Universidade de Lisboa

Faculdade de Farmácia

Development and characterization of Polyacrylic acid nanofibers

Madalena Freixial Ramalho

Trabalho de Campo orientado pela Professora Doutora Špela Zupančič, Professora Associada da Universidade de Liubliana e coorientada pela Professora Doutora Maria Henriques Lourenço Ribeiro, Professora Associada da Faculdade de Farmácia da Universidade de Lisboa.

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Trabalho Final do Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

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Declaro ter desenvolvido e elaborado o presente trabalho em consonância com o Código de Conduta e de Boas Práticas da Universidade de Lisboa. Mais concretamente, afirmo não ter incorrido em qualquer das variedades de fraude académica, que aqui declaro conhecer, e que atendi à exigida referenciação de frases, extratos, imagens e outras formas de trabalho intelectual, assumindo na íntegra as responsabilidades da autoria.

Resumo

A produção de nanomateriais, e mais especificamente de nanofibras, tem ganho um destaque significativo na indústria farmacêutica. A sua versatilidade e facilidade de produção são algumas das vantagens que tornam esse tipo de produto farmacêutico tão interessante.

O ácido poli(acrílico) (PAA) pode ter diferentes aplicações e, neste estudo, desenvolvemos e caracterizamos nanofibras de PAA produzidas por *electrospinning*. A formulação das nanofibras em questão sofreu modificações com a introdução de Tween 80 e metronidazol (MTZ), um antibiótico utilizado no tratamento de infecções vaginais.

As nanofibras foram caracterizadas por microscopia eletrónica de varrimento para análise da morfologia e do diâmetro, espectroscopia de infravermelho por transformada de fourier (FTIR) para avaliar a estrutura química, análises térmicas por calorimetria exploratória diferencial e termogravimetria, além de estudos de libertação do fármaco utilizando cromatografia líquida de ultra eficiência.

Embora a análise morfológica tenha mostrado nanofibras uniformes e estáveis, tanto nas nanofibras que tem MTZ como nas que não tem, quando o MTZ é adicionado às soluções, os diâmetros são mais heterogêneos e as nanofibras são mais grossas. Com o FITR tivemos a confirmação que o MTZ foi bem incorporado nas nanofibras. As análises térmicas mostraram a estabilidade das nanofibras até uma certa temperatura.

Quanto aos estudos de libertação, estes indicaram uma eficiência de encapsulação relativamente baixa, 34.23%, o que significa que 65.77% do MTZ é perdido durante o processo de *crosslinking*.

De modo geral, os resultados deste estudo mostram o potencial que as nanofibras de PAA têm para sistemas de libertação de medicamentos eficazes, nomeadamente para tratar infeções vaginais.

Palavras-chave: Nanofibras, Ácido poliacrílico, *Electrospinning,* Metronidazole

Abstract

The production of nanomaterials and more specific nanofibers has gained significant attention in the pharmaceutical industry. Their versatility and easy way of production are some of the advantages that make this type of pharmaceuticals so interesting.

Polyacrylic acid (PAA) can have different applications, in this study we developed and characterized nanofibers of polyacrylic acid produced by electrospinning. The formulation of the nanofibers in question suffered changes with the introduction of Tween 80 and metronidazole (MTZ), an antibiotic used in vaginal infections.

The nanofibers were characterized by scanning electron microscope for morphology and diameter, Fourier-transform infrared spectroscopy (FTIR) for chemical structure composition, thermal analysis with different scanning calorimetry and thermogravimetric, and the drug releases studies using ultra performance liquid chromatography.

Although the morphological analysis revealed uniform and stable nanofibers on both nanofibers with MTZ and without MTZ, when MTZ is added to the solutions the diameters are more heterogeneous and the nanofibers are thicker. Using FTIR, we had the confirmation that MTZ was well incorporated in the nanofibers. Thermal analysis showed the stability of the nanofibers up to a certain temperature. The DSC results showed that the nanofibers were stable up to a certain temperature and the TGA revealed gradual mass loss, attributed to the evaporation of moisture. Both results indicated suitability for pharmaceutical applications.

The release studies indicated a relatively small encapsulation efficiency, 34.23% which means that 65.77% of MTZ was lost during crosslinking.

Overall, the findings of this study show the potential of PAA nanofibers for effective drug delivery systems, particularly for treating vaginal infections.

Key words: Nanofibers, Polyacrylic acid, Electrospinning, Metronidazole

List of Abbreviations

DDS	Drug delivery systems
DSC	Different scanning calorimetry
EE	Encapsulation Efficiency
FTIR	Fourier- transform infrared spectroscopy
MTZ	Metronidazole
PAA	Polyacrylic acid
SEM	Scanning electron Microscopy
TGA	Thermogravimetric analysis
UPLC	Ultra performance liquid chromatography
UV-vis	Ultraviolet- visible spectroscopy
XRPD	X-Ray powder diffraction

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1. Introduction

1.1. Nanofibers and their biomedical applications

Nanotechnology has been growing and developing over the last 20 years and deals with the production and characterization of various nanomaterials. Their use is already well-established in various industrial areas and is expected to achieve even more profound impact in the future (1). Nanofibers are one of the many forms of nanomaterials and their structure is characterized by two external dimensions in the nanoscale and a third dimension that is larger than one micron (1). They can be produced through different methods such as electrospinning, drawing, forcespinning, interfacial polymerization, melt blowing, phase separation, self assembly, template phase extrusion and template synthesis (2). Over the last twenty years, nanofibers have been used for numerous biomedical applications (3). Tissue engineering is one of the most common applications, where nanofibers scaffolds can be used for regeneration and engineering of different types of tissues and organs like skin, cartilage, bone, collagen, dentin and liver. 2D and 3D scaffolds have been designed and created by adjusting the nanofibers structure and properties such as the diameter, alignment, layering, porosity, surface functional groups, mechanical properties, patterning and biodegradability (3, 4). Due to the high porosity of nanofibers, they allow the oxygen, moisture and nutrient exchange without provoking dehydration while preventing the entrance of microorganisms due to the small pore size and thus are also used in wound healing (5).

Another major biomedical application of nanofibers is their use as drug delivery systems (DDS) and, within this context, nanofibers are usually produced by electrospinning (5). Nanofibers represent a promising DDS since they are easy to produce and we can tune their properties, such as wettability, elasticity and surface to volume ratio (6). Many different drugs can be incorporated into nanofibers: antibiotics, proteins, anticancer and anti-inflammatory agents and DNA. Due to their high surface area which enables efficient drug release, as well as, the ability to load different hydrophilic and hydrophobic drugs and the potential to use various excipients, nanofibers enable us to modulate drug release kinetics, only by adjusting the composition of the nanofibers, the concentration of the components and the preparation process (6,7).

