Scientific Report

regarding the implementation of the project between September 2020 – August 2022

The overall objective of the proposal is to continue previous research and find the best way to deliver irradiated solutions for wound dressings to obtain the best antimicrobial effect. In this respect, it is proposed to use UV photo-crosslinked hydrogels.

Within stage I, the objectives were: *update the concept of the project to last-minute published progress to adapt the research to reach the aims specified in the project proposal; *irradiation of chlorpromazine solutions using 266 nm from Nd:YAG at 6.5 mJ.

Within stage II, the activities were: *photoinitiator rate of free radical production during irradiation; *fabrication of hydrogel by photopolymerization; *characterization of hydrogel: swelling measurements, UV-Vis and FTIR absorption spectroscopy, scanning electron microscopy, LIF; *unirradiated and irradiated CPZ loading into film hydrogels; *characterization of the loaded hydrogel: UV-Vis and FTIR absorption spectroscopy, SEM, LIF, drug release assay, *selection of Gram-positive/-negative bacteria sensible and resistant to antibiotics, *susceptibility test of Gram-positive/-negative bacteria to hydrogels loaded with unirradiated and irradiated CPZ.

Within stage III, the activities were: *photopolymerize the mixture of polymer-photoinitiator-CPZ in view of hydrogel formation, *characterization: swelling measurements, UV-Vis and FTIR absorption spectroscopy, SEM, LIF, LIF lifetime, and drug release assay, *photopolymerize the mixture of polymer-CPZ in view of hydrogel formation, *characterization: UV-Vis and FTIR absorption spectroscopy, SEM, LIF, LIF lifetime, and drug release assay, *susceptibility test of Gram-positive/-negative bacteria to hydrogels loaded with unirradiated and irradiated CPZ

Within this project, the following were realized:

- Update the concept of the project to last-minute published progress to adapt the research to reach the aims specified in the project proposal.
- Irradiation of chlorpromazine solutions using 266 nm from Nd:YAG at 6.5 mJ
- Determination of the photoinitiator rate of free radical production during irradiation.
- Development of the experimental system for obtaining hydrogels.
- Fabrication of hydrogel by photopolymerization.
- Characterization of hydrogel: swelling measurements, UV-Vis and FTIR absorption spectroscopy, scanning electron microscopy (SEM), LIF.
- Unirradiated and irradiated CPZ loading into hydrogels.
- Characterization of the loaded hydrogel: UV-Vis and FTIR absorption spectroscopy, SEM, LIF and LIF lifetime, drug release assay.
- Susceptibility tests of Gram-positive bacteria to hydrogels loaded with unirradiated and irradiated CPZ.
- Photopolymerize the mixture of polymer-photoinitiator-CPZ in view of hydrogel formation and characterization by UV-Vis and FTIR absorption spectroscopy, SEM, LIF, and drug release assay.
- Photopolymerize the mixture of polymer-CPZ in view of hydrogel formation and characterization.
- Susceptibility test of Gram-positive bacteria to hydrogels loaded with unirradiated and irradiated CPZ.

1. Update the concept of the project to last-minute published progress to adapt the research to reach the aims specified in the project proposal.

In the case of chronic wounds, the potential for infection and colonization increases due to the presence of avascular ulcers, which provide a favorable environment for the uninhibited growth of microorganisms [1]. The rate of infection depends on the type of wound, its mode of care and the general health of the patient [2]. Infection is a major complication of burns and is responsible for 50-75% of deaths [3]. Thus, products are needed to treat these wounds, products that are more economical and effective and that provide an optimal healing environment for the wound.

In recent years, efforts have been made to develop new dressings that meet the requirements of treating major skin wounds. Research has focused mainly on making ideal dressings. In this sense, hydrogels have been created, both natural and artificial, in order to be used in healing wounds.

Of all the hydrocolloid dressings, alginate or hydrogels, each with its own advantages and limitations, hydrogels are the best and have all the characteristics necessary for an ideal dressing. Hydrogels are made of crosslinked polymers, natural or synthetic, and are used in a variety of biomedical fields. They consist of a matrix of insoluble polymers with a water content of ≈96%. Hydrogels can donate water to the wound site and thus help maintain a moist environment. They are used in the administration of medicines, wound dressings, contact lenses and as electrodes or sensors. [4]. Various polymers with good bio-compatibility are used to form hydrogels. The natural ones are alginate, chitosan, gelatin and collagen, and the synthetic ones are polyurethane, poly (ethylene glycol), polycaprolactone, poly (vinyl pyrrolidone), poly (lactide-co-glycolide), polyacrylonitrile, or poly (amino acids).

Hydrogels can provide spatial and temporal control over the release of the therapeutic agents, including small molecule drugs, macromolecular drugs, and even cells. Conventional drug administration often requires high doses or repeated administration to stimulate a therapeutic effect, which can reduce the overall efficacy and lead to severe side effects, even toxicity. [5], [6]. In this sense, hydrogels can make the most of on the beneficial results of therapy by improving their effectiveness and reducing the toxicity and dose.

Hydrogels can be produced by exposing polymers to ultraviolet radiation, this process being considered a suitable tool due to easier processing compared to chemical or freeze-drying techniques. Moreover, irradiation brings the possibility of hydrogel formation and sterilization in one step. Photo-polymerization can spatially and temporally control the exposure area and the exposure time at room temperature, allowing easy and fast production of complex matrices [7], [8]. Photopolymerization uses light to dissociate the initiator into free radicals, which react with the double bonds of the crosslinking monomers or pre-polymers. [9].

In the formation of hydrogels, the following factors must be taken into account:

- structure and physico-chemical properties of the monomer;
- the phenomenon of inhibition due to oxygen;
- the influence of stabilizers or other additives present in monomers;
- thickness of the polymerized layer;
- type and intensity of light source;
- viscosity of the composition.

