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Layde Teixeira de Carvalho, Ana Julia R.M. Teixeira, Rodolfo Moraes, Rennan F.S. Barbosa, Rafaela Queiroz, et al.. Preparation and characterization of cationic pullulan-based polymers with hydrophilic or amphiphilic characteristics for drug delivery. *Reactive and Functional Polymers*, 2022, 181, pp.105441. 10.1016/j.reactfunctpolym.2022.105441 . hal-03831672

HAL Id: hal-03831672

<https://imt-mines-albi.hal.science/hal-03831672>

Submitted on 1 Dec 2022

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Preparation and characterization of cationic pullulan-based polymers with hydrophilic or amphiphilic characteristics for drug delivery

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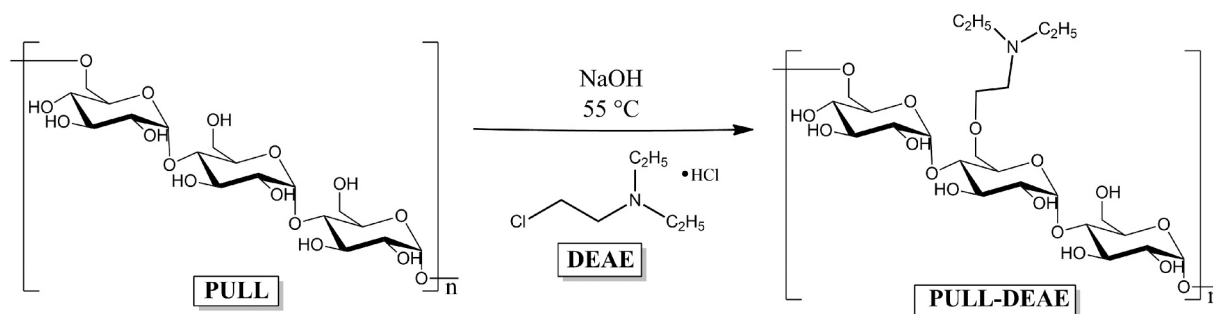
Pullulan has attracted considerable attention due to its immunogenicity, biodegradability, biocompatibility, and hydrophilic nature, making it useful in cosmetics and pharmaceutical applications. In addition, pullulan is considered a biopolymer capable of modification via chemical reactions, which is a promising strategy to expand the range of its applications. In this study, cationic pullulan-based polymers with hydrophilic or amphiphilic nature were successfully synthesized by nucleophilic replacement and ring-opening polymerization. Firstly, pullulan was partially modified with 2-Chloro-N,N-diethylethylamine (DEAE) to generate DEAE-modified pullulan (PULL-DEAE), a polymer with cationic and hydrophilic nature. Secondly, the remaining hydroxyl groups of PULL-DEAE were reacted with N-carbobenzyloxy-L-lysine N-carboxyanhydride (Lys(Z)-NCA), producing the grafted copolymer pullulan-DEAE-g-poly(Z-L-lysine) (PULL-DEAE-g-PZLL), a copolymer with cationic and amphiphilic properties. For the copolymerizations, different monomer concentrations (25, 40, and 55% w/w, relating to pullulan) were used, resulting in three copolymers with different sizes of hydrophobic segments. All polymers were characterized in terms of chemical composition, molar mass, thermal properties, critical aggregation concentration, and cytotoxicity. NMR confirmed the graft copolymerization, and the degree of substitution obtained for each copolymer was 0.13, 0.54, and 0.63, respectively. The results suggest that the physicochemical properties of both pullulan-based cationic derivatives (PULL-DEAE and PULL-DEAE-g-PZLL) could expand the application of modified pullulan for pharmaceutical fields such as drug delivery.

1. Introduction

Polysaccharides have been intensively studied and applied in various biomedical areas since they are regarded as natural biomaterials with promising properties such as biodegradability, biocompatibility, and non-toxicity [1,2]. However, in most cases, their physicochemical properties are insufficient for specific drug delivery requirements. Therefore, polysaccharide modification and grafting are available alternatives for inserting specific functional groups and tuning the properties of these biological polymers [3]. Pullulan (PULL) is a non-toxic and non-ionic polysaccharide obtained from the fermentation of

Aureobasidium pullulans, which chemical structure consists of maltotriose units connected by $\alpha(1-6)$ glycosidic linkages [4]. PULL is especially attractive since it presents hydroxyl groups along the polymer backbone, which can be easily modified into cationic pullulan by reacting with compounds containing amine groups [5-8]. Cationic polysaccharides, for example, have gained attention due to their ability to form electrostatic complexes with anionic biomolecules, nucleic acids, and proteins. The pullulan modification with a cationic molecule can promote bioactive properties such as antioxidant, antitumor, antimicrobial, pH-responsiveness, anti-inflammatory, and immunomodulatory activities, which justify their therapeutic potential [9,10].

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Scheme 1. Synthesis route of PULL-DEAE via nucleophilic displacement by DEAE.

Consequently, they have been mainly used as carriers for nucleic acid vaccines [11] and chemotherapeutics [12]. Diethylaminoethyl (DEAE) is one example of a molecule capable of grafting onto pullulan to obtain a polyelectrolyte, which is known as a macromolecule with a functional group that can be ionized in the aqueous medium. It can promote the interaction of the polysaccharide with other neutral or oppositely charged material [13]. The polymers' properties, such as charge density, pH sensitivity, and water affinity, can be modulated by the amount of DEAE introduced in the polysaccharides' chains. At low concentrations, the presence of DEAE does not alter the hydrophilicity of pullulan. Different polysaccharides have been evaluated with different degrees of DEAE modification, such as dextran [13–15], chitosan [11], and pullulan [16], for example. Thus, it holds a promising perspective for the future development of matrices for controlled delivery systems, with further investigations and deeper understanding [17,18].

