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STABILITY OF THE NEOMYCIN ANTIBIOTIC IN IRRADIATED POLYMERIC BIOMATERIALS

Angelica Tamiao Zafalon*[1], Vinicius Juvino Dos Santos[1], Ademar B. Lugão[1], Vijaya Rangari[2], Samuel Temesgen[3], Duclerc Fernardes Parra[1]

[1]Nuclear and Energy Research Institute (IPEN-CNEN/SP)-Av. Prof. Lineu Prestes, 2242, Cidade Universitaria, Zip Code 05508-000 São Paulo, SP, Brazil.
[2]Department of Material Sciences, College of Engineering, Tuskegee University, Tuskegee, AL 36088, United States.
[3]Department of Pathobiology, Tuskegee University, College of Veterinary Medicine, 1200 W. Montgomery Road, Tuskegee AL 36088, United States.

***Corresponding Author: Angelica Tamiao Zafalon**
Nuclear and Energy Research Institute (IPEN-CNEN/SP)-Av. Prof. Lineu Prestes, 2242, Cidade Universitaria, Zip Code 05508-000 São Paulo, SP, Brazi.

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ABSTRACT
Hydrogels can be produced by gamma radiation at 25 kGy. Free radicals from water radiolysis are responsible by crosslinking of the polymer and chain scission. On the other hand, gamma radiation can lead degradation of pharmaceuticals. Neomycin loaded hydrogel was prepared and the stabity of neomycin was evaluaded. Excipents were used to measure radioprotector potencial each one. HPLC-MS/MS method and thermal analysis were used to analysis effect of gamma irradiation in neomycin stability. PEG was a excipients in hydrogel formulation and showed to be a radioprotector. Neomycin was stable under gamma radiation when loaded in hydrogel.

KEYWORDS: Hydrogel, gamma radiation, degradation, antibiotic, neomycin, stability.

INTRODUCTION
Hydrogel is a class of biomaterials, composed crosslinked polymer, insoluble with ability to absorb large quantities of fluids and water without losing its integrity. These materials are extensively used pharmaceutical applications as drug delivery systems (Rosiak, 1993; Ahmed, 2013; Galante et al., 2018)]. Hydrogels can be produced by physical or chemical crosslinking methods. Gamma irradiation using ^{60}Co sources has been used to obtain hydrogel because its advantages as providing crosslinking, penetrating power, allows operating at low temperature, chemical-free and the promote sterilization single step (Abuhanoglu 2010; Kazakis, 2016; Tipnis, 2017; Galante et al., 2018) Dose of 25 kGy has been applied in industrial of drug and food for the sterilization of products in their final package, mainly of heat sensitive pharmaceutical formulation (Nakamura et al., 2015; Kazakis, 2016; Tipnis, 2017; Mozalewska, 2017; Davison et al., 2018; Vallotto et al., 2017).

During the irradiation process, water radiolysis produces free radicals as: $e^-_{aq.}$; H^+; OH^-, they interact with other molecules and several chemical reactions are initiated resulting in the formation of different products (Albarran, G. 2018). When those radicals interact with polymer chain initiating the crosslinking of the polymer, chain scission occurs leading to produce covalent insoluble compounds (Kalubowski, 2013). In case, this reaction is expect to obtain hydrogel by gamma radiation method (Park et al., 2012). On the other hand, sterilization by gamma radiation can lead to degradation of the material, compromising the drug effectivity and performance. (Kazakis, 2016; Galante et al., 2018). There are several studies that discuss organic compounds degradation by Advanced Oxidation Process (AOP). Gamma radiation is one method of AOP that produces reactive species mainly hydroxyls radicals able to degrade organic compounds such as pharmaceutical during treatment wastewater (Csay et al., 2012; Alkhuraiji, 2017; Albarran, 2018; Chu, 2018, Alsager et al., 2018).

