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LICENCIADA EM BIOQUÍMICA

FIBER FUNCTIONALIZATION FOR BIOMEDICAL APPLICATIONS

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Aos meus pais.

Ao Dr. Babú.

The limits of the possible can only be defined by going beyond them into the impossible.

Arthur C. Clarke

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"Ser humilde não é ser mais que alguém, é saber que não somos mais do que ninguém."

(Unknown)

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ABSTRACT

Deep-eutectic solvents (DES) are considered novel renewable and biodegradable solvents, with a cheap and easy synthesis, without waste production. Later it was discovered a new subclass of DES that even can be biocompatible, since their synthesis uses primary metabolites such as amino acids, organic acids and sugars, from organisms. This subclass was named natural deep-eutectic solvents (NADES).

Due to their properties it was tried to study the interaction between these solvents and biopolymers, in order to produce functionalized fibers for biomedical applications. In this way, fibers were produced by using the electrospinning technique. However, it was first necessary to study some physical properties of NADES, as well as the influence of water in their properties.

It has been concluded that the water has a high influence on NADES properties, which can be seen on the results obtained from the rheology and viscosity studies. The fluid dynamics had changed, as well as the viscosity.

Afterwards, it was tested the viability of using a starch blend. First it was tested the dissolution of these biopolymers into NADES, in order to study the viability of their application in electrospinning. However the results obtained were not satisfactory, since the starch polymers studied did not presented any dissolution in any NADES, or even in organic solvents. In this way it was changed the approach, and it was used other biocompatible polymers.

Poly(ethylene oxide), poly(vinyl alcohol) and gelatin were the others biopolymers tested for the electrospinning, with NADES. All polymers show good results, since it was possible to obtain fibers. However for gelatin it was used only eutectic mixtures, containing active pharmaceutical ingredients (API's), instead of NADES. For this case it was used mandelic acid (antimicrobial properties), choline chloride, ibuprofen (anti-inflammatory properties) and menthol (analgesic properties).

The polymers and the produced fibers were characterized by scanning electron microscope (SEM), Transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR). With the help of these techniques it was possible to conclude that it was possible to encapsulate NADES within the fibers.

Rheology it was also study for poly(ethylene oxide) and poly(vinyl alcohol), in a way to understand the influence of polymer concentration, on the electrospinning technique.

For the gelatin, among the characterization techniques, it was also performed cytotoxicity and drug release studies. The gelatin membranes did not show any toxicity for the cells, since their viability was maintained. Regarding the controlled release profile experiment no conclusion could be drawn from the experiments, due to the rapid and complete dissolution of the gelatin in the buffer solution. However it was possible to quantify the mixture of choline chloride with mandelic acid, allowing thus to complete, and confirm, the information already obtained for the others characterization technique.

Keywords: Natural Deep Eutectic Solvents, biopolymers, starch, poly(caprolactone), poly(ethylene oxide), poly(vinyl alcohol), gelatin, therapeutic deep eutectic solvents, electrospinning

RESUMO

Deep-eutectic solvents apresentam-se como solventes renováveis e biodegradáveis, sendo a sua síntese barata, simples e sem produção de resíduos. Posteriormente foi descoberta uma subclasse destes solventes que, para além de ter todas estas vantagens, consegue ainda ser biocompatível, uma vez que usa na sua síntese metabolitos primários de organismos, como aminoácidos, ácidos orgânicos e açúcares. Esta subclasse designou-se por *natural deep-eutectic solvents* (NADES).

Devido às suas propriedades, tentou-se estudar a sua interação com biopolímeros, de modo a produzir fibras funcionalizadas para aplicações biomédicas, através do uso de eletrofiação. Começou-se então por estudar as propriedades físicas destes solventes, bem como a influência da água nas propriedades dos mesmos. Conseguiu-se concluir que a água tem uma grande influência sobre as propriedades dos NADES, através da realização de estudos da reologia.

Posteriormente utilizaram-se misturas de biopolímeros, contendo amido, tendo-se procedido a estudos de dissolução nos solventes eutécticos, de modo a testar a sua aplicabilidade na eletrofiação. Porém nenhum dos biopolímeros teve sucesso na dissolução em NADES, nem em solventes orgânicos. Tentou-se então alterar a abordagem e recorreu-se a outros polímeros também de carácter biocompatível.

Poli(óxido de etileno), poli(vinil álcool) e gelatina foram os biopolímeros testados para a técnica de eletrofiação, juntamente com NADES. O poli(óxido de etileno) e o poli(vinil álcool) produziram as primeiras fibras com NADES, através da eletrofiação. Contudo a gelatina apenas produziu fibras para misturas eutécticas, os THEDES, que continham ingredientes farmacêuticos ativos (API's). Estes THEDES foram sintetizados misturando ácido mandélico (propriedades antibacterianas) com cloreto de colina, e ibuprofeno (propriedades anti-inflamatórias) com mentol (propriedades anestésicas).

Recorreram-se a várias técnicas de caracterização como microscopia eletrónica de varrimento (SEM), microscopia eletrónica de transmissão (TEM) e espetroscopia de infravermelho (FTIR), de modo a caracterizar as fibras obtidas, para os três polímeros. A informação obtida, e conjugada, permitiu então averiguar que o NADES e o THEDES conseguiam ser impregnados no interior das fibras, ou então encapsulados em casulos.

A reologia foi também uma propriedade estudada para o poli(óxido de etileno) e poli(vinil álcool), de modo a compreender a influência da concentração do polímero no processo de eletrofiação.

No caso da gelatina estudou-se citotoxicidade e tentou-se realizar um perfil de libertação controlada dos fármacos. As membranas de gelatina testadas não demonstraram qualquer toxicidade para as células, tendo mantido a viabilidade celular. Porém os testes de libertação não foram bem-sucedidos, devido à rápida desintegração das membranas quando colocadas em tampão. Para este último teste, porém, conseguiu-se quantificar o ácido mandélico com cloreto de colina, corroborando os resultados obtidos anteriormente, para as técnicas de caracterização.

Palavras chave: Natural deep-eutectic solvents, biopolímeros, amido, poli(caprolactona), poli(óxido de etileno), poli(vinil álcool), gelatina, therapeutic deep-eutectic solvents, eletrofiação

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NOMENCLATURE

API's	Active Pharmaceutical Ingredients
CA	Citric Acid
ChCl	Choline Chloride
CA:Suc	Citric Acid : Sucrose
ChCl:CA	Choline Chloride : Citric Acid
ChCl:MandAc	Choline Chloride : Mandelic Acid
DES	Deep-eutectic Solvents
DMF	Dimethylformamide
FTIR	Fourier transform infrared spectroscopy
Glu	Glucose
Ibu	Ibuprofen
Ibu:Men	Ibuprofen : Menthol
ILs	Ionic liquids
MandAc	Mandelic Acid
Men	Menthol
NADES	Natural Deep-eutectic Solvents
PCL	Polycaprolactone
POE	Poly(ethylene oxide)
PVA	Poly(vinyl alcohol)
SEM	Scanning electron microscopy
SPCL	Starch-Polycaprolactone
SCA	Starch-Cellulose Acetate
Suc	Sucrose
TEM	Transmission electron microscopy
THEDES	Therapeutic Deep-eutectic Solvents
Xyl	Xylose
RT	Room Temperature
Glu	Glucose

Chapter 1

OVERVIEW

OVERVIEW

PART 1: GREEN CHEMISTRY - THE NEED FOR SELF-SUSTAINABLE PROCESSES

Since the beginning of times that renewable processes allowed the replacement of various compounds, preventing the exhaustion of needed natural resources and the accumulation of possible toxic mixtures.

The passage of time led to a growth in various areas and men started to comprehend the mechanisms behind the processes. In this way the need of knowledge became to grow and the production of new compounds led to an increase of hazard materials. Thus the accumulation of non-biodegradable mixtures increased and natural resources also started to be used without control. As a consequence problems emerged associated with the abuse of renewable and natural resources. It became impossible to replace them with the same rate at which they were used. But with the fast development of the chemical industry, so did the awareness of society for environmental and health hazards that these processes could cause so they started to require all the benefits that these industries would provide but without the negative problems and with more sustainable processes.

It was following this line that the concept of Green Chemistry arose in the 90's[1] where can also be known as sustainable chemistry and it is defined as the process of eliminating, or reducing, the use of hazardous substances or their generation[1]. It involves twelve principles that help the user to adapt and adjust his practices for achieving the goal of sustainability.

The Twelve Principles of Green Chemistry[1] (see figure 1.1) embrace important points such as prevention, atom economy, less hazardous chemical synthesis, designing safer chemicals, safer solvents and auxiliaries, design for energy efficiency, use of renewable feedstock, reduce derivatives, catalysis, design for degradation, real-time analysis for pollution prevention and inherently safer chemistry for accident prevention. Through a careful plan that goes from chemical synthesis to molecular design[1], these principles serve as a guideline for the design of new chemical processes and compounds by applying the life-cycle from raw materials, the safety of the transformations and the toxicity and biodegradability of the materials used[1]. It also allows to demonstrate that it is possible to design products and processes that are profitable with all the benefits for human and environment health[1].

Although the success that the green chemistry is having in all research sectors, it is important to notice that these principles must be applied as a consistent system and not as independent goals[1] since it is only possible to achieve a sustainable process if they were applied as an integrated guideline[1].

The use of safer solvents can be the more active principle in the Green Chemistry since they are the main reason for the large production of waste due to their process and their applications, without counting with the properties such as flammable, corrosive and toxic profile that the most conventional solvents have[1]. In this way chemist started to work on a way to overcome these problems by searching for safer solvents that led to a new class of green solvents from water to supercritical fluids and ionic liquids[1].

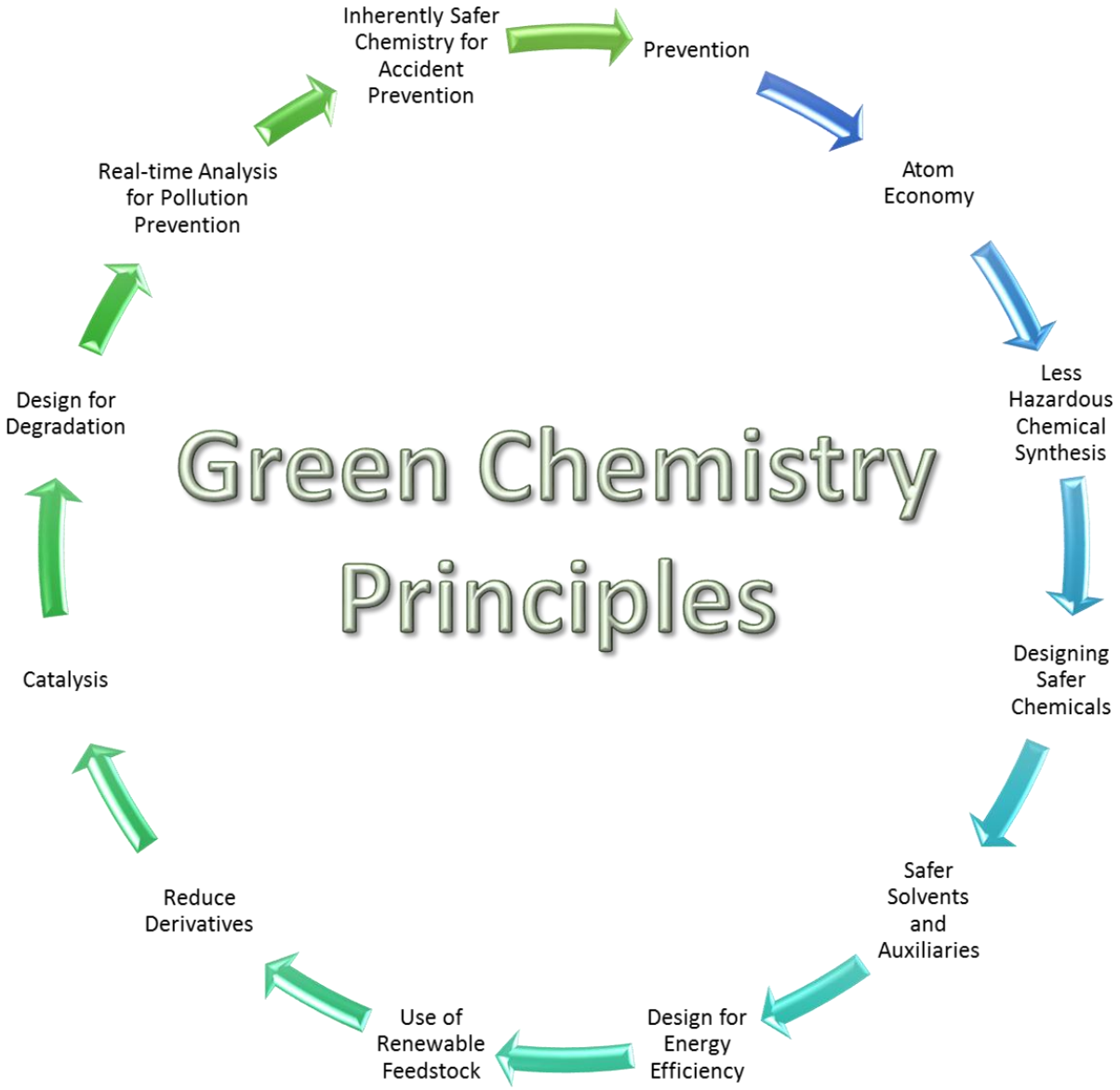

Figure 1.1. The Twelve Principles of Green Chemistry

PART 2: IONIC LIQUIDS – A NEW GREEN SOLVENT

Ionic liquids arose as the substitutes for VOCs (volatile organic compounds) due to various reasons such as the high use of these solvents and their volatility[2]. Although the existence of governmental policies that control the emission and the abusive use of VOCs it is important to develop more efficient and environmental friendly process in order to decrease these pollution[2].

So the ionic liquids have been seeing as the green solvents that could help to minimize the risks of using VOCs. They are made of organic salts composed by cations and anions, which present a liquid state at temperatures below 100°C[3], where some of them are liquid at room temperature[2] and thus called room temperature ionic liquids (RTILs).

Usually called as "designer solvents", their properties can be managed according to the needs of the scientist. The green character is conferred by unique properties typical of these solvents that go from the negligible of vapor pressure to the thermal stability and polarity[2,3]. These properties make it possible to use them under high temperature conditions, since they do not decompose over a large range of temperature[2], preventing the production of atmospheric VOCs even at low pressures[2]. The miscibility of the ionic liquids is another property that can be modified since they can go from immiscible to fully miscible[2] depending on the nature of the cation or the anion used.

It is due to all these properties, and the possibility to adapt the properties of ionic liquids, that make them interesting solvents to be used in all industries sectors and scientific areas, such as chemical engineering, energy, electrochemistry, catalysis, extraction, separation and organic reactions, among others[2] (see figure 1.2).

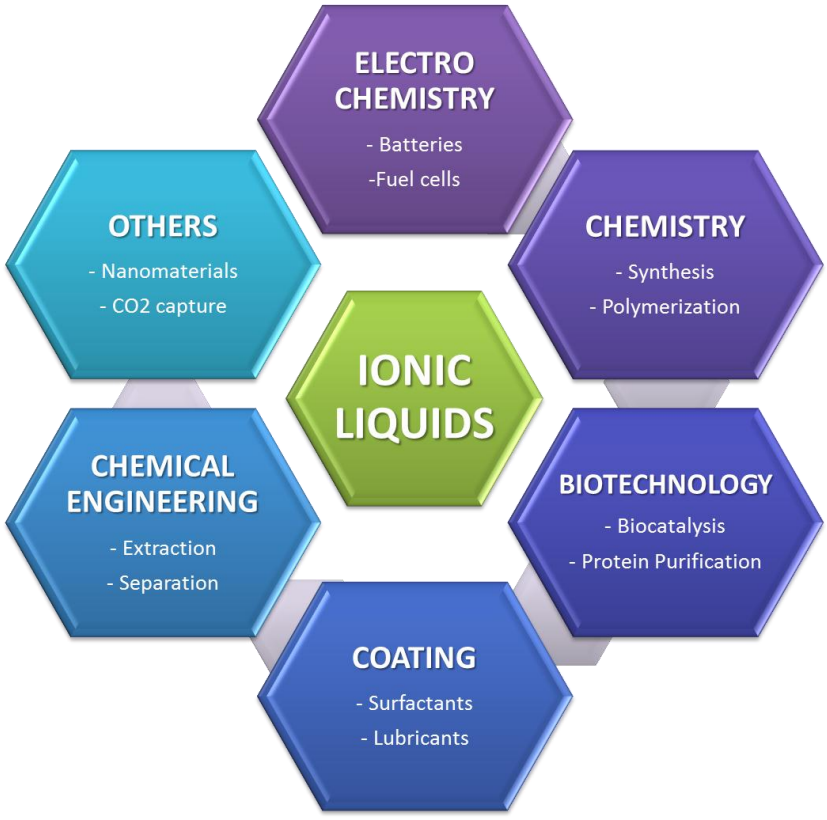

Figure 1.2. Possible ionic liquids applications. (Adapted from Pharm *et al*[4]*).*

Due to their vast applications the attention among these solvents had a suddenly increase that led to a rapid growth in publication rate[5]. Literature search shows that from the end of the 20th century the number of publication on ionic liquids increased over a 70 growth factor, according to web of knowledge database on the search for ionic liquids results (see figure 1.3).