The grafting of hydrophobic segments into the pullulan backbone is another alternative to introduce new properties to this biological polymer without changing its biocompatibility and biodegradability; however, giving it a self-aggregating behavior [19]. The physicochemical properties of pullulan-based amphiphilic polysaccharides are highly dependent on the nature, size, and hydrophobic characteristics of a grafted macromolecule, as well as the environmental parameters, such as pH and temperature [20]. The self-aggregation of amphiphilic copolymers increases their potential application as drug delivery systems in the possibility of forming nano and microparticles in an aqueous medium [21–23]. [19]. Poly(Z-L-Lysine) (PZLL) is a polypeptide with growing interest in the introduction of hydrophobic segments in the pullulan backbone because of its stable structure with a unique biodegradable and biocompatible property, appropriate for biomedical applications [24,25]. The presence of aromatic carbamate (Z), capable of conjugating other substances such as lipophilic drugs, also contributes to the interest in PZLL for biomedical applications [26]. Different polymerization routes are used to obtain PZLL; however, ring-opening polymerization (ROP) of amino acid N-carboxyanhydride (NCA) monomers is the most common synthesis [27,28]. Polypeptides exhibit advantages of natural protein properties, such as secondary and tertiary

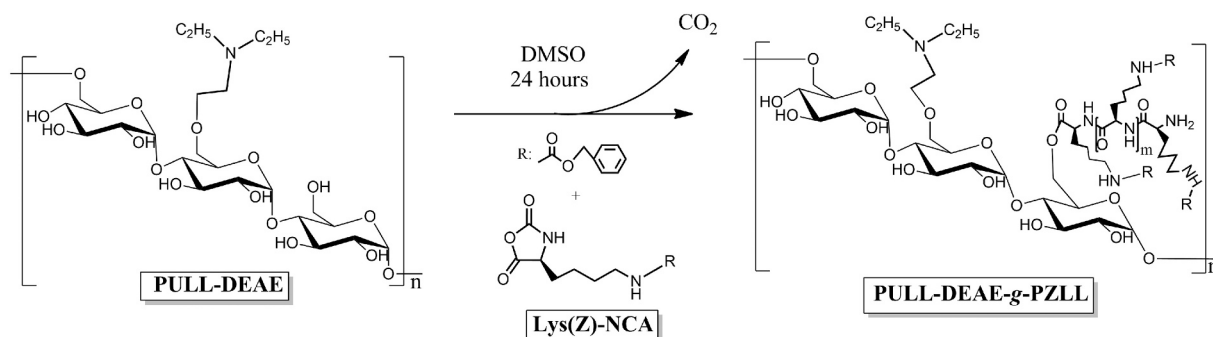
structures, and α -helical conformation, promoting self-assembly into complex three-dimensional structures [29,30]. In this context, PZLL is a promising candidate for grafting onto the pullulan backbone to obtain a versatile polypeptide-based copolymer [24,30].

In this work, cationic pullulan-based polymers with hydrophilic or amphiphilic nature were successfully synthesized by nucleophilic replacement and ring-opening polymerization. Firstly, the hydrophilic cationic pullulan-2-Chloro-N,N-diethylethylamine (PULL-DEAE) polymer was obtained by partially modifying the hydroxyl groups from pullulan. Then, the amphiphilic cationic pullulan-based was obtained after the copolymerization with N-carbobenzyloxy-L-lysine N-carboxyanhydride (Lys(Z)-NCA) in the remaining hydroxyl groups of PULL-DEAE, resulting in a grafted copolymer of pullulan-DEAE-g-poly(Z-L-lysine) (PULL-DEAE-g-PZLL). This study aims to evaluate the effect of the DEAE modification on the pullulan's backbone and the influence of different chains length of poly(Z-L-lysine) on the physical-chemical properties of the modified polysaccharide. All chemical modifications were successfully confirmed via usual chemical analysis and their impacts on chain length, thermal properties, cytotoxicity, and critical aggregation concentration were evaluated. The results represent an important contribution from a bibliometric approach for future investigation concerning the use of hydrophilic and amphiphilic pullulan derivatives for the encapsulation and controlled release of drugs from biomembranes, particles in aqueous suspension, etc.

2. Materials and methods

2.1. Material

The reagents used in this work were pullulan (PULL, $M_n = 120.000 \text{ g mol}^{-1}$, food grad) provided by Hayashibara Biochemical Laboratory (Japan), ϵ -benzoxycarbonyl-L-lysine-N-carboxyanhydride (Lys(Z)-NCA, 98%, AECHEM Scientific Corporation) and 2-chloro-N,N'-diethylethylamine hydrochloride (DEAE, 98%, Sigma-Aldrich). Dimethyl sulfoxide (DMSO, 99%, Synth) was purified by vacuum distillation. Sodium hydroxide (NaOH, P.A., Synth), hydrochloric acid (HCl, 37%, Synth), and



Scheme 2. Synthesis route of PULL-DEAE-g-PZLL via ring opening polymerization of the N-carboxyanhydride ring.

diethyl ether (99%, Synth) were used as received. The water used in all experiments was distilled and deionized.

2.2. Synthesis of PULL-DEAE

To prepare hydrophilic DEAE-modified pullulan (Scheme 1), the methodology was adapted from San Juan et al. (2007) [31], in which 1 g of PULL (6.2 mmol in glucose unit) was solubilized in 6 mL of deionized water. Then, 5 mL of 10 mol L⁻¹ NaOH solution was added, and the system was submerged in an ice bath. 0.7306 g (4.2 mmol) of DEAE was added to the reaction system. The medium was kept in a glycerin bath at 55 °C for 50 min. At the end of the reaction, the medium was washed 3 times with 20 mL diethyl ether, and then the pH was adjusted using concentrated hydrochloric acid, reaching approximately pH 7. The final product was dialyzed against deionized water for 3 days and then lyophilized to obtain PULL-DEAE.

2.3. Synthesis of PULL-DEAE-g-PZLL

To prepare the grafted copolymer of pullulan-DEAE-g-poly(Z-L-lysine) (PULL-DEAE-g-PZLL) (Scheme 2), different amounts of the Lys (Z)-NCA were mixed into a solution of PULL-DEAE (DS = 0.15) in DMSO. In brief, 25, 40, and 55 wt% of the Lys(Z)-NCA, relative to the PULL-DEAE, was introduced to synthesize the grafted pullulan. The reaction medium was submitted to nitrogen gas flow during the first hour of the reaction to carry away the carbon dioxide formed during the process. The system was sealed and kept under stirring at room temperature for 24 h. After the reaction time, the material was dialyzed for 24 h against distilled water and lyophilized according to the adapted methodology of Park and coauthors [32].

