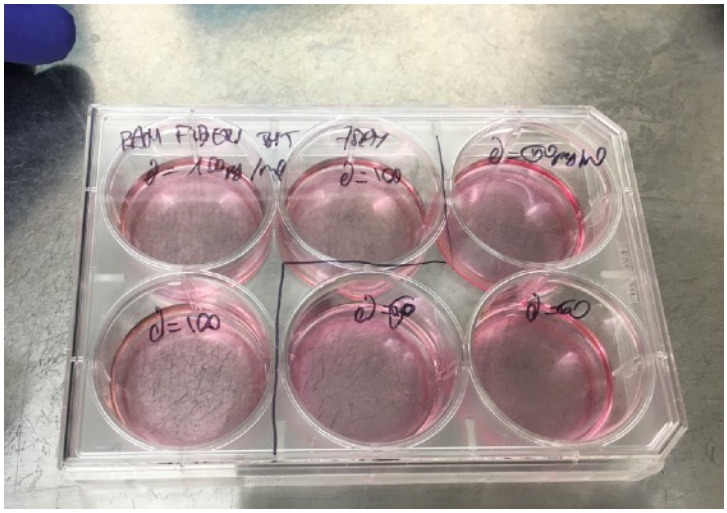


Figure 27: Example of seeded sample

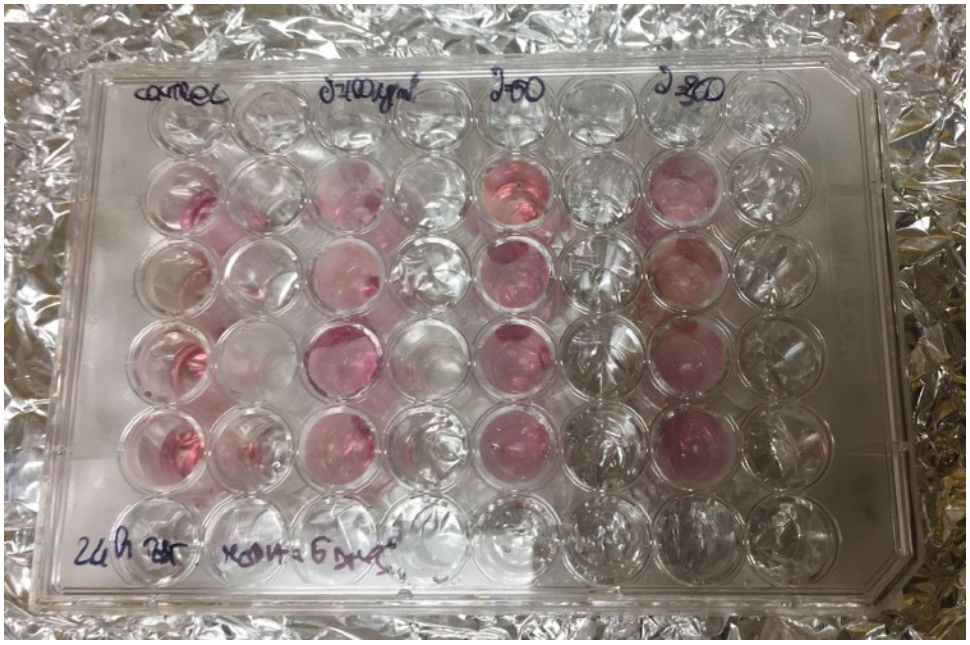


Figure 28: Example of indirect contact sample

4.11.2 Direct contact

A similar procedure for indirect contact testing has been conducted for direct contact test where 2000 cells have been seeded in every vial and the following day, in details 24 hours after seeding, the layer of PAN or Carbon has been deposited into the well in direct contact with cells. After the time scheduled(1, 3 or 7 days) the layer of the material has been removed and the procedure for MTT test or DAPI test has been followed in order to test the viability of cells. The well plates used and the seeding procedure of the sample is really similar as the indirect contact sample; figure 28, which reported indirect contact sample test, shows the usual arrangement of evaluation of samples similar for both indirect than direct test.

4.12.1 MTT test

The MTT assay, where the acronym indicates the compound bromide of 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium, is a standard colorimetric assay for measuring the activity of enzymes that reduce MTT a formazano, giving the substance a blue / violet color. In figure 29 is shown the chemical formula of MTT. This happens mainly in the mitochondria; it can be used to determine the cytotoxicity of drugs or other types of chemically active and potentially toxic substances. In fact, cell viability and cytotoxicity assays are used, for example, for drug screening and tests of chemicals or for others tests.

This reaction is evaluated and measured by the spectrophotometric reading of the sample, at a wavelength of 570 nm.

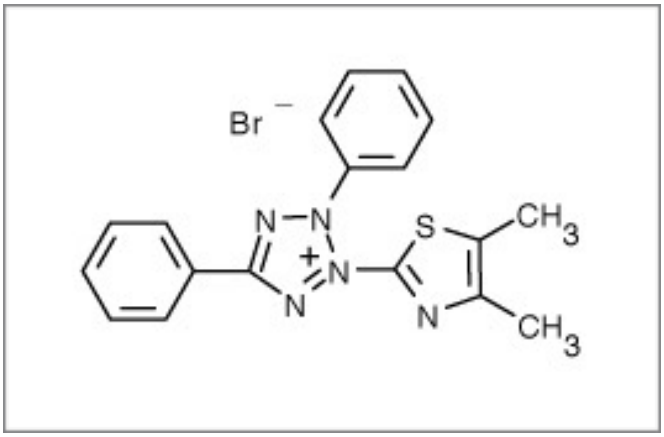
MTT test is a vitality test in fact, to assess mitochondrial activity, a tetrazolium salt test was used which is a colorimetric test capable of estimating the number of cells still having mitochondrial activity, and therefore cell viability. This test is based on a metabolic indicator, the soluble salt of

tetrazole which, in viable cells, is reduced in the

mitochondria by active dehydrogenase enzymes, to form a water-insoluble violet-colored crystal.

Figure 29: MTT Chemical Structure

Molecular formula: $C_{18}H_{16}BrN_5S$
Molecular weight: 414.32



Various reagents used for cell viability detection are based on various cell functions such as enzyme activity, cell adherence, cell membrane permeability, molecules production(for instance: ATP), coenzyme production(NADH of FADH2), and nucleotide uptake activity or enzyme-based methods using MTT cannot be used to distinguish between the healthy cells and the cells that are alive but losing cell function but they rely on a reductive coloring reagent and elements as dehydrogenase in alive cells to determine cell viability with a colorimetric method; while this test method consists of a protocol utilizing 3-(4,5-Dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reduction as methods for estimation of cytotoxicity.

This method is one of the most used in cytotoxicity test due to its characteristics as high reproducibility, safety and simple use.

In details, the method is used for determining mitochondrial dehydrogenase activities in living cells because MTT is reduced to a purple formazan by NADH. . However, MTT formazan is hydrophobic and insoluble in water; it always forms purple needle-shaped crystals inside cytoplasm. Therefore before measuring the absorbance, an organic solvent must be required to solubilize the crystals; for that reason Dymethil Sulfoxide(DMSO) which is an organic

compound belonging to the category of sulfoxides, is used to solubilize the crystals.

This method is dehydrogenase-based and it reflect cell condition, in fact it has higher sensitivity than other assays because it depend on mitochondrial activity (Figure 30).

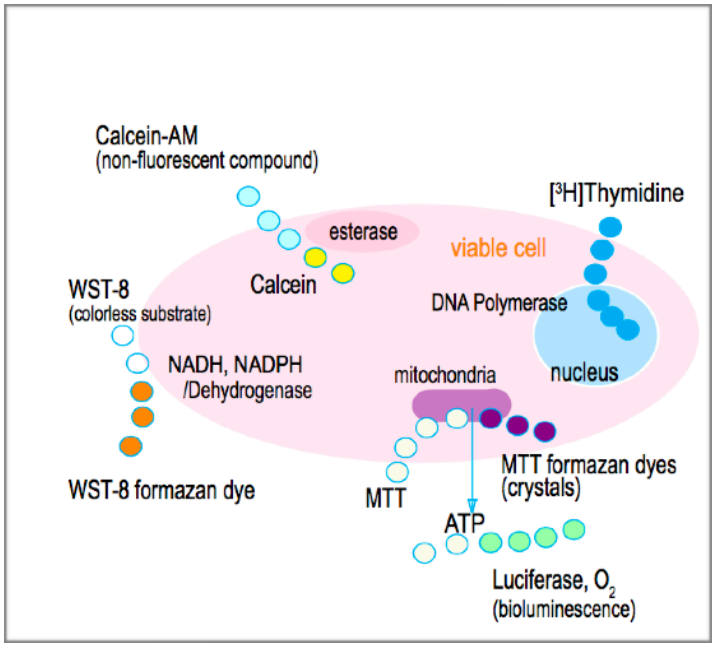


Figure 30: Reagents for cell viability detection

MTT reduction is a quantitative assays used to estimate cytotoxicity and it is prepared by diluting MTT in PBS with a concentration of 5 mg/ml.

Then 10 volume% MTT solution was added to each well-plate which was incubated for 4 hours at 37 °C in the dark. Subsequently, equal volume as cell seeded media, DMSO was added to each well to solubilize formazan salts and the well plate is ultrasonicated at room temperature in the dark for 15 minutes.

In the end, the well plates have been put in the Microplate Readers: Multi-Mode and Absorbance Readers(Model: Synergy H1 Hybrid Multi-Mode Microplate Reader from BioTek

) an absorption was assessed by a microplane reader at 570 nm of wavelength as cells viability is directly proportional to the absorption value so the percentage of surviving cells has been obtained.