Nanomaterials have also shown chemical and physical properties suitable to develop biomedical materials and biosensors. These types of materials need to have specific characteristics, such as biocompatibility to prevent harmful events, bioactivity

to develop an effect in the cells, tissues or organism and are easy to process. Electrospun nanofibers exhibit characteristics like high porosity, superior specific surface area, easy modification and cost-effectiveness that are suitable to develop biomedical devices. In terms of biosensors, nanofibers offer some advantages over traditional biosensors such as improved sensitivity, embellish responsiveness, cost effectiveness and greater detection capabilities (8).

1.2. Electrospinning technique

Among all the different ways to produce nanofibers, electrospinning is the most commonly employed due to its simplicity (2). Electrospinning was first observed in 1902 by J. F Cooley and its popularity has been rising due to the many different applications uses of electrospun nanofibers (9).

The machine for electrospinning technique has three essential components (exemplified in Figure 1), a syringe with the polymer solution or melt, a metal nozzle and the needle, a high source of voltage and a collector (2).

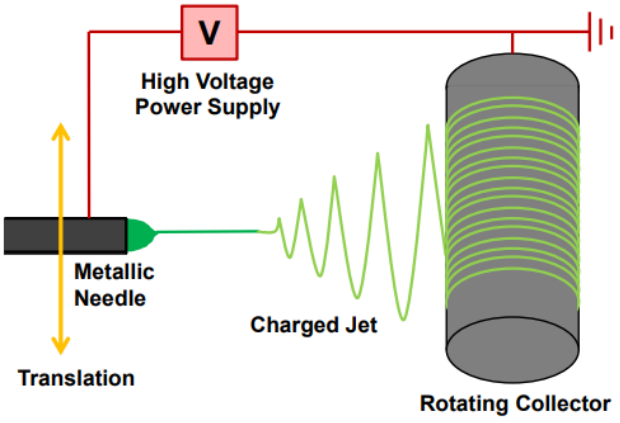


Figure 1 - Electrospinning process (Adapted from (10)).

The chosen polymer solution is pumped through the syringe, meanwhile a high voltage is used which induces a charge movement in the polymer creating a pendant drop. When the electrostatic repulsion of the polymer overcomes the surface tension, Taylor cone is formed (ideally has a conical shape) and a jet of polymer is created. To maintain the ideal Taylor cone and polymer jet, both the applied voltage and the flow rate need to be balanced and adjusted according to the polymer solution that is being used (11).

1.3. Effects of different parameters on electrospinning

The electrospinning process is modulated by different parameters. They can be separated into formulation parameters and process parameters. Process parameters are: voltage, flow rate and the distance between the needle and the collector. The formulation parameters are: polymer type, molecular weight, polymer concentration, viscosity, viscoelasticity, conductivity and surface tension of the polymer solution (7,12).

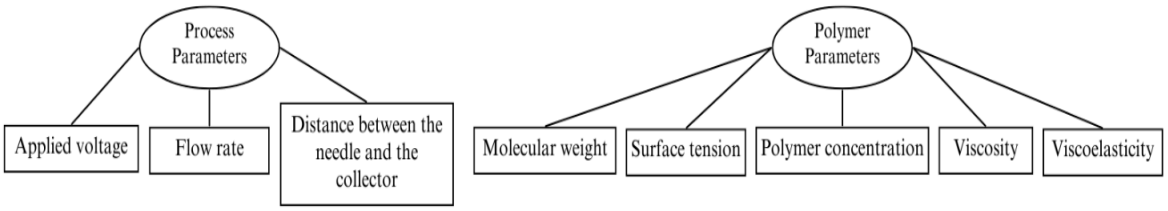


Figure 2: Parameters that affect the process of electrospinning.

1.3.1. Formulation Parameters

1.3.1.1. Effect of polymer molecular weight

The polymer molecular weight is one of the parameters that affect the process of electrospinning. When the molecular weight is low the electrospun nanofibers tend to have more beads, whereas, when the polymer has a higher molecular weight, the nanofibers are smoother and uniform (12).

1.3.1.2. Effect of polymer concentration

The polymer concentration influences the stretching of the charged jet, the viscosity and the surface tension. When the solution has a low polymer concentration, the jet is generally more unstable because the applied electric field causes the polymer chains to break resulting in beaded nanofibers. On the other hand, when the solution has a higher polymer concentration, the viscosity and the entanglement within the polymer chains increases, leading to the formation of larger and more uniform nanofibers. A maximum critical polymer concentration exists where the viscosity increases above the desired value and the nanofibers no longer have the desired morphology or the electrospinning process is not possible at all (13).

1.3.1.3. Effect of viscosity

The viscosity of the solution has a big impact on both the morphology and the structure of the nanofibers. When the solution used has low viscosity, there's difficulties producing continuous and smooth nanofibers resulting in a lot of beads. When the solution used is very viscous, the jet is produced very rapidly and there are difficulties to form the nanofibers (12). It's important to have an ideal viscosity that can be found by adjusting the polymer concentration (12, 13).

1.3.1.4. Effect of viscoelasticity

Viscoelasticity is also a characteristic of the polymer solution that influences the process of electrospinning. Polymers have both elastic (G´) and plastic (G´´) properties and the balance between these two is essential. To have a uniform jet without droplets, the elastic forces should be sufficient so that the jet does not break into droplets but not too high so that it can be formed at all. The plastic forces which increase much more rapidly with increasing polymer concentration should not be too high as they would prevent jet formation due to the solution's resistance to stretching, but they also should not be too low to not result in insufficient resistance of the solution to its flow which could result in spraying instead of spinning or formation of beaded nanofibers (2).

1.3.1.5. Effect of surface tension

The surface tension of the solution is very important because it significantly impacts the process by opposing the formation of the Taylor cone and therefore the jet. Usually, lower surface tension leads to electrospun nanofibers without beads and the possibility of using lower voltage. This parameter can be adjusted with the addition of surfactants to the solution (2).