2.4. Characterizations

2.4.1. Nuclear magnetic resonance (NMR)

The chemical structures of the materials were evaluated by NMR technique, using Avance III equipment, with 600 MHz for hydrogen frequency, equipped with an automatic sample changer and cryo-probe TCI. This technique was also used to estimate the degree of substitution of PULL-DEAE. ¹H and ¹³C nuclear magnetic resonance analyses were performed using deuterated dimethyl sulfoxide (DMSO-*d*₆) as a solvent.

To calculate the substitution degree of hydroxyl groups present in PULL, the ¹H NMR results obtained for the PULL-DEAE compound were used, and Eq. (1) proposed by Teramoto and Shibata [33] was applied:

$$\frac{A}{6x} = \frac{B}{1 + (3 - x)} \quad (1)$$

Where *A* refers to the resonance of the methyl groups on the DEAE molecule inserted into the PULL chain; *B* refers to the resonance of the protons present in the hydroxyls of the PULL glycosidic unit plus the protons attached to anomeric carbons; The variable *x* represents the number of hydrogens substituted in the reaction. Thus, by isolating *x* and considering it as the degree of substitution (DS), we obtain Eq. (2).

$$DS = \frac{4A}{6B + A} \quad (2)$$

The equation proposed by Teramoto and Shibata [33] was adapted to determine the hydroxyl groups' substitution degree of PULL after copolymerization with PZLL. The proton amount was modified due to the integrated area, as represented in Eq. (3).

$$\frac{A}{5x} = \frac{B}{1 + (3 - x) + 2} \quad (3)$$

Where *A* refers to hydrogen from the aromatic ring in PZLL chains grafted into the PULL backbone; *B* refers to the hydroxyl resonances presented plus the protons from anomeric carbons from the PULL glycosidic unit, besides two protons from PZLL structure that overlap in

the integration; The variable *x* represents the number of substituted hydrogens in the reaction, called degree of substitution (DS), which was obtained by Eq. (4).

$$DS = \frac{6A}{5B + A} \quad (4)$$

2.4.2. Gel permeation chromatography (GPC)

Number average molecular weight (*M_n*) and dispersity (*Đ*) of the polymers were determined by gel permeation chromatography (GPC) with an automatic injector (Sil-20A) and a Shimadzu (Japan) refractive index detector. Two Phenomenex, PolySep - SEC GFC P5000 and P3000 columns (3000–400,000 g mol⁻¹ separation range) and one Phenomenex, PolySep - SEC GFC P pre-column was used for separation. PULL and PULL-DEAE were solubilized in phosphate buffer solution (50 mmol L⁻¹, pH 7), containing 0.2 mol L⁻¹ sodium azide (fungistatic) and 0.05 mol L⁻¹ potassium nitrate, and filtered on a modified PVDF polyvinylidene difluoride membrane filter (0.45 μm). The copolymers were solubilized in DMSO and filtered on a modified PVDF polyvinylidene. The buffer solution was used as a mobile phase for all polymers, with a fixed flow rate of 0.5 mL min⁻¹ and injection volume of 25 μL.

2.4.3. Fourier transformation infrared (FTIR)

The polymers' chemical composition was evaluated using Spectrum 100 FTIR (PerkinElmer, USA) equipped with an Attenuated Total Reflection accessory (ATR). The FTIR technique was used with a diamond crystal as the internal reflection element. 32 scans at a 4 cm⁻¹ resolution were co-added in the IR range of 4000–500 cm⁻¹.

2.4.4. X-ray diffraction (XRD)

X-ray diffraction analyses were obtained by a PAN-analytical Empyrean ACMS 101 (Malvern, UK) diffractometer operating at room temperature. The determination was performed using a copper (Cu) X-ray tube with Kα radiation. The current was settled at 40 mA, and the voltage was 40 kV. The experiments were conducted with a scan range from 10 to 90° (2θ), a step size of 0.02° (2θ), and a counting time of 50 s.

2.4.5. Thermal analysis

Differential scanning calorimetry (DSC) was performed using a TA instrument – Waters LLC, Model Q20. The samples (about 9 mg) were heated at a rate of 10 °C min⁻¹. The first ramp was performed from 0 °C to 150 °C to remove thermal history and water from the samples, and the second one from 0 °C to 300 °C, both under a nitrogen atmosphere.

The thermal stability of homo and copolymers was investigated using a thermal analyzer STA 6000 (Perkin Elmer® Inc., Massachusetts, USA). The temperature range was from 30 to 600 °C in N₂ flow (20 mL min⁻¹) and a heating rate of 10 °C min⁻¹.

2.4.6. Critical aggregation concentration (CAC)

The critical aggregation concentration (CAC) of the PULL-DEAE and copolymers was determined by fluorescence spectroscopy using the Varian Cary Eclipse fluorimeter. Pyrene was used as a hydrophobic probe according to the methodology adapted from Constantin et al. (2017) [34]. For sample preparation, a solution of pyrene in acetone was added to a 10 mL volumetric flask, keeping the concentration of the probe in all flasks at 6.0 × 10⁻⁷ M. Then, the acetone was evaporated, and polymers solutions in an aqueous medium were added to the flask at different concentrations, ranging from 7.5 × 10⁻⁴ to 2.5 × 10⁻² mg mL⁻¹ to PULL-DEAE and PULL-DEAE-g-PZLL 25%, and 7.5 × 10⁻⁶ to 5.0 × 10⁻³ mg mL⁻¹ to PULL-DEAE-g-PZLL 40% and PULL-DEAE-g-PZLL 55%. The solutions were stored at room temperature for 24 h, and readings were taken in the equipment at an emission wavelength of 390 nm. The ratio between the emission intensity of the peaks at 333 nm and 337 nm (I₃₃₇/I₃₃₃) was used to determine the critical aggregation concentration.