The MTT test has been performed before on the PAN eluate and the Carbon Eluate; after obtaining a positive response by these two test it has performed for direct contact test both for PAN and Carbon fibers. (41), (42) and (43)

4.12.2 DAPI Test

DAPI is a nucleus-specific fluorescent dye that binds strongly to the regions rich in A-T of DNA.

The image of DAPI molecule is reported in Figure 31:

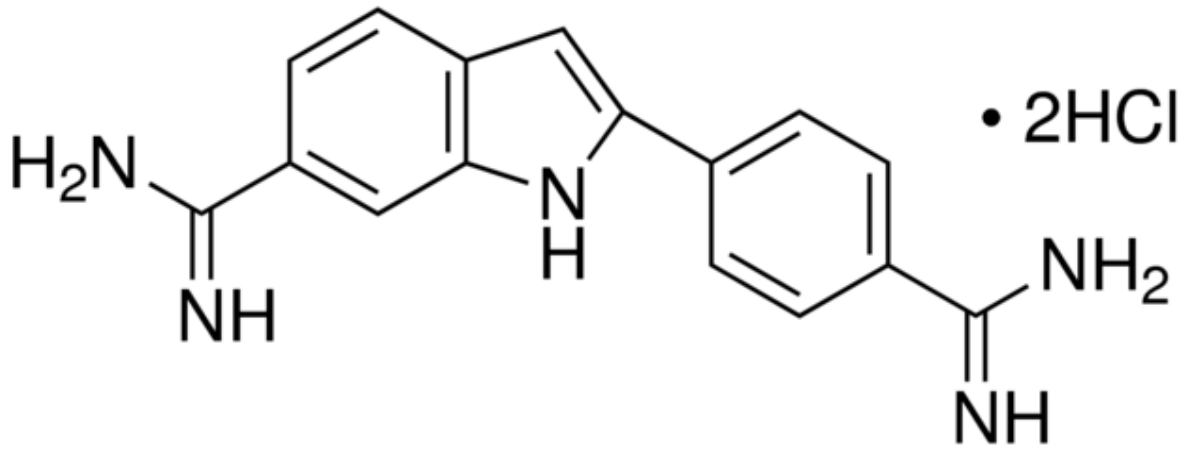


Figure 31: DAPI Molecule

Being a vital dye, DAPI is able to pass through the intact plasma membrane and color both live and fixed cells. This dye is excited by ultraviolet light and forms fluorescent complexes with

double-stranded DNA, showing a specificity for A-T bases. When DAPI binds to DNA, its maximum absorption is at 358 nm and its maximum emission is at 461 nm. The fluorescence emitted in the blue allows the characterization of the cell nucleus.

This dye, being excited by UV light, needs for the detection of a special laser or a mercury vapor lamp and this is why it is preferentially used in fluorescence optical microscopy. The morphology of the cell nuclei is observed. The nuclei have a normal phenotype when they emit homogeneous light signals, while the nuclei of apoptotic cells are identified by the fragmentation of the nucleus itself and by the condensation of the chromatin that is localized at the periphery of the membrane nuclear.

In Figure 32 are shown the excitation wavelength at 358 nm in blu and the emission wavelength at 461 nm in red.

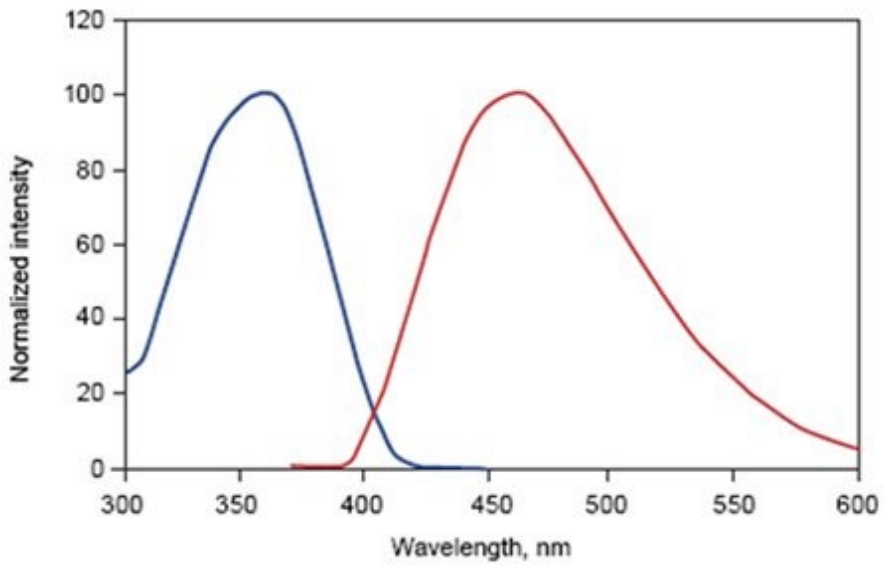


Figure 32: DAPI Excitation and emission wavelengths

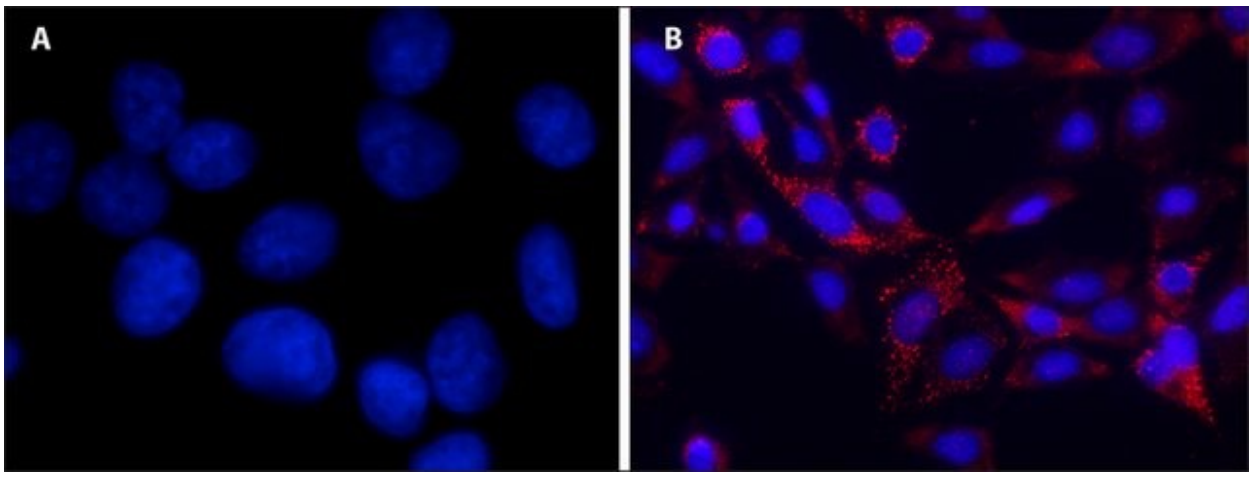


Figure 33: Cells evaluated with DAPI die

DAPI test has been used only to evaluate direct contact between cells and both PAN and Carbon materials.

Similarly to MTT test, 3 samples have been grown: the 1 day, the 3 days and the 7 days samples with 2000 cells for every well.

The real test is divided in two steps:

1. Fixation of cell culture plate

   The operations have been performed in biosafety cabinet.

   The culture medium was removed from the plate and washed carefully 3 times with PBS in order to remove non-adherent cells. Then 4 % Paraformaldehyde (PFA) has been added to each well and fixed for 30 minutes at room temperature.Then each well was washed twice with PBS.

2. Staining of cells with fluorescent dyes and quantification of fluorescence

   This part has been performed in dark biosafety cabinet.

   Enough diluted staining solution containing DAPI dye has been added to each well and then every well plate has been covered with aluminum foil in order to protect the sample from

light and then was incubated at room temperature for 30 minutes.

Subsequently, for each plate every well was washed three times with PBS and the last PBS wash was left in the well in order to keep cells hydrated for imaging.

In the end, the fluorimeter Olympus U-TB190 has been used to read the fluorescence. The machine uses and excitation wavelength of 350 nm and the emission is around 450 nm.

Two images are reported in Figure 34 and Figure 35 in order to show how cells appear through DAPI analysis. The nuclei are colored in blu thanks the die. It is possible to obtain the shape of cells through a high order of magnification as can be seen in Figure 35.