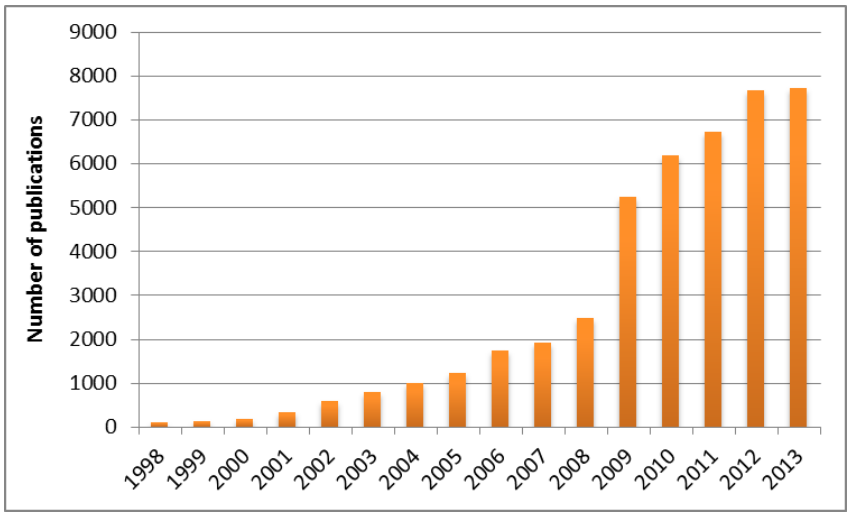

Figure 1.3. Evolution of the number of publications on ionic liquids, according to the Web of Knowledge database.

As said before, the applications of these solvents depend on the imagination of the user, leading to more than one hundred applications in all areas. But for the interest of this thesis theme spoken here, the use of these green solvents will be directed to a more specific area, electrospinning technique.

An example of an application of this technique is the production of ion jelly® fibers[6] (see figure 1.4). These fibers combine the properties of one polymer, gelatin, with ionic liquid, allowing the creation of membranes with a high area/volume ratio and high porosity[6]. This process enabled the increase the applications with ionic liquids.

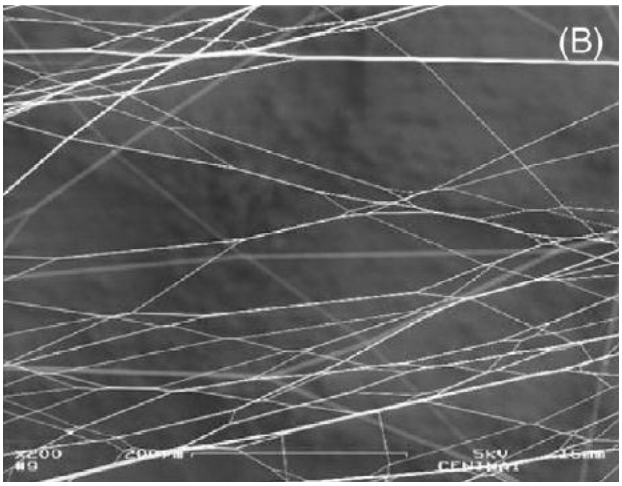

Figure 1.4. SEM image of ion jelly fibers. Adapted from Pimenta *et al*[5].

The advantage of using ionic liquids is the fact that it is possible to design their properties according to the application wanted by combining different cation/anion modifying thus their properties. However it is important to notice that ionic liquids are widely applied into the electrospinning technique along with others polymers as cellulose[7] and others[8,9].

PART 3: THE NEED OF NEW GREEN SOLVENTS

Designer property is the main characteristic that makes ionic liquids so appealing, since it enables to adjust the physicochemical properties of the solution. However the evolution of the science led to an advance on the information about ILs allowing grouping these solvents into various generations. The first generation comprehends physical properties that are often unique in ILs, such as decreased vapor pressure and high thermal stability. This generation did not attract much interest due to the oxygen-sensitive that did not allowed an easy handling.

Thus the research was directed to new synthesis of others ILs that led to the second generation, which have a more stable behavior in presence of water or oxygen. These solvents present interesting properties (lower melting points, viscosity, different solubility, etc.) enabling their use as reaction mediums. This generation is still target of study providing interest and novel applications in different areas.

Although the vast applications and interest on these green solvents, the green character of ionic liquids have been challenged due to the lack of information about their biodegradability and toxicity[10]. Moreover the sustainability of the production process is still challenging, since the synthesis of ionic liquids includes the production of by-products that can be harmful, leading to an increase of waste thus influencing the E-factor[11]. The E-factor highlights the waste produced in one process and it can be determined by dividing the total waste produced by the total amount of the desired product[12]. The smaller the E-factor the more sustainable will be the reaction.

The literature shows that the most common ionic liquids used are indeed toxic to nature[4] distancing them from the green image typically found. The question now relies on the possibility of designing environmentally acceptable ILs that will maintain their excellent application capability among with the capacity to fulfill the environmentally safety criteria.

In this way it has been proven that the addition of polar groups to the structure enables a decrease in toxicity and an increase in biodegradability[4]. But being ionic liquids known as designer solvents maybe the true solution passes through the creation of novel ionic liquids based on more environmentally friendly compounds, thus fulfilling all the criteria needed to guarantee the green character of these solvents.

Recently a new generation of ILs was created based on active pharmaceutical ingredients in order to produce ILs with biological activity. The development of antimicrobial Ion Jelly fibers[13] is one example of this application. Through the use of a biocompatible polymer and the combination of choline chloride and antimicrobial acids, it was possible to obtain electrospun fibers that demonstrated antimicrobial properties against *E. coli* and *B. subtilis*[13]. This discovering altered the non-green character that ILs had obtain previously since the cholinium cation presents a low toxicity and good biodegradability[11].

However there is a weakness on the use of this new generation of ILs. Besides the high costs in the synthesis and characterization of these ILs, halides are produced as by-products. Therefore, it is necessary to overcome these disadvantages in order to improve the viability of these new ILs.

Chapter 2

STATE OF THE ART

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PART 1: DEEP-EUTECTIC SOLVENTS: THE "NEWS IONIC LIQUIDS"

Deep eutectic solvents can be considered as the new ionic liquids in nowadays research evidencing close properties and applications to ionic liquids. They both share some physical properties that go from their non-flammability, due to the lack of measurable vapor pressure[14], and a non-reactive behavior with water[14]. They can also provide reaction conditions, like ILs, in areas such as liquid-liquid extraction[15], removal of glycerol from biodiesel fuel[15], dissolution and separation of components[16], catalysis[16], organic synthesis[16], and electrochemistry[16] among others. However deep-eutectic solvents result from the mixture of two safe compounds that in known proportions are able to form an eutectic mixture, being this the main reason why deep-eutectic solvents offer certain advantages over ionic liquids[14].

The components used for the DES preparation are cheap, renewable and biodegradable, overcoming the high price and sometimes the toxicity that some ionic liquids exhibit[16]. Beyond that these solvents do not need a post-synthesis purification since the purity that they have depends only on the purity of its individual components[14]. Finally they also present a low cost to the users making DES more desirable than ILs for a large-scale synthesis applications[14].

1.1. DEEP EUTECTIC SOLVENTS PRODUCTION

DES are a result of an eutectic mixture that occurs between a quaternary ammonium salt and a hydrogen-bond donor[14]. Choline chloride is an example of a quaternary salt and is also the most common reagent used for the formation of DES since it is a cheap, biodegradable and non-toxic compound having the advantage of being extracted from biomass or synthesized from fossil reserves[16]. The combination of this salt with hydrogen-bond donors such as urea, renewable carboxylic acids and polyols, results in an easy synthesis of deep-eutectic solvents[16]. This final solution presents a melting point that is far lower than the melting point of the individual components of the mixture. These phenomenon can be explained by the charge delocalization that occurs through the hydrogen bond that exists between the halide anion and the hydrogen-donor[14].

In general DES are characterized by the formation of a liquid phase with a very large depression of freezing point usually higher than 150°C[16]. However it is important to note that DES are liquid in a range of temperatures that goes from room temperature to 70°C[16].

Since they have close properties to ionic liquids it is possible to include DES in this class of solvents[17]. Still DES cannot be considered ILs since they haven't in their composition ionic species and can be formed with non-ionic compounds[16].

1.2. EUTECTIC MIXTURE

Deep eutectic solvents are compared to an eutectic mixture since the latter has in his composition two or more compounds[18] that result in a final solution with different properties than the initial compounds, being the melting point the property that suffers a stronger modification[14,19]. These type of mixtures present a lower melting point when compared to the initial components[18], and it is called the eutectic temperature[18]. However it is important to refer that this process only occurs when the compounds are in a fixed molar ratio[20], at an unique temperature[20], since it is the modification of the concentrations of the compounds in the mixture that are responsible for the

creation of this eutectic mixture. This phenomenon is then called the eutectic point and can be observed in Figure 2.1.

In case of changing the ratio of the compounds or if the temperature is modified, the eutectic mixture does no longer exist[20]. It is this temperature – ratio process that allows the formation of alloys in areas such as metallurgy and biomedicine being able to make them stronger and when needed, biocompatible.

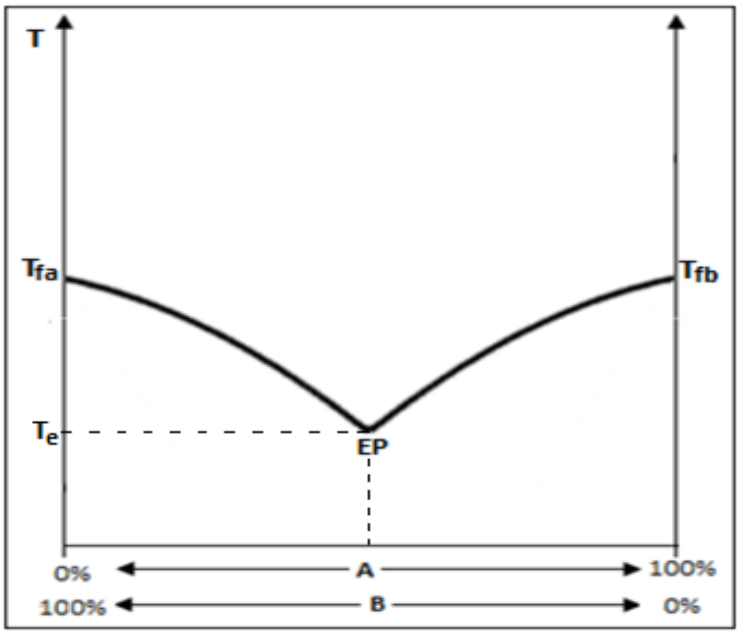

Figure 2.1. A typical diagram of a eutectic mixture made with two compounds. In this figure it is possible to see the molar ration as well as the temperature needed to achieve the eutectic point (EP).

PART 2: FROM DEEP-EUTECTIC SOLVENTS TO NATURAL DEEP-EUTECTIC SOLVENTS

The green character of deep-eutectic solvents is the principal quality that makes these solvents so interesting in the research area by means of replacing the harsh organic solvents. However the applications of DES and ILs in the real chemistry industry have some limitations due the toxicity of these solvents against humans and the environment, the high cost of ILs and the solid state of some DES at room temperature[19]. In this way it is important to overcome these disadvantages and turn these solvents more friendly for the various areas of application.

2.1. AN ALTERNATIVE MEDIUM

According to Dai *et al*. hypothesis[19], there are is alternative medium to water and lipids since there are various biological processes that occur in living organisms that are difficult to explain[19]. The survival of organisms in extreme environmental conditions, or the biosynthesis of poorly water soluble metabolites[19], are some of the biological processes that can confirm the existence of an alternative medium. This theory is supported by Choi *et al* [21] that raises some questions about the significance of large amounts of some simple molecules that can be found in various organisms such microbial, mammalians and plants. Due to their quantities the authors think that these molecules must serve a basic function in the living cells and not only as intermediates in metabolic pathways[21].

The application of NMR technique allowed a clear view of the major molecules present in these organisms that go from sugars to amino acids, choline and some organic acids[21]. In this way it is believed that these compounds can form a third new type of liquid separated from water and lipids[21].

For proving this hypothesis some experiments were made through mixture several of these molecules in known molar ratios that proved, in the end, to be liquid[19]. Table 2.1 shows various combinations of the most common metabolites that provide more than a hundred of possible combinations[19].

This new class of medium demonstrates the same abilities of ionic liquids and deep-eutectic solvents, since they can explain the biosynthesis and storage of poorly water soluble compounds giving thus the discovery of a new type of solvent that are named Natural Deep-eutectic Solvents (NADES).

Table 2.1. List of the various compounds that can be used to formed natural deep-eutectic solvents and their molar ratios[19].

Components			Mole ratio
Component 1	Component 2	Component 3	
Choline chloride	Lactic acid		1:1
Choline chloride	Malonic acid		1:1
Choline chloride	Maleic acid		1:1, 2:1,
Choline chloride	DL-Malic acid		1:1, 1.5:1,
Choline chloride	Citric acid		1:1, 2:1,
Choline chloride	Aconitic acid		1:1
Choline chloride	L-(+)-Tartaric acid		2:1
Choline chloride	Glycol		1:1,1:2
Choline chloride	1,2-Propanediol		1:1, 1:1.5, 1:2, 1:3
Choline chloride	1,2-Propanediol		2:1[a]
Choline chloride	Glycerol		1:1, 3:2
Choline chloride	*meso*-Erythritol		2:1[a]
Choline chloride	Xylitol		5:2
Choline chloride	Adonitol		5:2
Choline chloride	Ribitol		5:2
Choline chloride	D-Sorbitol		3:1, 5:2
Choline chloride	D-Xylose		2:1, 3:1
Choline chloride	A-L-Rhamnose		2:1
Choline chloride	D-(+)-Glucose		1:1, 2:1[a]
Choline chloride	D-(+)-Glucose		5:2
Choline chloride	D-(−)-Fructose		1:1, 1:1.5, 1:2[a]
Choline chloride	D-(−)-Fructose		5:2
Choline chloride	Sorbose		5:2, 1:1
Choline chloride	D-Mannose		5:2
Choline chloride	D-(+)-Galactose		5:2
Choline chloride	Sucrose		4:1, 1:1
Choline chloride	D-(+)-Trehalose		4:1
Choline chloride	Maltose		4:1
Choline chloride	Raffinose		11:2
Choline chloride	Proline	DL-Malic acid	1:1:1
Choline chloride	Xylitol	DL-Malic acid	1:1:1
Choline bitartrate	D-(+)-Glucose		1:1
Betaine	D-(+)-Glucose		5:2 [a]
Betaine	Sucrose		4:1, 1:1[a]
Betaine	Sucrose		2:1
Betaine	D-(+)-Trehalose		4:1
Betaine	D-Sorbitol		3:1[a]
Betaine	DL-Malic acid		1:1
Betaine	L-(+)-Tartaric acid		2:1
Betaine	D-Mannose		5:2
Betaine	Inositol	Raffinose	9:1:1[a]
Betaine	Sucrose	Proline	1:1:1
Betaine	Sucrose	Proline	5:2:2
Betaine	D-(+)-Glucose	Proline	1:1:1
Betaine	DL-Malic acid	D-(+)-Glucose	1:1:1
Betaine	DL-Malic acid	Proline	1:1:1
Betaine	DL-Malic acid	Inositol	1:1:1[a]
Betaine	Oxalic acid	D-(+)-Glucose	1:1:1
Betaine	Citric acid		1:1
Lactic acid	D-(+)-Glucose		5:1
Lactic acid	β-Alanine		1:1
DL-Malic acid	D-Xylose		1:1[a]
DL-Malic acid	D-(+)-Glucose		1:1, 1:2[a]
DL-Malic acid	Sucrose		1:1
DL-Malic acid	D-(−)-Fructose		1:1[a]
DL-Malic acid	D-Mannose		1:1
DL-Malic acid	Sucrose		1:1, 2:1
DL-Malic acid	Maltose		2:1[a]
DL-Malic acid	D-(+)-Trehalose		2:1
DL-Malic acid	Lactose		2:1, 1:1
DL-Malic acid	Raffinose		3:1[a]
DL-Malic acid	Xylitol		1:1[a]
DL-Malic acid	Adonitol		1:1[a]
DL-Malic acid	D-Sorbitol		1:1
DL-Malic acid	D-(+)-Glucose	D-(−)-Fructose	1:1:1
DL-Malic acid	D-(+)-glucose	Glycerol	1:1:1
DL-Malic acid	Sucrose	Glycerol	1:1:2
DL-Malic acid	L-Proline	Choline chloride	1:1:1
Citric acid	D-Xylose		1:1[a]
Citric acid	D-(+)-Glucose		2:1[a]
Citric acid	D-(−)-Fructose		1:1
Citric acid	Sorbose		1:1[a]
Citric acid	D-Mannose		1:1
Citric acid	D-(+)-Galactose		1:1[a]
Citric acid	Sucrose		1:1
Citric acid	Maltose		2:1
Citric acid	D-(+)-Trehalose		2:1
Citric acid	Raffinose		3:1
Citric acid	D-Sorbitol		1:1
Citric acid	Ribitol		1:1
Citric acid	Xylitol		1:1
Citric acid	Adonitol		1:1
Citric acid	L-Proline		1:1, 1:2, 1:3
Citric acid	DL-Malic acid		1:1[a]
Phytic acid sodium	Betaine		1:6
Phytic acid sodium	DL-Malic acid		1:6
Phytic acid sodium	Glycerol		1:6
Phytic acid sodium	L-Proline		1:6
Phytic acid sodium	D-(+)-Glucose		1:6
Phytic acid sodium	Choline chloride		1:3
D/L-Proline	Sucrose		2:1, 3:1
D/L-Proline	Sucrose		4:1,1:1[a]
D/L-Proline	D-Sorbitol		1:1
D/L-Proline	D-(+)-Glucose		1:1, 5:3
D/L-Proline	Lactic acid		1:1
D/L-Proline	DL-Malic acid		1:1
D/L-Proline	Citric acid		1:1, 2:1
D/L-Proline	Malonic acid		1:1[a]
D-Proline	D-(+)-Glucose		5:3
L-Proline	D-(+)-Glucose		5:3
L-Serine	DL-Malic acid		3:2, 1:1
L-Serine	D-(+)-Glucose		5:4[a]
L-Glutamic salt	Sucrose		2:1
L-Glutamic salt	D-(+)-Glucose		1:1
D-(+)-Glucose	DL-Malic acid		1:1[a]
D-(+)-Glucose	Citric acid		1:1
D-(+)-Glucose	L-(+)-Tartaric acid		1:1
D-(+)-Glucose	D-(−)-Fructose	Sucrose	1:1:1[a]
D-(−)-Fructose	Sucrose		1:1
β-Alanine	DL-Malic acid		3:2, 1:1
β-Alanine	Citric acid		1:1

[a] Not stable; solid precipitate within 7 days.