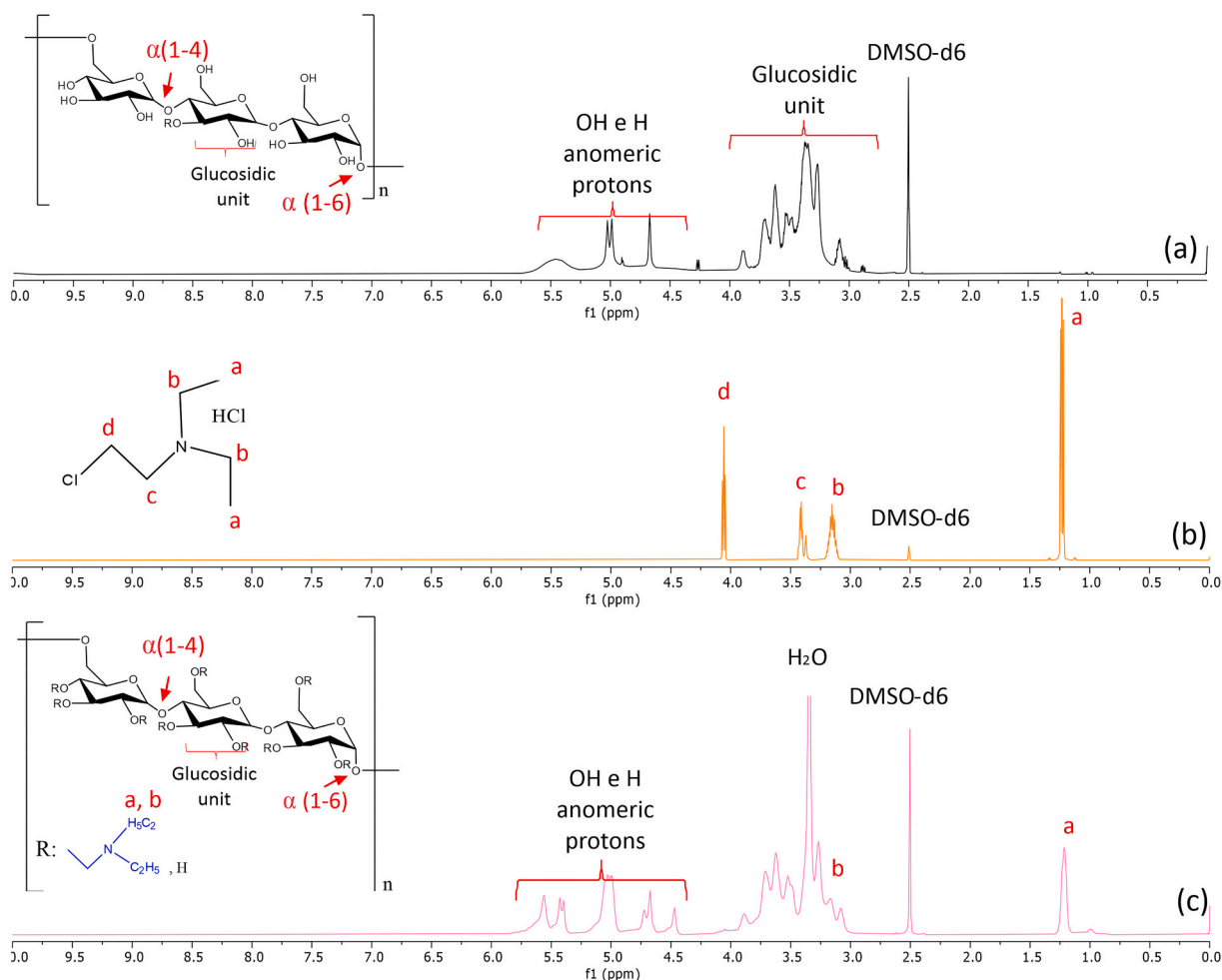


Fig. 1. ¹H NMR spectra of starting PULL (a), pure DEAE (b), and PULL-DEAE (c).

2.5. Cytotoxicity test

Cytotoxicity assay was performed by indirect method. To prepare the samples extracts, three samples were incubated at $1000 \mu\text{g mL}^{-1}$ in DMEM culture medium. To be used as a negative control solution, one flask of DMEM without any sample remained in the incubator for 24 h. Afterward, the medium and extracts were filtered separately using sterile syringe filters ($0.22 \mu\text{m}$ PDVF Millipore). Then, the extracts were used at $1000 \mu\text{g mL}^{-1}$ or diluted to $100 \mu\text{g mL}^{-1}$, $10 \mu\text{g mL}^{-1}$, and $1 \mu\text{g mL}^{-1}$ by using the incubated culture medium. L929 cells were plated in a 96-wells plate at 104 cells/well. After 12 h, the culture medium was replaced by 250 μL of the extracts at different concentrations or by the control solutions (DMEM as negative control and DMSO as positive control). One plate of cells was incubated for 24 h and another for 72 h. Cytotoxicity of the copolymer was evaluated by using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Kašpárková and co-workers [35]. After incubation, the extracts were withdrawn, and each well was washed with Phosphate Buffered Saline solution (PBS). Then, 100 μL of MTT aqueous solution at 0.5 mg mL^{-1} were added to the cells, and the cells were incubated for 3 h. Then, the MTT solution was withdrawn, and the formazan salt formed in the cells was solubilized with 100 μL of DMSO. The absorbance of the wells was measured at 540 nm in a Plate Reader Synergy H1 (Biotek, Santa Clara, USA). The experiment was performed in 6 replicates for each concentration, and the cell viability was calculated by considering the negative control as 100% of cell viability.

In order to further evaluate the cytotoxicity of the sample extracts, the cells were incubated with the extracts at $1 \mu\text{g mL}^{-1}$ for 72 h and

analyzed by optical microscopy regarding cell morphology. In this assay, 104 cells were plated on glass slides placed inside Petri dishes. After 12 h, the culture medium was replaced by sample extracts. The cells were incubated for 72 h and then fixed with ethanol. The cells were incubated with ethanol at 10, 25, 50, 75, and 90% for 20 min each. Finally, the samples were immersed in ethanol 99% for 1 h. Then, the cells were analyzed by optical microscopy at 40 X and photographed using a cell phone camera.

