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New Studies *In vitro* Antibacterial Evaluation and Cell viability in PMMA-G-PEG 4000 Derivatives with Encapsulated Erythromycin

Novos Estudos de Avaliação Antibacteriana in vitro *e Viabilidade Celular em PMMA-G-PEG 4000 Derivados com Eritromicina Encapsulada*

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Synthetic polymers have been extensively studied in several areas of knowledge, one of which is the pharmaceutical. The drug when encapsultated into the PMMA-g-PEG 4000 matrix can produce fewer side effects. The most frequent side effects of erythromycin are gastrointestinal and are dose related. Nausea, vomiting and diarrhea occur in low frequency with the usual oral doses. The drug used in this study was Erythromycin, an antimicrobial that acts directly on bacterial protein synthesis. Erythromycin was encapsulated into PMMA-g-PEG 4000 derivatives. All copolymers afther encapsultaions were characterized and identified by atomic force microscopy (AFM). *In vitro* assays of release, antimicrobial activity and cytotoxicity were performed for the copolymers obtained. The copolymers PMMA-g-PEG 4000 HAL and PMMA-g-PEG 4000 ACET showed drug controlled release profiles. All PMMA-g-PEG 4000 derivatives showed antibacterial activity against *Staphylococcus aureus* and did not show a cytotoxic effect on human fibroblasts.

Keywords: Copolymers; PMMA-g-PEG 4000; Erythromycin; controlled release; antibacterial activity.

1. Introduction

Polymers are very versatile for a series of applications including pharmaceutical applications.[1] Medicated polymeric films have found great application in topical therapy, being easily applied and avoid the troubles encountered in oral dosage forms.[2] Due to their broad range of applications, particularly for the controlled release of drugs, polymers are among the most widely used excipients in pharmaceutical technology.[3] Synthetic polymers are employed as excipients in the manufacture of cosmetics and systems for conventional and modified delivery of drugs. More recently, polymers have been developed to deliver drugs to target places. The advances of new drug delivery systems will only be possible with the development of polymers specifically for the pharmaceutical field.[1] In addition, the selection of the polymer is also influenced by the desired mechanism for drug release. To achieve specific drug release profiles, synthetic polymers may be chemically modified. Future developments in polymer sciences will be based on modifications of the chemical and physical properties of the polymers and on new combinations of copolymers able to provide a programmed and controlled release of a wide variety of drugs. Medicated polymeric films have found great application and can avoid the troubles encountered in oral dosage forms and are designed to deliver the drug at a controlled rate.[2] Controlled releases are applied to pharmaceutical forms solids and developed to release the drug gradually, maintaining the plasma concentration at therapeutic levels for a prolonged period and increasing the patient's adherence to the treatment. They also reduce fluctuations in the drug's blood concentration, avoiding subtherapeutic or toxic levels.[4] The drug when encapsulated into the PMMA-g-PEG 4000 copolymer can produce fewer side effects. The drug used in this study was Erythromycin, an antimicrobial that acts directly on bacterial protein synthesis. The Erythromycin is indicated as drug of first choice in the treatment of the acne and other illnesses that affect the skin. PEG 4000 and PMMA-g-PEG 4000 have shown promise for enhanced drug delivery and can be applied to drug formulations to prolong the half-life of the drugs.[5] In this work, encapsulation and controlled release of the drug Erythromycin in the PMMA-g-PEG 4000 and derivatives were performed.[6-8] In vitro antibacterial evaluation was made against Staphylococcus aureus and cytotoxic effect study in human fibroblasts.

2. Experimental Part

2.1. Materials and Methods

The reagents used for encapsulation of Erythromycin in PMMA-g-PEG 4000 derivatives were Commercial Erythromycin (Figure 1 (3)), dichloromethane (CH_2Cl_2), poly (vinylalcohol) were purchased from Sigma–Aldrich. The PMMA-g-PEG 4000 derivatives (Figure 1(2)) were synthesized by our research group and publicly available.[5-7] Atomic Force Microscopy (AFM) analyzes was carried out on a Bruker MultiMode 8 SPM, using the intermitent contact mode. Si cantilevers (NSC35/ALBS from MikroMash), with spring constants of 5–15 Nm^{-1} and a tip radius of curvature ~10 nm, were used throughout the study for in PMMA-g-PEG 4000 derivatives imaging. In vitro Controlled Release of the Erythromycin were done using UV/VIS spectrophotometer. Model FEMTO800Xi. The evaluation of the release on the ultraviolet spectrophotometer was performed in the wavelength of 320nm with Quartz bucket and the blank used was the buffer solution at a pH 7.2.