The photoinitiator must have the following characteristics:

- compatibility between the absorption characteristics of the photoinitiator and the emission characteristics of the light source;

- high quantum efficiency;
- soluble in water;
- non-cytotoxic;
- must not cause yellowing of the hydrogel;
- thermal and temporal stability.

Polymers and initiators selection

To meet the project objectives, the following polymers and initiator were chosen for the production of hydrogels by photopolymerization: natural polymer - methacrylate gelatin, synthetic polymer - polyethylene glycol diacrylate, photoinitiator type I - Irgacure 2592 and photoinitiator type II - riboflavin (co-initiator - L-ar).

Methacrylate gelatin (GelMa) has emerged in particular as an attractive option to mimic the extracellular matrix when the matrix is composed mainly of collagen. [10]. Gelatin is the hydrolysis product of collagen that can retain relevant fragments, such as Arg-Gly-Asp (RGD) which are important sites in cell attachment. The addition of methacrylate and/or methacrylimide groups results in versatile materials that mimic the cell matrix. [11].

Irgacure 2959 is a type I photoinitiator where photofragmentation generates pairs of radicals through the -α cleavage process [12]. After the absorption of a UV photon, the singlet state is transferred to a triplet state (inter-system transition), and from this state it is de-excited to form benzoyl and cetyl radicals. [13]. It is one of the few water-soluble photoinitiators, but has a low water solubility of less than 2%.

Polyethylene glycol diacrylate (PEGDA) contains double-bonded acrylate groups at each end of the polyethylene glycol chain, giving it the ability to undergo free radical light curing in the presence of a photoinitiator [14] to produce a photocrosslinked system [15], [16]. PEGDA is non-toxic, generating only a minimal immune response [17], [18] and is a biodegradable polymer.

Riboflavin (vitamina B_2) is a natural yellow pigment, which is widely used in biomedical applications due to its high solubility in water and biocompatibility [19]. The spectral characteristics are favorable to UV light curing, having a total of four absorption maxima at 223 nm, 267 nm, 373 nm and 444 nm [20]. Being a type II photoinitiator, it requires the presence of a co-initiator as an electron donor during the initiation of the polymerization reaction to generate a free radical. In this sense, L-arginaine is a possible candidate, being biocompatible, biodegradable and water soluble.

Hydrogel formation protocol

Prior to photopolymerization, the mixture of precursors is:

1. mixed with a magnetic stirrer at 200 rpm for 30 min at a temperature of 70 °C to allow the proper dissolution of each component

2. filtered with 0.2 μm filter

Photopolymerization

1. molds made of polylactic acid (PLA) using a 3D printer, 0.7 cm in diameter and 1 mm high

2. excitation source: Nd laser: YAG Excel Technology, Surelite II model, having the following characteristics: 266 nm wavelength of the emitted radiation, 6 ns temporal width of the pulse at half-height and 10 Hz pulse repetition rate

3. intensity between 2-10 mW/cm^2

4. The photopolymerization time is to be determined

After photopolymerization, the hydrogel is:

1. immersed in water for 24 hours at room temperature to remove precursor residues and to achieve absorption equilibrium (detection of non-reactive compounds)

2. Dried in the desiccator 8 hours before use

3. stored in the desiccator at 4 ºC

Physico-chemical methods for characterizing hydrogels

The hydrogels to be formed in stages II and III of the project will be characterized by at least the following methods:

- Swelling behavior study
- Drug loading study
- Drug release study
- FT-IR absorption spectroscopy by attenuated total reflection
- Electron microscopy
- UV-Vis spectroscopy
- Laser induced fluorescence
- In vitro evaluation methods
  - Cytotoxicity assay
  - Antimicrobial behavior

2. Irradiation of chlorpromazine solutions using 266 nm from Nd:YAG at 6.5 mJ.

Chlorpromazine (CPZ) solutions at a concentration of 2 mg/mL and a volume of 2 mL were irradiated with a laser beam emitted at 266 nm by the Nd: YAG laser (10 Hz, 6 ns FWHM). Laser radiation exposure times were 1, 5, 15, 30 and 60 minutes. The samples were investigated by UV-Vis and FTIR absorption spectroscopy and laser-induced fluorescence. The fluorescence was collected during irradiation of CPZ solutions using an optical fiber and a spectrograph was used to record the signal (Acton Research, SpectraPRo SP-2750).

The absorption spectra were recorded for the concentration of 0.2 mg/mL because at the concentration of 2 mg/mL the signal in the range of 200-290 nm is saturated. The UV-Vis absorption spectrum of the unirradiated CPZ in Figure 1a is characterized by two absorption bands with maxima at 254 nm and 307 nm. Following irradiation, the intensity of the band at 254 nm shows a hypochromic shift until the end of irradiation, the intensity of absorbance decreasing as follows: 1 minute - 8.6%, 5 minutes - 16.7%, 15 minutes - 31.3%, 30 minutes - 35.7% and 60 minutes - 38.4%. The band with maximum absorption at 315 nm shows a bathochromic shift of 20 nm during the first 60 minutes of irradiation being accompanied by its broadening.

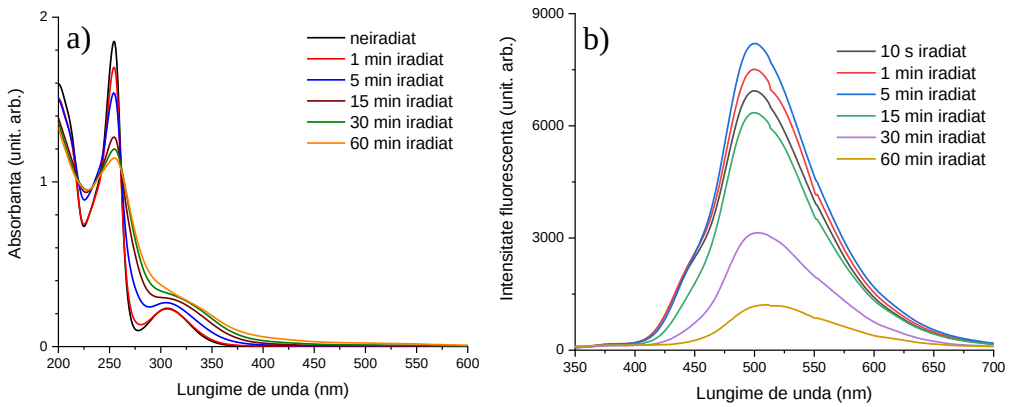

Figure 1. a) Absorbance spectra of unirradiated CPZ and irradiated CPZ solutions for 1, 5, 15, 30, 60 min. b) Fluorescence spectra of unirradiated COZ and irradiated CPZ solutions for 1, 5, 15, 30, 60 min