2.2. THE IMPORTANCE OF NADES IN RESEARCH

NADES became an important issue as they present a pharmaceutically acceptable toxicity profile[19]. Moreover, they brought an improved knowledge in the area of biosynthesis in organisms and also lead to an increase in the number of applications[19].

The molecular interactions responsible for the formation of NADES are exactly the same as in DES. It is the hydrogen bond, confirmed by NMR technique[19], that it the responsible for the molecular interactions. Table 2.2 demonstrates that in some NADES solutions, they have in their composition, known molar ratios of water. It is believed that water has an important role in the structure of NADES, influencing the stability of the structure and even their physical properties[19].

Table 2.2. Natural Deep-Eutectic Solvents with known ratios of water in their composition. Adapted from Y. Dai et al. (2013)[19].

Composition	Mole ratio
Malic acid:choline chloride:water	1:1:2
Glycerol:choline chloride:water	2:1:1
Malic acid:β-alanine:water	1:1:3
Proline:malic acid:water	1:1:3
Fructose:choline chloride:water	2:5:5
Xylose:choline chloride:water	1:2:2
Sucrose:choline chloride:water	1:4:4
Fructose:glucose:sucrose:water	1:1:1:11
Glucose:choline chloride:water	2:5:5

Although the presence of water can influence the physical properties of NADES it also modifies their solubility capacity (as well as in DES and ILs). However in some cases the addition of water is a needed step since most of DES are not liquid at room temperature. In order to overcome this problem and achieve liquid NADES at room temperature the addition of water is the main solution[19]. But beyond the decrease of viscosity the addition of water also leads to the loss of hydrogen bonds between the compounds and thus destroying the structure responsible for the formation of NADES[19]. In order to control all this conditions the addition of water must be controlled and in known amounts since the hydrogen bonds play key role in structure stabilization.

Dai *et al.* showed that the number of hydrogen bond donor or acceptor groups, the spatial structure of the groups and the position of the bonds are critical to the stability of NADES structure[19]. This phenomenon can be seen in the preparation of solution with organic acids such as succinic acid and citric acid since a liquid solution is not achieved when prepared with succinic acid. However if the solution was prepared with citric acid a liquid solution is achieved. This can be explained by observing the acid structure presented in figure 2.2 where citric acid presents an extra carboxylic group allowing the formation of an extra hydrogen bond thus influencing the structure of the NADES[19].

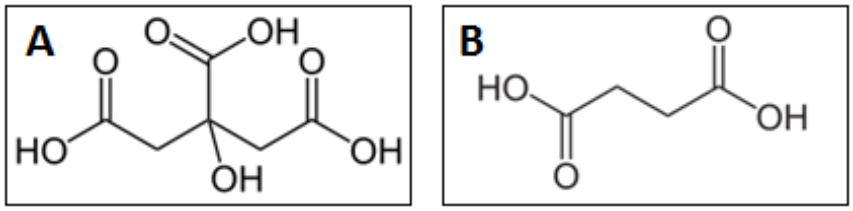

Figure 2.2. Chemical structure of the citric acid (A) and the succinic acid (B) permitting the comparison between these two organic acids.

Due to their different physical characteristics it is possible to use these solvents as an alternative medium in various applications such as extraction [22], organic[23] and enzymatic[24] reactions and it can also be used for increasing the solubility of compounds such as DNA, gluten and starch with values higher than in water[19].

The application of these green solvents in this work relies on the fabrication of new polymers and extends their applications for various research fields.

PART 3: ELECTROSPINNING – A FIBER MAKING TECHNIQUE

The advance in the areas such as nanotechnology forced the creation of new and improved techniques in order to promote the fabrication of nanostructures with unique properties and applications[25]. It is in this way that electrospinning arises as a new and improved method capable of producing fibers with a higher contact area in a nanoscale production. Beyond that this process makes it even possible to control the fiber characteristics such as the diameter, composition and length[25].

The fiber technology appears in the 30's when Formhals discovered an apparatus capable of performing polymer filaments with the use of electrostatic repulsion between surfaces[26] that was called back then electrical spinning[26]. However, due to the technology limitations, this process was not capable of accomplish nanofibers, neither having significant applications[27].

The application of this technology into nanoscale only occurred in the 90's when the electrospinning process was re-introduced, in order to characterized it and to find out what were the parameters behind it[28]. Therefore Doshi and Renenker were responsible for renaming it and so the electrospinning name[28] appears. Among that they were capable of conjugate the electrospinning process with SEM, allowing then to demonstrate the significance of nanofibers and their applications.

3.1. ELECTROSPINNING SETUP

The setup behind the electrospinning process is based on three primary components: a high-voltage power supply , a metallic needle and a grounded collector[25] (see figure 2.3). The high-voltage supply typically has a range between 1 and 30 kV. The metallic needle is linked to a syringe, with the polymeric solution within, attached to a syringe pump that enables the continuous fed at constant rate[25]. The pump is an important component since it creates a pendent drop in the end of the needle that will be electrified when an electric field is applied, yielding an highly electrified solution with the creation of equal charges distributed all over the surface of the drop[25] . Due to the equal distribution of charges, the liquid drop formed at the tip of the needle suffers a repulsive interaction at the same time that an attractive force, exerted by the collector, takes place. This electrostatic forces leads to a deformation of the drop that achieve a conical shape known as the Taylor cone[25]. However for the

formation of a liquid jet is important that the strength of the electric field overcomes the surface tension of the liquid. Only these conditions enable the formation of a long and thin fiber that will be pushed out from the Taylor cone and then attracted to the grounded collector[25].

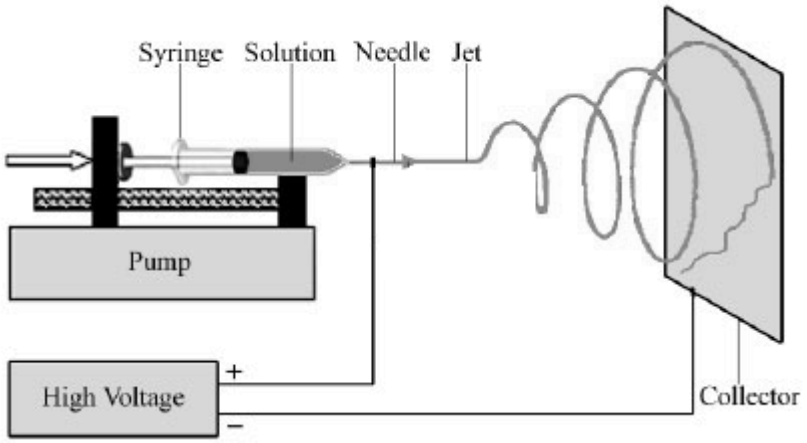

Figure 2.3. Representation of the electrospinning setup (adapted).

3.2. ELECTROSPINNIG PARAMETERS

In order to achieve good results from this process it is important to pay attention to various parameters that influence the fiber formation. These parameters can be separated into three groups which are related with the solution, the process itself and environmental conditions[29].

In the solutions parameters, the concentration of the polymer has an important role in the electrospinning process since for lower concentrations there are not conditions for the elongation of the polymer and so a electrospray process is obtained (see figure 2.4-A)[29]. But an higher concentration of polymer is also not good to produce nanofibers since the final result is a microscale fiber (see figure 2.4-B)[29].

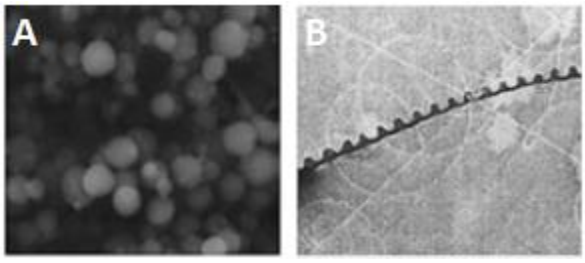

Figure 2.4. A) SEM images that demonstrate the electrospray process due to low concentration of polymer; B) SEM image from a microscale fiber due to a higher concentration of polymer. (Amplification: a) non-defined; b) 100x) Adapted from Li and Wang (2013)[29]

Beyond this scale modification the concentration also influences the viscosity of the solution[29]. When the solution presents a very low viscosity it is not possible to obtain a continuous jet leading to the electrospray process. On the other hand, for a very high viscosity it becomes hard to eject the solution through the needle[29] and so the formation of a jet may also be impossible. This property can be changed by adjusting the polymer concentration. It is also important to consider the

molecular weight since this parameter also influences the process. The presence of beads can be a result of a low molecular weight polymer. In order to obtain smooth fiber for the same concentration it is necessary to increase the molecular weight of the polymer[29]. However for higher values of molecular weight microfibers can be obtained with the possibility of existence of beads[29].

Furthermore, it is also important to consider the surface tension of the solution in the electrospinning process[29]. This parameter plays an important role in the existence or not of beads in the final product and according to literature it is possible to convert fibers with beads to smooth fibers with the decrease of the surface tension[29]. This process can be achieved by changing the mass ratio of the solvents mix or the solvent used, since it also contributes to the surface tension[30].

The process parameters to take into account include voltage, flow rate, collector and the distance between the collector and the needle[25,29]. It is known that the electric field applied has indeed a role on the fiber diameter. However it is not a linear parameter since various studies demonstrate that in some polymers exist alterations of the diameter whereas for other polymers the change of the electric field does not have any effect[29]. Other parameter that also influences the diameter is the flow rate since is related to the time needed for solution polarization[25,29]. Thus normally is recommended the use of a low flow rate in order to give enough time for the polarization of the polymer solution occur.

The distance between the collector and the tip of the needle has also an influence on the diameter and morphology of the fiber[25,29]. Here the distance is an important parameter since it influences solvent evaporation time, enabling the formation of the fiber. So if a short distance is used there will not be enough time for the solidification of the fiber. Although long distances does not help either since it can disrupt the polymer elongation thus leading to the formation of beads[29]. Distance also influences the electric field. However, as stated above, its effect on fiber diameter is not clear. The collector is also an important feature since it acts as the conductive substrate allowing the collection of the fibers[25].

At last the environmental conditions, which include the humidity and temperature used for this process[25,29]. The conjugation of these two properties influence others such as the evaporation rate of the solvent that will increase at low humidity conditions[29]. Some workers have already demonstrated that in some cases these parameters can also influence the diameter and the morphology of electrospinning fibers[29].

PART 4: POLYMERS – FROM PLASTIC BAGS TO BIOMEDICAL APPLICATIONS

Polymers can be grouped into two groups depending on their source, which may be natural or synthetic. This latter arose during the world wars two due to the lack of natural polymers, which have led to the development of polymers such as nylon, acrylic, polyester and Teflon, among others[31]. These manufacture process increasing the popularity of these kind of polymers since there are unlimited and economic possibilities for fabricate them with specific properties[31].

Synthetic polymers derive from petroleum resources which leads to an exhaustive use of these fossils fuels and also the accumulation of plastics in the environment, since the chemical stability of these polymers does not allow an easy decomposition of the material[31].

Therefore scientists directed their way into the track of natural polymers due to their biodegradability and also their renewable source. Natural polymers can be categorized into eight different categories that include polysaccharides, proteins, lignin and nucleic acids, among others[32].

Nonetheless there is a non-win situation on using some biodegradable polymers obtained from natural derived polymers. They present predominant hydrophilic nature, which results on a fast degradation rate, and poor mechanical performances. In this way it very common to improve these characteristics by blending these natural polymers with other biopolymers with synthetic origin[32].

By definition a biomaterial is any natural or synthetic material that can interact with biological systems as like supplementation or substitution of functions of living tissues[33,34].Over time various natural polymers were tested as replacement for tissues from living forms but the response from the host were variable since the material was not always tolerated by the human body[33]. In this way it was tested the interactions between tissues and the natural materials for a better understanding.

In order to be a biomaterial with an appropriate host response, it should also be biocompatible for performing its function[33]. By being biocompatible, the biomaterial can be broken down and excreted, or reabsorbed by the human body, without any need of surgical revision or removal[34].

However it is important to notice that the host response depends on the chemical, physical and biological properties of the biomaterial[33,34]. This is an important question to address for the design of biodegradable biomaterial since they must not induce an inflammatory response, presenting a degradation time according to their function, possessing adequate mechanical properties according to their use[34]. Beyond that the products of the degradation cannot be toxic and have to be readily reabsorbed or excreted by the organism and should include appropriate permeability and processability for designed application[34].

The best known resources capable of making biodegradable plastics are starch and cellulose[31], that by blending with other polymers are very used for biomedical applications[35,36]. But according to clinical experience it is possible to ensure that not all the engineered materials can be used for biomedical applications[33].

The possibility of adapt and the various advantages that the biodegradable biomaterials have allows to apply them in various sectors, that will depend on their final use.

Chapter 3

MATERIALS AND METHODS

MATERIALS AND METHODS

PART 1: PREPARATION AND CHARACTERIZATION OF NADES AND THEDES

Being known as the substitutes for ionic liquids, NADES (subclass of DES) should share similar properties with ILs. There is, however, very limited data available on the properties of these solvents. Thus it became important to study some physical properties of these solvents, in order to acquire more information about them.

1.1. CHEMICALS

The preparation of NADES results from the use of individual compound without any additional step of purification after their preparation. It was used choline chloride (Sigma-Aldrich, ≥98%), citric acid (Merck, >99%), sucrose (Cmd Chemicals), D-(+)-glucose (Cmd Chemicals), D-(+)-xylose (Merck, >99%), R-(-)-mandelic acid (Alfa Aesar, 98%), (±) - menthol (Aldrich, ≥98%) and ibuprofen sodium salt (Fluka, ≥98%). The synthesis of ibuprofen was prepared by following the protocol, in appendix I, and store at -20ºC[37], according with Stott *et al.*

1.2. PREPARATION OF NADES

In this study NADES were prepared in different molar ratios as shown in Table 3.1. Concentrated solutions of glucose (Glu) 1M, sucrose (Suc) 1M, xylose (Xyl) 1M and citric acid 1M (CA) were previous prepared. For solutions contained these compounds it was added the volumes needed to achieve the molar ratios.

For NADES containing chloride choline (ChCl), it was weight and the needed volume of the second compound was added for the molar ratio wanted. After achieving clear solutions they were dried through the vacuum evaporating method, at 40 °C and 75-65 mbar. This process occurred until a viscous solution was obtained.

Table 3.1. List of Natural Deep-Eutectic Solvents used for this study.

Composition	Molar Ratio
Choline Chloride: Citric Acid (ChCl : CA)	1:1
Choline Chloride: Citric Acid (ChCl : CA)	2:1
Choline Chloride: Glucose (ChCl : Glu)	1:1
Choline Chloride: Sucrose (ChCl : Suc)	1:1
Choline Chloride: Sucrose (ChCl : Suc)	4:1
Choline Chloride: D(+) – Xylose (ChCl : Xyl)	2:1
Choline Chloride: D(+) – Xylose (ChCl : Xyl)	3:1
Citric Acid: Sucrose (CA : Suc)	1:1
Citric Acid: Glucose (CA : Glu)	1:1

1.3. PREPARATION OF THEDES

Two THEDES solutions were prepared, mandelic acid with choline chloride (ChCl:MandAc) and ibuprofen with menthol (Ibu:Men). It is possible to see in Table 3.2 the molar ratios and the temperatures used for their preparation.

Table 3.2. Therapeutic Deep-eutectic solvents (THEDES) used for this study, with the molar ratio and the temperature used for their preparation.

	Molar Ratio	Temperature
ChCl:MandAc	1:2	40 °C
Ibu:Men	1:3	40 °C

The solutions were stirred until obtaining a clear solution. For ChCl:MandAc mixture it was added a small amount of water, to ensure the completely dissolution. In the end the water was remove with the help of a vacuum evaporator.