Antibiotics are classified according their chemical structure and structural complexity degree, such as presence of heterogeneous atoms, single cyclic, aromatic ring, different molecules sizes, which could results in center of the hydroxyl radicals attack (Alsager et al., 2018, Chu et al., 2018). Drug degradation with aromatic structure is known, but there is a knowledge gap about drugs containing amine in their structure (Chu et al., 2018). It has been found that hydroxyl free radical from radiolysis is the main responsible to induce expressive structural changes in compound, which results in the loss of structural and functional integrity of various antibiotics, including betalactam, carbapenems, cefaclor,

sulfamethoxazole. In addition, it has been established that hydroxyl free radical from radiolysis is an electrophile high powerful oxidant and the main responsible by inducing expressive structural changes in compounds of higher electron density (Chu et al, 2018). This interaction can result in initial hydroxylation, ring opening and backbones fragmentation reaction, followed by the loss of structural and functional integrity of various antibiotics, including betalactam, carbapenems, cefaclor, sulfamethoxazole, amoxicillin, doxycycline, ciprofloxacin and other pharmaceutical like salicylic acid, diclofenac and ibuprofen (Yu et al., 2008; Banaschik et al., 2018; Chu et al., 2018, Kim et al., 2017; Albarran 2018; Rubasinghege et al., 2018; Reina et al., 2018, Alsager, et al., 2018).

Although gamma radiation is well known method employed to obtain hydrogels, few studies of drug interaction under radiation are available to elucidate its stability and applicability in the hydrogel. In most case, antibiotics were loaded hydrogels by swelling equilibrium method, where hydrogel were sought in saturated solution. Recent studies have shown antimicrobial hydrogels containing antibiotic as chlorhexidine, moxifloxacin, gentamicin, ampicillin, amoxicillin, tetracycline and amikacin. In these examples, the drugs were loaded after irradiation or the hydrogels were obtained by chemical crosslinking method. In both cases, there were not antibiotics degradation by radiolysis. (Singh, 2009; Namazi et al., 2016; Singh, 2016; Singh et al., 2016; Hu et al., 2017; Flores et al., 2017; Sood et al., 2017 Alsager et al., 2018; Anirudhan 2018;). As already mentioned above, the hydrogel will be placed on the wound and it is extremely important that it maintains its sterility until the moment of use, so as not to be a contamination loader for the wound. When the drug is loaded after the radiation process, the sterility can be compromised by the handling and immersion of the hydrogel in the solution saturated with the drug, since the studies do not describe if there was concern about the sterility.

When incorporating the drug into the hydrogel formulation and then irradiating at 25 kGy dose in its final package, the hydrogel will be sterile. This dose is indicated for both polymer crosslinking and material sterilization.

Therefore, proposing drugs stability under radiation should be individually investigated before using gamma radiation method to obtain hydrogel as controlled release of antibiotic. In this context, three factors can influence

In this context, there are three factors can affect the drug stability under radiation reported as percentage of water, concentration of the sample and presence of radioprotectors. The generation of the free radicals depends on presence of water for being produced during irradiation process. After generated, free radicals interact to the drug, this interaction is dependent of drug initial concentration, once the increased competition reaction of reactive species with target compounds. In additional, radioprotectors can absorb generated radicals and so inhibiting drug degradation (Nakamura, K. et al, 2015; Chu, L. et al, 2018). Several excipients can be applied in formulation as radioprotector, such as polyethylene glycol (PVP), buffers, salts and surfactants (Nakamura et al., 2015; Capanema et al., 2018). Theses compounds are known scavenger and absorb free radicals produced by radiolysis. If present in the formulations, they could protect the drugs against the deleterious effect of radiation. Suitable information is not available in the literature about radiation effects of many products, especially of drugs that are unstable in presence of water (Varshney 2004). Drug effectivity and stability after radiation are the main point of interest of this study. Degradation of until 5% has been observed in pharmaceutical solids under gamma irradiation at 25 kGy dose, but this average is higher when drug is irradiated in aqueous solutions until completely degradation (Varshney, 2004; Yang 2017; Kim, et al., 2017; Tegze et al., 2018).

Neomycin is an aminoglycoside antibiotic widely used for topic infections treatment. This antibiotic has a hydrophilic property and it has effect against Gran positive and Gran negative bacteria (Abuhanoglu 2010; Stypulkowska et al., 2013; Wan et al., 2018). In this case, two points can be sought. First, in the production of hydrogels drug encapsulated for topical uses is the retention of encapsulated drug interfering with the efficacy of drug release into the hydrogel and, second, it evaluates drug stability undergoes radiation. Drug degradation with aromatic structure is known, but there is a knowledge gap about drugs containing amine in their structure, and neomycin has nitrogen in its structure (Chu 2018).