The LIF spectrum of the CPZ solution, with a concentration of 2 mg/mL, irradiated for 60 minutes is characterized by the presence of a single band with a maximum at 499 nm (Figure 1b). The fluorescence intensity increases by 7.6% after the first minute of irradiation and by 15.4% after 5 minutes, then begins to decrease with 82.5% compared to the fluorescence intensity recorded in the first 10 s.

The IR spectrum was recorded in the range 3600 - 750 cm^{-1} at a resolution of 4 cm^{-1} and an average on 32 spectra. The CPZ samples in aqueous solutions were dried on a KRS-5 support, applying 20 µL of the sample, and the spectrum of the support was subtracted. The IR spectra of unirradiated and irradiated CPZ solutions are shown in Figure 2.

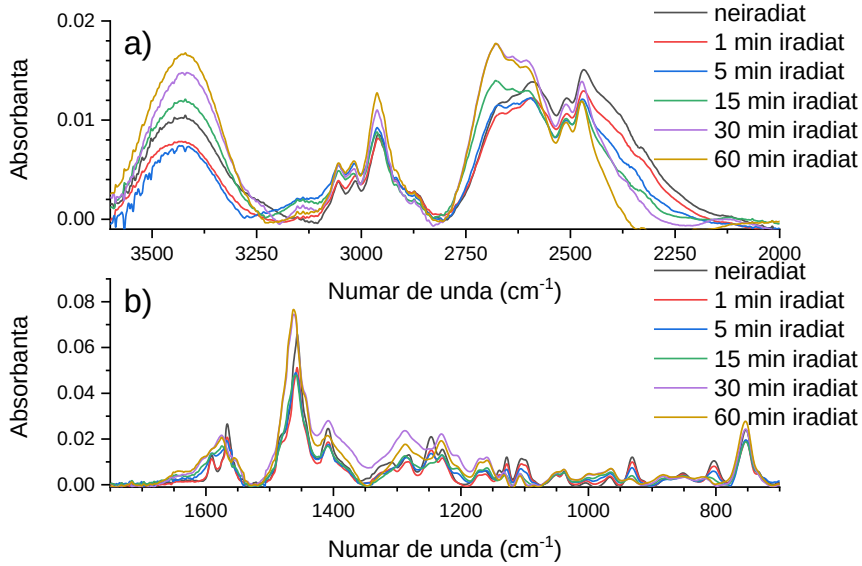

Figure 2. FTIR spectra of unirradiated CPZ and irradiated CPZ for 1, 5, 15, 30, 60 min in the domain: a) 3600-2000 cm^{-1} and b) 1750-700 cm^{-1}.

It was observed that the bands at 1140 cm^{-1} and 1098 cm^{-1}, in the unirradiated CPZ spectrum, attributed to the deformation vibration of the C−H bond, are no longer found in the IR spectra of irradiated CPZ solutions resulting in the cleavage of the N−CH_3. It was also observed the appearance of bands at 1205 cm^{-1}, after 5 minutes of irradiation, attributed to the vibration of the O−H bond deformation and the stretching vibration of the C−O bond within the phenol group and at 1086 cm^{-1},

after 30 min irradiation, attributed to the stretching vibration of the S=O bond within the sulfoxide group. The appearance of these two bands indicates the presence of phenol groups to the molecular structure of CPZ and the generation of oxidative forms.

3. The experimental system developed for obtaining hydrogels

The irradiation source was a Continuum Nd: YAG laser (10 Hz, 6 ns FWHM) emitting at 266 nm. Photoinitiator solutions and photoinitiator-polymer mixtures were exposed at 266 nm for 1, 5, 10, 20, and 30 min at 0.25 mJ (irradiance, I = 6.6 mW/cm^2), 0.45 mJ beam energies (I = 11 mW/cm^2), 0.75 mJ (I = 19.7 mW/cm^2), and 1 mJ (I = 26.3 mW/cm^2). The mold was 3D printed from polylactic acid and has an inside diameter of 0.7 cm and a height of 0.1 cm. The mold had a total volume of 35 µL.

The laser-induced fluorescence (LIF) signal was collected using an optic fiber and recorded using a SpectraPRo SP-2750 (Acton Research) spectrograph. Absorption spectra were recorded with a Perkin Elmer spectrophotometer, Lambda 950 model and FTIR spectra with Nicolet iS50 FTIR spectrometer.

The surface morphology of deposited films was inspected by scanning electron microscopy (SEM) with an FEI Inspect S electron microscope at 20 kV acceleration voltage in high vacuum using top-view and cross-section modes.

4. Fabrication of hydrogel by photopolymerization

Photopolymerization with pulsed laser radiation has various advantages, including a quick crosslinking time due to the ability to vary the beam energy and select the excitation of chromophores due to monochromatic light. Fluorescence emission provides various advantages over conventional crosslink monitoring methods, including fast response time, high sensitivity, and non-invasive in situ research.

Irgacure 2959 (2-hydroxy-1- [4- (2-hydroxyethoxy) phenyl] -2-methyl-1-propanone), riboflavin, methacrylate gelatin, GelMa, (natural polymer), polyethylene glycol diacrylate, PEGDa, (synthetic polymer) and L-arginine were purchased from Sigma-Aldrich. The solvent used was ultrapure water. Concentrations of 0.05%, 0.35% and 0.7% were used for Irgacure 2959. The concentration of 0.7% is the maximum amount that could be dissolved in ultrapure water.