1.4. WATER QUANTIFICATION

The water was quantified through the Karl Fischer method using an 831 KF Coulometer (Metrohm). This coulometer titration is based on the classical method developed by Karl Fischer, which allows determining the water content in solutions. It is based on a reaction between iodine and water. This reaction occurs at a 1:1 molar ratio[38], which will allows to quantify the total amount of water present in the solution tested. This process was carried out at room temperature where the final value was the mid value of three measures.

1.5. VISCOSITY

Viscosity is important for the characterization of NADES, since it can influence the final result on the electrospinning experiment. In this way viscosity was measured with a help of two rheometers: Malvern, Kinexus Prot (at 3B's Research Group, Universidade do Minho) and AR-G2 TA instruments stress controlled oscillatory rheometer (at CENIMAT, FCT/UNL), since the studies were performed in different places. For the rheology it was used the parallel plates technique and a temperature of 25°C. The results were interpreted with the help of appropriated software.

PART 2: STARCH BLENDS PROCESSING

Starch blends are biocompatible polymers with various applications. However their interaction with these new solvents is new, being thus necessary to perform different experiments such as: dissolving the polymers into organic solvents and NADES, testing the possibility of applying them into electrospinning, and even the possibility of modifying their structure.

2.1. CHEMICALS

The chemical used were acetic acid glacial (Scharlab, >99%), chloroform (Carlo Erba, >99%), dimethylformamide (Scharlab, >99%), dioxane (Merck, >99%), toluene (Sigma-Aldrich, >99%), starch-polycaprolactone (SPCL; Novamont, Italy), starch-cellulose acetate (SCA; Novamont, Italy), polycaprolactone (PCL; Sigma-Aldrich, Mn 70000-90000), propionic anhydride (Sigma-Aldrich, >99%) and ibuprofen anhydride, which was synthesized according to Bartoli *et al.* All chemicals were used without any purification.

2.2. DISSOLVING THE POLYMERS IN VARIOUS SOLVENTS

Predetermined amounts of polymer were added to various NADES and stirred, in order to test their dissolution. The influence of temperature it was also tested. Table 3.3 demonstrates the weight fraction and the temperature tested for each polymer.

Table 3.3. Polymers tested for the dissolution into NADES, with the weight fraction used and the range of temperature tested, in order to improve dissolution.

Polymers	Weight fraction (% w/v)	Temperature Range
SPCL	5	RT to 60°C
SCA	5	RT to 60°C
PCL	7	RT to 60°C

2.3. CHEMICAL MODIFICATIONS OF SPCL

Beyond testing the dissolution of starch blends, chemical modifications to starch were also performed. Two protocols were followed for chemical modifications on SPCL, known as acetylation. The first one involved ibuprofen anhydride. For this process it was added a 10% (w/v) of SPCL for 1ml of chloroform. For the reaction it was used a weight ratio of 1 starch for 3 anhydride. The solution was left to stir during 48h at room temperature. The steps followed for both these modifications can be seen of appendix II.

The second protocol involved the modification of starch with propionic anhydride. A 5% (w/v) of SPCL was added to a round-bottom flask with 1ml chloroform, and 500µl of propionic anhydride, and left to stir at room temperature.

PART 3: ELECTROSPINNING OF BIOCOMPATIBLE POLYMERIC SOLUTIONS

Beyond starch polymers it was also tested the viability of conjugate others biocompatible polymers with NADES. Here it will be also study their applicability into electrospinning and also their viability as functionalized polymers.

3.1. CHEMICALS

The polymers used were poly (ethylene oxide) (POE; Sigma-Aldrich, Mv 5000000), poly (vinyl alcohol) (PVA; Acros Organics, 95% hydrolyzed, Mv 95000) and gelatin (Gel; Panreac, CULTIMED.403902). The NADES used were choline chloride with citric acid (ChCl:CA) and citric acid with sucrose (CA:Suc).

3.2. SAMPLES PREPARATION

A known amount of polymer was dissolve into water, then NADES was added and the mixture was left to stir, until complete dissolution. Table 3.4 shows the conditions used for PEO and PVA.

Table 3.4. Biopolymers used for the electrospinning technique. It shows the volume of NADES added, as well as the polymer weight fraction added to each sample. The temperature is related to the dissolution of the polymer, in water.

		NADES Volume fraction %(v/v)	Polymer Weight fraction %(w/v)	Temperature
PEO	ChCl:CA (1:1)	2	2	Room temperature
			3	
			4	
	CA:Suc (1:1)	2	2	
			3	
PVA	ChCl:CA (1:1)	2	7,8	Preheated water 80°C
			9,8	

For preparing the gelatin samples, it is possible to see on Table 3.5 that different concentrations of gelatin were experimented. First it was only dissolved gelatin in preheated water. After that it was tested the influence of the addition of THEDES, on the aqueous gelatin solutions. All these processes were performed at 40°C, and the solutions were all stirred during 1h30.

Table 3.5. Conditions used to prepare gelatin samples, for the electrospinning. It indicates the volume of NADES and the polymer weight used for each sample.

		THEDES Volume fraction (v/v)	Polymer Weight fraction (w/v)
GEL	Aqueous Solutions	-	30
		-	35
		-	40
	ChCl:MandAc (1:2)	2	30
			35
	Ibu:Menthol (1:3)	0,5	30
		1	
		1,5	
		2	

3.3. ELECTROSPINNING EXPERIMENTAL SET-UP

The high voltage supply used, for the electrospinning voltage, was a Glassman EL 30kV, that was linked to the metallic needle. The solution, in the syringe, was pumped with a KDS100 from KD Scientific. The metallic needles had various diameters and they were from ITEC. For collecting the fibers a fixed collector was used. All these equipments were located into an acrylic box, in order to help to control the ambient parameters.

Among that it was used an electric heater, for cases where the temperature needed to be higher. For controlling humidity, the air conditioning of the room was used.

3.4. ELECTROSPINNING CONDITIONS

For producing fiber it was necessary to test various parameters in order to achieve the best electrospinning conditions. Table 3.6 shows the parameters range tested for each polymer, the temperature and the humidity inside the box, the flow rate of the polymeric solution, the distance between the tip of the needle and the collector and the voltage applied to the solution. Later, on chapter 4, it will be discussed the parameters chosen and how they influence the process.

Table 3.6. Electrospinning range parameters tested for each polymer sample.

		RANGE OF PARAMETERS TESTED				
		Temperature	Humidity	Flow rate	Distance	Voltage
PEO	CA:Suc (1:1)	RT-30°C	30-45%	0,05-0,3 ml/h	15-30 cm	5-15 kV
	ChCl:CA (1:1)	RT-30°C	30-50%	0,1-0,7 ml/h	15-35 cm	5-25 kV
PVA	ChCl:CA (1:1)	RT-30°C	40-50%	0,1-0,2ml/h	11-18cm	15-25kV
GEL	ChCl:MandAc (1:2)	28-42°C	15-40%	0,1-0,5 ml/h	15-20 cm	15-25 kV
	Ibu:Men (1:3)	28-32°C	15-40%	0,1-0,4 ml/h	15-20 cm	15-25 kV

PART 4: POLYMERS CHARACTERIZATION

4.1. FOURIER TRANSFORM INFRARED SPECTROSCOPY

Being an absorption technique, FTIR is commonly used to identify chemical compounds or substituents groups, in samples. Infrared spectra results from transitions of vibrational states, due to molecular vibrations[39].

This process happens due to interactions that occur between the infrared light and the molecule analyzed. The sample absorbs the energy from the light, and the molecule starts to vibrate[39].

Capable of analyze gas, liquid or even solid samples, FTIR became an important technique on the identification of compounds, since every molecule has vibrational modes that differs slightly from other molecules (with exception of enantiomers)[39]. It is commonly used the region between 4000 and 400 cm^{-1}, since most of the organic compounds or inorganic ions absorbs the radiation within this region[39]. The term Fourier transform comes from the use of a mathematical process, the fourier transform, that is responsible for converting the raw data, into the known spectra.

For the study it was used a Tensor 27 FTIR spectrometer, from Bruker Optic GmbH, with the help of the OPUS 6.0 software, for data analysis.

4.2. SCANNING ELECTRON MICROSCOPY

This technique is a type of microscopy that can image and analyze bulk specimens. It uses an electron beam that is created by the acceleration of electrons, and is responsible for tracing over the object, and creating an exact replica of the original object[40]. Once the electron beam hits and interacts with the sample, it is created a complete 3D image. Beyond image signal, others signals are produced (like secondary electrons), that will be responsible for the registration of different levels of brightness[40].

This microscope operates in vacuum and it relies on electric field to work. In this way it became important that the sample has conductivity, or otherwise they must be coated with a conductive material, such as gold or platinum[40].

For sample coat it was used a Polaron SC502 sputter coater. The microscope used was a Zeiss DSM 962. For fixing the samples it was used a carbon conductive tape purchased from Agar Scientific.

4.3. TRANSMISSION ELECTRON MICROSCOPY

This other type of microscopy also uses the electron beam to analyze the sample. However in here the electrons interact strongly with the atoms of the sample, passing through it, which implies the use of a thin thickness sample[41]. It is then by this interaction that it is possible to form an image, that will be a two-dimensional projection of the sample.

The most common image obtained is through a contrast formation, where the image is formed directly by occlusion and absorption of the electrons[41], by the sample. Regions where the electrons cannot pass through the sample, appears in dark[41]. However for regions where the electrons are unscattered, the image appears brighter[41]. Among it may also be a grey scale between them, which will depend on the interaction between the sample and the electrons[41].

For this study it was used a Hitachi 8100 with ThermoNoran light elements EDS detector and digital image acquisition (at MicroLab/IST-UL).

PART 5: BIOLOGICAL AND DRUG DELIVERY STUDIES

5.1. BIOLOGICAL STUDIES

Extracts from membrane formed by gelatin fibers with THEDES were prepared according to the ISO/EN 10993 norm. L929 cells from mouse fibroblast, with a $1{,}5x10^4$ cell/mL concentration were grown during 24h at 37°C. After that period the medium was substituted by gelatin membranes, and the cellular viability was evaluated through the MTS assay, after 72h.

5.2. DRUG DELIVERY PROFILE

The gelatin membranes, obtained by electrospinning, were weighed and suspended in 10mL of phosphate buffer solution. Then it was stirred at 60 rpm at 37 °C.

Aliquots of 500 µL were withdrawn in predetermined time intervals and the same volume of fresh medium was added to the suspension.

The samples were analyzed by UV/Vis spectroscopy, in a microplate reader, at 250 nm.

Chapter 4

RESULTS AND DISCUSSION

PART 1: PHYSICAL PROPERTIES OF NADES AND THEDES

The influence of water content in NADES in their physical properties, namely, in viscosity was studied. It became clear that the water had a high influence on NADES properties.

1.1. WATER CONTENT

The water removal from NADES is not an easy process, due to the interactions that can occur between NADES and water. The water content was measured to all the NADES and THEDES with the help of the Karl Fischer method, and the results can be seen on Table 4.1.

Table 4.1. Percentage of water content present in each NADES.
The values were obtained through the Karl-Fischer method.

	Water (%)
ChCl : CA (1:1)	12,8/7,2*
ChCl : CA (2:1)	2,3
ChCl : Suc (1:1)	0,2
ChCl : Suc (4:1)	0,2
ChCl : Glu (1:1)	5,5
ChCl : Xyl (2:1)	0,2
ChCl : Xyl (3:1)	4,4
CA : Suc (1:1)	5,2
ChCl:MandAc (1:2)	0,9
Ibu:Men (1:3)	0,11

*value obtained for the dry process with nitrogen, after the vacuum method

The high water content in the ChCl:CA (1:1) was used to study the influence that the water could have in NADES properties. In this way it was make a rheology and dry study with this NADES that will be discussed forward.

1.2. VISCOSITY

Viscosity becomes an important property to study due to the influence that water content may have on solutions. The rheology study was made after drying the solutions. First the solution was only dried by using the vacuum evaporator. After that the efficiency of using nitrogen as drier was tested by bubbling nitrogen in the solution that was previously dried in the vacuum evaporator.

The use of nitrogen is a traditional method of removing oxygen from liquids. In the solution in question is insert nitrogen bubbling and the oxygen is removed. This process has some efficiency on removing water because along with deaerating, the entrainment of water occurs.

As said before, interactions between water and NADES can hinder its removal from the solution. In this way it was used a second drier method, in order to explore if it would be possible to extract a higher content of water. Figure 4.1 compares the result obtained for the appliance of only the vacuum evaporator and the result obtained for the conjugation of using vacuum and nitrogen. This experiment was only performed for one NADES, ChCl:CA. Nitrogen was insert into the solution, where it was bubbled for 2 hours.

The water content of each experiment was measure and it showed a difference of 5% of water between the two processes (vacuum evaporator: 12,8% of water; vacuum with nitrogen: 7,2% of water). This difference of water content led to a 5x fold increase on the viscosity value. Both NADES as a Newtonian behavior since viscosity does not change, with the increase of shear rate.

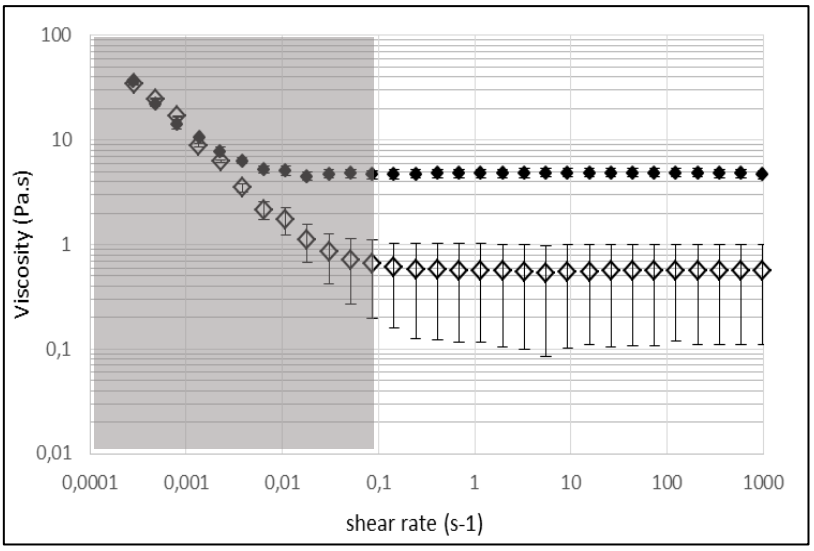

Figure 4.1. The viscosity obtained for ChCl:CA, dried with nitrogen (closed symbols), and ChCl:CA, dried only with vaccum (open symbols) at 25°C. For ChCl:CA (close symbols) solution the viscosity was 4,801 Pa.s, and for ChCl:CA (open symbols) was 0,558 Pa.s. Data in the grey area are considered outliers.

PART 2: STARCH BLENDS PROCESSING

Although starch based polymers have already been used in other experiments, with different solvents, their application with NADES is new. It was study their dissolution in various organic solvents and also NADES. Among that it was tested the viability of using organic solvents as co-solvents. The main objective of this study was to apply the electrospinning technique in order to obtain fibers with solutions. At the end it was tested the possibility of modifying the structure of starch, in order to try to improve the solubility of the polymers into NADES, and even see if it was possible to apply the electrospinning technique. It was only known that for SPCL case, it was needed a minimum concentration of 7 % of PCL to produce fibers. Therefore a concentration slightly lower of 5 % was experiment to determine the viability of producing starch base biopolymer solutions in NADES.

2.1. DISSOLVING THE POLYMERS IN NADES

According to the literature starch presents a good solubility in NADES, demonstrating even a better solubilization in these new solvents than in water[19]. In this way it was tested the solubility of SPCL and SCA in NADES. The procedure used was the addition of a known amount of the polymer into NADES and then stirred, at different temperatures, in order to obtain a clear and soluble solution. It is possible to see in Table 4.2 the concentrations used for each polymer and the results obtain for each.

Table 4.2. Results obtained for the dissolution test made for SPCL and SCA polymer, in NADES, at various temperatures (x symbol – occurred dissolution; - symbol – presence of powder in NADES, after stirred 16h).

	SPCL 5%(w/v)		SCA 5%(w/v)	
	RT	60°C	RT	60°C
ChCl : CA (1:1)	-	x	-	-
ChCl : CA (2:1)	-	x	-	-[1]
ChCl : Suc (4:1)	x	[2]	-	-[1]
ChCl : Glu (1:1)	-	-	-	-
ChCl : Xyl (2:1)	-	-	-	-[1]
ChCl : Xyl (3:1)	-	x	-	x
CA : Suc (1:1)	-	-[1]	-	-[1]

[1]The solution became yellow due to the sucrose degradation

[2]This temperature was not tested due to the previous knowledge of the sucrose degradation

For the cases were total dissolution was achieved the obtained solutions were tested for fiber production through electrospinning, nevertheless in all cases only droplets were projected to the collector. Higher concentrations were not used due to high viscosity of NADES with polymers, a disadvantage for their handling.

2.2. TESTING ORGANIC COMPOUNDS AS SOLVENTS

The low solubility of the polymers led to an alternative approach that consisted on the use of organic solvents as co-solvent. Since PCL presented a good solubility in organic solvents[42], maybe the use of them could help the dissolution of starch blends, and PCL, in NADES.