Motivated by this purpose, this work evaluated the stability of a neomycin under gamma radiation. Differential scanning calorimetry (DSC), Termogravimetry, and high-performance liquid chromatography coupled mass detector (HPLC-MS/MS) were used to measure and compare the stability and structural integrity of neomycin before and after irradiation. Antimicrobial activity was measured by disc diffusion.

MATERIAL AND METHODS

Materials

N-vinyl-2-pyrrolidone (PVP) K-90 (Mw 360.000), polyethylene glycol – 300 (PEG), agar and neomycin sulphate were obtained from Exodo Cientifica, Oxiteno, Oxoid and Sigma Aldreich, respectively in pharmaceutical degree.

Insert Figure 1

Preparation of hydrogels

The crosslinking of the PVP hydrogel was induced by irradiation process. The hydrogels syntheses were

performed on the mixture of PVP polymer (6% wt), PEG (0,45%) and agar (1,5% wt). The compounds were mixed in water and heated to 85°C for 5 min, then cooled to 50°C for addition of neomycin. The solution was placed in *polyethylene terephthalate* (PET) molded forms in volumes of 10 mL and vacuum sealed in the sequence. The sealed samples were irradiated at 25 kGy gamma irradiation dose at 6 kGy/h rate in order to crosslink and sterilize the polymers gelified solution.

Neomycin analysis by HPLC-MS/MS

The standard of neomycin sulfate was weighed into volumetric flasks and dissolved with the distilled water to produce stock solution at concentration 1 mg mL^{-1} (1000 ppm) and successively dilutions were done to obtain five required concentration (20, 50, 100 200 and 400 µg mL^{-1}). Neomycin concentration was measured by HPLC-MS/MS (Agilent Technologies 1290 Infinity) coupled with a C18 Aquasil column (100 x 2.1) 5µm and MS–MS system used was AB Sciex 3200 Q Trap equipped with an electrospray interface (ESI). Mobile phase was a mixture of acetonitrile, water and formic acid and a flow rate of 250 µL min^{-1}. The MS-MS analysis were carried out in the positive mode and full scan acquisition 250 – 620 m/z. Zhaobing Guo.

Neomycin Stability study

Standards of neomycin sulfate with mannitol, PEG and isopropilic alcohol (1:3 w/w) were irradiated at 25 kGy dose. The samples were analyzed by HPLC-MS/MS.

Thermal analysis

The hydrogels were lyophilized and thermogravimetric analysis (TG) and derived termogravimetry (DTG) were carried out from Mettler Toledo equipment. A sample mass of 5.0 ± 0.1mg was weighted. The program consisted in heating rate of 10°C min^{-1} in the range of 25 to 600ºC, under N_2 atmosphere flowing at 10 mL min^{-1}. The DSC curves analyses were carried out using a Mettler Toledo equipment. The samples mass of 5.0 ± 0.1 mg were heated at 10°C min^{-1} from 25 to 350°C in nitrogen atmosphere at 10 mL min^{-1}.

Antimicrobial activity

Antimicrobial effect of the hydrogel/neomycin was carried out by disc diffusion with Gram negative (*P. aeruginosa)* strain. Bacteria was incubated at 37°C in LB broth overnight. The sterile cotton swab was dipped three times in bacteria suspension gently spread onto ISO-Agar plates. The hydrogels were cut in small circular disks and gently placed on the bacteria lawn. The plate was incubated overnight at 37°C. Ciprofloxacin was used as positive control and hydrogel without neomycin, as negative control. The inhibition halos were observed and measured with ruler. Images of the plates were taken using Aplha Imager HP. The experiments were carried out in triplicate.