2.2. Encapsulation of Erythromycin in PMMA-G-PEG4000 derivatives

In order to obtain the polymeric Erythromycin films (Figure 2), 1.00 g of copolymer and 0.40 g of Erythromycin were added to a beaker with 10.0 mL of dichloromethane at approximately 30 °C (Figure 2 (1)). This system was kept mixing until complete dissolution. In a other beaker (Figure 2 (2)) containing 40.0 mL of water at aproximately 70 ºC, 0.40 g of polyvinyl alcohol (PVA) were poured into the organic phase (Figure 2 (1 and 2)). The mixture was subjected to agitation at 500 rpm for 6 h at 40 °C (Figure 2 (3)). Then, the products were taken to the oven at 40 °C for 24 h to evaporate the solvente dichloromethane (Figure 2 (4)). After this time, white films were obtained.

2.3. *In vitro* antibacterial evaluation

The in vitro antibacterial property was evaluated against Gram-positive bacteria *Staphylococcus aureus* ATCC 25923 by broth microdilution method to establish the minimum inhibitory concentration (MIC)[9]. Bacterium was cultivated in Müeller-Hinton agar medium at 37 °C, for 24 h. The inoculum was prepared using the direct colony suspension method by means of a saline (0.9 % NaCl) suspension of colonies selected from a 24 h agar plate, before each assay. The suspension was adjusted to reach turbidity equivalent to a 0.5 of the McFarland standard scale (1 x 10^8 CFU/mL). The inoculum were diluted 1:100 in broth in order to obtain a final assay with 5 x 10^5 CFU/mL. The polymers solutions were made in Müeller-Hinton broth to obtain final concentrations from 1000.00 to 0.49 µg/mL. 50 µL of sample and 50 µL of inoculum were added in a 96 wells

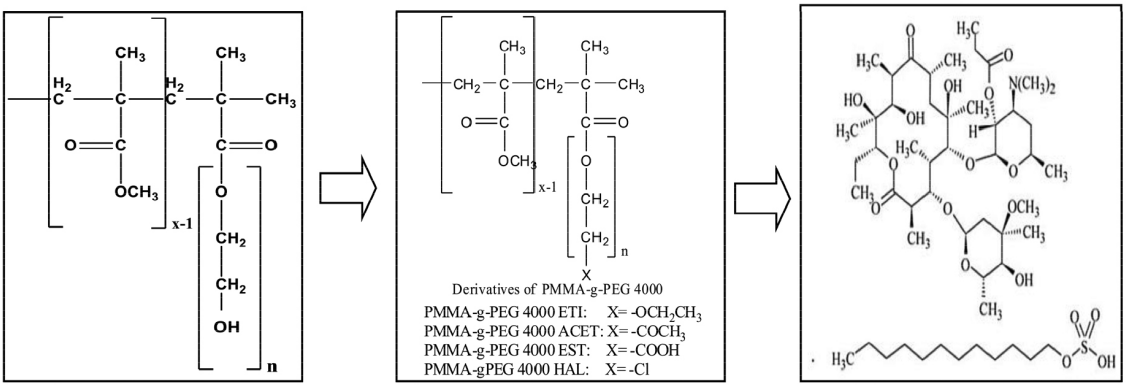

Figure 1. Chemical structure of PMMA-g-PEG 4000 (1), PMMA-g-PEG 4000 (2) derivatives and Erythromycin (3)[6-8]