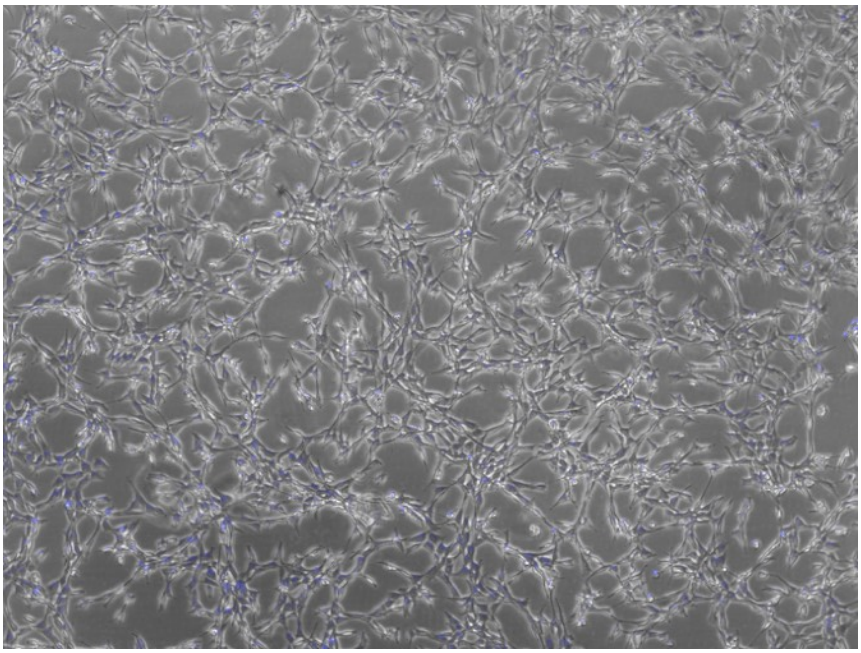


Figure 34: Example of cells evaluated with DAPI

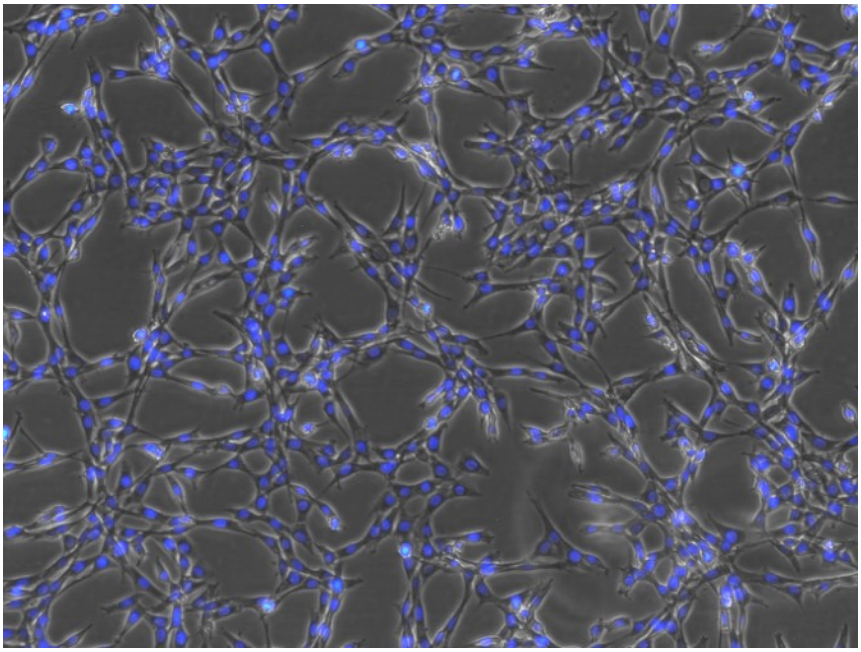


Figure 35: Example of cells evaluated with DAPI

4. RESULTS AND DISCUSSION

In details, three different tests have been performed and results are reported; thanks to observation of cells through optical microscope it has been possible to observe the shape of cells in contact with different types of media and it has been observed that cells in contact with carbon media have a less physiological behavior respect to cells in contact with PAN media and normal media.

More over, in order to confirm previous observation the proliferation rate of cells has been calculated obtaining following results:

- Normal proliferation rate: 22, 19 hours
- Proliferation rate with PAN Fibers media: 48,079 hours
- Proliferation rate with Carbon Fibers media: 73,93 hours

The bigger is the time of proliferation rate the harder is living for cells in that environment, that is why it can be stated that Carbon media fibers affected proliferation rate and Carbon fibers have a harmful behavior.

4.1 Indirect test

The release test (also called leaching test or leaching test) is a test during which the solid material is put in contact with a leacher (which acts as a separating agent) and from which a liquid is produced (called eluate).

The aim is to determine the release of the constituents of the materials and the potential pollution of the environment with these constituents over a long period of time. The vitality of cells has been evaluated through MTT test.

The Indirect contact test has been performed with the eluate of both Pan and Carbon fibers and the test is focused on the release of particles by Pan and Carbon fibers and toxicity is evaluated after 24, 72 hours and 7 days.

Media and carbon fibers have been in contact for 5 days.

Four different concentrations have been evaluated:

- Control sample: cells grow with normal media

- ∂ = 500 µg/ ml: the density of carbon fibers is 500µg per 1 ml of Media.

- ∂ = 100µg/ ml: the density of carbon fibers is 100µg per 1 ml of Media.

- ∂ = 50 µg/ ml: the density of carbon fibers is 50µg per 1 ml of Media.

24 hours PAN fibers eluate test

•First sample (∂= 50 µg/ml): 87%

The value means that, respect to the control sample, 87% of cells are alive.

• Second sample (∂= 100 µg/ml): 69%

The value means that, respect to the control sample, 69% of cells are alive.

•Third sample(∂=500µg/ml): 42%

The value means that, respect to the control sample, 42% of cells are alive.

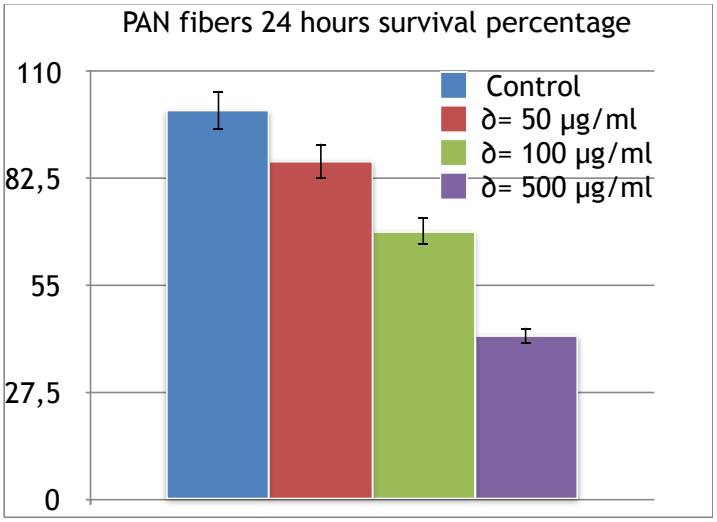


Figure 36: 24 hours survival rate with PAN media

72 hours PAN fibers eluate test

•First sample (∂= 50 µg/ml): 54%

The value means that, respect to the control sample, 54% of cells are alive.

• Second sample (∂= 100 µg/ml): 22%

The value means that, respect to the control sample, 22% of cells are alive.

•Third sample(∂=500µg/ml): 15%

The value means that, respect to the control sample, 15% of cells are alive.

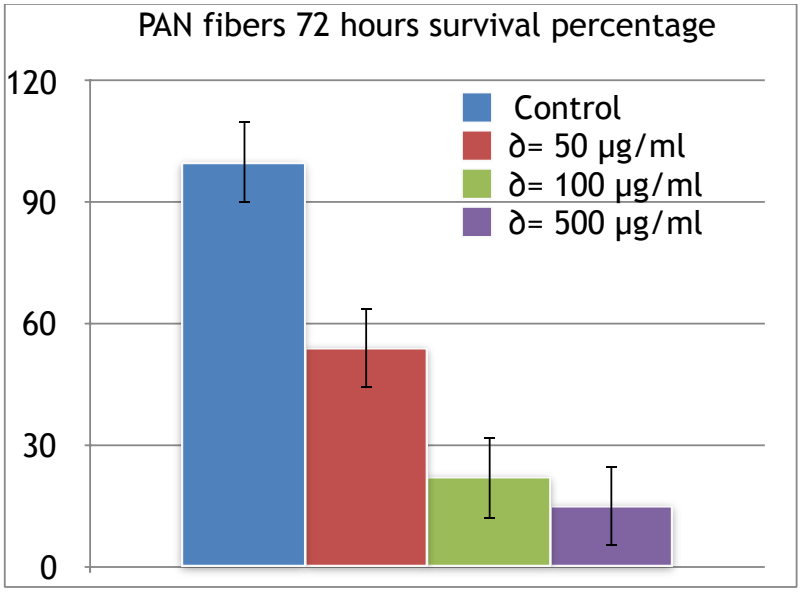


Figure 37: 72 hours survival rate with PAN media

7 days PAN fibers eluate test

•First sample (∂= 50 µg/ml): 64%

The value means that, respect to the control sample, 64% of cells are alive.

• Second sample (∂= 100 µg/ml): 55%

The value means that, respect to the control sample, 55% of cells are alive.

•Third sample(∂=500µg/ml): 42%

The value means that, respect to the control sample, 42% of cells are alive.

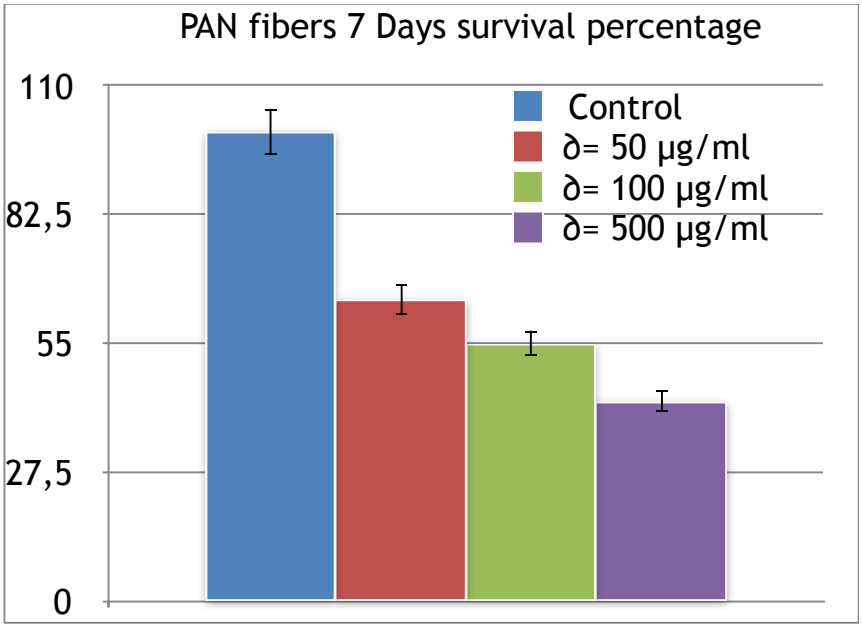


Figure 38: 7 days survival rate with PAN media

24 hours Carbon fibers eluate test

- First sample (∂= 50 µg/ml): 98%

The value means that, respect to the control sample, 98% of cells are alive.