RESULTS AND DISCUSSION

Effect og gamma irradiation on the neomycin stability

Gamma irradiation has been explored to produce hydroxyl free radical for oxidation pharmaceuticals in treatment wastewater to avoid bioaccumulation environmental (Alkhuraiji 2017). In this way, it is possible to compare the dose used in this method to degrade these compounds in water and to discuss the relation of the impact of the radiation on the drugs during crosslinking. In summary, drug degradation rate increases with increasing radiation dose when drug were irradiated in aqueous solutions (Chu et al., 2018). Previous studies have been evaluated and the results showed that amphetamine solution was completely degraded at 2,8 kGy and anti-inflammatory drugs such as ketoprofen, and diclofenac were stable until 5 kGy dose. Antibiotic degradation in aqueous environment is well documented, with many research evidenced chlorafenicol was destroyed with 7 kGy, while penicillin and amoxicillin were degraded with dose above 12 kGy. (Csay 2012; Madureira et al., 2017; Alkhuraiji 2017, Chu et al., 2018).

In this context, gamma radiation has revealed to be an important method to degradate pharmaceuticals in wastewater. However, it remains unclear what impact that radiation has on the stability of the drug when it is irradiated with excipients and polymers for obtaining hydrogels. Considering neomycin loaded hydrogel; drug stability was evaluated under radiation in excipients presence such as mannitol, PEG, isopropyl alcohol, and analyzed by the HPLC-MS/MS method. It can be observed in Fig. 2 that the irradiation process influenced in a different way the stability of each sample. The non-irradiated neomycin obtained the highest intensity, followed by the irradiated powder sample. Aduhonoglu (2010) observed in their studies a reduction of 5% in neomycin concentration as a result of irradiation at 25 kGy dose. In our work, neomycin was irradiated in powder, no presence water, so it can be affirmed that no free radical from radiolysis was produced and there was not degradation.

HPLC-MS/MS analysis showed that neomycin solution decreased 80% its intensity after irradiated at 25 kGy. Gamma radiation is known as an effective method to degrade pharmaceutical in water presence due mainly hydroxyl radical produced from radiolysis. Therefore, it is important to study the behavior of neomycin under the radiation, as well as its interaction of the other components of the formulation. It is thus possible to correlate if it is possible to use neomycin for the production of hydrogel by gamma irradiation.

In pharmaceutical industry, non-pharmacological active substances, known as excipients, may be incorporated into the production of medicine. When present, excipients improve the properties active pharmaceuticals. It has been reported that some excipients may interact

with free radicals from water radiolysis decreasing degradation of pharmacological agents under radiation (Nakamura et al., 2015; Vallotto et al., 2017; Chu et al., 2018).

Mannitol is an alcohol from mannose family vastly used as excipient. Neomycin sample was irradiated with mannitol at 25 kGy dose. Stability curve obtained similar result than irradiated neomycin solution, thus indicating there was degradation in both samples. This result indicates that mannitol had no protective function. Although, Sleger (2006) studied metoprolol degradation under gamma radiation and suggested that radiolysis of drugs in the solid-state, is not enough to the degradation of metoprolol powder at the dose of 25 kGy. And when metropolol was irradiated in solution and in presence of mannitol, this reacts quite well with hydroxyl radicals and protected drug against gamma degradation. Indicating the appropriate choice of excipients, contributed to radioprotecting pharmaceutical. Therefore, mannitol can act as radioprotector at high concentration but in this study was used 1.5 mg of mannitol, this concentration may have been insufficient for the desired effect.

Insert figure 2
Irradiated Neomycin solution in the presence of PEG and isopropyl alcohol presented peak intensity reduction of 9.01 and 16.17%, respectively. These results indicate that PEG and alcohol showed a protective effect of neomycin against the free radicals produced by the gamma radiation.

The influence of PEG as radioprotector has also been studied, and the results are illustrated in Fig.2, the Intensity neomycin in presence of PEG was very high around 91%; so the results have showed PEG radioprotector function, it can be easily explained once PEG has high solubility. When it is in aqueous solution, PEG chains remained around the neomycin and this effect can be utilized to control the free radical attack of radiation and protecting neomycin from degradation during the crosslinking process. (Lugao el at, 2002; Park J S et al, 2012). Different concentrations of PEG on the hydrogel formulation have also been employed to decrease or reduce the gelation % and swelling of the prepared hydrogel according to the irradiation dose. Studies have reported that the addition of PEG to the hydrogel composition could improve efficiency of the hydrogel barrier against microrganism from environment and prevent secondary infection (Lugão et al., 2002; Ajji et al., 2005; Singh et al., 2016; Goncalez-Torres et al., 2018).