1.3.2. Process parameters

1.3.2.1. Effect of applied voltage

The applied voltage during electrospinning has a crucial effect on the formation of the nanofibers. Usually, solutions with high surface tension, low conductivity or high viscosity tend to need a higher voltage, while solutions with the opposite properties require lower voltages. As the voltage increases, the repulsive electrostatic forces also increase, leading to the stretching of the jet and therefore, thinner nanofibers. However,

when the applied voltage is too high, the chance of spraying and forming beads is higher because the Taylor cone and the jet become unstable (2).

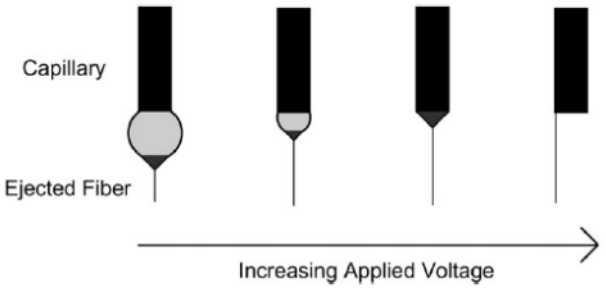


Figure 3 - Effect of varying the applied voltage on the formation of the Taylor cone (Adapted from (14)).

1.3.2.2. Effect of flow rate

The flow rate used during electrospinning influences the porosity, shape and the size of the electrospun nanofiber (14). This parameter is mainly influenced by the volatility of the solvent that is used. Solvents with high volatility need a higher flow rate (2). As the flow rate increases, the porosity of the nanofibers and the diameter also increases. Although, when the flow rate is too high, there's a more likely formation of beads, because the nanofibers don't have time to dry before reaching the collector. This also flatterns the nanofibers (14).

1.3.2.3. Effect of the distance between the needle and the collector

The distance between the needle and the collector also influences the electrospun nanofibers. It affects both the morphology and the diameter of the nanofibers. When the distance is too short, there's not enough time for the nanofibers to dry and solidify. On the other hand, when the distance is too big, it's more likely to have beads (2).

1.4. Polyacrylic acid (PAA): Properties and application

1.4.1. Chemical properties

Polyacrylic acid (PAA), formerly recognized as poly(1-carboxyethylene), is a high molecular weight synthetic polymer produced by polymerization of acrylic acid. It contains a carboxylic group in each monomer (9) and has the following chemical formula:

$\left[- CH_2 - CH(C0_2H) -\right]_n$ where n is the number of repeating monomer units (15).

The carboxylic groups are reactive and can undergo esterification, amidation and ionic crosslinking with cations. Another characteristic that carboxylic groups have is that they can form hydrogen bonds, making PAA highly hydrophilic and hygroscopic, being capable of absorbing and retaining water (15). Its solubility depends on the pH: in acidic conditions PAA is less soluble due to the largely protonated state of the the carboxylic acid functional groups, while, on the contrary, in alkaline conditions, PAA is more soluble because these groups exist in a largely deprotonated state. In addition to water, PAA is also soluble in alcohol, DMF, methanamide and formamide (15). In water, PAA forms a salt, polyacrylate, which has a net negative charge affecting the reaction with certain cations (15).

1.4.2. Physical properties

PAA usually appears as a white powder, but it can be transformed into translucent films (15). The molecular weight can differ a lot depending on the number of repeating units, which affects solubility, viscosity and mechanical properties. The glass transition temperature is one of the characteristics that varies with the molecular weight, and is usually above 100ºC (15).

1.4.3. Application

PAA has been used in numerous different ways and because of its biocompatibility and biodegradability it is widely used in drug deliveries. It can also be used as a food additive due to its low toxicity (9).

The ionizable properties of PAA, originating from the presence of carboxylic functional groups, enable the formation of a hydrogel. Such hydrogels can be loaded with different compounds like low molecular weight drugs, proteins, or peptides and can be

produced as injectable gels, films or tablets. PAA can also be used as a mucoadhesive, making it a good choice for delivering drugs to surfaces like eyes, nose and skin as well as it can extend the duration of these drugs in those areas making PAA more bioavailable. Furthermore, PAA has stimuli-responsive behavior which can be used when the release of the drug depends on a certain parameter that works like a stimuli like change in pH, temperature or ionic forces (16). If cations are used to facilitate the hydrogel formation, such hydrogel possess improved mechanical properties and can remain stable from degradation for longer periods of time. Positively charged ions such as Ca2+ or Fe3+ can be used for this purpose (17, 18).

1.5. Metronidazole (MTZ): Properties and application

1.5.1. Chemical properties

Metronidazole (MTZ) is formerly recognize as 2-(2-methyl-5-nitro-1H-imidazol-1-yl) ethanol, and has the following chemical formula: $C_6H_9N_3O_3$ (19).

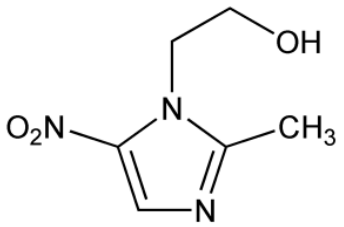


Figure 4 - Structure of metronidazole (Adapted from (20)).

Its molecular weight is 171.15 g/mol and it has a nitroimidazole ring that is responsible for its antimicrobial activity. It's slightly soluble in water (at 20 ºC), 1 g/100 ml, in ethanol, 0.5 g/100 ml and in chloroform, less than 0.005 g/100 ml (19).

1.5.2. Physical properties

It is in the form of a white crystalline powder, its melting point is in the range of 158-160 ºC and its pKa around 2.2 (due to its nitroimidazole ring). It has low lipophilicity (logP is -0.02) and it's stable at room temperature (20).

1.5.3. Application

MTZ is mostly used for the treatment of anaerobic bacterial infections, protozoal infection and microaerophilic bacterial infections. It has the ability to diffuse into the organism and cause the cell death of these susceptible organisms. The nitro group is reduced by nitroreductase enzymes, which produce nitro radicals that are highly reactive. These radicals inhibit the protein synthesis by interacting with the DNA which causes the loss of the helical shape of the DNA and strand breakage (21).

MTZ is indicated against protozoal infections, such as Tr*ichomoniasis vaginalis*, *Entamoeba histolytica*, *Giardia lamblia*, blastocysts, and *Balantidium coli* and bacterial infections caused by Bacteroides species, Fusobacterium species, Clostridium species, *Gardnerella vaginalis*, *Helicobacter pylori*, Prevotella species, Porphyromonas species, and *Biophilia Wadsworth* (21,22).