- Second sample (∂= 100 µg/ml): 30%

The value means that, respect to the control sample, 30% of cells are alive.

- Third sample(∂=500µg/ml): 20%

The value means that, respect to the control sample, 20% of cells are alive.

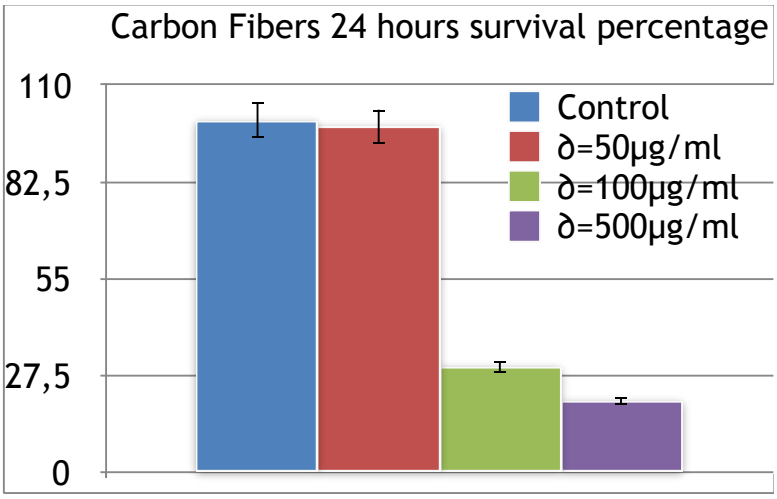


Figure 39: 24 hours survival rate with Carbon fibers media

72 hours Carbon fibers eluate test

- First sample (∂= 50 µg/ml): 76%

  The value means that, respect to the control sample, 76% of cells are alive.

- Second sample (∂= 100 µg/ml): 20%

  The value means that, respect to the control sample, 20% of cells are alive.

- Third sample(∂=500µg/ml): 6%

  The value means that, respect to the control sample 6% of cells are alive.

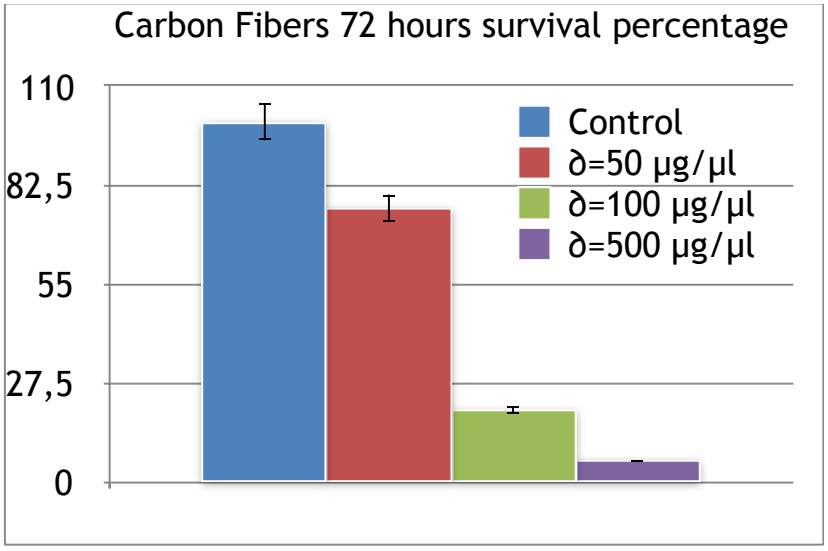


Figure 40: 72 hours survival rate with Carbon fibers media

7 days Carbon fibers eluate test

- First sample (∂= 50 µg/ml): 77%

  The value means that, respect to the control sample, 77% of cells are alive.

- Second sample (∂= 100 µg/ml): 23%

  The value means that, respect to the control sample, 23% of cells are alive.

- Third sample (∂= 500 µg/ml): 10%

  The value means that, respect to the control sample, 10% of cells are alive.

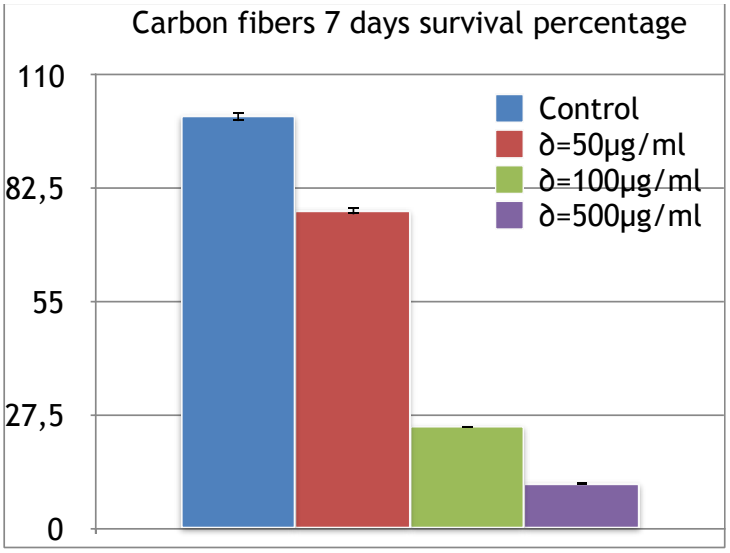


Figure 41: 7 days survival rate with Carbon fibers media

Conclusions of indirect citotoxicity

Quantitative changes in cell vitality are reported as it follows:

1) Non-cytotoxic (> 90%),

2) Slightly cytotoxic (60-90%),

3) Moderately cytotoxic (30-59%),

4) Severely cytotoxic (<30%)

The viability and growth of cells are always compared to the control group.

PAN Fibers have:

- for ∂= 50 µg/ml a slightly cytotoxic behavior

- for ∂= 100 µg/ml a moderately cytotoxic behavior

- for ∂= 500 µg/ml a moderately cytotoxic behavior

Carbon fibers have:

- for ∂= 50 µg/ml a slightly cytotoxic behavior

- for ∂= 100 µg/ml a severely cytotoxic behavior

- for ∂= 500 µg/ml a severely cytotoxic behavior

Due to the behavior with eluate the direct contact test can give more information on cytotoxicity of the materials but due to moderately cytotoxic behavior and severely cytotoxic behavior respectively of PAN Fibers and Carbon Fibers a qualitative evaluation of survival cells is needed before performing direct contact MTT test.

4.2 Direct test

4.2.1 DAPI

Qualitative evaluation of presence of cells and survival of cells during direct contact test have been performed before the quantitative evaluation due to the fact that it was not known for sure that cells can survive to direct contact test.

The qualitative screening is the common methodology used before quantitative screening in order to figure out if are present cells in the samples.

In details as for indirect contact test 1 day, 3 days and 7 days samples have been evaluated but only two samples for every time scheduled have been used: the control sample and the direct contact sample.

After the removal of PAN or Carbon layer and after fixing cells the fluorescent DAPI die has been added to every well plate and then the presence of cells is measured.

The general overview for the samples is reported and then details for comparison.

The white spots in the images are the nuclei of cells that can be detected through the luminescence of the fluorescence die. The values of wavelengths are reported:

- Excitation wavelength 358 nm
- Emission wavelength 461 nm. (40)

4.2.2 Evaluation of PAN Fibers:

<u>1 day direct contact test</u>

The control sample is reported is reported in Figure 42. As it can be seen many nuclei of cells are present in the image which means that the seeding procedure has been successful.

The nuclei of cells, which are made fluorescent by the die, can be observed in the figure which is synonym of alive cells.

The of 24 hours direct contact sample is reported in Figure 43.

What can be declared is that cells have survived to direct contact test. The difference between the control sample and the direct one is difficult to see through images even if is clear a lower distribution of cells in the direct contact sample.

Figure 42: DAPI evaluation of PAN Fibers control test 1 day

Figure 43: DAPI evaluation of PAN Fibers Direct contact test 1 day

3 days direct contact test

The control sample is reported in Figure 44. What can be declared is that cells have survived to direct contact test for 3 days. The difference between the control sample and the direct one is difficult to see through images even if is clear a lower distribution of cells in the direct contact sample and it is even clear the lower distribution of cells respect to the 1 day contact sample.

More over, it is observed the migration of cells from the centre of the fiber to the side of there fiber where they are subjected to less physical and chemical stress respect to the centre of the well plate where they have been seeded. This light migration is reasonable, in fact, cells feel the environment and moves in order to fine a more stable and quiet place where stay. Anyway the migration is not so marked which is symptom of not so harmful behavior of the fibers.
An image of this behavior is reported in Figure 46 , it is clear the more dense distribution of cells on the top of the image where the wall of the well plate is present.

Figure 44: DAPI evaluation of PAN Fibers control test 3 days

Figure 45: DAPI evaluation of PAN Fibers Direct contact test 3 days

Figure 46: Cells migration Direct contact test 3 day

7 days direct contact test

The control sample is reported in Figure 47 The direct contact sample is reported in Figure 48.

What can be declared is that cells have survived to direct contact test for 7 days. The difference between the control sample and the direct one is clear to see through images because is clear a lower distribution of cells in the direct contact sample and it is even clear the lower distribution of cells respect to both 1 day and 3 days contact samples.

More over, the migration of cells observed in the 3 days samples from the centre of the well to the side of there fiber where they are subjected to less physical and chemical stress respect to the centre is more marked as can be seen in Figure 49 Cells have moved from the center of the well to the side o the well on the left of the image where can be observed a more presence of fluorescent nuclei of cells.