These characteristics of PEG provide important information and explain the importance of the presence of the PEG in the hydrogel formulation.

It is well known that drug stability also depend on chemical reactions that can occur during the manufacturing process. Theses chemical reactions include exposure to heat, moisture, light, or an oxidizing atmosphere for example free radical from radiolysis. Many excipients are employed to increasing shelf life of drugs and protecting them against humidity, atmospheric oxygen and thermal degradation.

When an interaction occurs between excipient and drug, the physicochemical properties such as solubility, hydrolysis, oxidation and polymerization, may be modified, and thus the stability can be changed. A DSC is frequently used to provide information of the thermal properties of the solid pharmaceuticals and it has been used to evaluate drug such as angiotensin converting enzyme, lisinopril and enalapril (Lin 2012). Thermal stability study of the solid pharmaceutical drugs is important to evaluated losing the drug activity, degradation rate or also toxins generation that can decrease the effectiveness and increase the toxicity of the drug (Lin 2012).

Thermal stability of neomycin was evaluated by DSC. Neomycin was irradiated at 25 kGy dose, and its stability was compared with no irradiated sample. In the results shown in Fig. 3, two crystalline melting peaks can be observed with maximum values of 154.42 and 158.63°C.

Insert figure 3
Neomycin is composed of two isomers (B and C), and their concentration can change according to the production process (Stypulkowska 2013). However, the B isomer concentration is always higher than C. In the DSC profile, the first peak corresponds to the crystalline fusion of the C isomer, and the second is associated to B isomer.

Non-irradiated neomycin curve overlapped with date of the irradiated neomycin. Irradiated neomycin powder has shown minimal degradation when undergoing gamma radiation dose of 25 kGy.

In the Fig. 4, it can be observed irradiated and non-irradiated neomycin DSC profiles. In irradiated samples curve, the C isomer melt has a T_{onset} 146.32°C, and the B isomer, 163.10°C, where 398.86 mJ was required to complete the C isomer melting and 2666.53 mJ, for the B isomer.

In the non-irradiated neomycin curve, the C isomer melting was found to have T_{onset} 149.75°C, and the B isomer, 160.97°C, where 402.57 mJ was required for C isomer and 1967.28 mJ for B isomer.

According these results, it was possible to inquire the ratio between B and C isomers. The ratio was 87/13 (w%) for non-irradiated neomycin and 83/16 (w%) for irradiated neomycin. Similars results were reported by Roetz (1995) in their studies for the determination of neomycin isomers by liquid chromatography.

The difference melting point between samples reported in Fig. 4 can be attributed by the effect of gamma irradiation. Irradiated neomycin curve had a shift to the left, with a melting peak of 3 degrees lower than non-irradiated neomycin curve. Fig. 4 shows the TG / DTG profile of the non-irradiated neomycin, hydrogel and hydrogel/neomycin. The mass loss and their corresponding temperature range are shown in Table 1.

Insert figure 4
Insert Table 1
No irradiated neomycin exhibited a mass loss of 9.5% to 179.9°C, which is mainly due to water loss and it was stable up to 185.8°C, above which it begins to decompose into two distinct peaks around 233.3 and 290.5°C, with a residual mass of 43.5%.

It can be explained perhaps owing to thermal degradation of a new material formed by residual of the first stage of the degradation process, and the large mass loss in air in this step indicates the oxidation of residual mass. In the TG/DTG curves of hydrogel/neomycin, second step is shifted toward lower temperature compared to the hydrogel, indicating the presence of neomycin degradation. From Table 1, it can clearly see that neomycin present in hydrogel exhibit higher thermal stability than pure. The major mass losses are observed in the third decomposition step, in the range of 343.1-499.6°. This peak is characteristic of degradation PVP.

DSC and TG profiles clearly showed that neomycin could be heat and gamma radiation sterilized because its melting peak and Tonset were above sterilization temperature by autoclave, where it is applied 121°C as sterilization temperature and hydrogel showed Tonset above 176°C, resulting high thermal stability.