The organic solvents were tested, first, as solvents for SPCL, SCA and PCL. After that, it was tested their behavior as co-solvents for polymers. Dissolving starch biopolymers in organic solvents was determined in order to test the viability of applying them to the electrospinning.

The organic solvents used, as well as the results acquire for each polymer, can be seen in table 4.3. It was also tested various temperatures.

Table 4.3. Organic solvents tested for dissolving SPCL, SCA and PCL polymers, at various temperatures (x symbol – occurred dissolution; - symbol – presence of powder in solvents, after stirred 16h).

	SPCL		SCA		PCL	
	RT	40°C	RT	40°C	RT	40°C
2-Propanol	-	-	-	-	-	-
Acetic Acid	-	x[1]	x[1]		-	X
Chloroform	x[1]		-	-[2]	x	
Dichloromethane	-	-[2]	-	-[2]	x	
Dioxane	-	x[1]	x[1]		-	X
DMF	-	x[1]	-	-	-	X
Ethanol	-	-	-	-	-	-
Toluene	-	-	-	-	x	

[1]The solution appears a mat colour without full dissolution of the polymer

[2]Evaporation of the solvent at 40°C

For SPCL and SCA a clear solution was never obtained. Probably the starch fraction in the polymer blend was not dissolved in the organic solvent, forming a milky liquid at the end.

According to literature, starch can be soluble in glacial acetic acid, if the solution is heated at 100 °C[43]. This information led to the conclusion that for a full solubilization of the starch blends, it would be necessary to use higher temperatures.

It is reported that it is possible to produce nanofibers-based scaffolds[44], by mixing SPCL in acetic acid. *Jukola et al.* produced SPCL construct composed of large bead connected by thin fibers[44]. The SPCL solutions obtained were experimented for electrospinning, nevertheless only electrospray was achieved for all cases. It is possible that as in the case of the work from *Jukola et al.* constructs with nanofibers connecting the large bead could have been achieved. By optical microscopy only particles could be identified, to confirm the presence of fibers SEM analysis should be performed.

2.3. MISCIBILITY OF NADES IN ORGANIC SOLVENTS

However it was thought that by mixing together NADES and organic solvents, it would be possible to improve the dissolution. So, in order to carry out this idea, it became necessary to first determine which solvents were miscible with NADES. Thus, it was added to a vial, a 1:2 volume proportion of NADES and organic solvents. The solvents used, as well as the result obtained, can be seen on Table 4.4. For this step all the NADES, present on Table 3.1, were tested. However the result obtained were independent of NADES used.

Table 4.4. List of organic solvents tested in order to achieving the compatibility between them and NADES, in a 1:2 volume ration. The organic solvents are organized from the less polar to the more polar.

Organic Solvent	Miscibility
Toluene	Formation of two phases
Dioxane	Formation of two phases
Chloroform	Medium miscibility
Dichloromethane	Formation of two phases
Dimethylformamide	Miscible
2-Propanol	Formation of two phases
Acetic Acid	Miscible
Ethanol	Miscible
Water	Miscible

A general analysis allows concluding that NADES are more similar with polar solvents. Being an important factor, the polarity did not justify the results obtained, since NADES shows miscibility for dimethylformamide, a solvent less polar than 2-propanol. Thus it is not possible to obtain information about NADES polarity, from these results.

However it is important to notice that among NADES the polarity changes[12]. This property can be study through the use of a probe dye (*e.g.* Reichardt's Dye 30) along with the UV/Vis technique[12,16].

2.4. DISSOLVING POLYMERS WITH CO-SOLVENTS

According to table 4.3, it seems that polymers present a higher solubility in less polar solvents, than ethanol or acetic acid. This hypothesis can help to explain the difficulty in dissolving these polymers in NADES. But, since organic solvents are able to dissolve a certain extent of these starch blends, it was tested the option of using them as co-solvents. However it became necessary to

use a middle term solvent, since NADES and polymers do not present the same solubility properties, in organic solvents.

After result analysis, chloroform and acetic acid were the solvents chosen to use as co-solvents, since they presented miscibility with NADES and could also dissolve the polymers.

However, when NADES and polymers were used together it was not possible to obtain a clear and homogeneous solution. This result was observed for both solvents used. The final result was the formation of two phases. It is believed that a solvent separation between the organic solvent and NADES occur. The polymer stayed on the organic phase, due to its properties.

PART 3: CHEMICAL MODIFICATION OF SPCL

As mentioned before, starch presents a very low solubility, due to its rigid structure. So to improve the use starch, each industry adapts this molecule, according to their final application.

Acetylation is a reaction that is responsible for introducing an acetyl functional group into a chemical compound (see Figure 4.2).

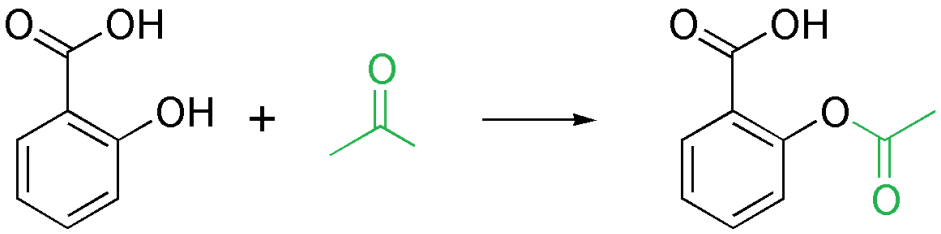

Figure 4.2. Example of an acetylation reaction. The acetyl group is represented green.

So it was thought that by modifying the structure of starch, by replacing hydrogen bonds, it would be possible to improve the solubility of starch on NADES or organic solvents. The modification of starch with ibuprofen anhydride enables the substitution of hydroxyl groups with the ibuprofen molecules. In this way it would be possible to obtain a polymer with anti-inflammatory properties in the end. The protocol used for this experiment can be seen in appendix II. The efficiency of the reaction was followed by FTIR, by comparing the SPCL spectrum (Figure 4.3) with the spectrum obtained for the reaction (Figure 4.6).

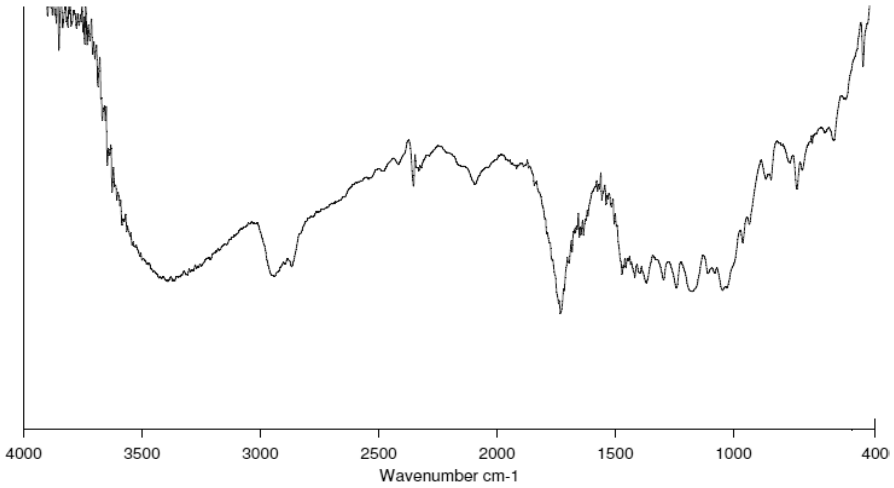

Figure 4.3. FTIR spectrum of SPCL.

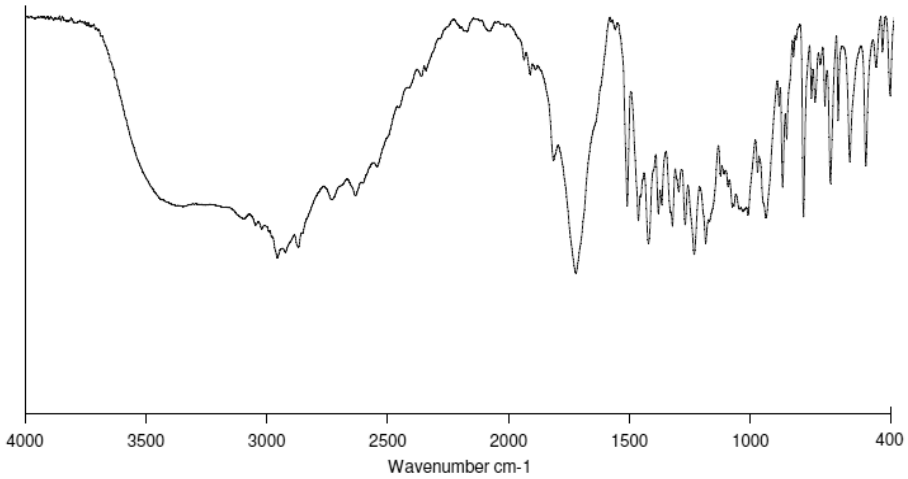

Figure 4.4. FTIR spectrum of the reaction between ibuprofen anhydride with SPCL, in chloroform.

The wavenumber ranging between 2800 and 3100 cm^{-1} it is due to the aromatic ring of ibuprofen, and the 1815 cm^{-1} peak is assigned to the anhydride. Thus a first look to the FTIR spectrum shows a successfully modification. However the final result demonstrated a sticky appearance, that it was thought to be non-reactive anhydride. Washing with ethanol was performed, for remove the non-reactive anhydride, and a new FTIR spectrum was made, showed on Figure 4.5.

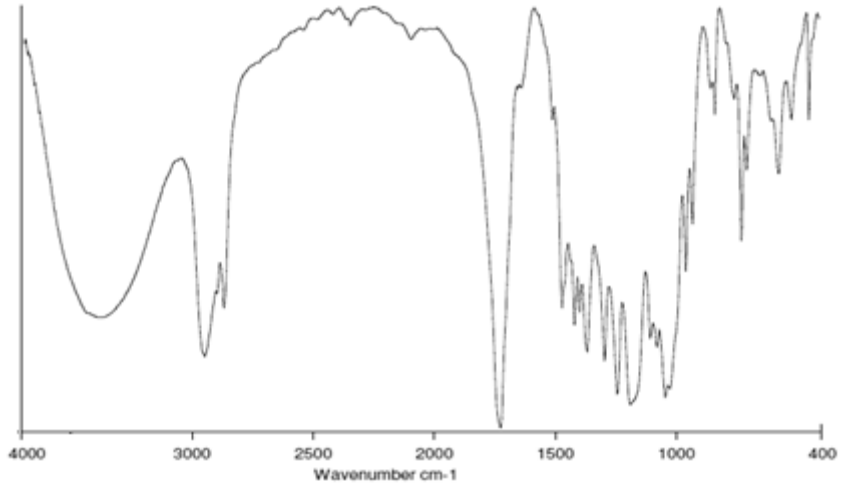

Figure 4.5. FTIR spectrum of the reaction between ibuprofen anhydride with SPCL, but after washing the product reaction with ethanol.

It can be seen that there are some differences between SPCL with anhydride spectrum and the after ethanol washed spectrum. It can be seen a good similarity between the SPCL spectrum (Figure 4.3) and the one obtained for the washing with ethanol. Thus it is possible to conclude that the reaction did not occurred, since the anhydride was dissolved by the ethanol during washing.

The substitution of hydroxyl groups was also tried, after the ibuprofen anhydride reaction, with anhydride propionic. Using the same conditions as the one describe on appendix II, the FITR spectrum of this reaction can be seen on Figure 4.6.

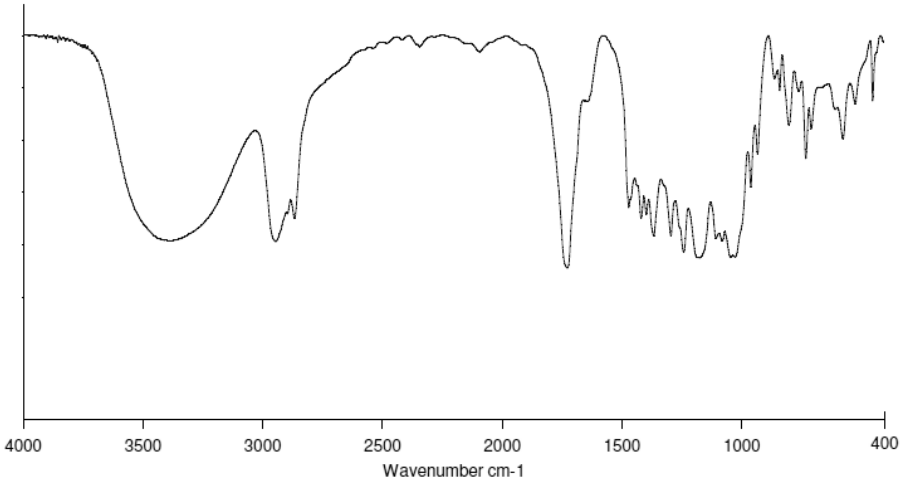

Figure 4.6. FTIR spectrum of the reaction between SPCL and propionic anhydride, in chloroform.

There are resemblances between SPCL spectrum and the anhydride propionic reaction spectrum, leading to the conclusion that the modifications within these conditions are not the ideal one. However this presents a good way for starch modification, being necessary to perform more experiments, in order to achieve and optimize better reaction conditions.

PART 4: ELECTROSPINNING OF BIOCOMPATIBLE POLYMERS

The poor results obtained for the dissolution of starch blends, led to a change on the method used until now. In this way it became necessary to use others biocompatible polymers, and test their viability on electrospinning. Thus, poly (ethylene oxide) (POE), poly (vinyl alcohol) (PVA) and gelatin were the polymers chosen for this process.

4.1. POLY (ETHYLENE OXIDE)

Being a water soluble polymer[45], PEO is a synthetic polymer that is available in various molecular weights. Due to this property, and to chain flexibility, this is a polymer commonly used for biomedical applications (drug delivery, tissue engineering scaffolds, among others)[45]. Further than its biocompatibility, it is nontoxic being a polymer approved by FDA[45] for use in various sectors, such as pharmaceutical formulations, food and cosmetic.

4.1.1. DISSOLVING NADES IN PEO SOLUTION

Experiments were performed in order to determine the solubility of PEO directly into NADES, at various temperatures. None of the solutions showed any dissolution of PEO. But knowing that it is a water soluble polymer, the approach was changed.

It was prepared a water solution of PEO. Then NADES have been added, until achieving a volume ratio of 2:1 of PEO for NADES. However there was a formation of a precipitate in all cases, with the expectation of ChCl:CA (1:1) and CA:Suc (1:1). Hereafter, all the experiments were performed with these two NADES.

4.1.2. FIBERS OPTIMIZATION

Now with the possibility of preparing polymeric solutions with NADES, it was tested their viability at electrospinning technique. It is known that this technique has various parameters that

must be accounted for. From literature, it was possible to get a range of ambient and process parameters, and also the concentration needed, which would allow the production of fibers. Thus, with all this information, it was possible to construct Table 3.6, which shows the range tested for each parameter. The fraction of NADES in the solution was maintained constant at 2% (v/v) for all the experiments. The first parameter that has been taken into account was the polymer concentration. For 0,5% (w/v) of PEO in water the result was electrospray. Thus the concentration was tuned for 2% that demonstrated immediately the formation of fibers, for both NADES. However a closer look with the help of an electron microscope demonstrates the existence of beads, in all fibers (Figure 4.7). In this way, it was necessary to increase the polymer concentration. As can be seen on Figure 4.7 for 3% (w/v) of PEO in water no beads were observed, with smoother and thinner fibers.