UV-Vis absorption spectroscopy was used to analyse Irgacure 2959 solutions, and the absorption spectra of unirradiated and irradiated solutions at energies of 0.25 mJ, 0.45 mJ, 0.75 mJ, and 1 mJ are shown in Figure 1.

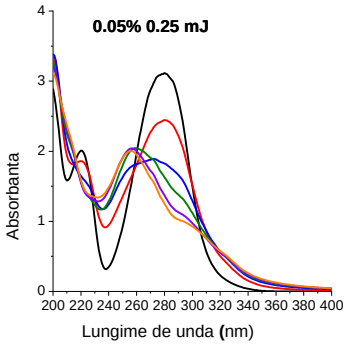

Figure 1. UV-Vis absorption spectra of Irgacure exposed to 266 nm laser pulsed radiation at various energies and concentrations.

Changes in the UV-Vis absorption spectra of Irgacure 2959 suggest photo-decomposition in the 25 benzoyl and hydroxyalkyl radicals.

The loss of the 1663 cm^{-1} band and the emergence of the 1725 cm^{-1} band was the first evidence that Irgacure 2959 had been photo-degraded (Figure 2). Both bands are attributed to the stretching vibration C = O, however the shift of the band to higher wave numbers supports ketone group cleavage. The disappearance of the band with a maximum of 1254 cm^{-1} also confirmed this (vibration of the C-C-C skeleton from the aromatic ketone). Furthermore, the bands responsible for C-H vibration and C-CH3 skeletal vibration in C-CH3, such as 1392, 1276, 1011, 985, and 976 cm^{-1}, promote Irgacure2959 photo-degradation into benzoyl and hydroxyalkyl radicals.

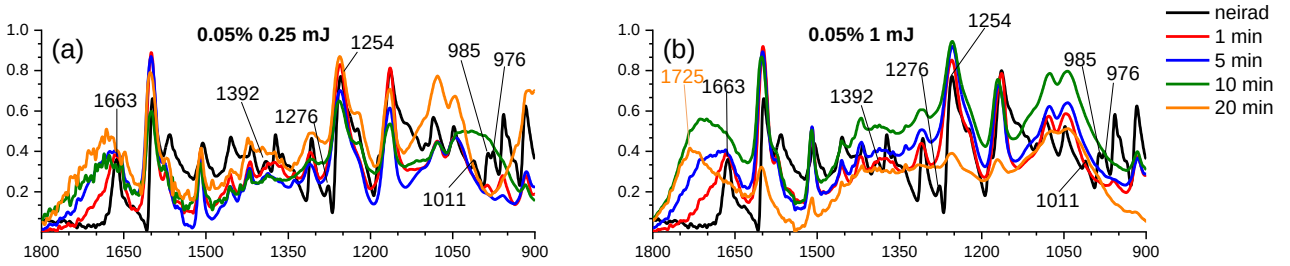

Figure 2. FTIR spectra of unirradiated and irradiated Irgacure 2959 solutions: (a) concentration 0.05% and energy 0.25 mJ; (b) 0.05% concentration and 1 mJ energy;

During irradiation, laser-induced fluorescence (LIF) was measured in real time. This method has the advantage of monitoring the photo-degradation of the Irgacure 2959 molecule in real time. Every 5 seconds, the LIF spectrum was collected. This approach offers the irradiated solution's fluorescence spectrum as well as its fluorescence kinetics profile (Figure 3).

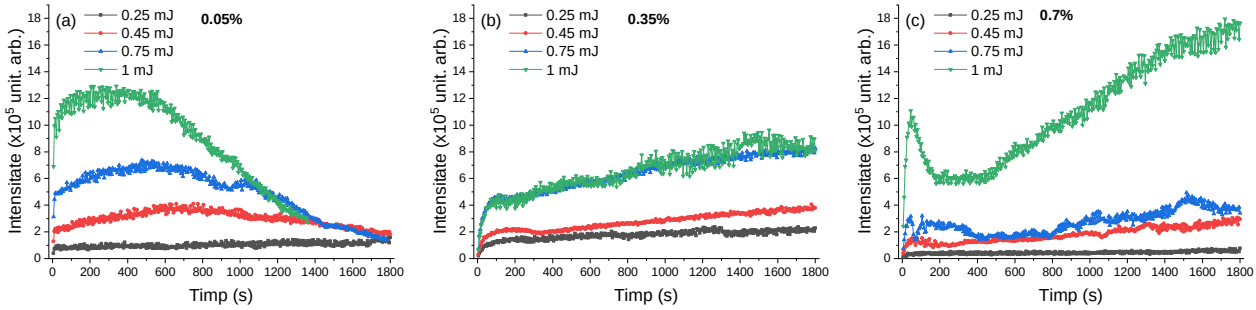

Figure 3. LIF kinetics profile for Irgacure 2959 irradiated solutions at energies of 0.25, 0.45, 0.75 and 1 mJ at concentrations: (a) 0.05%, (b) 0, 35%, (c) 0.7%.

To allow proper dissolution of each component, the polymer-photoinitiator solutions were stirred with a magnetic stirrer at 200 rpm for 30 min at 70 ° C. A 35 µL volume of the polymer-photoinitiator mixture solution was placed in the mold and exposed to pulsed laser radiation emitted at 266 nm under the same conditions as the simple photoinitiator solution, with exposure times of 1, 5, 10, 20, and 30 min and energies of 0.25 mJ, 0.45 mJ, 0.75 mJ, and 1 mJ.

Hydrogels were removed from the mold and immersed in ultrapure water (3 mL) at room temperature for 24 h to eliminate precursor residues and reach absorption equilibrium (detection of non-reactive compounds). They were dried in a desiccator for 24 h before being stored in a dark desiccator at 4^{o}C.