Therefore, neomycin is an antibiotic topic, it is not necessary to be sterile, but wound dressing should be it. As gamma radiation at 25 kGy is responsible for crosslinking and sterilization in one step, DSC and TG analysis indicated no changes in thermal stability after neomycin irradiation process. It is important to add other event in this discussion. The initial solution concentration could influence in degradation rate (Chu 2018, Hayrabolulu et al., 2018). The solute increase could increase targets number for the free radicals from radiolysis, in competitive effect. In view of the fact that hydroxyl radicals and hydrogen atoms interact with polymer to crosslink reaction, few reactive radicals were available to degrade the neomycin.

Antimicrobial assay was carried out by diffusion disc method. Hidrogels samples were cut, placed in bacterial lawn and incubated by 24h. The inhibition zones can be observed in Fig. 5.

Insert figure 5
The positive control (Fig. 5a) did not exhibit inhibition halo, indicating that the hydrogel formulation itself did not able to kill bacteria.

Inhibition zone diameters Hydrogel/neomycin against *P. aeruginosa* obtained the values of 22 ± 2 mm (Fig. 5c).

In summary, results indicated that neomycin was released from hydrogel, once hydrogel was used with a drug delivery system. Inhibition halo presence around hydrogel/neomycin sample demonstrated that neomycin has effectiveness against bacteria. Even with degradation around 9% after irradiation at 25 kGy, neomycin remained effective against bacteria. These results consolidate the importance of the excipients used in the pharmaceuticals manufacture, since excipients can act many ways in formulation, including, as radioprotectors act against the degradation coming from free radicals.

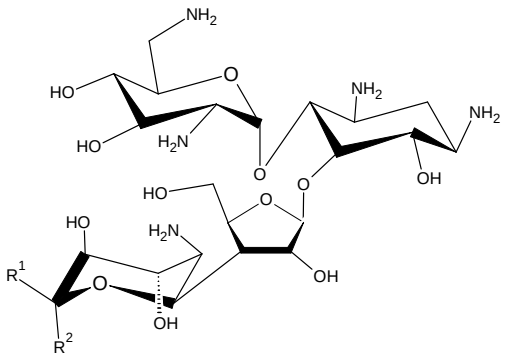

Fig. 1: Neomycin chemical structure; R1= CH_2NH_2; R2 =H_2.

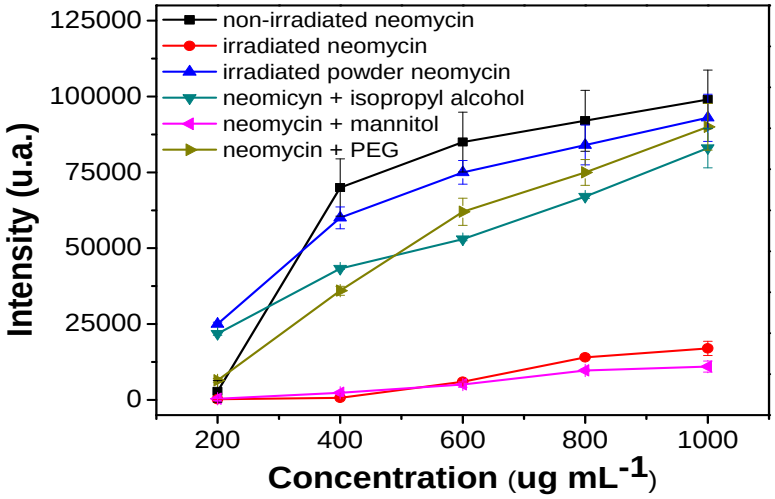

Fig. 2: Neomycin stability irradiated of at 25kGy. Comparison between different radioprotectors.