Therefore, MTZ is indicated in the treatment of intestinal and liver amebiasis, intra-abdominal infections, bacterial septicemia, lower respiratory tract infections, central nervous system infections like meningitis and brain abscess, bone and joint infections, endocarditis, surgical prophylaxis and skin structure infections. Also against gynecologic infections like endometritis, tubo-ovarian abscess and bacterial vaginosis, which was the aim of the nanofibers we were producing (21,22).

Due to its anti-inflammatory properties, metronidazole can also be used topically for rosacea and off-label for Crohn's disease (21).

2. Aims of this study

In this study, nanofibers of PAA were developed by electrospinning. During our study, different parameters, more importantly, the formulation was changed (Tween 80 and MTZ were added) in order to develop more stable and uniform nanofibers.

For the characterization, different methods were used, such as scanning electron microscope to evaluate the morphology and to measure the diameter of the nanofibers, Fourier transform infrared spectroscopy was used to evaluate the crosslinking, thermal analyst like differential scanning calorimetry and thermogravimetric analysis to characterize the way the nanofibers behave under a large range of temperatures. For the quantification of MTZ we first did release studies and later used ultra performance liquid chromatography.

The main goal of this study was to produce nanofibers that could be used vaginally to treat bacterial vaginosis.

3. Materials and Methods

3.1. Materials

Polyacrylic acid, Tween 80, iron chloride and metronidazole were all obtained from Merk, Darmstadt, German.

The ultrapure water was obtained from a millipore A10 advantage system (Merk)

3.2. Methods

3.2.1. Preparation of polymer solutions

3.2.1.1. Polyacrylic acid

The polymer solutions were prepared by dissolving the appropriate mass of PAA in ultrapure water and stirred at room temperature overnight on a magnetic stirrer. The PAA solutions were prepared at the following concentrations: 5% (w/v), 6% (w/v), 7% (w/v), 7.5% (w/v), 8% (w/v) and 9% (w/v).

3.2.1.2. Polyacrylic acid with Tween 80

In order to improve the electrospinning process, Tween 80 was added to the PAA solutions of 7% (w/v) and 7.5% (w/v) in concentrations of 0.05% (w/v), 0.1% (w/v) and 0.3% (w/v). The solutions were prepared by dissolving the mass of PAA in purified water and then the Tween 80 was added in the concentrations above. All solutions were under magnetic stirring overnight.

3.2.1.3. Polyacrylic acid with Tween 80 and Metronidazole (MTZ)

MTZ was added to the 7.5% (w/v) PAA and 0.01% (w/v) Tween 80 in pre-mixed weigh ratio of PAA:MTZ (95:5), PAA:MTZ (92.5:7.5) and PAA:MTZ (90:10). MTZ and PAA were weighed and added to the purified water. Then the Tween 80% was added and the solutions stayed under magnetic stirring overnight.

3.2.2. Electrospinning process

The machine used for the electrospinning was the model Fluidnatek LE by Bioinicia (Spain).

The syringe used contained 10 mL of solution and a needle was attached. Before setting up the process, all bubbles of air inside the syringe were taken off to prevent

problems during electrospinning. The distance between the tip of the needle and the collector was set to 13 cm.

The voltage and the flow rate were different depending on the solutions used. The voltage range was between 7.4 kV - 8.9 kV and the flow rate between 350 mL/min - 550mL/min. In table 1 we have the voltage and flow rate applied in the solutions that were the most stable and followed with the experiments.

Table 1- Applied voltage and flow rate

Concentration (w/v)	Flow rate (mL/min)	Voltage (kV)
PAA 7.5%	500	8.3
PAA 7.5% +Tween 0,01%	480	8
Tween 0,01% + PAA:MTZ (95:5)	520	8.4

3.2.3. Crosslinking of Polyacrylic acid with Tween 80 and Metronidazole (MTZ) nanofibers

Following the production of PAA nanofibers by electrospinning, the samples were crosslinked. For this process, pieces of nanofiber mats (previously weighed) were submerged in iron (III) chloride solution with the concentration of either 15 mmol/L or 150 mmol/L for different times. After taking the samples out of the crosslinking solution, the mats were submerged in ultrapure water in order to remove the excess $FeCl_3$. After that, some of the samples were either dried at room temperature, or at 50ºC, or transferred directly into PBS as the first step in the quantification of MTZ loading, or the release studies.

3.2.4. Characterization

3.2.4.1. Scanning electron Microscopy (SEM)

Nanofibers were visualized under scanning electron microscope (Supra 35 VP; Carl Zeiss, Oberkochen, Jena, Germany). Pieces of nanofiber mats were fixed onto the metal pins using double-sided conductive tape. The imaging process was conducted at a 1 kV accelerating voltage with a secondary electron detector. Nanofiber diameters were

measured using ImageJ 1.44p software (National Institutes of Health, Bethesda, MD, USA) on 50 randomly selected nanofibers in a single view field of an image.

3.2.4.2. Fourier-transform infrared spectroscopy (FTIR)

Fourier Transform Infrared Spectroscopy (FTIR) was used to evaluate the formation of bonds during crosslinking. The spectra were obtained in the spectral range of 4000 cm^{-1} to 600 cm^{-1} with 64 scans recorded at a resolution of 2 cm^{-1}.

3.2.4.3. Thermal analysis

3.2.4.3.1. Differential scanning calorimetry (DSC)

Differential scanning calorimetry (DSC, Mettler Toledo, Greifensee, Switzerland) was used to evaluate the solid-state characteristics of the nanofiber mats. The samples (around 5 mg) were weighed into aluminum pans with a pinhole heated at the rate of 10 K/min within a temperature range of 30 to 250 °C under a nitrogen flow of 50 mL/min.

3.2.4.3.2. Thermogravimetric analysis (TGA)

Thermogravimetric analysis (TGA, Mettler Toledo, Greifensee, Switzerland) was used to evaluate thermal stability and how the mass of the nanofibers mats behave under different temperatures. The samples (around 5 mg) were weighed into aluminum pans with a pinhole heated at the rate of 10 K/min within a temperature range of 30 to 250 °C under a nitrogen flow of 50 mL/min.

3.2.4.4. Quantification of MTZ

3.2.4.4.1. Uncrosslinking nanofibers

For the uncrosslinking of metronidazole we had different approaches, first tried putting the weighted pieces of nanofibers that were crosslinked in iron chloride into chloridric acid to directly determine the amount of MTZ that was loaded

Secondly, we tried to indirectly measure the amount of MTZ by the difference of weight. We crossedlinked the nanofibers in iron chloride 15 mmol/L or 150 mmol/L for different times, after that they were dried, either at room temperature or 50ºC and then they were washed with water, dried again and weighted to determine the % mass loss.