The following samples were prepared: Irgacure 2959 (0.05%, 0.35% and 0.7%) + GelMa (10%), Irgacure 2959 (0.7%) + GelMa (15%), Riboflavin (0, 05%, 0.7%) + L-arginine (0.1%) + PEGDa (10%), Irgacure 2959 (0.05%, 0.35% and 0.7%) + PEGDa (10%). These were subjected to beam intensities of 0.25, 0.44, 0.75, and 1 mJ at time periods of 1, 5, 10, 20, and 30 minutes. Irgacure 2959 (0.05 %) + GelMa were the only suitable hydrogel formed. Even after 60 min of 1 mJ exposure, no

hydrogels were generated for Riboflavin+L-arginine+PEGDa. For the remaining combinations, hydrogels in the form of thin films were produced.

The dry hydrogels were incubated for 24 h in one mL of unirradiated CPZ. After that, the hydrogels were removed, placed on a vessel, and dehydrated. These hydrogels were subjected to the following testing methods: swelling behavior, UV-Vis and FTIR absorption spectroscopy, scanning electron microscopy (SEM), and laser induced fluorescence (LIF).

The irradiated samples 1 min had the greatest degree of swelling, depending on the irradiation time. After 1 min, the light curing process produces longer polymer chains, causing the hydrogels to stiffen and absorb less water. The best swelling ratio was observed when Irgacure 0.05 % + 10% GelMa was irradiated for 1 min at 0.75 mJ. The hydrogels turned yellow when exposed above 5-10 min, hence these conditions were deemed unsuitable.

Before usage, the CPZ loaded hydrogels were dehydrated. They were immersed in 1 mL of Mueller-Hinton culture medium and phosphate buffered saline and incubated at 37 ° C for varying times (2, 4, 8, 24 and 48 h). Figure 4 presents the CPZ release in Mueller-Hinton culture medium

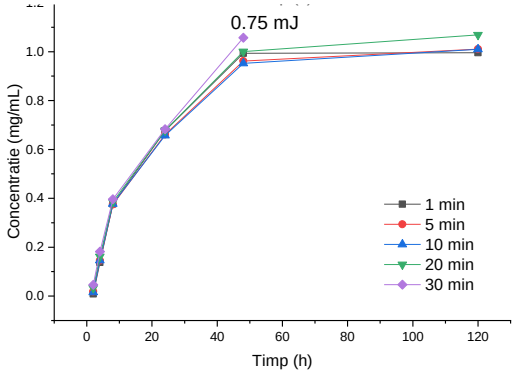

Figure 4. CPZ release in Mueller-Hinton culture medium

For all energies employed, a continuous release of CPZ in Mueller-Hinton culture medium and PBS was seen during a 48-hour period. Hydrogels formed at energies of 0.25 mJ in both Mueller-Hinton culture medium and PBS had the lowest released concentration.

In the IR spectra of dry hydrogels, only the presence of GelMA is observed, with no changes between the hydrogels resulting from different irradiation periods. As a result, the residues of the precursors were eliminated from the hydrogels. In addition, after the CPZ release studies, the IR spectra of the hydrogels were examined, and no CPZ was found in the dried hydrogels. At the same time, the FTIR spectrum of the hydrogels revealed no substantial changes in the polymer structure during the photopolymerization process.

For the mixture of Irgacure (0.7%) and GelMa (10%) solutions, the changes induced in the fluorescence kinetics profiles are different from those resulting from the irradiation of the Irgacure-GelMa mixture where the photoinitiator concentration was 0.05%. And in this case, for the samples containing GelMa, a significant decrease of the fluorescence intensity was observed compared to the one resulting from the irradiation only of the Irgacure 2959 solution, suggesting the formation of the hydrogel. As in the case of using only Irgacure 2959 solution at a concentration of 0.7%, water evaporation was observed.

After immersion of the CPZ unirradiated and irradiated loaded hydrogels in one mL of bacterial suspension of S. aureus ATCC 29523, the number of colonies was expressed in CFU/mL. It was observed that both CPZ unirradiated-loaded hydrogel and CPZ irradiated -loaded hydrogel, completely prevented the adhesion of bacterial colonies and the formation of biofilms on the surface of the samples.

5. Photopolymerize the mixture of polymer-photoinitiator-CPZ in view of hydrogel formation and characterize them by UV-Vis and FTIR absorption spectroscopy, SEM, LIF, and drug release assay.

Taking into account the swelling rate, the amount of CPZ released and the appearance of the hydrogel, the solution containing Irgacure 2959 (0.05%) and GelMa (10%) irradiated for 1 min at 0.75 mJ generated the hydrogel with the best properties. Thus, the concentration of 0.05% for Irgacure 2959 and 10% for GelMa were kept constant throughout the studies, varying only the concentration of CPZ. The concentrations of CPZ tested were 1, 2 and 4 mg/mL. In order to be sure of obtaining antimicrobial products from CPZ irradiation during the formation of hydrogels, the exposure time to laser radiation was extended up to 5 min. The samples were characterized by UV-Vis and FTIR absorption spectroscopy, SEM, and LIF.

The absorption spectra of the irradiated Irgacure 0.05% - CPZ (1, 2, 4 mg/mL) - GelMa 10% samples show a higher absorbance compared to those of Irgacure 0.05% - CPZ (1, 2, 4 mg/mL) due to the influence of GelMa, which provides a more viscous environment than ultrapure water. Also, during the irradiation, for Irgacure 0.05% - CPZ (1, 2, 4 mg/mL) - GelMa 10%, a decrease in absorbance was observed with increasing irradiation time, suggesting that during the photopolymerization, CPZ is transformed into photoproducts, even if Irgacure also participates through its radicals in the crosslinking of the hydrogel.