Figure 47: DAPI evaluation of PAN Fibers Control test
7 days

Figure 48: DAPI evaluation of PAN Fibers Direct contact test

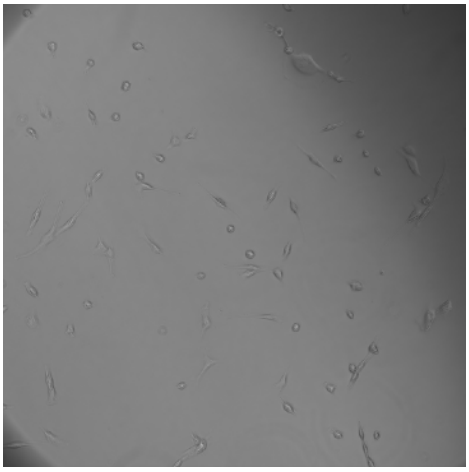
Figure 49: Cells migration Direct contact test 7 day

4.2.3 Evaluation of Carbon Fibers

A comparison between tho images is reported in Figure 50 and in Figure 51 which report the same portion o well evaluated.

The first image is what can be seen with ved through the microscope without using the fluorescence tool while the second image is what can be observed through the microscope without using the fluorescence tool.

It can be observed in Figure 50 that cells in direct contact with carbon do not completely behave in a physiological way. In fact only few of them are completely sticked to the well while most of them have a round shape and do not have the characteristic scattered shape of fibroblast. This behavior can be correlated to a non completely healthy state of cells which do not try to occupy as much surface as possible. Most of the cells has a round shape which is symptom of still attachment state of cells that can be observed after few hours after seeding and should no be observed during days after seeding.



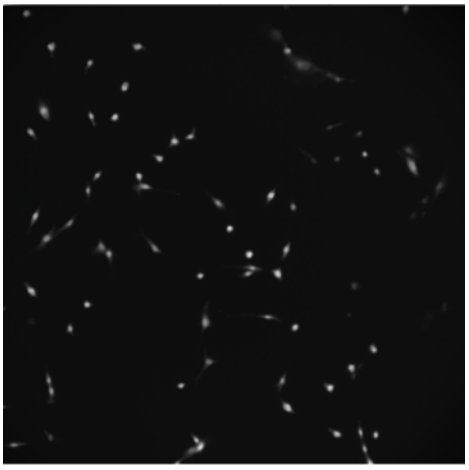


Figure 50, Figure 51: DAPI evaluation of Carbon Fibers Direct contact test

It has been observed a significantly decrease of survival of cells in direct contact with carbon fibers. Fibers have affected vitality of cells even after a short time of direct contact and can be

observed in 24 hours samples.

Two explanatory images are reported in Figure 52 and Figure 53 which are respectively the 24 hours contact sample and the 72 hours contact sample. The first image shows that vitality if affected and the second image shows that the survival rate after three days is significantly affected, if fact, only few spots can be observed in the image.

In the end, it has been observed through optical microscope that cells are not anymore alive after seven days of direct contact with Carbon fibers.

In order to evaluate the vitality of cells and quantify it with numbers the quantitative MTT test has been performed to study how much vitality of cells is affected by Carbon Fibers.

Figure 52:DAPI evaluation of Carbon Fibers control test 3days

Figure 53: DAPI evaluation of Carbon Fibers Direct contact test 3days

4.2.4 MTT

After direct contact for 1,3 and 7 days the samples of Control and Direct contact have been evaluated through MTT die similarly as the indirect contact test.

In fact, 2000 cells has been seeded in every well(1 day test in 48 well plate, 3 days test in 24 well plate and 7 days test in 6 well plate) and in the direct contact wells a layer of PAN fibers or Carbon fibers has been deposited. Before the addition of dies during the test the layer of material has been removed.

The media in the well plates has been changed every two days after seeding.

<u>1 day Pan fibers direct test</u>

- Direct test: 64%

The value means that, respect to the control sample, 64% of cells are alive.

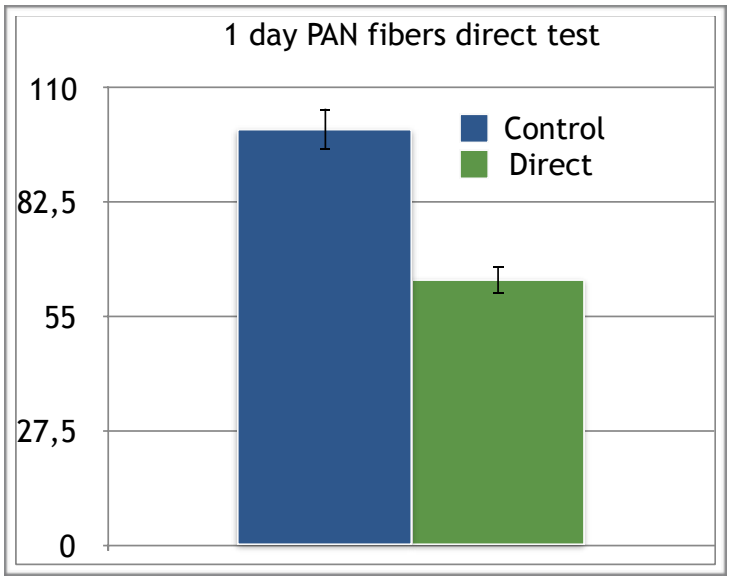


Figure 54: Cells survival rate with PAN Fibers Direct contact test 1 days

3 day Pan fibers direct test

- Direct test: 49%

The value means that, respect to the control sample, 49% of cells are alive.

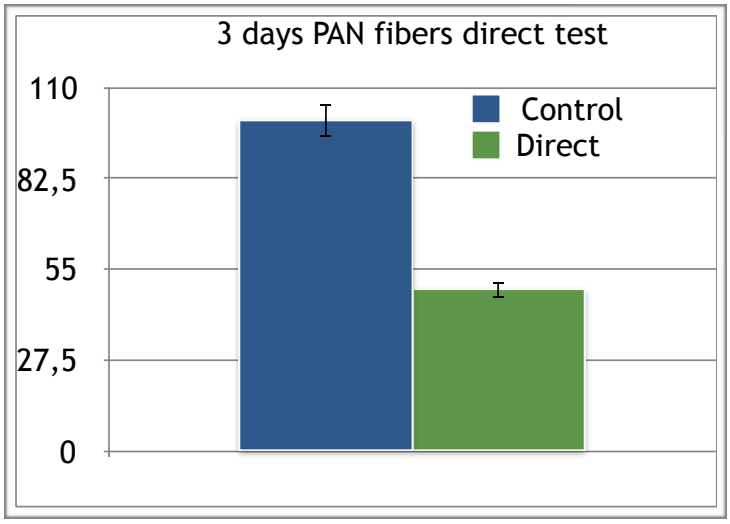


Figure 55: Cells survival rate with PAN Fibers Direct contact test 3 days

7 day Pan fibers direct test

- Direct test: 30%

The value means that, respect to the control sample, 30% of cells are alive.

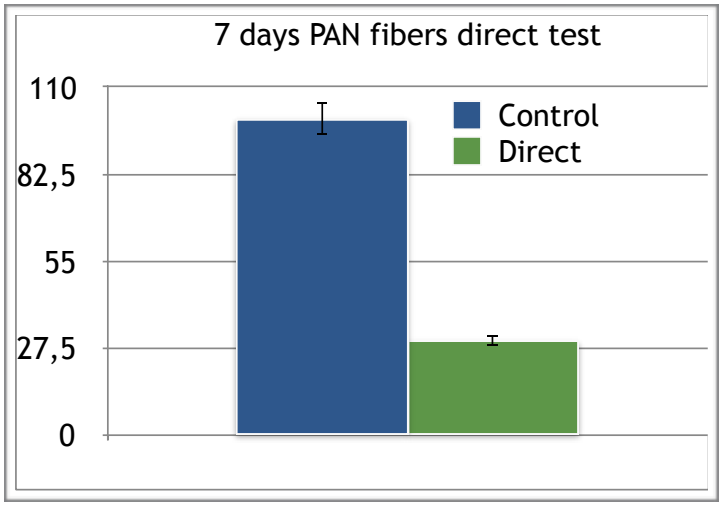


Figure 56: Cells survival rate with PAN Fibers Direct contact test 7 days

4.2.5 Results

PAN fibers affect the vitality of cells; in details the direct contact test has been evaluated with the same parameters of the indirect contact test and the results are reported; PAN Fibers have:

- for 1 day contact a slightly cytotoxic behavior
- for 3 day contact a moderately cytotoxic behavior
- for 7 day contact a moderately cytotoxic behavior

Even if vitality is affected it is observed that cells are alive after 7 days of contact with PAN fibers which have a slightly cytotoxic behavior for short period contact and moderately cytotoxic behavior for long contact period.