3. Results and discussion

3.1. ¹H NMR and ¹³C NMR

Aiming to prepare two types of cationic pullulan, one with hydrophilic and another with amphiphilic properties, the polysaccharide was first chemically modified by 2-chloro-N,N'-diethylethylamine hydrochloride (DEAE), resulting in the hydrophilic PULL-DEAE. After, PULL-DEAE was grafted with different amounts of Poly(Z-L-Lysine), obtaining the copolymers PULL-DEAE-g-PZLL 25, 40, and 50%. In the first step, DEAE reacted with pullulan by nucleophilic displacement, resulting in a carbon-oxygen bond that takes place from a carbon-chlorine bond, as shown in Scheme 1. The DEAE group into the pullulan backbone provides an ionizable tertiary amino group which could result in a pH sensitivity in the polysaccharide. The reaction was conducted in a solution containing concentrated sodium hydroxide, which can neutralize the hydrochloride released from DEAE in the reaction and transform the hydroxyl groups in pullulan's backbone into more reactive ones and consequently accelerating the etherification [20,36]. However,

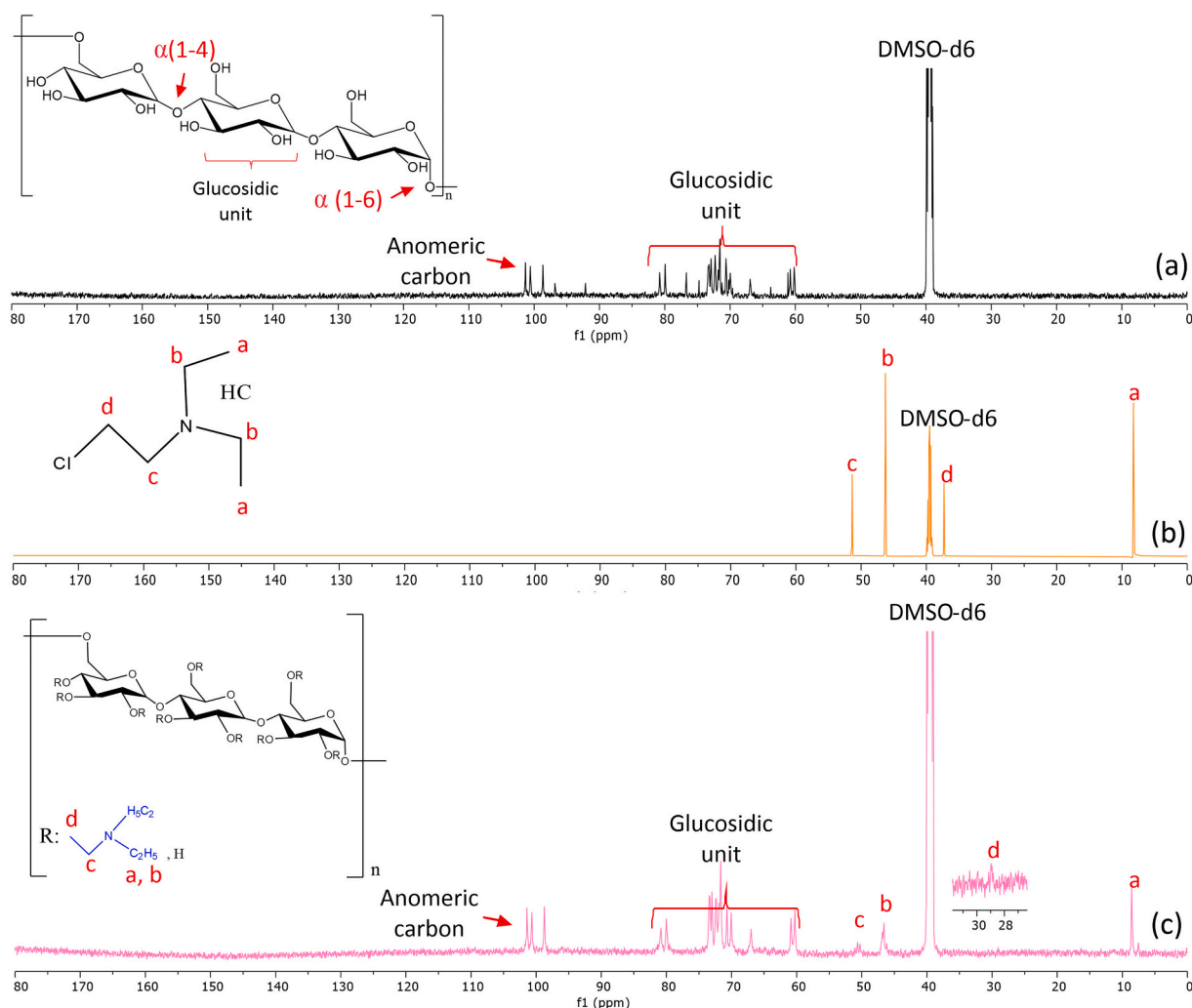


Fig. 2. ^{13}C NMR starting PULL (a), pure DEAE (b), and PULL-DEAE (c).

according to Nichifor et al. 2010, when the concentration of NaOH exceeds 0,4 mol/anhydroglucose unit, it can inactivate the reagent by hydrolysis or even degrade the polysaccharides [21], even though pullulan presented a good stability front alkaline hydrolysis [37]. As the etherification reaction proceeds through a nucleophilic displacement, it is expected that the reaction occurs preferably in carbon C6 of the PULL, as it presents the most reactive hydroxyl group in the pullulan backbone. In this process, the arrangement of PULL chains may be impacted since the DEAE molecules impose a steric hindrance to a compact arrangement, which is interesting to promote further modification in the latter process. However, it is not expected that all the available hydroxyl groups would be modified since the amount of DEAE used in the reaction was 0,67 mol/anhydroglucose unit. Besides that, the pullulan chain also presents a strong intra and intermolecular hydrogen bonding, which can affect the chemical modification yield. Thus, an important aspect is evaluating the substitution degree to understand the extension of the etherification process.