3.2.4.4.2. Release Studies

For the release studies we prepared the samples as following: weighted the mat pieces of the nanofibers, crosslinked them in iron chloride for 10 seconds, washed them

in water for a couple of seconds and the transferred them into a vial (A) containing 10 mL of PBS into the incubator to shake a 150 rpm at 37ºC. At predetermined points 5 minutes, 15 minutes, 30 minutes, 1 hour and 2 hours, we took the vials from the incubator pipetted 500 µL into smaller vials (B), then added 500 µL of fresh PBS into the previous vial (A) making sure the volume was always the same and returned to the incubator. To the second vial (B) we did a 4x dilution (we added 1500 µL of PBS) in half the samples and on the other half a 5x dilution (we added 2000 µL of PBS).

After that we filtered all the dilution solutions (first the one that were 4x dilutes), starting from the one that was the fewest time in the buffer to the one that stayed there the 2 hours into UPLC vials. In each filtration we discarded the first 0.5 mL and filtered the rest. In between filtration we always cleaned the filter with PBS and filled the syringe with air and gently pushed through to make sure that the filter didn´t have any remaining solution. Then we did the same thing for the 5x diluted.

3.2.4.5. Ultra performance liquid chromatography (UPLC)

Ultra performance liquid chromatography (UPLC, system Acquity Waters, USA) the column (Waters Acquity CSHTM C18 1,7 µm, 2,1 x 50 mm, Irska).

For the calibration curve we prepared a stock solution of MTZ with a concentration of 1000 µg/mL by weighing 100 mg of MTZ into a 100 mL flask and diluting it to the mark with phosphate buffer at pH 7.4. To ensure the active substance was fully dissolved, we placed the solution in an ultrasonic bath for 1 minute. By appropriately diluting the stock solution of MTZ, we prepared solutions with concentrations of 0.05 µg/mL, 0.1 µg/mL, 0.2 µg/mL, 0.5 µg/mL, 1 µg/mL, 2 µg/mL, 5 µg/mL, 10 µg/mL, 15 µg/mL, 20 µg/mL, and 30 µg/mL, which were used as standards to create a calibration curve. For method validation, we prepared three additional MTZ stock solutions using the procedure described above, and from each, we prepared standards with concentrations of 0.5 µg/mL, 10 µg/mL, and 20 µg/mL.

For the quantitative determination of MTZ, we used UPLC. For the gradient elution method, we used mobile phases A1 (10% (V/V) MeOH, 0.1% (m/V) H3PO4) and B1 (98% (V/V) ACN). The gradient of the mobile phase was as follows: 0-3 min 0% B, 7 min 20.0% B, 7.40-8.20 min 50.0%, and 9.80-11 min 0% B. The column used was thermostated at 50°C. Each sample was injected once and the analysis time for each sample was 11 minutes. Each injection had a volume of 5 µL, with a flow rate of 0.250 mL/min. The detection of metronidazole was performed at a wavelength of 320 nm. The

area under the chromatographic peak is proportional to the concentration, which was considered in the calculation of the active substance concentration in the tested samples.

3.2.5. Statistical analysis

To compare the sample without MTZ and the ones that had MTZ in their composition we did a T-test with the results of the diameter of the nanofibers. This statistical analysis helps us determine if the presence of metronidazole causes a statistical difference in the morphology of the nanofibers or not. The type of T-test was the independent samples T-test since the groups were independent from each other.

The formula used was as following: $t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{s^2(\frac{1}{n_1} + \frac{1}{n_2})}}$,

$\overline{x_1}$ and $\bar{x}_2$ are the means of the samples; s^2 is the pooled variance and n_1 and n_2 are the sample sizes of the groups.

The test was performed using Microsoft excel. If the result is $p<0.05$ then there is statistical difference between the groups.

4. Results and Discussion

4.1. Effect of Polyacrylic acid concentration on the nanofibers development

To assess the influence of PAA concentration on the nanofibers development, 6 different solutions were prepared: 5% (w/v), 6% (w/v), 7% (w/v), 7.5% (w/v), 8% (w/v) and 9% (w/v). The stability of the process was evaluated according to the following parameters: Taylor cone and jet formation, the existence of droplets and preliminary observations of nanofiber morphology under a light microscope.

In the 5% (w/v), 6% (w/v) and 7% (w/v) solutions, the Taylor cones were unstable, forming in a pulsatile manner, stretching a bit and then falling/cutting off and the jets were discontinuous leading to the formation of a lot of droplets. In the 7,5% (w/v) solution the Taylor cone formation was stable and the jet was mostly uniform resulting in fewer droplets. The 8% (w/v) and 9% (w/v) resulted in very viscous solutions and therefore, the formation of unstable Taylor cones and jets.

4.2. Effect of Tween 80 concentration on the nanofibers development

To have a more stable jet during the electrospinning, Tween 80 was added to the more stable solutions of PAA, (7% (w/v) and 7.5% (w/v)) because of it´s surfactant properties. The concentrations used were 0.05% (w/v), 0.1% (w/v) and 0.3% (w/v).

Addition of 0.3% (w/v) Tween 80 resulted in a very viscous solution and in an unstable electrospinning process. In the solution with 0.1% (w/v) Tween 80, the Taylor cone was the most stable and the jet was uniform. With the addition of Tween 80 at 0.05% (w/v) there was no improvement in either the Taylor cone formation or the jet formation compared with only PAA at 7.5% (w/v).

4.3. Effect of Metronidazole concentration on the nanofibers development

To evaluate if MTZ was a good fit to incorporate into the nanofibers, pre-mixed ratio of MTZ such as 5:95, 7.5:92.5 and 10:90 were added to the most stable nanofibers so far, 7.5% (w/v) PAA and 0.01% (w/v) Tween 80.

The solution of PAA:MTZ (95:5) worked really well, the jet was stable and the Taylor cone was in the ideal conical shape and overall the electrospinning was uniform

and with almost 0 droplets. On the other hand, the solutions PAA:MTZ (92.5:7.5) and the PAA:MTZ (90:10) weren´t stable enough to have a uniform jet and a good Taylor cone.