<u>1 day Carbon fibers direct test</u>

- Direct test: 27%

The value means that, respect to the control sample, 27% of cells are alive

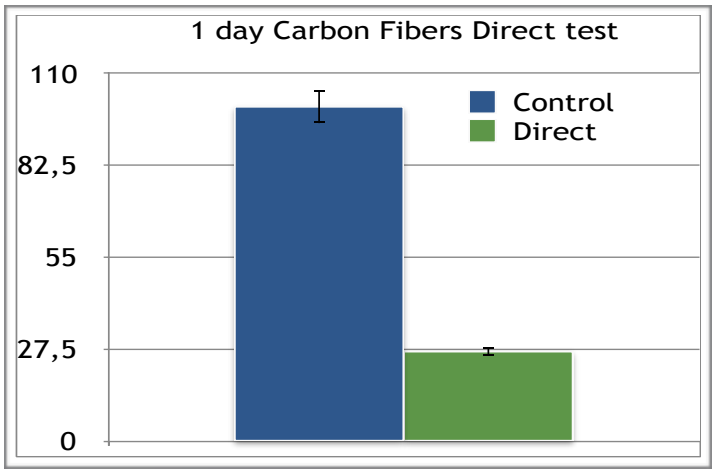


Figure 57: Cells survival rate with Carbon Fibers Direct contact test 1 days

3 day Carbon fibers direct test

- Direct test: 12%

The value means that, respect to the control sample, 12% of cells are alive

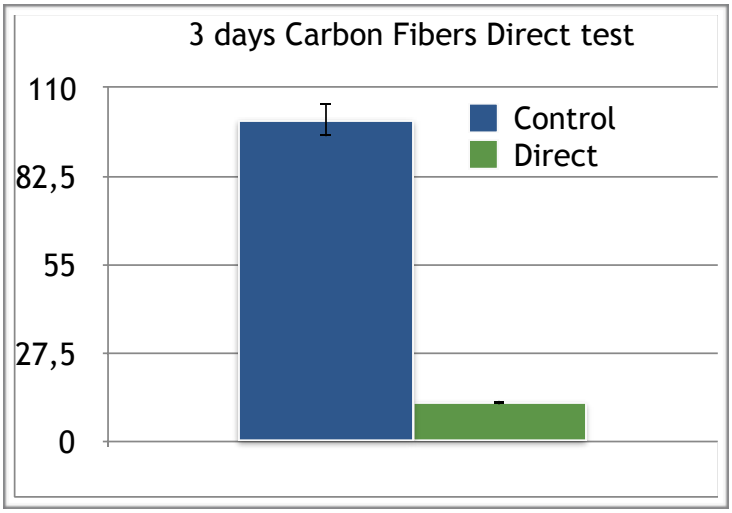


Figure 58: Cells survival rate with Carbon Fibers Direct contact test 3 days

7 day Carbon fibers direct test

Graphs are not reported because it is observed through optical microscope that after 7 days of direct contact test cells are dead.

4.2.6 Final Results

Carbon fibers affect the vitality of cells; in details the direct contact test has been evaluated with the same parameters of the indirect contact test and the results are reported; Carbon Fibers have:

- for 1 day contact a severely cytotoxic cytotoxic behavior

- for 3 day contact a severely cytotoxic behavior

- for 7 day contact a completely cytotoxic behavior

Vitality of cells is highly affected after short period contact with Carbon Fibers and after 7 days it is observed that cells are not alive which means that fibers have a completely cytotoxic

behavior for long periods.

Every test on nanoparticles obtained through carbon fibers has a support not only must be detached from the support but also must be purified before testing them with cells.

4.2.7 Cleaning step

Due to results obtained a cleaning step is suggested in order to reduce the toxicity of compounds layers to test. It is very important to understand precisely what is meant and what operations are performed to obtain industrial washing. The industrial washing consists in the preparation of the surfaces in view of subsequent specific treatments, or of the final finishing of the product before packaging and sale, in accordance with the nomenclature proposed by the UCIF (Union of Finishing Equipment Manufacturers) and with the terminology adopted by the CEN / TC27 I / WG I (working group of the European Committee for Standardization for the safety of industrial washing plants).

In a more specific sense we define industrial washing as a process consisting of one or more operations aimed at removing and eliminating from the surface of industrial products, and with the degree of cleaning required, the contaminants of an organic and / or inorganic nature, through interventions and actions of chemical and physical nature (mechanical, thermal, electrical) variously combined together. The industrial washing process generally understood can consist of several operations, each of which has its own very specific function.

In the industrial washing process the following phases can be distinguished:

- Pre-wash
- Real wash
- Rinse
- Drying

Surface treatment actions are defined as washing agents those chemicals that are used in the removal of contaminants from an artifact.

In the treatment of surfaces, the washing agents can be divided into two groups:

1) Detergents, generally produced in aqueous solution that exert a chemical-physical action on the contaminant, causing their detachment from the surface and preventing them from being redeposited;

2) Solvents, products that interact with the contaminant, allowing them to dissolve.

Due to impurities of working procedure a washing step of final product is suggested in order to obtain a clean final layer of carbon fibers.

One of the most common and useful techniques used in industry for cleaning is Ultrasonic Cavitation which is the phenomenon by which it is possible to understand the principle of ultrasonic cleaning. In a cleaning liquid, the ultrasonic waves created by a special ultrasonic electronic generator and by a particular installed properly transducer under the bottom of a stainless steel tank, generate compression waves and depression at very high speed. This The working frequency of the ultrasonic generator create a wave proportional to its own value. In general, the working frequency is between 28 and 50 Khz. The "ultrasonic cavitation" is the phenomenon generated.

4.2.8 Surface tension, viscosity and vapor pressure

To better understand the phenomenon, we must call to our attention some fundamental concepts such as the "surface tension", the "viscosity" and the "vapor pressure or pressure". In fact, liquids are characterized by the fact that the particles are more free to move than in solids, although they are subjected to forces of attraction much higher than those existing in gases. In particular, water is a molecular liquid, it evaporates at all temperatures but boils at a well-defined

temperature called the "boiling temperature" which for distilled water is 100 degrees centigrade to which the vapor pressure reaches the value of 1 atmosphere.

The technical procedure of what happens is described in following lines. The first phase is the the depression phase (figure 59 A) where a multitude of gas bubbles are created inside the liquid which become larger until the acoustic depression phase (negative pressure) lasts. This formation of microscopic bubbles of gas is the beginning of cavitation (ie formation of gaseous cavities within the liquid). The following phase or second phase is characterized by ultrasonic compression of the bubble (figure 59 B, the enormous pressure exerted on the bubble which has just expanded, compresses the volume enormously increasing the temperature of the gas contained in it (figure 59 C). Then the bubble collapses on itself and implodes releasing huge impact energy (figure 59 D). The energy released due to the collapse of the gas bubble, hits the surface of the object to be cleaned interacting both physically and chemically. Physically we will have a phenomenon of “micro-brush" at very high frequency (about 50,000 times per second for a machine that operates at 50 Khz) and chemically with the detergent effect of the chemical substance present in the ultrasonic bath detergent.

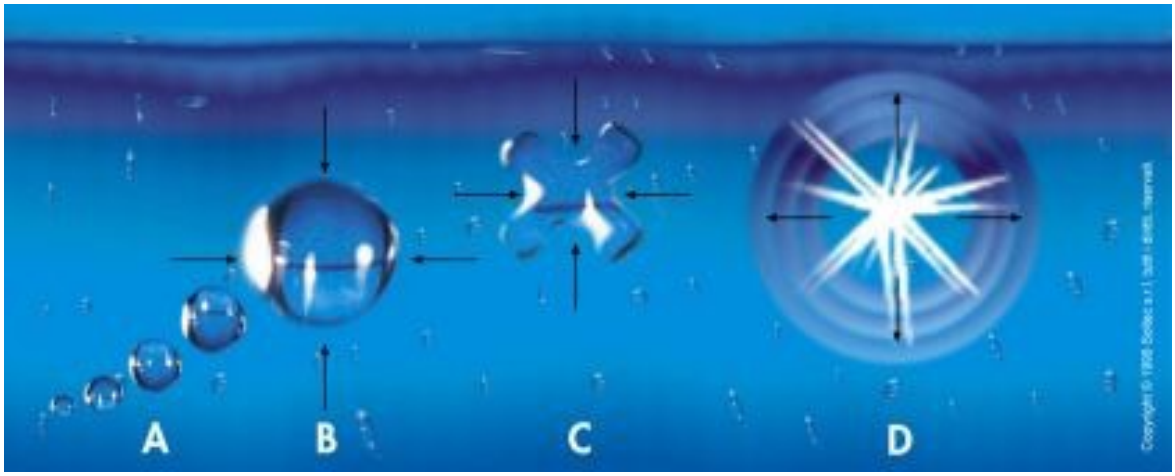


Figure 59: Sonication cleaning description

4.2.9 The choice of the type of detergent and the working temperature

The detergent used and its physical-chemical properties are fundamental for the ultrasonic washing machine. When choosing an ultrasonic and detergent washing machine, there are many parameters to consider. First of all, it is the type of contaminant which has to be removed and consequently the choice of chemicals that can remove the dirty part. Of course the chemical detergent used has to be able to cavitate efficiently, must be a watery solution with a low surface tension and with high vapor pressure, more over it should be used at a working temperature around 50 ° - 60 ° C. The cavitation intensity varies with the temperature and so the working temperature in an ultrasonic cleaning bath of the aqueous solution is very important;. The cavitation intensity is directly proportional to temperature, up to about 70 ° C and then decreases and disappears at the boiling temperature of the liquid. The vapor pressure of the detergent solution is another important parameter to consider. By pressure or vapor pressure is meant the following concept: the surface molecules of a liquid in a closed and thermostatted container have enough energy pass to the vapor state and arrange themselves in the available space above the liquid.

At a constant temperature, some vapor molecules return to the liquid state when the equilibrium speed is reached and the evaporation rate reach the condensation rate. Vapor pressure is defined as the pressure exerted by the vapor molecules, in these conditions. Its value depends on the temperature and not on the amount of liquid present. Therefore if a liquid is heated, the boiling phenomenon occurs when the vapor pressure increases with the temperature and when the vapor pressure equals the external pressure. Each liquid therefore is characterized by its own vapor pressure and a different boiling temperature. For instance, water will have a vapor pressure much lower than ethyl alcohol at the same temperature. Water boils at 100 ° C with a vapor pressure of 1 atm while ethyl alcohol boils at 78 ° C with a vapor pressure of 1 atm. The temperature at which the vapor pressure of the liquid equals the pressure of 1 atm is defined

for normal boiling temperature. In the cavitation process the correct understanding of vapor pressure is important as it plays a predominant role. In fact, the vapor pressure of the liquid and the surface tension value are proportional to the energy required to form a cavitation bubble. Cavitation is hard when the liquid vapor pressure is low (for example with cold water). On the opposite side, cavitation bubbles implode with more energy, but nevertheless we have to raise the power applied very much to reach the minimum level of cavitation. Therefore the result is generally reduced in a lower bubble formation and fewer implosions. For instance, the vapor pressure increases when the temperature of the liquid raises making easier the vaporous cavitation. The minimum cavitation threshold is lowered by a high vapor pressure value, creating many more bubbles that collapse with lower energy proportional to the difference between internal and external pressure. Another parameter as the viscosity of the liquid is also to be evaluated. High viscosity values prevent cavitation, while the diffusion of ultrasonic waves and therefore the formation of cavitation bubbles is allowed by low viscosity values.