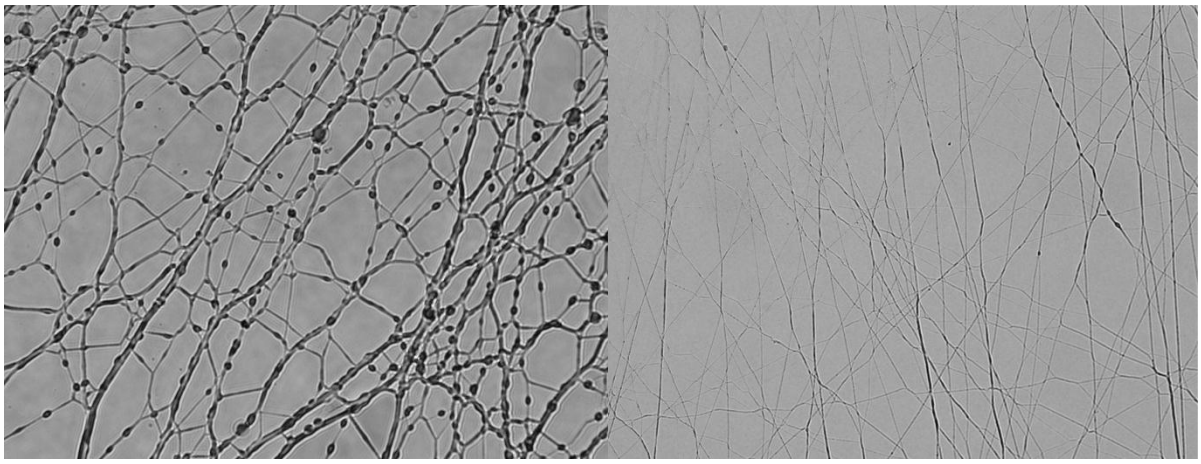

Figure 4.7. Electron microscope images for the electrospinning optimization parameters. **Left image** - PEO 2% (w/v) with CA:Suc 2% (w/v) fibers (conditions: 24cm, 10kv, 0,2ml/h, 45%, 23°C); **right image** - PEO 3% (w/v) with CA:Suc 2% (w/v) fibers (conditions: 24cm, 15kV, 0,2ml/h, 35-40% and 28-30°C). (Amplification of both images: 100x)

Concentration of PEO higher than 4% (w/v) did not produce fibers, due to the high viscosity of the solution. Knowing that viscosity is an important electrospinning parameter, it was study the viscosity for each NADES itself, as well as for each PEO concentration tested. Both results can be seen on Figure 4.8 and Figure 4.9**.**

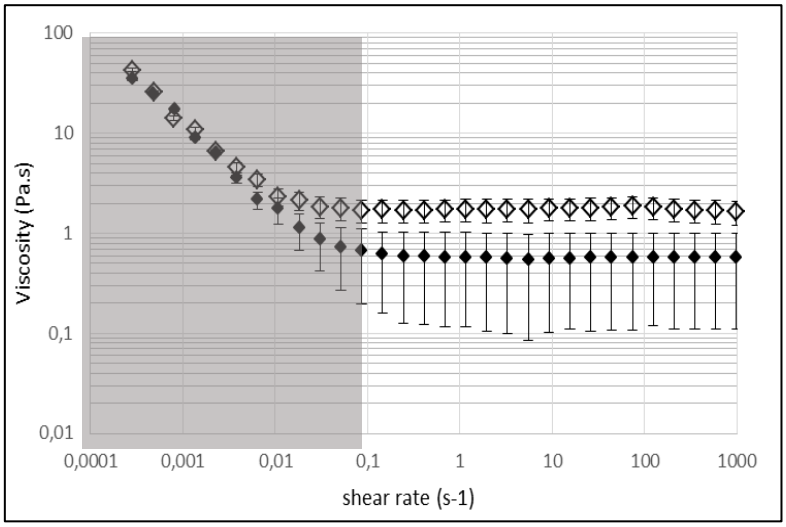

Figure 4.8. The viscosity parameter obtained for CA:Suc (open symbols) and ChCl:CA (closed symbols), at 25°C. For CA:Suc solution the viscosity was 1,743 Pa.s and for ChCl:CA was 0,558 Pa.s. Data in the grey area are considered outliers.

The results show that both NADES have a Newtonian fluid behavior, after a share rate of 0,1s-$^{-1}$, since they have a linear performance with the increase of the shear rate. However for shear rates below 0,1s^{-1} the values are not considered, since the measures were not performed at stationary state.

It is also possible to observe that CA:Suc presents a higher value of viscosity than ChCl:CA. Nevertheless, it is important to study the rheology of the NADES/polymer solution, for a better understanding of the results obtained in the electrospinning.

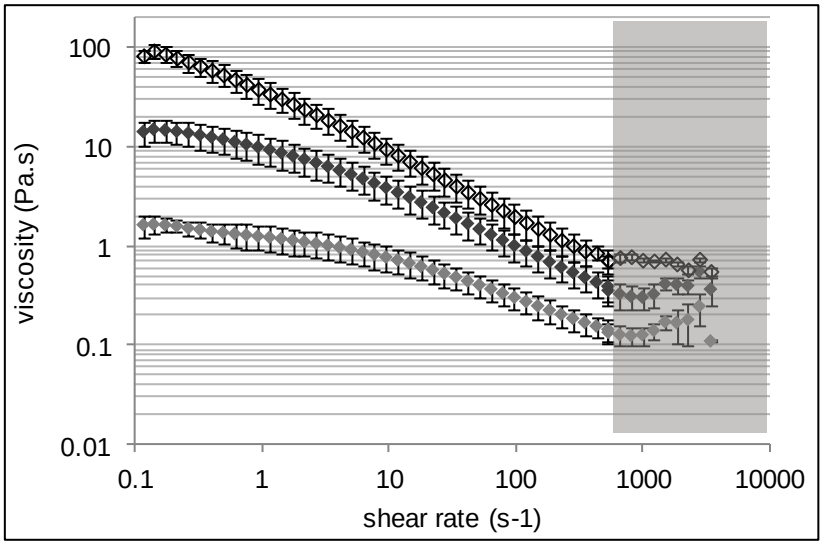

Figure 4.9. Effect of polymer concentration on the viscosity of the different POE solutions with 2% (v/v) ChCl:CA. PEO concentration of (2% (w/v): grey symbols; PEO concentration of 3% (w/v): black symbols; PEO concentration of 4% (w/v): open symbols. These experiments were realized at 25°C. Data in the grey area are not considered.

It is possible to see a 10 fold increase on the viscosity with the increase of polymer concentration. It is reported that for high values of viscosity the ejection of the solution can be difficult[29], preventing the formation of a continuous jet. However, with lower viscosity, the formation of beads may occur. Both these situations were seen, when the various solutions with different concentrations of PEO were tested.

This can explain the difficult on obtaining fibers for concentrations higher than 3% (w/v) and lower than 2% (w/v), being this last value the minimum concentration that can be used to produce fibers. The values inserted on the grey area are not considered, since the solution is no longer at stationary state.

Maintaining the concentration constant, other parameters were optimized, and can be seen in table 4.5. The needle used for both solutions had a caliber of 27G (0,25mm).

Table 4.5. Electrospinning optimized parameters, obtained for PEO samples, with CA:Suc and ChCl:CA NADES.

	RANGE OF OPTIMIZED PARAMETERS					
	Weight Fraction	**Temperature**	**Humidity**	**Flow rate**	**Distance**	**Voltage**
CA:Suc (1:1)	3% (w/v)	28-30 °C	35-40%	0,2 ml/h	24cm	15 kV
ChCl:CA (1:1)	3% (w/v)	28-30 °C	35-40%	0,5 ml/h	28 cm	20 kV

It was noticed that temperature and humidity had a high influence in the formation of fibers. By using humidity below 35%, along with temperatures above 28°C, it had occurred solution accumulation on the tip of the needle, by forming a drop. Consequently this prevented the elongation of the polymer, and thus the formation of fiber. In this way temperatures closer to 28°C, and humidity of about 40%, were the best conditions for obtaining fibers.

4.1.3. FIBERS CHARACTERIZATION

After finding the optimized conditions it was necessary to characterize the morphology and composition of the fibers. The morphology of the fibers was analyzed by SEM. The result obtained for each solution can be seen on Figure 4.10.

It is possible to see that the fibers obtained showed some kind of cocoons. From here it can be theorized that the solution was encapsulated inside those cocoons (Figure 4.10 – upper), instead of being inside the fibers. Among that it is also possible to see fractures along the fibers (Figure 4.10 – bottom right), which can mean that they have low resistance. Lastly it is also possible to see fiber fusion, which could help the encapsulation of NADES.

Although the encapsulation of NADES in cocoons could open several possibilities for the application of these fibers, the major problem on using this polymer is the fact that the fibers disappearing in less than 12h when left at room conditions. There are several protocols that can be used for modifying the fiber structure, making it more durable. But the main protocols uses UV photoinitiators[46], where most of them are hazardous to the human body.

It was then needed to search for other biocompatible and biodegradable polymer, with a cleaner crosslinking process.

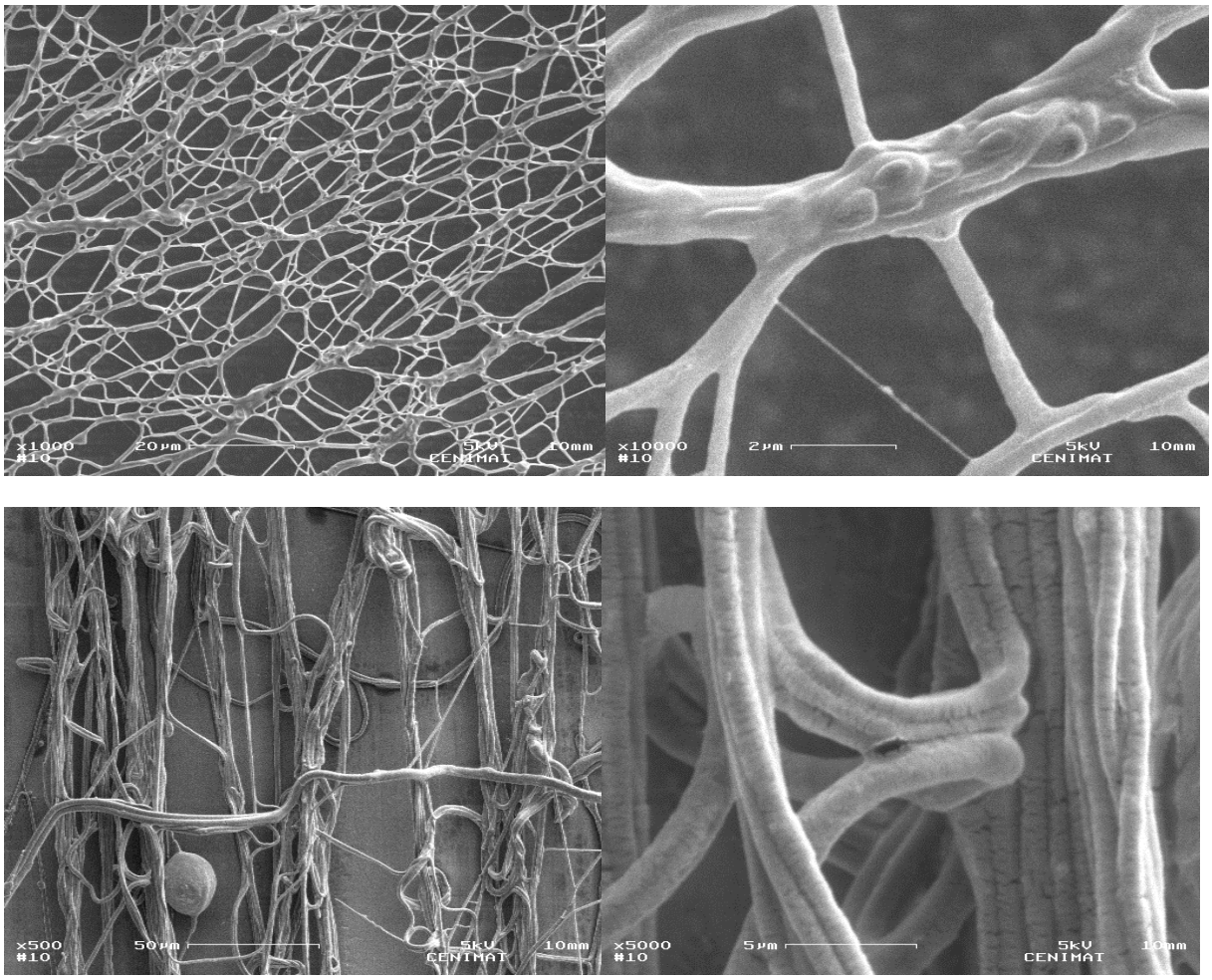

Figure 4.10. SEM images obtained for NADES fibers. **Upper left and right** - PEO 3% (w/v) with CA:Suc 2% (v/v) fibers (electrospinning parameters: 24cm, 15kV, 0,2ml/h, 35-40% and 28-30°C); **bottom left and right** - PEO 3% (w/v) with ChCl:CA 2% (v/v) fibers (electrospinning parameters: 28cm, 20kV, 0,2ml/h, 35-40% and 28-30°C).

4.2. POLY (VINYL ALCOHOL)

Poly (vinyl alcohol) is obtained through the hydrolysis of poly (vinyl acetate)[47], being produced industrially due to its non-expensive aquisition[48]. Among that this polymer presents very interesting properties that go from its biocompatibility[47,48], to non-toxicity[48] and non-carcinogenicity properties[48]. Due to these advantages PVA has been widely used for many applications on various research areas such as implants and artificial organs[49,50], antimicrobial films[51], contact lenses[52] and even in drug delivery[47,48]. It is also a hydrophilic polymer, allowing the absorption and swelling in water[48]. Furthermore it is easy to change its structure, modifying its properties turning it into a water insoluble polymer by a simple heat treatment [53,54].

4.2.1. FIBERS OPTIMIZATION

It is known that the electrospinning technique is affected by various parameters that influence the morphology of the fibers. However for the poly (vinyl alcohol) there is another property that is needed to be accounted for. Due to its production process, PVA is available with various degrees of hydrolysis. This characteristic affects the PVA behavior changing its mechanical properties, water resistance and even the dissolution of the polymer[55]. Thus for lower degrees of

hydrolysis PVA shows an easier dissolution, but with a lower mechanical resistance when compared with a PVA solution with a higher degree of hydrolysis[55]. In this way it was used a PVA powder with a high degree of hydrolysis for applying to the NADES/PVA electrospun fibers.

Literature shows a range of concentration tested for electrospinning[47,53,55], in some cases with the help of surfactants, in order to decrease the surface tension of the solution[55]. For this study it was experimented two concentration of PVA, 7,8% (w/v) and 9,8% (w/v), both with 2% (v/v) of NADES. For the 7,8% (w/v) concentration it was obtained fibers but with the presence of beads. But with the increase of concentration the morphology of the fiber changed from beaded fiber to a smoother and uniform fiber. The environmental and the process parameters were also taken into account. These parameters were also optimized, as can be seen Table 4.6. The needle used for both solutions had a caliber of 23G (0,41mm).

Table 4.6. Electrospinning optimized parameters obtained for PVA, with ChCl:CA.

	RANGE OF OPTIMIZED PARAMETERS					
	Weight Fraction	**Temperature**	**Humidity**	**Flow rate**	**Distance**	**Voltage**
ChCl:CA (1:1)	9,8**%** (w/v)	28°C	45-50%	0,2 ml/h	16 cm	20 kV

The viscosity plays an important role on the formation of smooth fibers. The rheology for these two solutions and the result can be seen on Figure 4.11**,** for PVA solutions of 7,8% (w/v) and 9,8% (w/v).

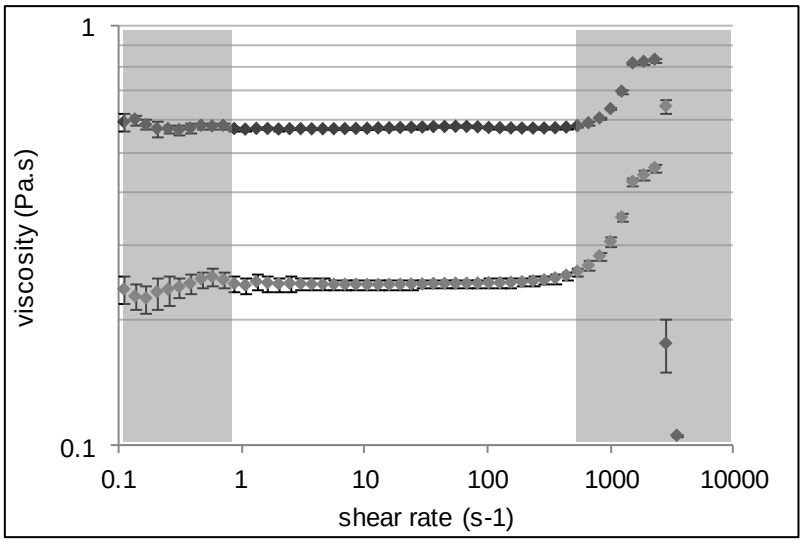

Figure 4.11. Effect of polymer concentration on the viscosity of the different PVA concentration with 2% (v/v) ChCl:CA (grey symbols: 7,8% (w/v); black symbols: 9.8% (w/v)). Both experiments were realized at 25°C. Data in the grey area are considered outliers.

The difference of the viscosity in these two solutions is sufficient for having different results on the fibers obtained. This can be due not to the viscosity itself but to the surface tension and the polymer concentration, since these properties are all related[29].

PVA demonstrates a typical behavior of a Newtonian fluid, since the viscosity remains constant with the increase of the shear rate[56]. Also the shear stress increases linearly with the increase of the shear rate[56] (see Figure 4.12).

From the grey area values, on figure 4.11, it is possible to see that the measures were not made at stationary state for shear stress lower than $1s^{-1}$. For shear stress higher than $800s^{-1}$, in both figures, 4.11 and 4.12, it is possible to see that the values also oscillate. For this case it can be considered that the solution may not be uniformly moving, due to the high speed used, and also to the viscosity of the solution. This is why the values are not considered.