4.4. Crosslinking of Polyacrylic nanofibers with FeCl3

For the crosslinking of the nanofibers, the chosen composition of the nanofibers was 7.5% (w/v) PAA + 0.01% (w/v) Tween 80 + (95:5) MTZ since it was the most stable formulation. Both the concentrations of iron chloride used, 15 mmol/L and the 150 mmol/L worked very well.

We tried different times of crosslinking such as only 10 seconds, 1, 2, 5, 10, 30 minutes, 1 hour and 2 hours. We noticed that the crosslinking was almost immediate, so for the release studies and the quantification of MTZ we started using only the 10 seconds of crosslinking. The washing with the water was also at different times, only a couple of seconds, 10, 30 seconds and 1 minute. As the washing was also practically immediate, for the continuous studies we only washed it for a couple of seconds. For the drying, there was no difference between the samples that were dried at room temperature or in the dryer.

As the following table shows, we conducted various tests measuring the percentage of mass loss during crosslinking to evaluate its effectiveness and if it created stable structures. Like the results show, the percentage of mass loss is minimum which indicates that the process of crosslinking the nanofibers with iron chloride creates a strong structure and therefore the crosslinking was successful.

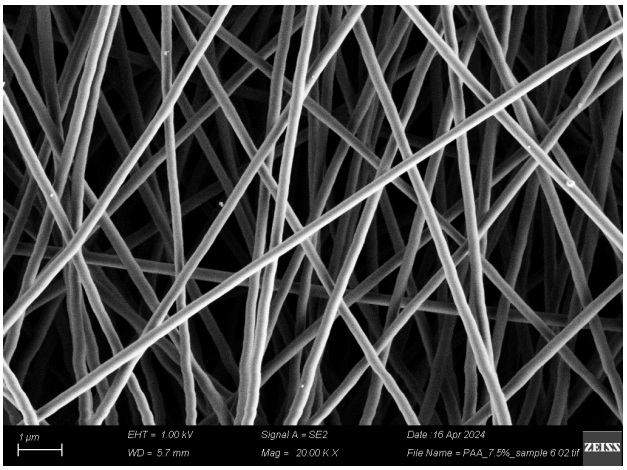
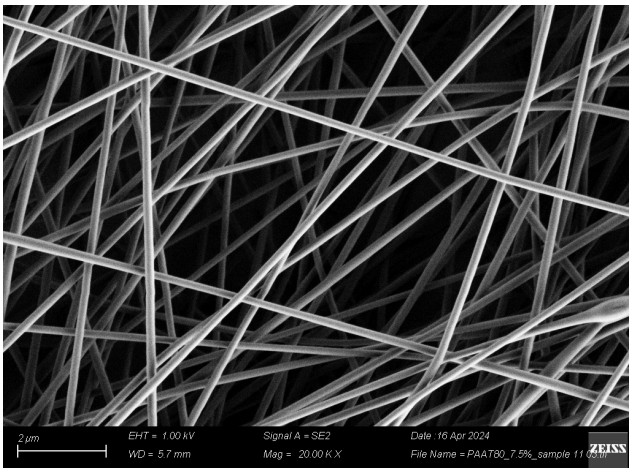
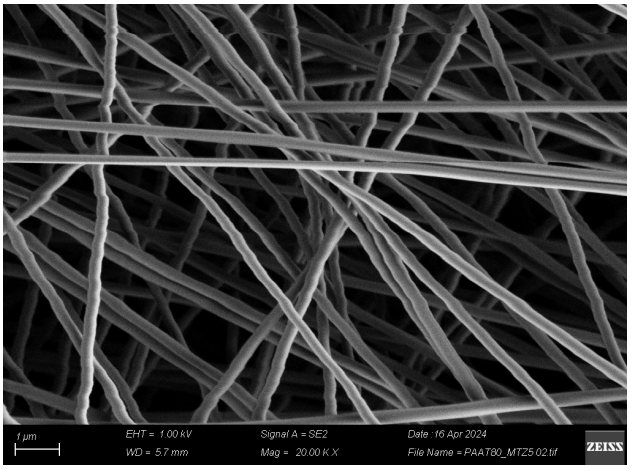
Table 2- Percentage of mass loss during crosslinking.

Mass of the sample (mg)	Time in $FeCl_3$	Mass of the sample after crosslinking (mg)	Mass after drying (mg)	% of mass loss
1.92	10 s	5.20	4.19	19.42
4.83	10 s	10.22	8.55	16.4
1.43	10 s	5.79	4.31	25.56
6.02	30 s	11.84	9.39	20.69
5.96	30 s	9.33	7.09	24.01
2.98	30 s	7.37	5.94	19.40
1.24	1 min	4.79	3.77	21.29
8.10	1 min	13.19	11.43	13.34
5.46	1 min	10.82	8.54	21.07
3.07	10 min	6.47	5.09	21.33
5.10	10 min	11.13	9.85	11.50
7.64	10 min	12.2	10,84	11.15
5.25	30 min	9.03	7.60	15.84
5.53	30 min	12.27	9.92	19.15
1.97	30 min	4.79	3.56	25.68
5.94	1 h	12.58	9.34	25.76
6.40	1 h	14.87	12.23	17.75
2.13	1 h	6.98	5.27	24.50

4.5. Scanning electron Microscopy (SEM) analysis

To access the morphology of PAA nanofibers, the samples were observed by SEM. In the following table are the SEM images of different nanofibers.

Table 3- SEM results of prepared nanofibers.

Composition Concentration (w/v)	SEM image
7.5% PAA	
7.5% PAA + 0.01% Tween 80	
0.01% Tween 80 + PAA:MTZ (5:95)	

As these images show, the nanofibers with only PAA and PAA with Tween 80 show well produced, uniform and smooth fibers with basically no defects. When MTZ is added there is a little change in the nanofibers morphology and uniformity.

To evaluate the effect of metronidazole in the nanofibers morphology, both the sample with only PAA 7.5% (w/v) with Tween 80 0.01% (w/v) and the sample with PAA 7.5% (w/v) with Tween 80 0.01% (w/v) plus (95:5) MTZ were analyzed. In the following table is the diameter of 50 randomly selected nanofibers in a single view field of an image.

Table 4 - Diameter of nanofibers

Composition of nanofibers Concentration (w/v)	Average diameter (nm)	Minimum diameter (nm)	Maximum diameter (nm)
7.5% PAA + 0.01% Tween80	205.37± 38	171.42	243.27
0.01% Tween80 + PAA: MTZ (95:5)	259.37± 46	214.19	306.17

As the results show, the PAA and Tween 80 nanofibers are more uniform and have a small range within their diameters. When MTZ is added to the solutions the diameters are more heterogeneous and the diameter increases, which indicates that the MTZ interacts with the polymer and makes thicker nanofibers.