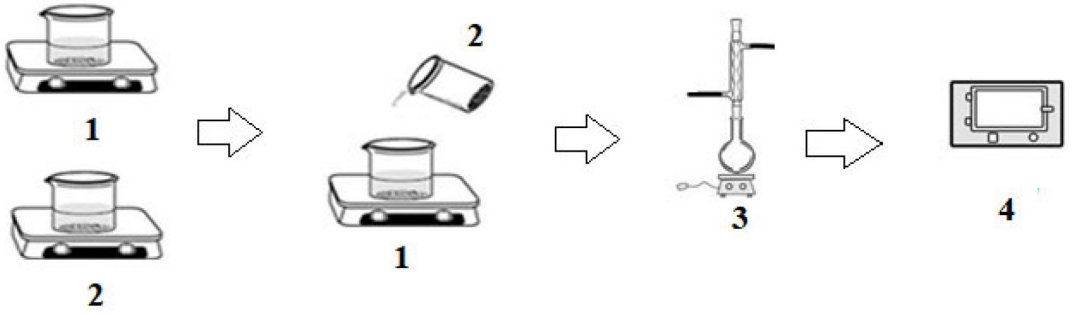

Figure 2. Encapsulation of Erythromycin in PMMA-g-PEG 4000 derivatives

plate. In the negative control were added 50 µL broth and 50 µL of inoculum. To verify the methodology efficacy, tetracycline (100.0 µg/mL) were used as positive control. Only 100 µL of broth were added in in the medium control. The plates were closed and incubated at 37°C, for 24 h. After the incubation period, 30 µL of triphenyl tetrazolium chloride (TTC, 0.25 mg/mL) were added and the plates were incubated for aditional 3 h color. The MIC were performed in triplicate and established as the smallest concentration in which no bacterial growth was detected (no visible color).

2.4. Cell viability

Human fibroblasts MRC-5 cells, cultivated in RPMI 1640 medium (Sigma®), were distributed in 96-well microtiter plate using a density of 5 x 10^4 cell/well and after they were incubated at 37°C with 5% of CO_2, for 24 h. Cells were treated with the samples dissolved in RPMI 2% DMSO, at concentrations ranging from 1000.0 to 62.5. Cell viability was evaluated using the sulforhodamine B assay (SRB).[11] After 24 h incubation, the media was removed and cells were fixed with cold 20% trichloroacetic acid for 1 h at 4°C. The microtiter plate was washed with distilled water and dried. Thereafter, fixed cells were stained for 30 min with 0.1% SRB dissolved in 1% acetic acid. The plate washed again with 1% acetic acid, again allowed to dry and 200 µL of 10 mM TRIS buffer (pH 10.5) were added to stain solubilization at room temperature for about 30 min. Samples absorbance was read in the spectrophotometer (490 nm) and the results were expressed as the percentage of viable cells over untreated cells.

2.5. *In vitro* Release Study of the Erythromycin

1.25 mg of each encapsulated PMMA-g-PEG 4000 derivatives were added in a buffer solution to prepare a solution of 25.0 mL with pH 7.2 and was left under agitation the room temperature. The concentration used for the release was 0.05 mg/mL. Every 15 minutes, an aliquot of the solution is placed in the quartz bucket was evaluated on the ultraviolet spectrophotometer at wavelength of 320 nm, starting from 0 (zero) to at most 4h.

3. Results and Discussions

3.1. AFM of PMMA-g-PEG 4000 derivatives with encapsulated Erythromycin

Samples for AFM analysis were prepared by the spread coating method, using 0.7 mg/ml of dichloromethane solution on a mica substrate and it was dried, after 30s, by a nitrogen flux. In all cases, considerable changes on the film organization can be seen, produced by a modification of the polymer chain and/or by encapsulation process. Here, it was possible to observe the isolated microspheres formation in Figure 3(a) the drug sample (diameter ~ 67±8 nm), in Figure 3(c) PMMA-g-PEG 4000 EST ERITR (diameter ~ 120±41 nm) and in Figure 3(e) PMMA-g-PEG 4000 ETI ERITR (diameter ~ 95±15 n). The PMMA-g-PEG 4000 HAL ERITR copolymer (Figure 3(d)) did not present the formation of isolated microspheres. The AFM image also shows significant variations in sample