Compared to absorption spectroscopy, where the absorption spectra of CPZ and Irgacure overlap and only CPZ spectra can be observed, LIF offers the possibility to visualize the fluorescence spectra of both CPZ and Irgacure. This is possible because CPZ has an emission maximum at 480 nm and Irgacure at 320 nm. The LIF spectra of the solutions of Irgacure 0.05%-CPZ 1, 2, 4 mg/mL were analyzed to elucidate how the laser radiation influences the production of radicals (Figure 5).

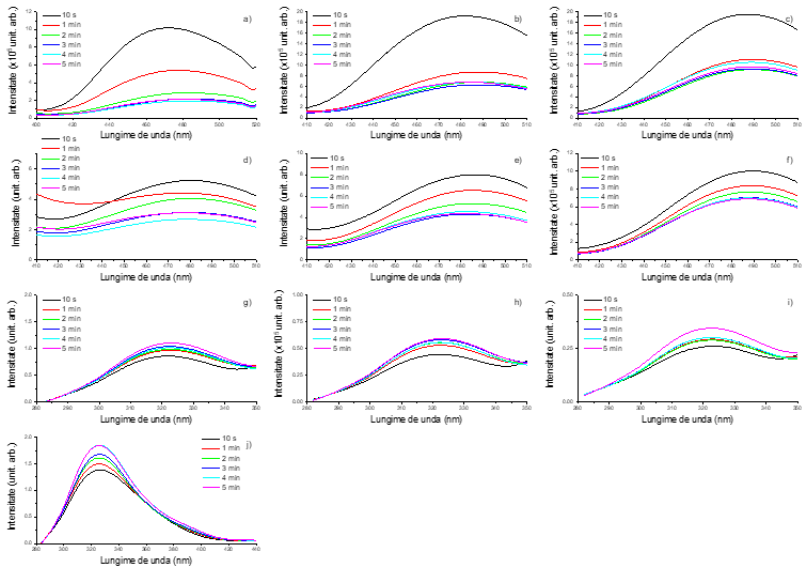

Figure 5. LIF spectra of solutions exposed to laser beams emitted at 266 nm, energy of 0.75 mJ, for 5 min: a) CPZ 1 mg/mL, b) CPZ 2 mg/mL, c) CPZ 4 mg/mL, d) Irgacure 0.05%-CPZ 1 mg/mL (CPZ) e) Irgacure 0.05%-CPZ 2 mg/mL (CPZ), f) Irgacure 0.05%-CPZ 4 mg/mL (CPZ) g) Irgacure 0.05%-CPZ 1 mg/mL (Irgacure) h) Irgacure 0.05%-CPZ 2 mg/mL (Irgacure), i) Irgacure 0.05%-CPZ 4 mg/mL (Irgacure), j) Irgacure 0.05%

Analyzing the impact of radiation on CPZ and Irgacure molecules, in both cases, a decrease in fluorescence intensity is observed when the Irgacure 0.05%-CPZ mixture is irradiated compared to those of CPZ or Irgacure solutions. As the concentration of CPZ increases, the fluorescence intensity for Irgacure decreases, more precisely with a decrease of 38% when CPZ has a concentration of 1

mg/mL and of 81% for CPZ 4 mg/mL. Also, a decrease in fluorescence intensity for CPZ is observed when Irgacure is added, from approximately 48% for CPZ 1 and 4 mg/mL and 58% for CPZ 2 mg/mL. Thus, the higher the concentration of CPZ, the less radicals of Irgacure are formed, which can affect the crosslinking of the hydrogel if CPZ irradiation does not also result in the generation of radicals that can adjust to the formation of polymer chains.

Figure 6 shows the profiles of the fluorescence kinetics for the Irgacure 0.05%-GelMa 10%-CPZ solutions (1, 2, 4 mg/mL), where both the CPZ band and the Irgacure band are analyzed. Only for Irgacure 0.05%-GelMa 10%-CPZ 1 mg/mL can be observed the same profile of fluorescence kinetics as that obtained in stage II for Irgacure 0.05%-GelMa 10%, which already provides an indicator that this hydrogel presents properties similar to those of the hydrogels obtained in stage II.

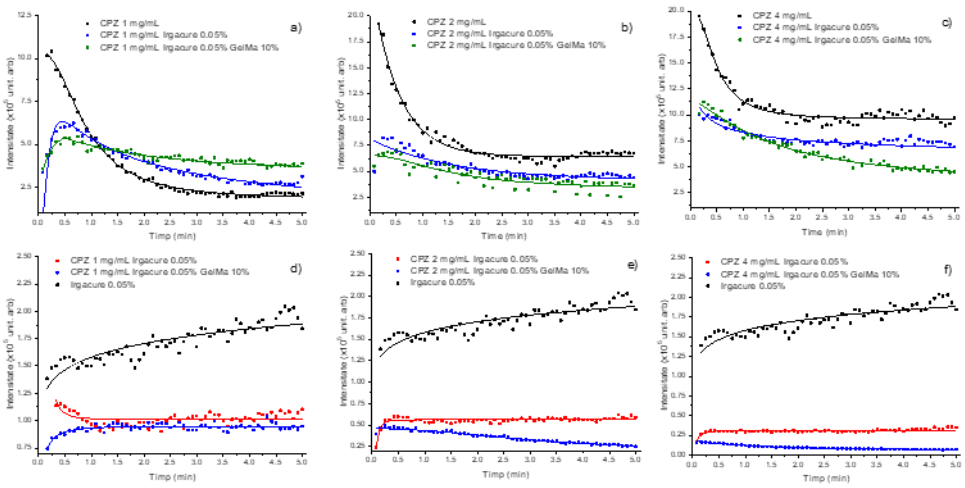

Figure 6. Fluorescence kinetics profile for Irgacure 0.05%-GelMa 10%-CPZ irradiated with 266 nm for 5 min, where CPZ has the following concentrations: a) 1 mg/mL (CPZ peak), b) 2 mg/mL (CPZ peak), c) 4 mg/mL (CPZ peak), d) 1 mg/mL (Irgacure peak), e) 2 mg/mL (Irgacure peak), f) 4 mg/mL (Irgacure peak).