As explained before, we did a statistical analysis using a T-test to compare the diameter of the nanofibers. The test was performed using Microsoft excel and the result was $p = 4.74 \times 10^{-21}$. As we can interpret, the addition of MTZ resulted in a larger diameter of the nanofibers and the difference in the mean diameters between the placebo and the MTZ-loaded nanofibers was statistically significant ($p<0.05$).

In a study that investigated the effect of varying PAA concentrations (5–10% w/v) on fiber morphology using electrospinning. Their results indicated that PAA concentration of 7.5% w/v produced uniform fibers with diameters ranging from 200–250 nm and when incorporated drugs like amoxicillin increased diameters to 300–400 nm due to drug-polymer interactions. (9) Comparing the results on this thesis 205 nm for placebo and 259 nm with MTZ, align with the trend of increased diameter and heterogeneity when drugs are incorporated.

4.6. Fourier-transform infrared spectroscopy (FTIR) analysis

As we can observe in figure 5, in the non-crosslinked sample, there's a sharp peak at 1700 cm^{-1}which corresponds to the $C = O$ stretching of the carboxylic group whereas in the crosslinked samples this peak reduces its intensity and we can observe additional peaks at 1545 cm^{-1} we can attribute this to the coordination of carboxylate groups of PAA with Fe^{3+} ions, a signal of the crosslinking.

PAA when crosslinked, the carboxyl groups deprotonate and form the carboxylate anion, which coordinates with the iron. There's two types of this stretching, asymmetrical and symmetrical. Typically, the asymmetrical one appear at 1500-1600 cm^{-1} and the symmetrical 1400-1450 cm^{-1} (23).

As we can see in figure 5 the new peaks prove the formation of the coordinate bond that enables crosslinking of PAA.

In terms of the peak around 2500-3500 cm^{-1} both spectra present it, typically this region shows the OH stretching. Although, in the crosslinker's PAA, the peak is smoother and broader. That can be attributed to the increase of hydrogen bonding during the crosslinking.

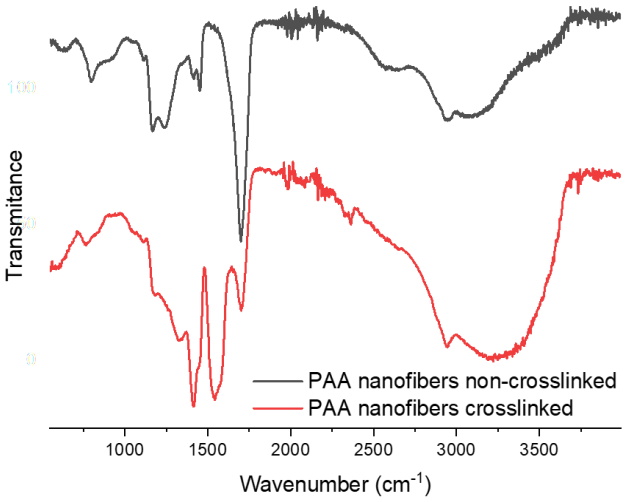


Figure 5 - FTIR spectra of PAA nanofibers non-crosslinked (black) and PAA nanofibers crosslinked (red).

4.7. Different scanning calorimetry (DSC) analysis

As we can interpret in figure 6, MTZ is crystalline in its powder as seen from the melting peak at approximately 160°C. Meanwhile the supplied PAA powder is not crystalline or semi-crystalline (absence of any melting endotherms). However, there is a thermal event occurring in the approximate range of 50-125°C, which we attribute to moisture removal.

MTZ is amorphized during electrospinning, evidenced by the disappearance of its melting endotherm peak. It must, however, be noted that this could also be a result of its relatively low loading (5 or 10 % w/w), which could result in the actual mass of crystalline MTZ in the electrospun samples to be below the detection limit of DSC. An additional analysis that could elucidate this further would be X-Ray powder diffraction (XRPD).

There was a study that evaluated DSC of PAA nanofibers crosslinked with Fe^{3+} ions that revealed no sharp melting peaks for encapsulated drugs, confirming amorphization (8).

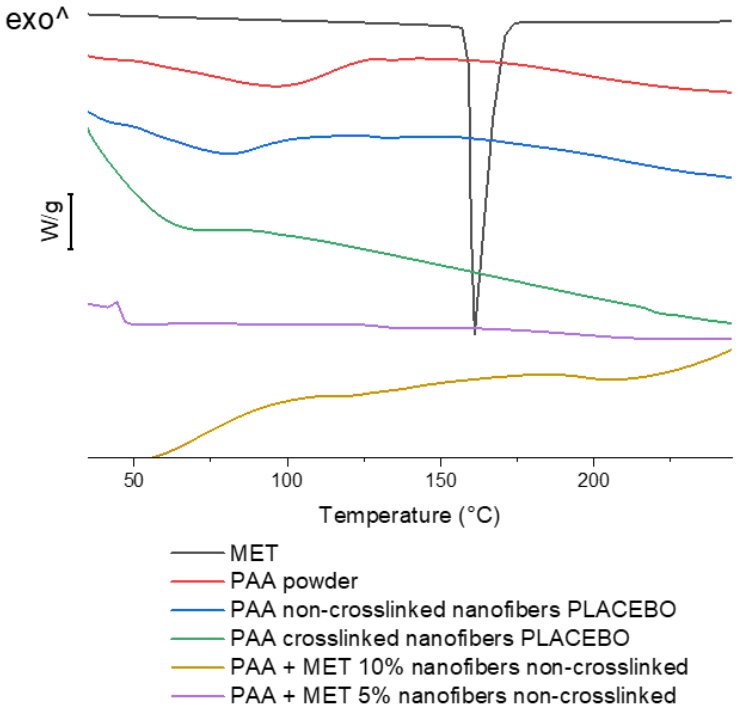


Figure 6 - DSC results.

4.8. Thermogravimetric analysis (TGA) analysis

As we can interpret from the TGA results, there is a slight and gradual decrease in the mass around 5 minutes that we attribute as the evaporation of moisture or remaining solvent. This drop is around 6% which is around 0.47 mg. After this initial decrease, the graphic slope tends to decrease which suggests continuous degradation of the sample, however there is not a relevant loss of mass up to the 26 minutes indicating that the sample is relatively stable with only minor decomposition.