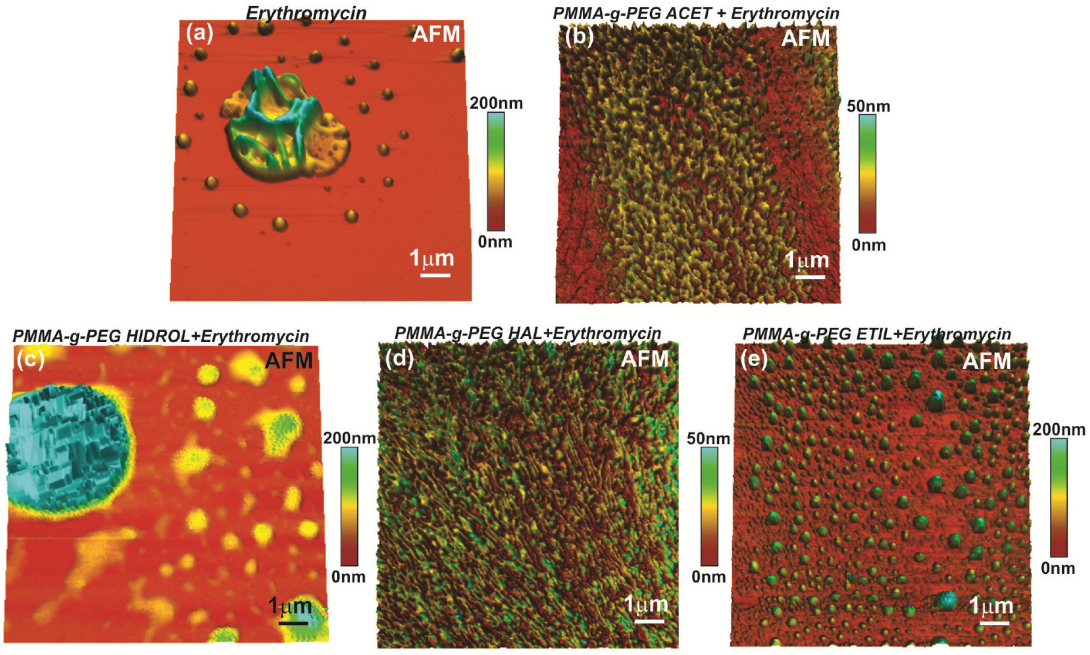

Figure 3. AFM images of Erythromycin sample (a) and PMMA-g-PEG 4000 derivatives: PMMA-g-PEG 4000 ACET ERITR (PMMA-g-PEG 4000 ACET + Erythromycin) , PMMA-g-PEG 4000 EST ERITR (PMMA-g-PEG 4000 HIDROL + Erythromycin), PMMA-g-PEG 4000 HAL ERITR (PMMA-g-PEG 4000 HAL + Erythromycin) and PMMA-g-PEG 4000 ETI ERITR (PMMA-g-PEG 4000 ETIL + Erythromycin), thin films after the encapsulation process (b-e)

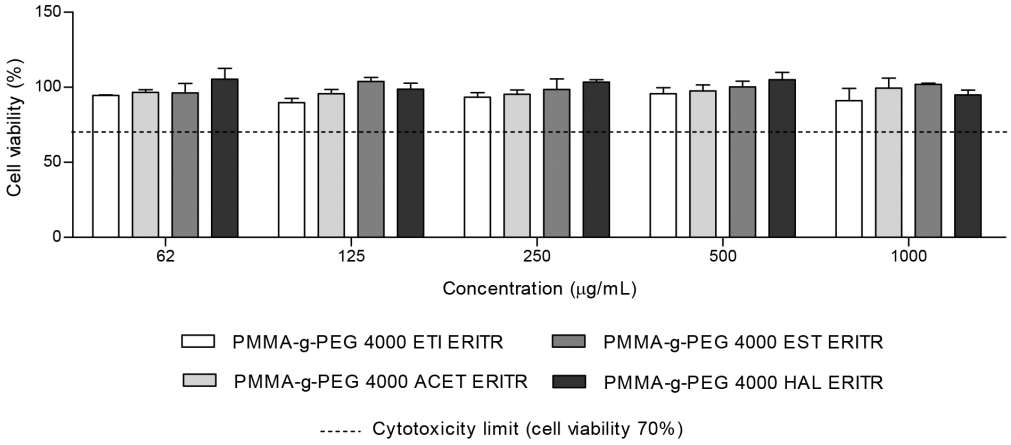

Figure 4. Cytotoxicity of the synthesized copolymers with encapsulated Erythromycin

roughness and in the film organization, probably due to interaction between the drug and the copolymer. The same behavior ocurrs to PMMA-g-PEG 4000 ACET ERITR copolymer (Figure 3(b)), which has an insatisfactory formation of microspheres.