4.2.10 Technology and advantages

The “Sweep System” is the principle through which the surface is cleaned during the washing step.

The frequency of the ultrasonic generator, the power, the use of the "Sweep System" type generator and finally the type of transducer used are important parameters for obtaining good levels of cavitation in a liquid. The size of the gas bubble inside the liquid subjected to ultrasonic sonification is determined by the frequency of the ultrasonic generator. The smaller the size of the cavitation bubble generated as the higher the frequency of the generator, on the contrary, the lower the frequency the greater the size of the bubble. It is clear that a larger bubble will need more energy to implode and consequently it will also have a greater impact energy, while a smaller bubble requires less energy to implode and consequently has an energy lower impact.

The advantage of using ultrasonic cleaning systems with high frequencies is due to the fact that there is a better cavitation distribution per unit area high due to many more bubbles in the unit of time.

For example, in a 20 Khz system, the distance between the nodes and the antinodes (or bells) of the acoustic wave is practically divided in half compared to that generated by the 40 Khz systems. Therefore, the 40 Khz systems generate in a second many more bubbles and above all smaller ones, allowing to reach even very small points per unit area. To give a practical example, I can compare the high-frequency fine cavitation to a very fine-grained emery paper while the low-frequency one, to a very coarse-grained emery paper. The purpose of emery paper is to sand, but it is clear that very different results can be obtained depending on whether a fine or coarse type is used. The piezoelectric or magnetostrictive type can be used as type of generators. Generally, piezoelectric transducers are used since it is possible to design transducers with higher frequencies than those ones obtained through of the magnetostrictive type. For example, high-power magnetostrictive transducers usually do not exceed 22 Khz. In the end, the distribution of ultrasonic cavitation is improved by the use of a “Sweep System" type generator. In fact, the generator’s frequency is modulated around a central frequency, with variations of more or less 1 Khz. For example, a transducer driven at 40 kHz will oscillate at a frequency between 39 and 41 kHz. This frequency variation prevents the so-called "stationary waves" that can generate acoustic interference phenomena inside the liquid when at least two but usually more than two wave trains cross in the same space region. The cleaning times is reduced by the "Sweep" system; more over it prevents damage to delicate parts, greatly increases the distribution of ultrasonic cavitation and facilitates the process in liquids that caving with difficulty. Generally, the "Sweep" system is used in industry with very professional washing systems, but today some manufacturers are beginning to provide it even on small ultrasound

washing machines.

An example of sonication cleaning system is provided reporting the patent number:

- US8584687B1 United States

4.2.11 Sterility: fundamental step in cell culture

Sterility is a fundamental requirement in all phases of cell culture. In fact, particularly nourishing culture media can be an excellent substrate for the growth of numerous contaminating microorganisms, which could permanently and inevitably compromise the viability of the crop itself. The contaminants that can infest a cell culture are generally: viruses, bacteria, fungi (yeasts and molds) and mycoplasmas, as well as cells of different types (cross contamination).

Viruses can cause massive culture lysis producing in this case macroscopic effects as foci of transformation; in other cases the viruses can integrate into the genome of the host cell, without giving themselves any sign, and are transmitted to the daughter cells. In any case, viral infections are rather rare and do not constitute the first cause of alarm in the context of contamination. For cell cultures, however, bacterial contamination is by far the most frequent.

The only thing to do, as soon as you realize the bacterial contamination is to kill the crop and start again, using all the necessary precautions and trying, above all, to identify the contamination vector, which can be directly the operator , or the materials and / or solutions used by him. As a preventive measure, a mixture of penicillin and streptomycin can often be added to the culture media. Mycoplasmas, on the other hand, are the smallest organisms with independent life and are included among the bacteria. They are not visible under an optical microscope and do not modify the morphology of the contaminated cells, however they alter its growth (reduction of mitosis, induction of apoptosis and decrease of cellular adherence) and both biochemical characteristics (pH reduction, chromosomal fragmentation, translocations and

reduction in the number of chromosomes) and antigenic. It is possible to observe the presence of mycoplasmas using the fluorescence microscope, after having highlighted the DNA with specific dyes. Fungi, on the other hand, can be unicellular (yeasts) or multicellular (filamentous molds provided with aerial mycelia). The yeasts appear to be optic microscopic like isolated and roundish cells, while, the pollution from mold is recognizable to the observation to the microscope for the presence of unmistakable filamentous formations that cover the monolayer of cells.

More over, a clean environment from dust and other contaminants is mandatory; a technical cleaning step is suggested in the production of carbon fibers.

4.2.12 Technical cleaning procedure

The residual contamination deriving from the various production processes can in some cases lead to high costs deriving from waste and possible failures on the final component with consequent opening of complaints and relative increase in costs deriving from reworking and returns.

As with all types of processes and analyzes, the determination of the degree of technical cleaning is also subject to a series of factors that can influence the correct expression of the analytical result. The technical cleaning analysis can be divided macroscopically into three phases:

- Washing and packaging
- Isolation of residual contaminants
- Determination of the level of contamination.

The first step, washing the components, takes place through a washing machine(Figure 60) which uses a jet of hot water under pressure (added or not with a suitable detergent) alternated

with air jets: during the process the contaminants are eliminated by effect of water friction on the surface of products. By adding a suitable detergent it is also possible to remove substances of an organic nature present at a superficial level soluble in an amphoteric environment. The cleaning of the surface can be effectuate through Sonication Cleaning previously described.

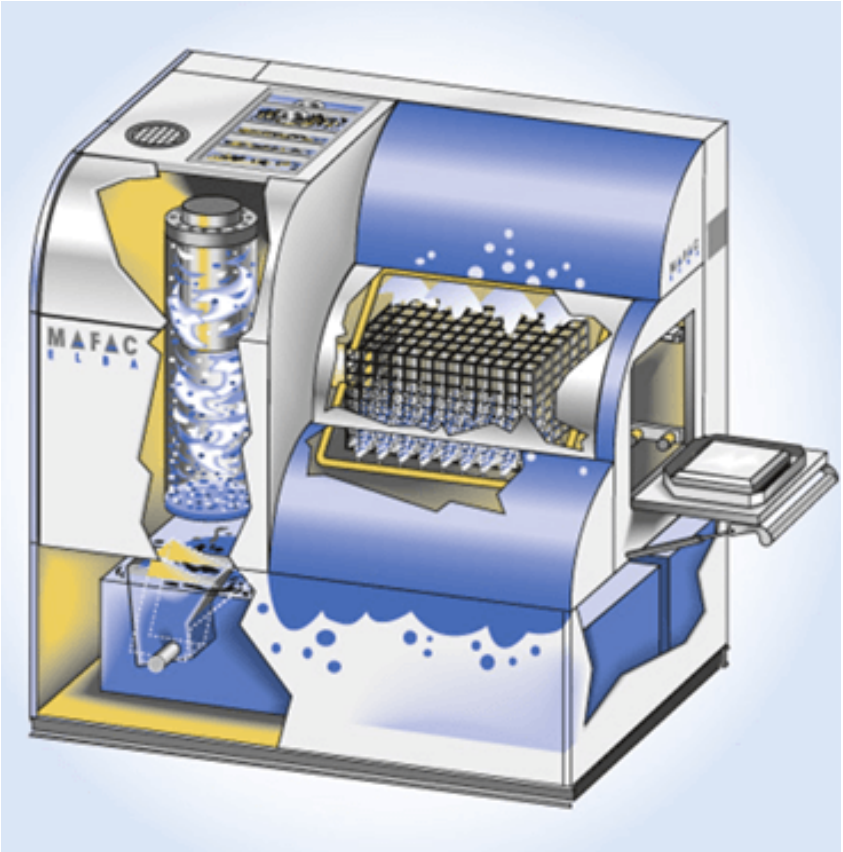


Figure 60: Washing machine

The critical steps of this phase are the chemical-physical treatment of the water and the post-washing packaging.

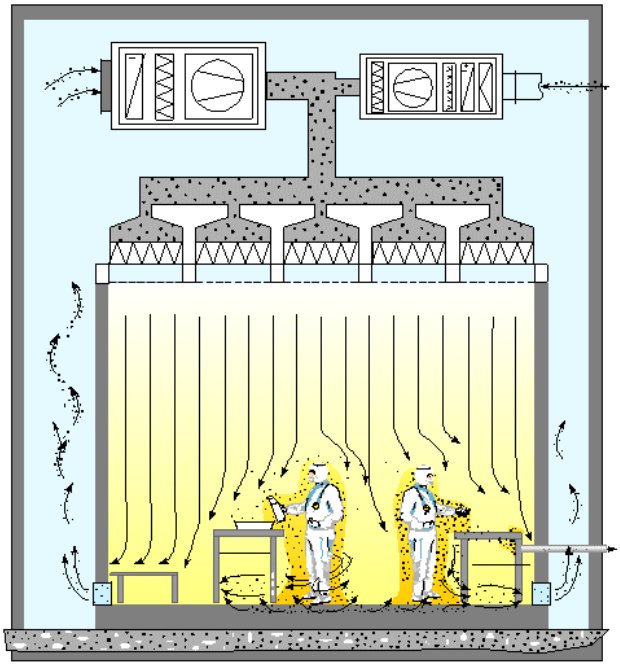
The cleaning of the incoming water is guaranteed both mechanically, through the presence of appropriate mechanical filters capable of eliminating solid contaminants above 50 microns, and through a water deionization system using ionic resins capable to eliminate most of the salts normally present in drinking water which, if present, could lead to fouling and then settle on washed items. This fact entails on the one hand the risk of return due to visual non-

compliance, on the other hand that these deposits can release solid contaminants on the finished component with possible damage on the final machinery.