In the second modification, the obtained PULL-DEAE reacted with the monomer Lys(Z)-NCA through a ring-opening polymerization (ROP) reaction, as illustrated in Scheme 2. In this process, it is expected that part of the unmodified hydroxyl groups in PULL-DEAE are replaced by the grafting of PZLL, which results in the reduction of hydrophilicity of PULL-DEAE and emerging of amphiphilic properties. During graft copolymerization, carbon dioxide is released due to the ring opening of the N-carboxyanhydride group. Thus, it was investigated if the different amounts of PZLL impacted the substitution degree.

The modification of the PULL structure was evaluated using NMR analysis. Fig. 1, Fig. 2 and Fig. S1 (Supplementary Material) present the ^1H , ^{13}C and HSQC NMR spectra, respectively, regarding the initial modification procedure. As observed in Fig. 1a, pullulan presents resonances at 4.3–5.7 ppm, assigned to the overlap region of PULL hydroxyl groups (-OH) with the (1–4) and (1–6) anomeric protons, and resonances at 3.0–4.0 ppm, related to hydrogens from polysaccharide ring [38]. In Fig. 1b DEAE molecule exhibits typical resonances at 1.22 ppm (6H, $-\text{N}(\text{CH}_2\text{CH}_3)_2$, “a”), 3.15 ppm (4H, $-\text{N}(\text{CH}_2\text{CH}_3)_2$, “b”), 3.40 (2H, $-\text{CH}_2\text{CH}_2\text{N}-$, “c”), and 4.05 (2H, $-\text{CH}_2\text{Cl}$, “d”) [39]. After the modification, it was observed in Fig. 1c that PULL-DEAE maintained all typical pullulan resonances, indicating that the glycosidic structure was preserved. The spectra presented the appearance of new resonance peaks at 1.20 and 3.17 ppm, which are related to DEAE structure (hydrogen “a” and “b”, respectively, as indicated previously), demonstrating the successful chemical modification of pullulan. The occurrence of secondary reactions between two DEAE groups was not evidenced in Fig. 1c since there are no peaks characteristic of two DEAE groups on a single hydroxyl. The secondary reaction was previously observed by San Juan and collaborators (2006) [40]; however, the amount of DEAE applied by them was significantly greater than the one used in this work since the main goal was not to modify all the hydroxyl groups on PULL. To understand the modification efficiency during the applied procedure, the DS was determined using Eq. 2, which was observed at a value of 0.15. DS obtained indicates that about 15 hydroxyls were replaced during the modification process for one hundred glucose units, which means a

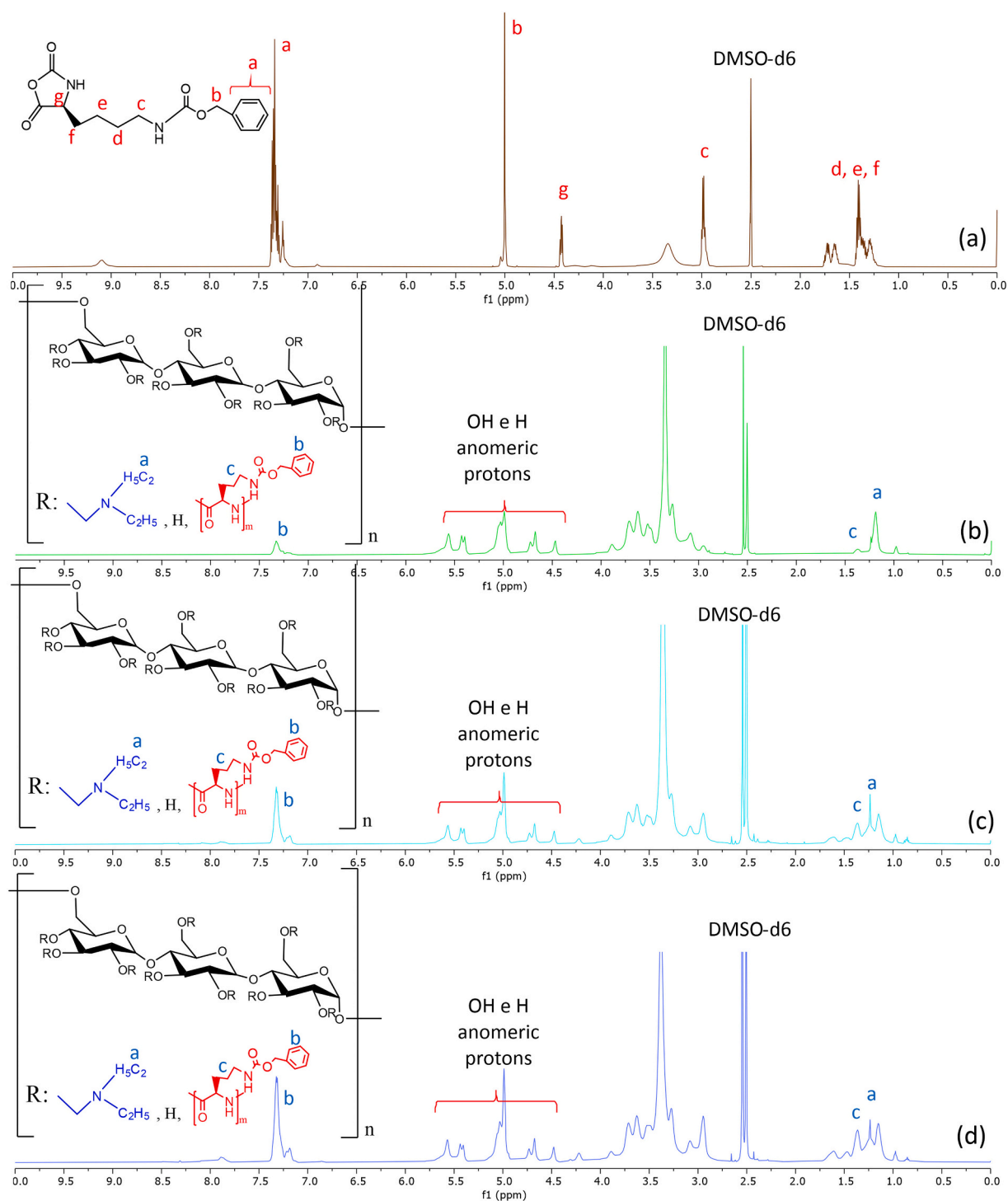


Fig. 3. ¹H NMR spectra of Lys(Z)-NCA (a) and PULL-DEAE-g-PZLL grafted with different proportions (25, 40 e 55 wt%, concerning pullulan) of PZLL segments (b-d).