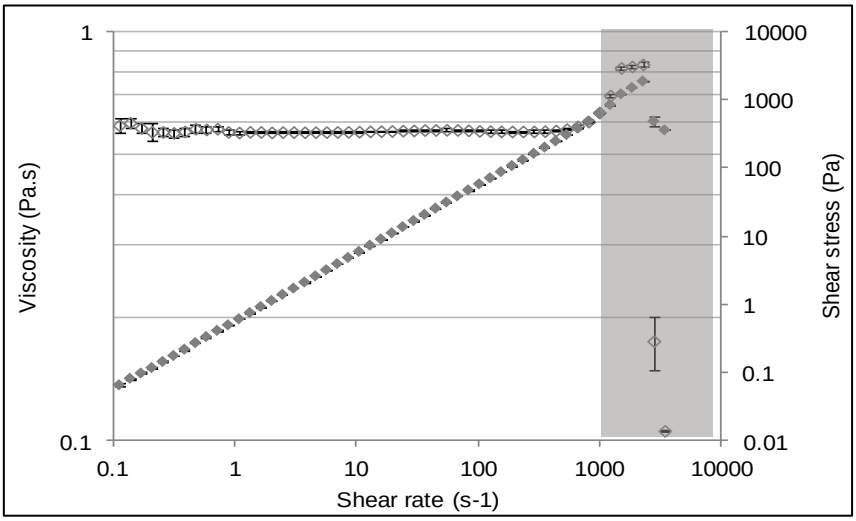

Figure 4.12. Viscosimetry of the sample PVA 9.8% with ChCl:CA (open symbols: viscosity; closed symbols: shear stress). This experiment was realized at 25°C. Data in the grey area are considered outliers.

4.2.2. FIBERS CHARACTERIZATION

Through the analysis of infrared spectroscopy it is possible to obtain the chemical functional groups that exist on the analyzed sample, thus allowing to determine if new interactions between compounds are taking place. For the PVA case, FTIR and TEM will help prove if it is possible to encapsulating NADES inside the fibers.

Through TEM images (Figure 4.13, left images) it is possible to observe a contrast difference. Due to the beam of electrons used for this technique, the interaction between the beam and the samples will create an image with darker or lighter regions, which will depend on the nature of the sample. Knowing that the polymer is thicker than NADES, regions with only polymer will appear lighter, and for NADES the regions will appear darker.

The FTIR spectrum only works as complementary information. Through these spectrums it is possible to observe that in fact both solutions are present on fibers. PVA spectrum (right image on Figure 4.13) shows an hydrogen bonded band[57], at ν=3442,93 cm^{-1}, C-H broad alkyl stretching band[57] at ν=2923,36 cm^{-1}. NADES are mostly compound by C-H and O-H bonds, being the main reason to explain the large band between 3500 and 2800 cm^{-1}. Among that, for the ChCl:CA case it also exits esters C=O bonds due to the citric acid (ν=1731,02 cm^{-1}). In the fibers spectrum it is possible to see all these principle bands (ν=3321,69 cm^{-1}; ν=2941,35 cm^{-1} and ν=1716,33 cm^{-1}) supporting thus the information obtained by TEM. The band that appear at ν=2350 cm^{-1} is typical of CO_2[58].

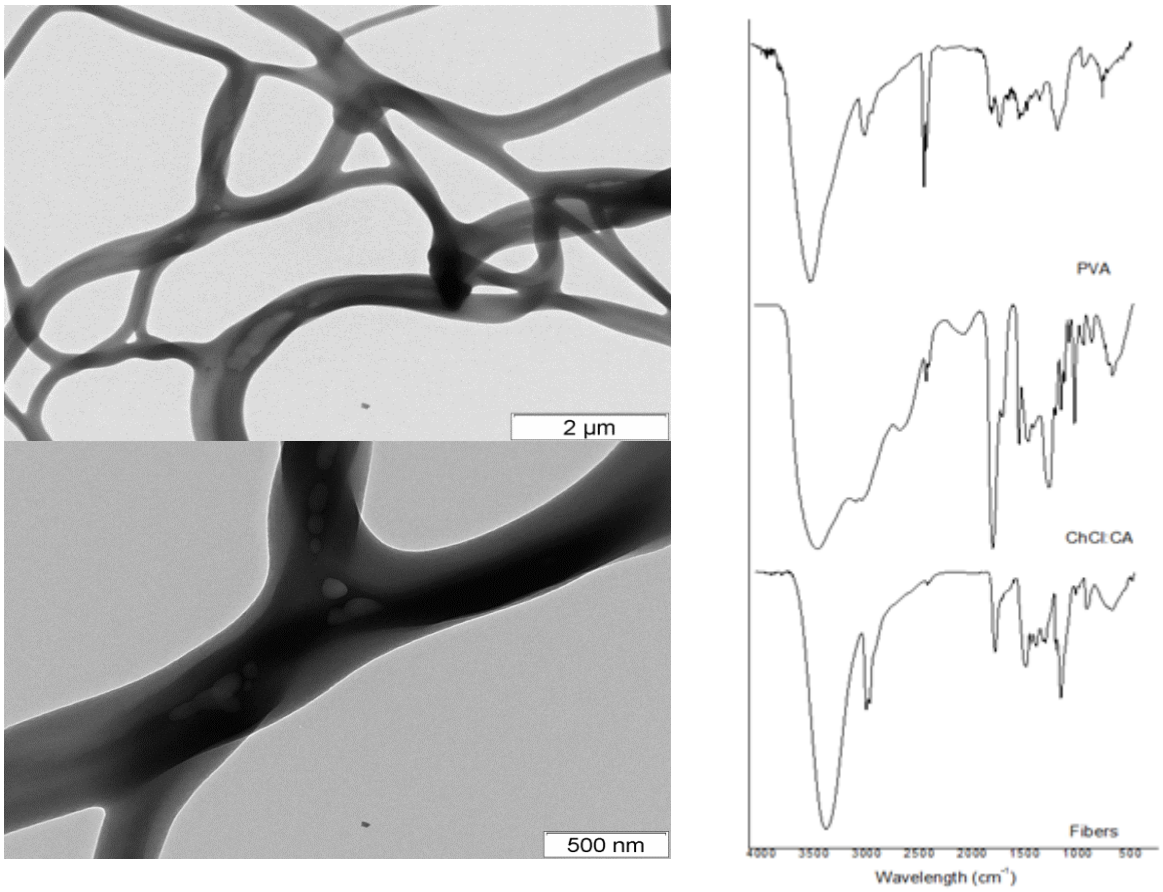

Figure 4.13. The left images shows the results obtained from the analysis of PVA 9,8% (w/v) with ChCl:CA 2%(v/v) fibers through TEM. The image on right shows the FTIR spectrum obtained from the analysis of the same fibers. (Electrospinning parameters: 16cm, 20kV, 0,2ml/h, 45-50%, 28°C).

4.3. GELATIN

Being the main point of this thesis the use of biopolymers for biomedical applications, it became necessary to, after understand the behavior polymer-NADES, starting to use active pharmaceutical ingredients (API's) as substitutes of NADES. It was used the same synthesis process of NADES to synthetize these new solvents, named THEDES, therapeutic deep-eutectic solvents.

For testing the possibility of using THEDES with biopolymers, gelatin was used as biopolymer. The change of polymer was due do to the fact that had been already tested the viability of gelatin fibers in similar solvents. Among that, gelatin also presents good properties that can be an advantage for this study.

Obtained by a controlled hydrolysis of collagen, gelatin is widely used in clinical processes due to its nonantigenicity, favorable absorbability and cost efficiency[59]. This polymer also accelerates the wound healing and tissue regeneration, being commonly used for making electrospun fibers[59]. Beyond that gelatin is also soluble in hot water[13,59].

4.3.1. FIBERS OPTIMIZATION

Table 3.6, on chapter 3, demonstrates the range of parameters tested for the optimization of the electrospinning process. Thus both gelatin solution, with ChCl:MandAc and Ibu:Men, had a distance between the collector and the needle of 18 cm and a flow rate of 0,2 ml/h. The charged

used was 20kV, and the ambient parameters include a range of temperature between the 30-32 °C and humidity between 35-40%. However, the temperature of gelatin inside the needle was higher than 40 °C, in order to avoid the jellification of the solution inside the syringe. The needle caliber was 23G (0,41nm) [13].

4.3.2. FIBERS CHARACTERIZATION

After parameter optimization, the characterization of the obtained fibers was performed, through various techniques. Thus it was analyzed the surface of the fiber, with the help of SEM. Figure 4.14 and Figure 4.15 demonstrate those images from fibers obtained for both solutions.

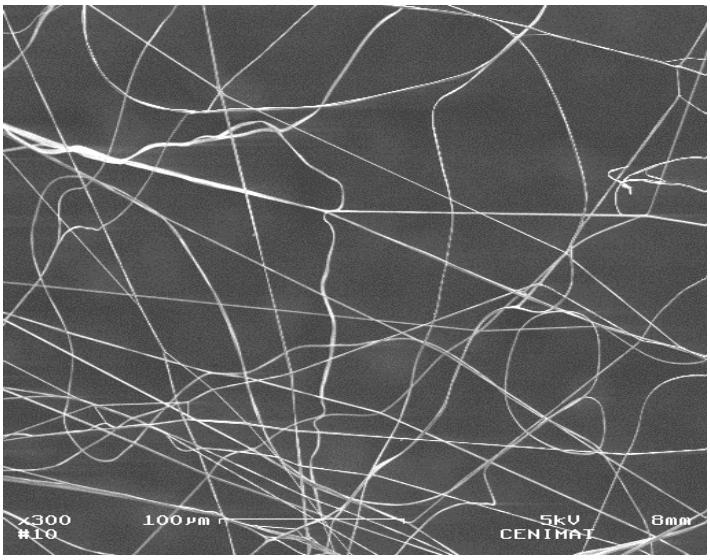

Figure 4.14. SEM image of the fibers obtained for 30% gelatin with ChCl:MandAc at 2%. Image amplification: 300x. (Electrospinning parameters: 18cm, 20kV, 0,2ml/h, 35-40% and 30-32°C).

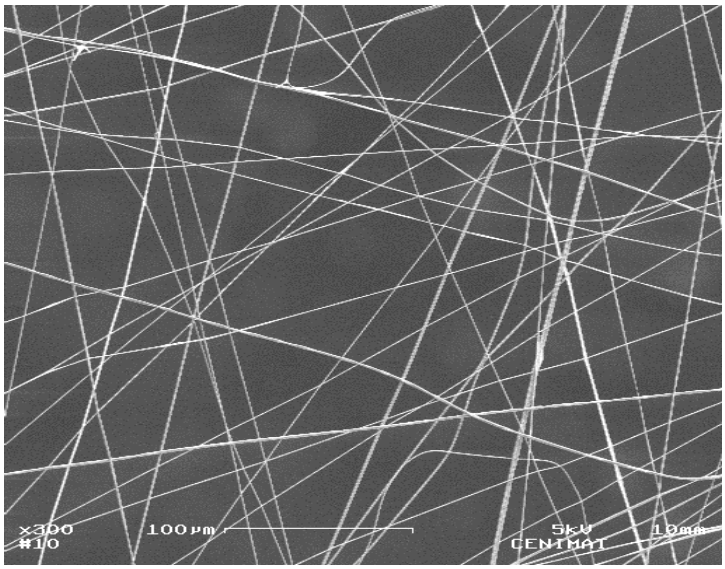

Figure 4.15. SEM image of fibers obtained for 30% gelatin with Ibu:Men at 1,5%. Image amplification: 300x. (Electrospinning parameters: 18cm, 20kV, 0,2ml/h, 35-40% and 30-32°C).

It is possible to notice, on Figure 4.15**,** that some fibers appear to have a helical shape. This characteristic can be proven on Figure 4.16 where it is used a higher magnification.

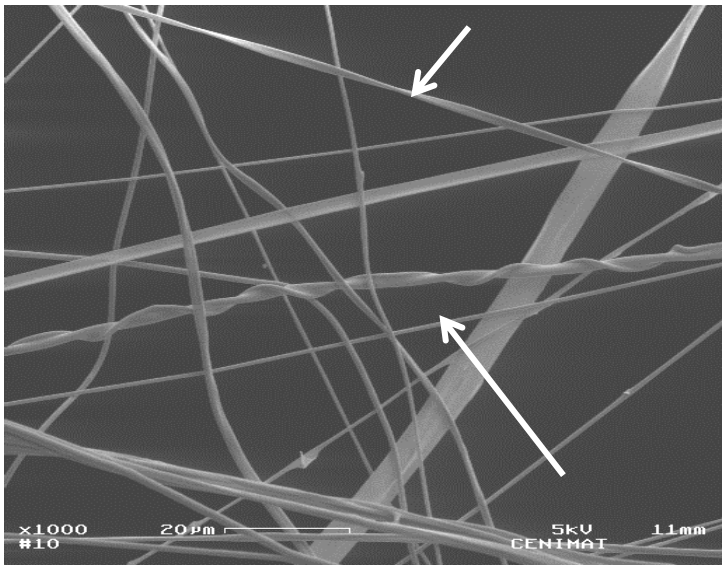

Figure 4.16. SEM image of fibers obtained for 30% gelatin with Ibu:Men at 1,5%, with a higher amplification (amplification: 1000x). The arrows indicate the helical form. (Electrospinning parameters: 18cm, 20kV, 0,2ml/h, 35-40% and 30-32°C).

The advantage of having this kind of helical fibers is the higher surface area. Fibers with high surface areas had gain a substantial attention due to their practical applications on various processes such as filtration, drug delivery, scaffolds for tissue engineering, among others[60]. But beyond the helical shape, it is also possible to see that fibers also can adapt others forms.

Koombhongse *et al*[61] shows that the variation of electrospinning parameters contributes for the variation of fibers shapes, and they can adapt shapes like the ones demonstrated on Figure 4.17.

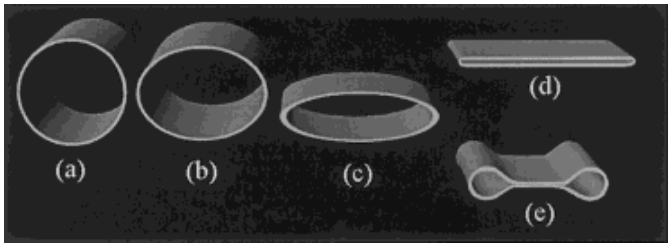

Figure 4.17. Possible shapes that fibers can have, according to Koombhongse *et al*[61]. The figure demonstrates the collapse of the fiber (4.8 a-c), with the formation of flat ribbons (4.8 d) and also the formation of ribbons with two tubes (4.8 e).

The electrospinning parameters that contribute for these shapes are electrical charge and the atmospheric pressure. The circular cross section present on figure (4.17-a) is the result of the solvent evaporation. However due to the atmospheric pressure, this section tend to collapse becoming elliptical.

Sometimes by skin connection it is possible to obtain a two tubes structure. Here with the electrical charge it is also possible to obtain a flat ribbon (figure 4.17-d). The charge tends to flow to the edges of the ribbon. This will cause a lateral force on the tubes, which will lead to the collapse and thus the formation of a flat ribbon.

According to this it is possible to see on Figure 4.18 those other shapes, that gelatin can adapt. In this case it is possible to see two kinds of shapes such as, flat ribbon shape (1) or even ribbon with two tubes (2)[61].

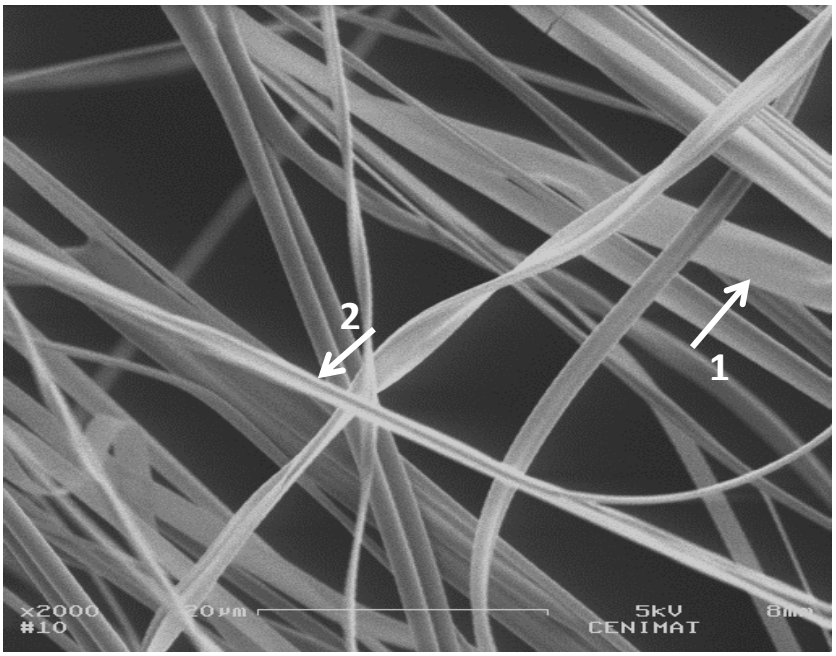

Figure 4.18. SEM image of fibers obtained for 30% gelatin with ChCl:MandAc at 2%, with a higher amplification (amplification: 1000x). It is possible to see the formation of flat ribbons (1) and also ribbons with two tubes (2). (Electrospinning parameters: 18cm, 20kV, 0,2ml/h, 35-40% and 30-32°C).

The internal composition of the gelatin fibers were characterized by FTIR and TEM. Figure 4.19 represent TEM images for both ChCl:MandAc and Ibu:Men fibers. Figure 4.20 and Figure 4.21 represent the FTIR spectrum obtained for each gelatin with THEDES fibers, as well.