Even the packaging phase presents some problems that could affect the final cleaning of the items. The fundamental problem lies in reducing as much as possible the cross-contamination between the samples just washed, the washing and packaging operator and the external environment: both the operator and the packaging bags and the surrounding environment, free or less than machinery, they are sources of pollution from solid particles.

To contain these problems, the washing and packaging operator wears special garments with reduced release of solid contaminants (overalls, masks, goggles and nitrile gloves), while the packaging bags are periodically subjected to technical cleaning analysis : for the analysis to be carried out correctly it is necessary to package the items first and then sample them again to perform the analysis.

In the laminar flow (Figure 3), unlike the turbulent flow (Figure 4), the infinitesimal layers of fluid, in this case filtered air with class 7 HEPA filters run quickly one above the other without any mixing taking place . The speed and acceleration of the fluid particles are completely described by two parameters: internal friction and pressure gradient.



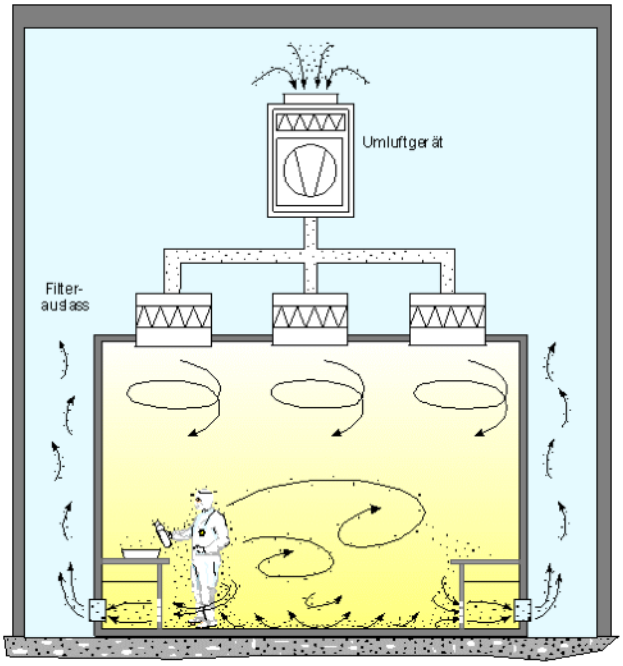


Figure 61: Laminar Flow

Figure 62: Turbolent Flow

In order to reduce the environment-sample cross-contamination phenomena, the area used for the washing-packaging process has been subjected to laminar flow: it is a flow whose behavior is determined by internal friction forces. In short, the function of the laminar flow is to form a protective barrier between the external environment and the work area (also thanks to the presence of PVC strips) and to break down the particulate present in the work area: in the case of a laminar motion the particles are conveyed downwards remaining at floor level without the possibility of mixing in the work area. In the case of a turbulent motion instead, the particles are not conveyed in a single direction, but in different directions and at different heights constituting a possible source of cross-contamination.

It must however be specified that, despite the presence of laminar flow in order to work correctly, the cabin must be switched on at least an hour before the washing operations and that operators perform slow movements in order not to run the risk of generating whirling motions in contrast to the laminar regime.

The phase of isolation of residual contaminants (post washing) takes place inside a laminar flow cabin, (which exploits the principles just set out for the washing area) positioned, as well as the optical microscope for the reading after isolation, in a laboratory with controlled environment placed, previously and during the analysis at an overpressure compared to adjacent rooms: in this way the opening of the door causes the air to escape from the laboratory thus preventing to the contaminants present in adjacent rooms to enter, thus constituting a source of possible contamination.

As previously stated, the samples for checking the degree of cleaning must be taken only after bagging so that the analysis evaluates the overall sample-bag contamination.
Inside the extraction booth the articles are placed inside a tank filled with a suitable liquid (it must not affect the material of the article, have certain values of viscosity, especially in which one opts for a method of isolation by agitation and possibly have low toxicity and flammability), ultrasonated and subjected to subsequent washing by pressurized liquid so as to more easily reach areas with blind spots that are difficult to reach only by immersion or that could cause contamination that cannot be determined later during the analysis.

Also the choice of the filter is of fundamental importance for a correct expression of the data: the filter must be made of a material resistant to the extraction solvent, while its porosity should generally be 1/3 compared to the smallest particle that can be determined. To give a practical example, having as specific minimum detectable particles of 15 microns, the filter should have a maximum porosity of 5 microns.

Another important factor related to filters, especially when a gravimetric determination of pollutants is required is the presence of humidity present in the filter itself which, if not

eliminated would lead to errors of determination.

To eliminate residual moisture, the filter is conditioned by heating it in the stove until constant weight. The operating temperature varies from material to material so it is necessary to refer to the technical specifications of the house that supplies them. It is a good rule not to exceed the drying temperature too much as, in addition to thermal degradation phenomena, there may be a widening of the porosity of the filter subjected to heating with the risk that the nominal porosity is no longer maintained with the consequent passage of particulate through the mesh. It is possible to maintain a lower temperature by increasing the time spent in the oven.
Having the possibility, it is suggested to filter a certain quantity of extraction liquid through the filter and then put it into drying. In this way the material will be perfectly conditioned and free from any organic residues present in the same with evaporation temperatures higher than those used for conditioning. This phase is verified by weighing up to constant weight. Once removed from the oven, the filter must be kept for at least an hour in a dryer with silica gel (it is never good to weigh hot objects, for which a certain period of cooling is required; the silica gel prevents the material from absorbing humidity).

Particular attention must be paid to the balance which must have certain construction and positioning characteristics that allow for the most accurate measurement possible.
In addition to the verification of accuracy, resolution and reliability, other factors must also be taken into account:

- Balance switch-on: due to their mechanics and degree of precision the analytical balances should always remain on or on at least a couple of hours before the determination.
- Weighing bench: it must not flex under the weight of the instrumentation and must be free of vibrations, antimagnetic and antistatic therefore, materials such as glass, plastic and steel should

be avoided, the first two above all for a speech of resistance, but also for the possible presence of electrostatic charges that would interfere with the equipment. This last problem can be contained with the application of ground masses.

The weighing bench must be placed on the floor or fixed to the wall; it is a good rule not to use a double fixing because the bench would be subjected to double vibration. Another trick linked to the workbench is its stability: the scale should not detect changes in weight by applying pressure to the bench on which it rests and the scale should be positioned close to the games of the bench, a point in which the phenomenon of vibrations is minimized.

- Work area: must be dedicated to the budget and in any case be in an area free of vibrations, away from passageways and drafts.
- Temperature: must be kept constant, possibly in an ideal range between -5 ° C and 30 ° C depending on the applications.
- Humidity: The ideal relative humidity value is between 45% and 60%. Where it is not possible to maintain this range it is advisable to remain within values between 20% and 80%.
- Light: the scale should be positioned away from windows and lights in general due to heat emission with formation of convective currents or temperature gradients that are not stable over time.

The determination of the level of contamination takes place by means of optical microscopy (other reading systems are possible, such as ACP, SEM, etc.) and other analytical techniques such chemical screening of residuals and contaminants present in the surface of the products; for example Infrared Spectroscopy is a common and widely used method to analyze surfaces.

If the sample cannot be directly analyzed the analysis of last liquids used to wash the samples can be analyzed in order to evaluate the still presence of contaminants.

During the extraction phases, the liquid passes through the previously conditioned filter

which will then be placed back in the oven to eliminate the analysis solvent and subsequently read by a stereo microscope.

Also in this phase, similarly for the washing and packaging passages, the environmental contamination plays a role of primary importance to provide a reliable result.

Before being able to perform the test it is essential to ascertain and assess its suitability: for this reason a validation of the analytical method is performed. Up to six washes are performed on the same sample, which in turn consists of a certain number of items. Each wash is collected on a different filter which will then be read under the microscope, obtaining six reading points.

4.2.13 Conclusion

The technical cleaning analysis is an analytical method for determining the quantity of pollutants present on the articles that is affected by a series of factors that contribute to the release of a reliable result.

As with all analyzes, even in this case, excluding random errors on the part of the analyst and a sampling of pieces to be subjected to analysis not congruent with the total number of printed articles, there are a series of measures to be implemented to ensure that the result obtained is as close as possible to the real situation.

5 Future developments

Future evaluation can be obtained performing again the tests previously reported. The main purpose is to investigate toxicity of carbon fibers due to impurities occurred during production procedure and that were not avoided in the previous test due to the fact that a purification step is missing.

It is advised a superficial characterization for carbon fibers before and after the purification treatment in order to visualize the chemical composition of the surface and to observe if there are any significant changes before and after the decontamination process. Infrared analysis to characterize the chemical composition of the surface are suggested in order to obtain a better knowledge of the chemistry of the sample. In addition, due to purification of sample it is assumed an increase in cell viability over 7 days of direct contact.

More over, having already a good level of knowledge of the results obtained on the toxicity of the materials, more specific analyzes are suggested such as the evaluation of the production of products such as protocollagen or collagen through ELISA tests.

In the end, it is suggested a morphological characterization of the surface for obtaining data regarding he defects on the surface because in that way is possible to correlate the attachment and others behaviors of cells to the superficial morphology os the are and/or the presence or absence of specific chemical compounds.

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