There is also on study that evaluated TGA of PAA nanofibers crosslinked with Fe^{3+} ions their results showed a 6–8% weight loss up to 100°C (moisture loss) and stability up to 200°C. Indicating that our results are accordingly. (8)

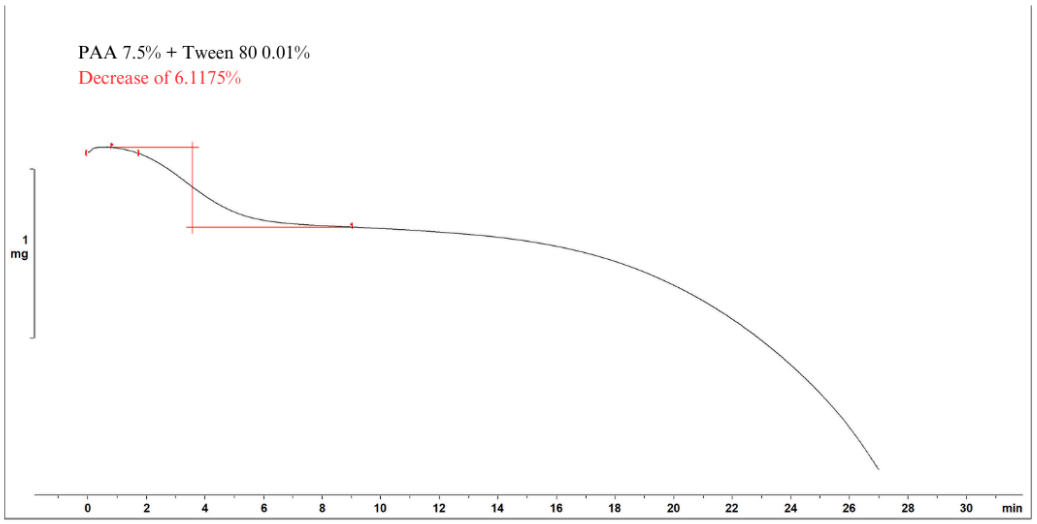


Figure 7 - TGA results (PAA 7.5% + Tween 80 0.01%).

4.9. Determination of encapsulation efficiency of metronidazole in nanofibers

For the quantification of MTZ loaded in the nanofibers the direct method with chloridric acid did not work, the nanofibers were tightly crosslinked and, therefore, the MTZ was not released. After these results we moved on for the indirect quantification of MTZ using the release studies to determine the encapsulation efficiency (EE).

First we did a calibration curve as shown in figure 8.

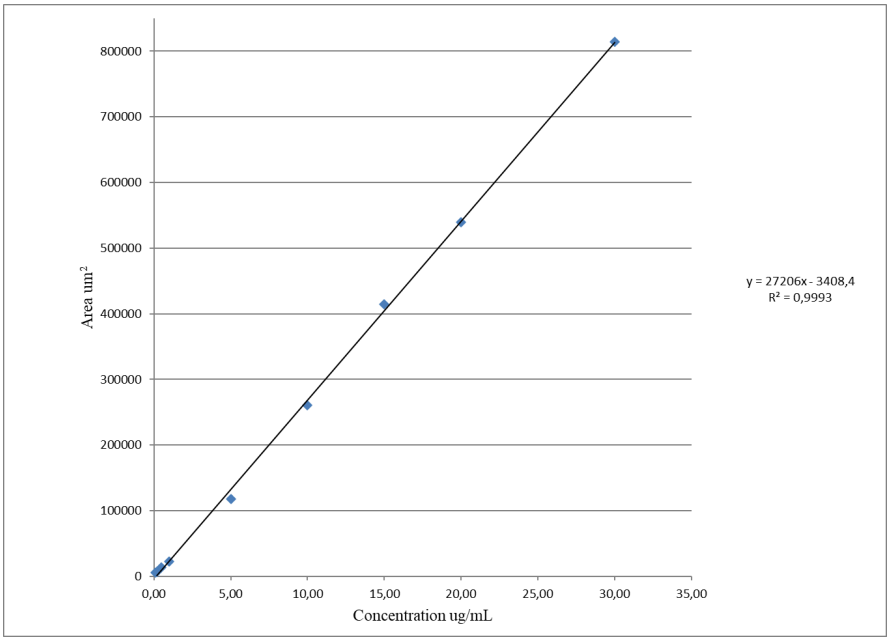


Figure 8- Calibration curve for MTZ quantification, correlation between MTZ concentration (ug/mL) and area (um^2).

Using the calibration curve we calculated the concentration of our samples, the crosslinking solution and the water used for washing the crosslinking. With these results we, and considering the mass of the sample, the dilution ratios and the ratio of MTZ we calculated the encapsulation efficiency, $EE\% = 34.23$. Which indicates that 65.77% of MTZ was lost during crosslinking.

Comparing with a study by Arkaban (15) that evaluated encapsulation of ciprofloxacin in PAA nanofibers for wound healing, they achieved an EE of 50-60%, which suggest that there is potential for optimization, such as adjusting crosslinking parameters or using surfactants to enhance stability.

5. Conclusion and Future work

This study showed that PAA can be successfully introduced in nanofibers that are produced by electrospinning. When Tween 80 and MTZ were introduced in the formulation we were able to improve the stability of the process and the properties of the nanofibers resulting in suitable pharmaceutical applications.

Speaking of the production process, we confirmed that electrospinning is a good and effective method to produce uniform nanofibers. The formulation modifications that were made showed improvements and potential applications, especially for treating infections. The morphology was analyzed using SEM that revealed smooth surface and consistent diameter within the nanofibers, characteristics that are really important for pharmaceutical uses.

The rightful incorporation of MTZ was confirmed with FTIR that showed effective integration of MTZ without significant chemical degradation. Both the DSC and TGA showed thermal stability of the nanofibers. The DSC results showed that the nanofibers were stable up to a certain temperature and the TGA revealed gradual mass loss, attributed to the evaporation of moisture. Both results indicated suitability for pharmaceutical applications.

Although the encapsulation efficiency wasn't the best, we confirmed a successful crosslinking of the nanofibers.

Overall, the findings of this study show the potential of PAA nanofibers for effective drug delivery systems, particularly for treating vaginal infections.

For the future, the most important thing would be continuing with the release studies under different pH and temperature conditions. Optimization of the crosslinking would also be a good process to improve since it could help with the encapsulation efficiency.

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