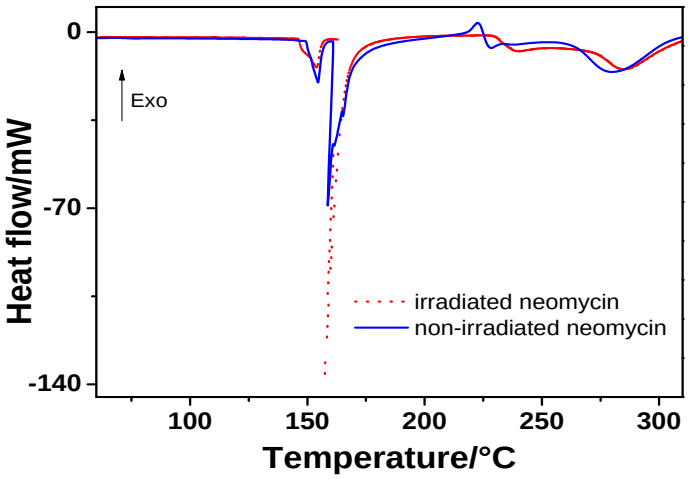

Fig. 3: DSC profiles of neomycin irradiated and non-irradiated.

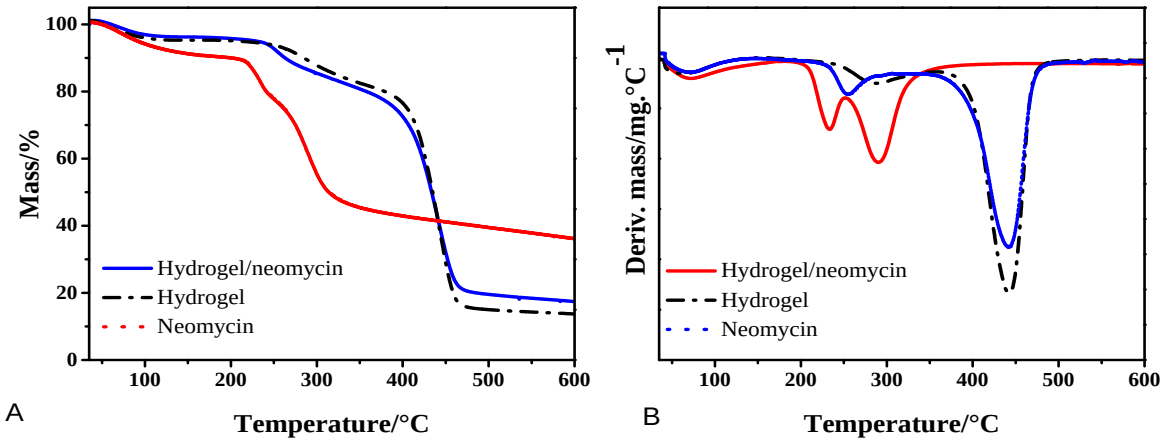

Fig. 4: (A)TG and (B) DTG curves for non-irradiated neomycin, hydrogel/neomycin and hydrogel, obtained under N_2 flow.

Fig. 5: Antimicrobial activity assay by diffusion disk in Hydrogel/neomycin against *P. aeruginosa.* (a) hydrogel no drug (negative controle); b) Ciprofloxacin disc (positive control) and c) hydrogel/neomycin sample.

Table 1: TG data of degradation for neomycin, hydrogel/neomycin and hydrogel under nitrogen flow.

Sample	Mass loss (%)	T *onset* (°C)	T *endset* (°C)	DTG max (°C)
Neomycin	9,5	35,9	179,9	71,8
	12,9	185,8	251,5	233,3
	33,8	251,5	384,4	290,5
Hydrogel/neomycin	3,7	40,6	142,2	70,8
	13,0	175,4	325,6	254,7
	61,8	343,1	499,6	441,7
Hydrogel	4,6	36,8	156,9	64,8
	12,7	229,2	347,7	289,4
	66,8	357,7	495,7	442,1

CONCLUSION

Neomycin showed different degree of degradation by free radicals from radiolysis when irradiated at 25 kGy. Thermal stability of neomycin was evaluated by DCS and TG. Thus, it can be inferred that the stability of neomycin under ionizing irradiation is due to a set of factors such as the presence of water, concentration and the presence of radioprotectors in the hydrogel formulation. Antimicrobial analysis illustraded effectiveness of neomycin released from hydrogel against P. aeruginosa.

This study was important to elucidate the stability of neomycin under radiation and whether the incorporation of this drug into the hydrogel could be performed before or after the radiation process. By determining the stability of neomycin, the gamma radiation method can be employed to obtain the hydrogel and also ensure sterility in a single step.

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