The overall composition of the samples was examined using FTIR absorption spectroscopy. The FTIR spectra were compared with those of GelMa powder, 2mg/mL CPZ solution irradiated for 30 min (used in stage II to load the hydrogels) and 0.05% Irgacure solution irradiated for 1 min. The IR spectra of the dry hydrogels show a major influence of GelMa, most of the IR bands of GelMa being present. The IR bands corresponding to CPZ solutions 2 mg/mL irradiated for 30 min can also be observed in the IR spectra.

The only hydrogels for which the release of irradiated CPZ was obtained at intervals greater than 2 h are Irgacure 0.05%-GelMa 10%-CPZ 1mg/mL resulting from exposure to laser radiation for 1 and 5 min (Figure 7). The rest of the hydrogels released irradiated CPZ in the first 2 h. In addition, the hydrogels Irgacure 0.05%-GelMa 10%-CPZ 1mg/mL obtained after irradiation for 5 min released irradiated CPZ only in the first 24 h.

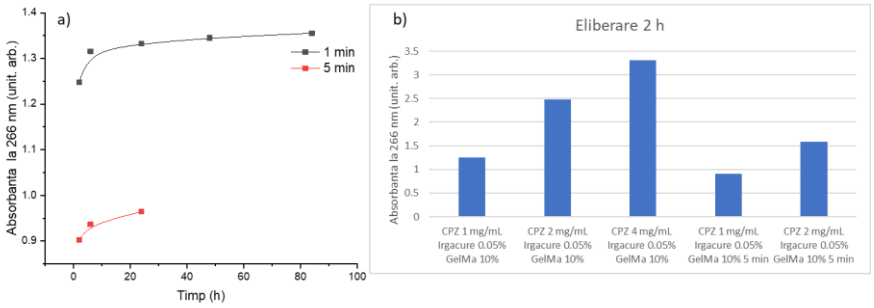

Figure 7. a) Release curves of irradiated CPZ in PBS for Irgacure 0.05%-GelMa 10%-CPZ 1mg/mL cross-linked by irradiating the solutions for 1 and 5 min. b) Absorbance measured at 266 nm for CPZ irradiated and released after 2 h of immersion of the hydrogels in PBS.

The SEM images show that Irgacure 0.05%-GelMa 10% has an almost smooth and homogeneous morphological structure, while the rest of the hydrogels have a less ordered structure. For the hydrogels Irgacure 0.05%-GelMa 10%-CPZ (1, 2, 4 mg/mL) obtained after irradiation for 1 min, a wavy and rough surface with folds and cracks is observed. As the concentration of CPZ increases, the surface of the hydrogels becomes more uneven. However, with the increase of the irradiation time, a more pronounced homogeneity is observed compared to the samples irradiated for 1 min. The rough surface can be produced by the fractional collapse of the polymer gel network during the drying process.

6. Photopolymerize the mixture of polymer-CPZ in view of hydrogel formation and its characterization.

Previous studies have shown that during CPZ irradiation, radicals are produced that help to form photo-products with antimicrobial activity [21], [22]. This study aims to investigate the possibility that these radicals can be effective in the cross-linking process. This approach will eliminate two steps from the hydrogel formation, namely the prior irradiation of CPZ and its loading into the hydrogels. Taking into account the swelling rate, the amount of CPZ released and the appearance of the hydrogel, the solution containing Irgacure 2959 (0.05%) and GelMa (10%) irradiated for 1 min at 0.75 mJ generated the hydrogel with the best properties. Thus, the concentration of 10% for GelMa was maintained throughout the studies, varying only the concentration of CPZ. The concentrations of CPZ tested were 1, 2 and 4 mg/mL. The samples were characterized by UV-Vis and FTIR absorption spectroscopy, SEM and LIF.

The absorption spectra of the irradiated GelMa 10% - CPZ (1, 2, 4 mg/mL) samples show a higher absorbance compared to those of CPZ (1, 2, 4 mg/mL) due to the influence of GelMa which gives a more viscous environment than ultrapure water. Also, during irradiation, a decrease in absorbance was observed for GelMa 10% - CPZ (1, 2, 4 mg/mL) with increasing irradiation time, suggesting that during photopolymerization of the hydrogel, CPZ is transformed into photoproducts, some of which participates in the crosslinking of the hydrogel.

The LIF spectra of GelMa 10% - CPZ solutions (1, 2 and 4 mg/mL) were analyzed and compared with those of CPZ 1 mg/mL, CPZ 2 mg/mL and CPZ 4 mg/mL to elucidate how which laser radiation influences the production of radicals (Figure 8).

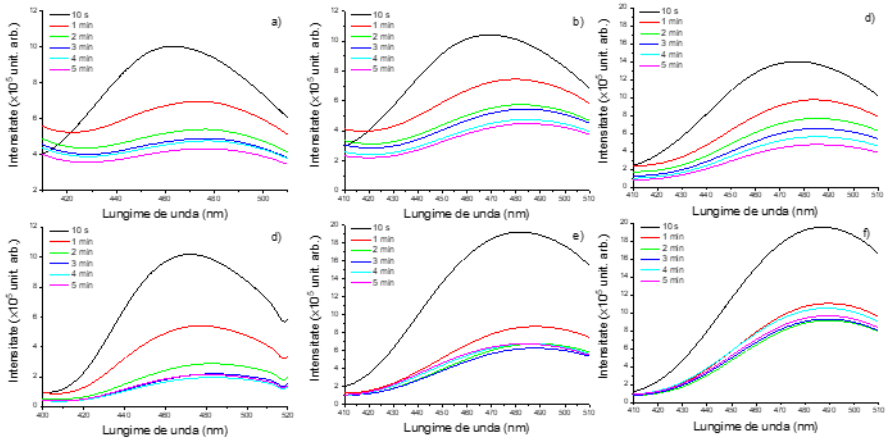

Figure 8. LIF spectra of solutions exposed to laser beams emitted at 266 nm, energy of 0.75 mJ, for 5 min: a) GelMa 10%-CPZ 1 mg/mL, b) GelMa 10%-CPZ 2 mg/mL, c) GelMa 10%-CPZ 4 mg/mL, d) CPZ 1 mg/mL, e) CPZ 2 mg/mL, f) CPZ 4 mg/mL.