lower substitution based on the amount of DEAE used at 0.67 mol/mol of anhydroglucose unit. Shibata et al. 2002, also investigated the etherification of pullulan by evaluating two different alkyl bromides, 1-bromopropane and 1-bromobutane, and three different amounts of each reagent. It was observed that the DS obtained was approximately half of the maximum DS available for the reaction [41]. Therefore, the low DS obtained was expected, demonstrating the effectiveness of DEAE modification in the PULL chain.

Fig. 2a shows that PULL possesses resonance peaks at 98, 100, and 101 ppm related to the anomeric carbons of pullulan structure and peaks around 60–70 ppm related to carbons of polysaccharide structure [42].

Evaluating the DEAE spectra in Fig. 2b it is observed characteristic carbon resonances at δ (ppm) = 8 (-N(CH₂CH₃)₂, "a"), 37 (-CH₂Cl, "d"), 46 (-N(CH₂CH₃)₂, "b"), and 51 (-CH₂CH₂N-, "c") as previously mentioned in the literature [11]. The PULL-DEAE spectra observed in Fig. 2c showed the presence of anomeric carbon resonances from pullulan and characteristic DEAE carbon resonances at 8, 46, and 51 ppm, represented by "a", "b" and "c", respectively. It is also observed that the DEAE methylene peak, represented by "d", slightly shifted from 37 to 29 ppm, which can be explained by the heteroatom neighboring to this carbon since the chlorine atoms are released during the reaction, and a new bond is formed with the oxygen atom, modifying the electron

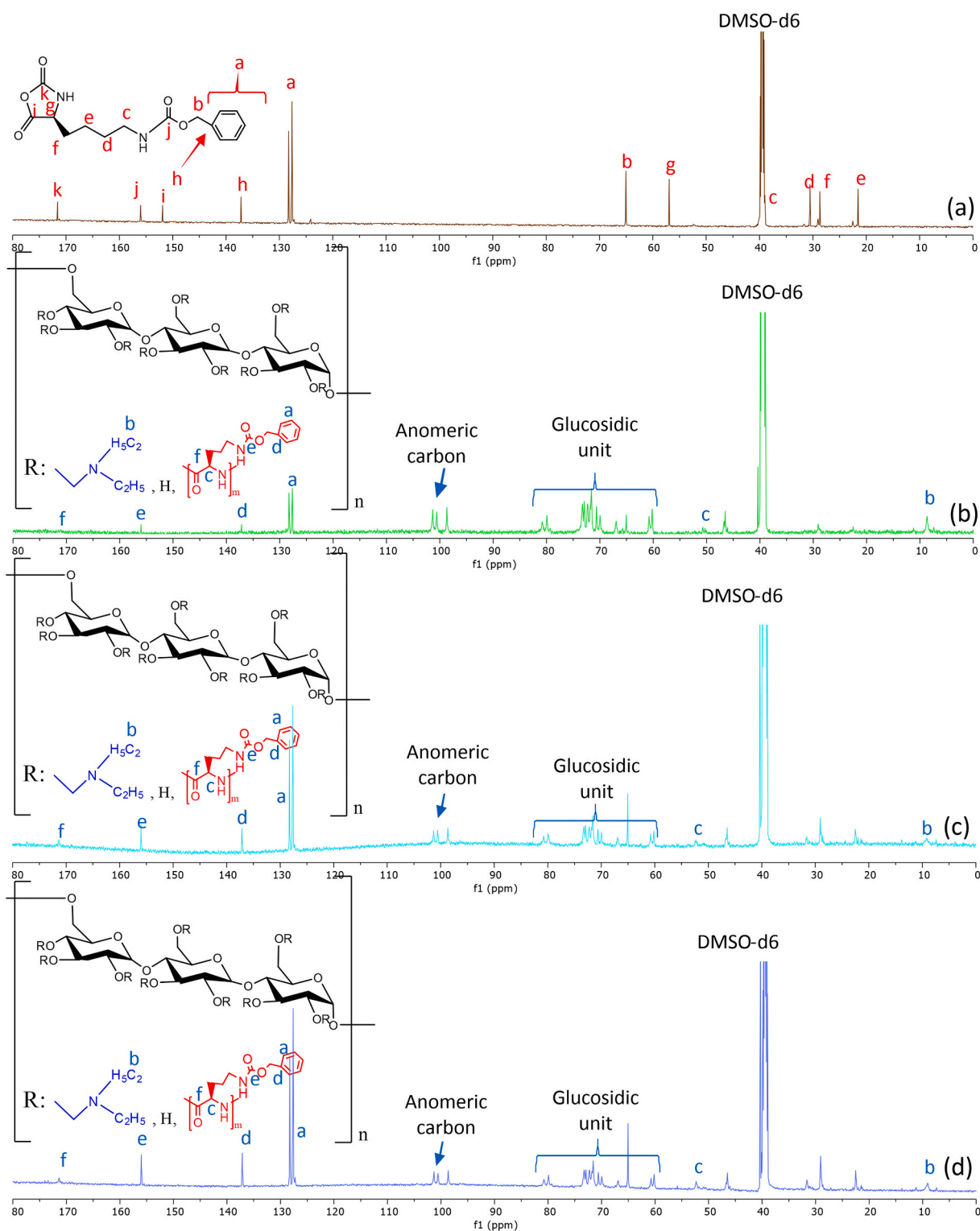


Fig. 4. ^{13}C NMR spectra of Lys(Z)-NCA (a) and PULL-DEAE-g-PZLL grafted with different proportions (25, 40 e 55 wt%, concerning pullulan) of PZLL segments (b-d).

clouds, and causing the chemical shift observed. These observations support that the reaction was successful in promoting pullulan modification.