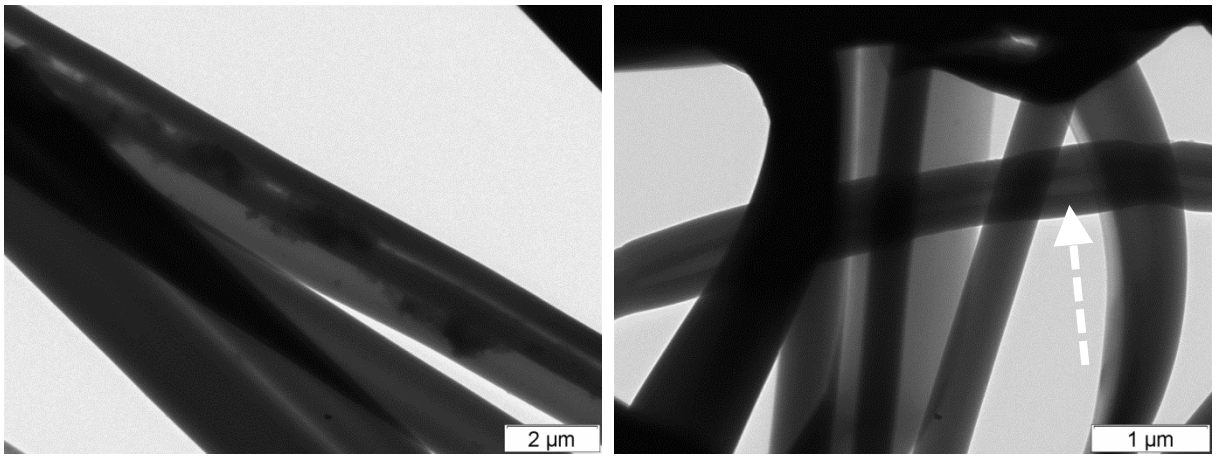

Figure 4.19. TEM images of gelatin fibers with THEDES, in optimized conditions. **Left** - gelatin 30% (w/v) with ChCl:MandAc 2% (w/v); **right** - gelatin 30% (w/v) with Ibu:Men 1,5% (w/v) (the arrow pointing the change of density). (Electrospinning parameters: 18cm, 20kV, 0,2ml/h, 35-40% and 30-32°C).

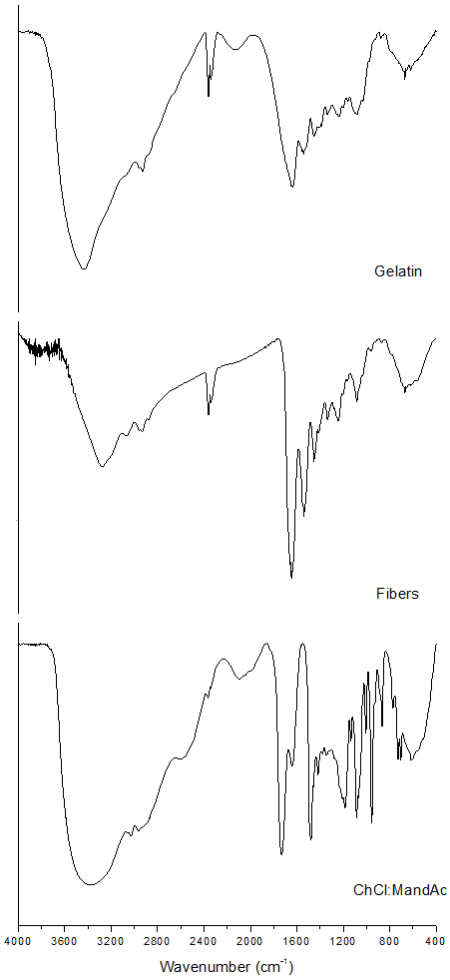

Figure 4.20. FTIR Spectrum of gelatin, ChCl:MandAc (THEDES) and the electrospun fibers obtained.

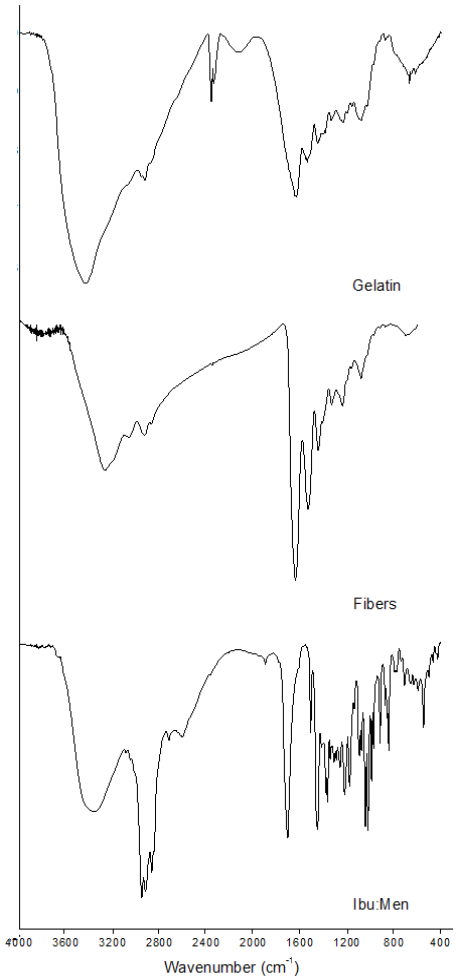

Figure 4.21. FTIR Spectrum of gelatin, Ibu:Men (THEDES) and the electrospun fibers obtained.

As already seen for PVA fibers it is possible to observe that the same situation occurred for gelatin fibers. Darker and lighter regions can be also observed, differencing regions where it is gelatin and where it is THEDES.

For the ChCl:MandAc fibers it is possible to see the existence of crystals like regions inside the fibres. This feature is due to solid state typical of mandelic acid, at room temperature and below. For Ibu:Men fibers it seems at first sight that there is no density difference between the fibers. However it is possible to notice a very light change.

Gelatin spectrum reveals the amide C=O ligation (v=1690-1630 cm^{-1}), the C-H bond (v=1450-1470 cm^{-1}) and the N-H bond (v=3325-3330 cm^{-1})[62]. From the FTIR spectrum it is not possible to conclude much information that can help to confirm the TEM information. However it is possible to see a shift on the position of N-H peak to lower frequencies, on the fibers spectrum. This could mean that the NH group is involved on hydrogen bonds[62], which can lead to the confirmation that THEDES can be present in the fiber.

As well as has appeared for PVA, gelatin also shows the typical CO_2 band. Both compounds were measured under the same conditions.

4.3.3. *IN VITRO* BIOLOGICAL STUDIES

The results present on Figure 4.22 shows the cellular viability obtained for the biological studies for each gelatin membrane tested, gelatin with the Ibu:Men and gelatin with the ChCl:MandAc mixture.

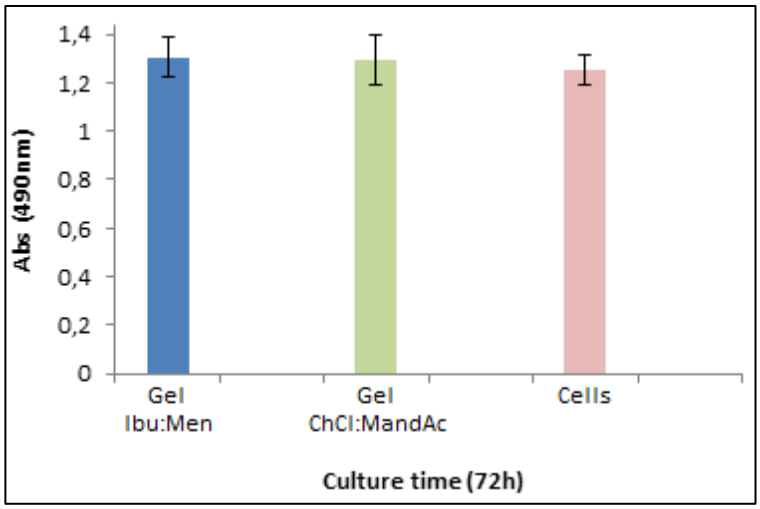

Figure 4.22. MTS assay for both gelatin membranes with THEDES tested (Ibu:Men and ChCl:MandAc)

These results demonstrate that the gelatin membranes do not compromise the cellular viability. In other words this material is not cytotoxic for the cells, since they grow in media replaced by these membranes made from gelatin fiber.

4.3.4. DRUG RELEASE PROFILE

For this study it was used a PBS buffer since its osmolarity and ion concentrations match with those found on human body. However it was not possible to reach any conclusion regarding the interaction of the THEDES with the polymer since it was completely dissolved in the buffer. Thus it was not possible to control the release of THEDES to the solution. Nevertheless it was possible to know that for the ChCl:MandAc membrane the release was 3,4±0,4%.

This study enable to prove the existence of ChCl:MandAc, inside the fibers, supporting the information obtained for TEM.

Although it was not possible to trace a profile of controlled release for the membranes used, it can be seen that gelatin fibers can have promising results in biomedical applications. The non-toxicity of these membranes associated to possibility of having API's, inside the fibers, allows there use for wound dressing applications.

Chapter 5

CONCLUSION AND FUTURE WORK

CONCLUSION

The development of functionalized fibers for biomedical applications has been a common topic in research. However the use of deep-eutectic solvents is completely novel.

The pharmacy industry uses for the processing of their products, drugs on their crystalline form. This implies the use of large amounts of the ingredient, for the manufacturing of the pills, in order to achieve the real drug effect on human organism. Consequently this increases the costs of production. The advantage of using these new solvents is the liquid state that they present. This feature gives them a non-crystalline structure, which increases their solubility in water, thereby increasing the uptake. This can help reducing the costs associated to the pharmaceutic industry, since it will be needed less quantity of the drug for the same application.

The aim of this work was the development of biocompatible fibers with active pharmaceutical ingredients, within them by electrospinning. Different biopolymers and DES were studied and the fiber produced were characterized by optical and electron microscope. The morphology of the fibers was accomplished with SEM, and the internal composition by TEM and FTIR.

The optimization process showed the influence of the various electrospinning parameters. It was possible to conclude that the polymer concentration had the highest influence in the production of viable fibers. The adjustment of this factor influenced the presence or the absence of beads, along the fibers. Among that, it was possible to determine that the distance and the voltage used were linearly dependent. Also the humidity influenced, in some cases, the jet formation. For the PEO case it was observed that for lower percentage of humidity occurred the formation of a drop, on the needle tip. The temperature proved to be also an important factor, for the gelatin case, since it was necessary to use higher temperatures in order to maintain the gelatin solution in its liquid state, avoiding the jellification of the polymer inside the syringe.

The composition of the inner fiber was determined through the use of TEM and FTIR, where it was possible to observe the encapsulation of NADES and THEDES. For the PEO polymer the encapsulation occurred due to the fiber fusion, making NADES encapsulated in cocoons. However for the PVA and gelatin, NADES and THEDES were encapsulated within the fiber.

The PVA polymer was used as concepts prove, showing that it was possible to obtain fibers with these new solvents. However for the use of API's it was used gelatin, due to good results that were obtained in the past. Gelatin showed good cytotoxicity results, since the cells maintained their viability. For the controlled drug release profile experiments significant results could not be obtained, due to the rapid dissolution of the gelatin membrane, when in contact with the PBS buffer. However it confirmed the results obtained with TEM. Although the failure as drug delivery polymer, it was possible to see that in fact the membrane had in its interior the THEDES.

Starch blend polymers were also studied. However their application to the aim of this work was not possible, since they did not demonstrate any dissolution in NADES or organic solvents necessary to produce fibers by electrospinning. Nevertheless new data regarding the interaction of NADES and different starch blend polymers was obtained.

Generally it was possible to demonstrate that the conjugation of a biopolymer with THEDES, and the production of bioactive nanofibers by electrospinning, is a viable process for biomedical applications, namely for wound dressing or drug delivery applications.

FUTURE WORK

In addition to the results presented in this thesis, there are some others techniques or others methods that could be used. In order to have a full characterization of the fibers it would be necessary to use the Differential scanning calorimetry with thermal gravimetric analysis (DSC-TGA) technique. Commonly used for determine the loss or gain of mass, due to decomposition, oxidation or loss of volatiles. In this way it will be possible to assure and quantify the presence of NADES/THEDES in the fibers.

Also it would be necessary to characterize the fibers through mechanical tests, in order to test their behavior under stress conditions (elongation and compressive strengths).

Lastly there is the possibility of changing the gelatin used. The rapid dissolution of gelatin was due to the temperature used, during the process. To counter this problem, the use of a gelatin with a higher melting point would be required to maintaining the membrane intact, when in contact with PBS buffer.

Nevertheless it is necessary to take into account the NADES. Although their simple synthesis, this process needs to be optimized, or the approach needs to be change as water removal still poses some issues. One possible method passes through the use of $scCO_2$ (supercritical carbon dioxide), for the extraction of water from the solutions. Other possible method is the optimization of nitrogen drying process, for studying the influence, and efficiency, of all these process on the water removing process.

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APPENDIX

APPENDIX I

IBUPROFEN PREPARATION

a) Dissolve 1g of ibuprofen sodium salt in 20ml of distilled water.

b) Add chloride acid to the aqueous solution until achieve a pH between 1 and 2. (This procedure can be controlled with the help of pH test strips).

c) Extract the aqueous solution by adding 10ml of dichloromethane. This step must be repeated three times.

d) Join all organic fractions, dry them with sodium sulfate anhydride, followed by filtration.

e) Evaporate the solvent from the aqueous solutions.

f) Analyze the final result with the help of FTIR.

APPENDIX II

SPCL ACETYLATION

a) Weight the empty vial.

b) Dissolve SPCL into the solvent.

c) Add the necessary quantity of anhydride compound.

d) Leave the solution to stir.

e) Evaporate the solvent from the medium reaction.

f) Weight the final result (here you should have a weight increase of SPCL).

g) Analyze the final result with the help of FTIR.

APPENDIX III

FITR SPECTRA OF THEDES AND ITS INDIVIDUAL COMPOUNDS

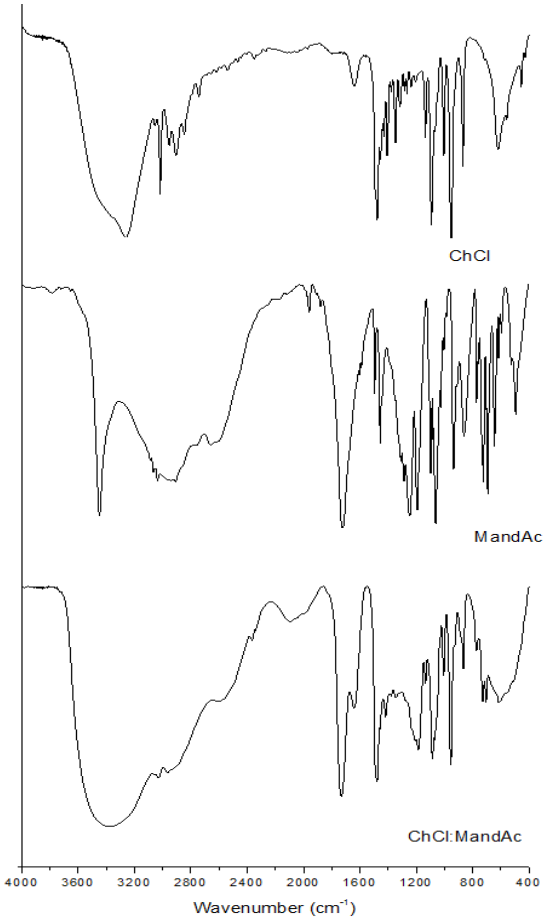

Figure A.1. FTIR Spectrum of choline chloride (ChCl), mandelic acid (MandAc) and the mixture of both, ChCl:MandAc (THEDES).

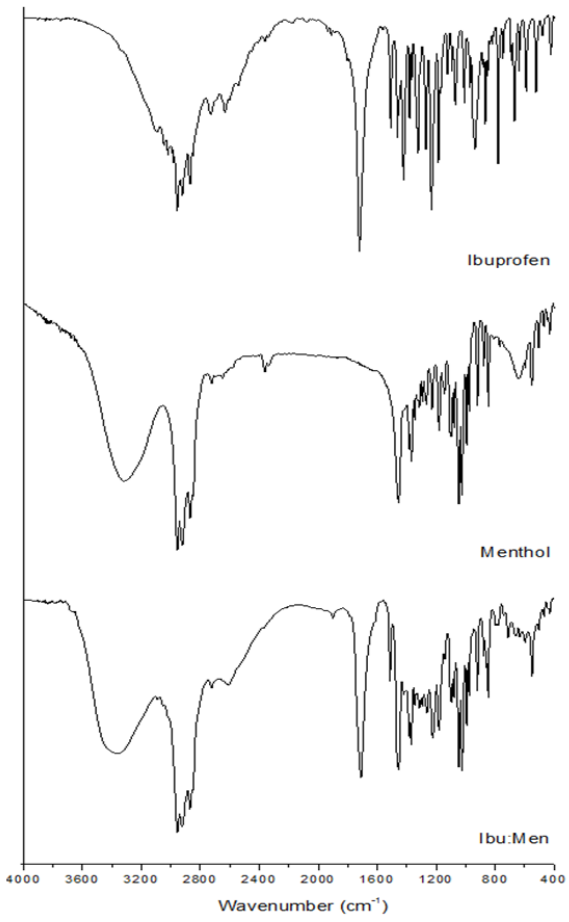

Figure A.2. FTIR Spectrum of ibuprofen (Ibu), menthol (Men) and the mixture of both, Ibu:Men (THEDES).