Analyzing the impact of radiation against the CPZ molecules in the first 10 s, an increase in the fluorescence intensity of GelMa 10%-CPZ is observed when the concentration of CPZ increases. After 1 min of irradiation, the fluorescence intensity of GelMa 10%-CPZ 1mg/mL starts to decrease similar to that of CPZ, while for GelMa 10%-CPZ (2 and 4) mg/mL the decrease in intensity is slower than that of CPZ (2 and 4 mg/mL). These changes suggest that the same photoproducts are also formed in solutions with GelMa 10%, photoproducts that can help to crosslink the hydrogel.

For all hydrogels the same profile of fluorescence kinetics as in the case of CPZ solutions is observed, but with a fluorescence intensity for hydrogels higher than that of CPZ solutions. The IR spectra of the dry hydrogels show a major influence of GelMa, most of the IR bands of GelMa being present. The IR bands corresponding to CPZ solutions 2 mg/mL irradiated for 30 min can also be observed in the IR spectra.

The hydrogels resulting from the irradiation of GelMa 10%-CPZ released all the amount of CPZ in the first 2 h. As expected, the higher the concentration of CPZ in the hydrogels, the greater the amount of CPZ released. After a 24 h immersion, the hydrogels begin to decompose, which suggests that the complete crosslinking of GelMa has not occurred.

The antimicrobial activity of unirradiated and irradiated CPZ loaded hydrogels against *Staphylococcus aureus* (*S. aureus*) ATCC 25923 and methicillin-resistant *S. aureus* (MRSA) was investigated by the disk diffusion method and the colony forming unit assay. For *in vitro* studies, L929 fibroblast cells were used to examine cell morphology, viability and proliferation following interaction with the hydrogels. Morphology was examined by optical microscopy, and cell viability and proliferation were quantitatively assessed by MTT and LDH assays. These analyzes were performed to determine the cytotoxic effect of the hydrogels.

For Irgacure 0.05%-GelMa 10% - CPZ (1, 2, 4 mg/mL) and GelMa 10% -CPZ (1, 2, 4 mg/mL) hydrogels it was observed they inhibit the growth of MRSA and are more effective than penicillin in preventing adhesion of MRSA strains. Supplementary, the CPZ irradiated hydrogels showed a non-cytotoxic effect against L929 fibroblast cell lines

GelMa 10% - CPZ hydrogels (1, 2, 4 mg/mL) obtained as a result of irradiation for 1 and 5 min, even if they showed antimicrobial activity against MRSA, began to degrade after 24 h immersion in PBS, which did not make them ideal candidates for controlled release of CPZ. For Irgacure 0.05%-GelMa 10%-CPZ hydrogels (1, 2, 4 mg/mL), even though all of them showed a strong antimicrobial effect, only Irgacure 0.05%-GelMa 10%-CPZ 1 mg/mL irradiated for 1 min released CPZ irradiated

up to 84 h, making it the best hydrogel in terms of antimicrobial activity and drug release obtained in this project.

In this way the objectives of the project for this stage were met and the estimated results were obtained.

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1. Negut, C. Ristoscu, T. Tozar, V. Grumezescu, C. Hapenciuc, C. Mihailescu, L. Floroian, I.N. Mihailescu; MAPLE double structures bioglass–PMMA as potential anticorrosive, antimicrobial and drug delivery platforms; "Tehnologii Emergente în Ingineria Materialelor – EmergeMAT", 29-30.10.2020, Bucuresti, Romania – poster presentation

2. T. Tozar; M. Boni; S. Nistorescu; ML. Pascu; A. Staicu; Photodegradation study of Irgacure 2959 during hydrogel formation via 266 nm pulsed laser radiation; OSA Biophotonics Congress: Optics in the Life Sciences, virtual conference, 12–16 April 2021, USA - poster presentation.

3. T. Tozar; M. Boni; S. Nistorescu; ML. Pascu; A. Staicu; Hydrogels photo-crosslinking by 266 nm pulsed laser radiation; 9th International Conference on Radiation in Various Fields of Research, 14-18 June 2021, Montenegro - poster presentation

4. T. Tozar; M. Boni; S. Nistorescu; ML. Pascu; A. Staicu; Irgacure 2959 photodegradation study during hydrogel synthesis using 266 nm pulsed laser light; 23rd International Conference Materials, Methods & Technology, 19-22 August 2021, Bulgaria - oral presentation

5. T. Tozar, M. Boni, S. Nistorescu, M. Bojan, A. Staicu; Photopolymerization of gelatin methacryloyl (GelMA) hydrogels: synthesis, properties, and biological application, 1st Central and Eastern European Conference on Physical Chemistry & Materials Science (CEEC-PCMS1), 26-30.07.2022, Split, Croatia – poster presentation

6. T. Tozar, M. Boni, S. Nistorescu, A. Staicu; Pulsed laser photo-crosslinking of gelatin methacrylate hydrogels for the localized delivery of chlorpromazine, 20th International Balkan Workshop on Applied Physics and Materials Science, 12-15.07.2022, Constanta, Romania – poster presentation

7. T. Tozar, M. Boni, S. Nistorescu, A. Staicu; Designing gelatin methacrylate hydrogels using pulsed UV radiation for controlled delivery of chlorpromazine, The International Conference on Lasers, Plasma, and Radiation – Science and Technology (ICLPR-ST), 7-10.06.2022, Bucharest, Romania – poster presentation

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