3.2. *In vitro* antibacterial evaluation

The encapsulated Erythromycin in the PMMA-g-PEG derivatives demonstrated antibacterial activity, being the PMMA-g-PEG 4000 ACET ERITR with lower activity (lower MIC) (Table 1).

Table 1. Minimal Inhibitory Concentration (MIC) of the PMMA-g-PEG derivatives with encapsulated Erythromycin against *Staphylococcus aureus*

PMMA-g-PEG 4000 derivatives	MIC (µg/mL)
PMMA-g-PEG 4000 ETI ERITR	3.9
PMMA-g-PEG 4000 ACET ERITR	125.0
PMMA-g-PEG 4000 EST ERITR	3.9
PMMA-g-PEG 4000 HAL ERITR	3.9

3.3. Cell viability

The cytotoxicity results show that all copolymers were no toxic to MRC-5 human fibroblasts at tested concentrations (Figure 4), with cell viability greater than 70%.[12] These results indicate the safe use of these copolymers since the concentrations to antibacterial activity were considered whithout cytotoxic effect (Table 1).

3.4. *In vitro* Release of the Erythromycin

Figure 5 shows the in vitro release profiles of Erythromycin from the encapsulated PMMA-g-PEG 4000 derivatives. There was a fast release of erythromycin from the PMMA-g-PEG 4000 EST ERITR copolymer in the first 15 minutes. This large amount of drug released initially is known as burst effect.[13] This release oscillated during 150 minutes. After, a growing drug release profile was observed up to the end of the assay. The copolymers PMMA-g-PEG 4000 HAL ERITR and PMMA-g-PEG 4000 ACET ERTR showed slow and controlled drug release profiles during the 4 hours of evaluation. The PMMA-g-PEG 4000 ETI ERITR copolymer also showed a slow release of the drug, although this release was slightly higher after 135 minutes. The gradual drug release maintains the plasma concentration at therapeutic levels for a prolonged period of time. Thus, drug administrations are required less frequently compared to conventional release drug (immediate release). Therefore, the modified release of a drug from polymeric matrices may allow greater patient adherence to treatment and a reduction of side effects.[14,15]

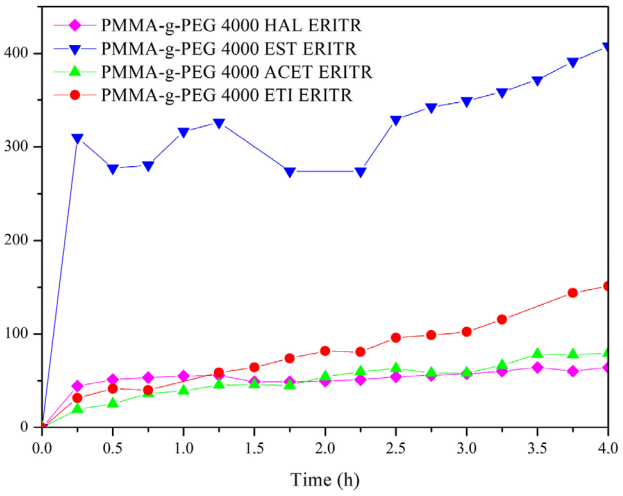

Figure 5. *In vitro* Erythromycin release profiles from the PMMA-g-PEG 4000 derivatives at pH 7.2 for 4 hours

4. Conclusions

All PMMA-g-PEG derivatives presented characteristics that identificated the encapsulation with Erythromycin. AFM images show that microsphere formation occurs for PMMA-g-PEG 4000 EST ERITR and PMMA-g-PEG 4000 ETI ERITR, which is indicative of a successful

encapsulation process. In the in vitro release study, it was observed that the drug was released in a controlled manner from the copolymers PMMA-g-PEG 4000 HAL ERITR and PMMA-g-PEG 4000 ACET ERITR, which is of great importance, since it can increase therapeutic efficacy and reduce toxic effects of the drug. Finally, the PMMA-g-PEG 4000 derivatives showed antibacterial activity against *Staphylococcus aureus* and did not show a cytotoxic effect on human fibroblasts.

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