The obtained PULL-DEAE was then grafted with PZLL through ROP reaction, which is expected to take place on the remaining hydroxyl groups from the pullulan backbone, as indicated in Scheme 2. The grafting process was also evaluated using NMR analysis, and Fig. 3 and Fig. 4 present the ^1H and ^{13}C NMR spectra, respectively. From Fig. 3a it

was observed that Lys(Z)-NCA presents resonances at δ (ppm): 1.23–1.76 ($-\text{CH}_2\text{CH}_2\text{CH}_2-$, “d, e, f”), 2.97 (2H, $\text{CH}_2\text{CH}_2\text{NH}$, “c”), 4.40 (1H, $\text{CHC}(\text{NH})\text{C}$ -, “g”), 4.99 (2H, $-\text{CH}_2\text{O}-$, “b”), and 7.33 (related to hydrogens from aromatic ring, “a”) [43]. The grafted PULL-DEAE-g-PZLL copolymers spectra with varied PZLL content are observed in Fig. 4b-d. The copolymers spectra show that all the materials preserved the hydrogens from anomeric and polysaccharide rings, indicating that the grafting process maintained the pullulan structure. After the grafting

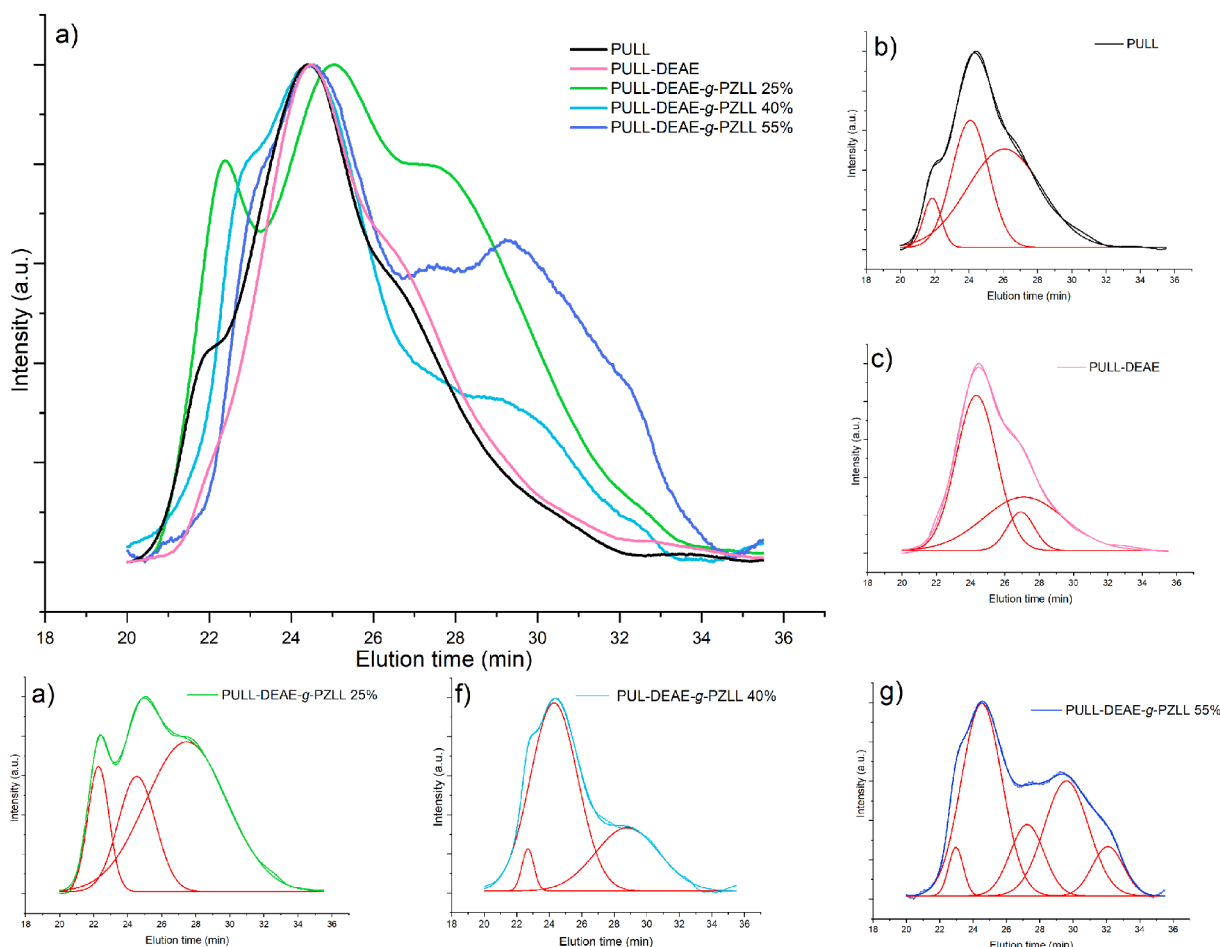


Fig. 5. Chromatograms of PULL, PULL-DEAE, PULL-DEAE-g-PZLL 25%, PULL-DEAE-g-PZLL 40%, and PULL-DEAE-g-PZLL 55%.

process, new resonance peaks were observed at 7.30 and 1.34 ppm, characteristic of the aromatic ring and methyl groups from PZLL segments, represented by “b” and “c”, respectively. Liu et al. reported similar results [44]. No significant differences were observed between the spectra of copolymers grafted with different PZLL amounts, indicating that the grafting process was similar for all the copolymers. The DS was then calculated using Eq. 4, and it observed values of 0.13, 0.54, and 0.63 for PULL-DEAE-PZLL 25, 40, and 55% w/w, respectively. The increase in DS value with the increased monomer content used during copolymerization indicates that the process was efficient, promoting the grafting of the pullulan backbone with poly(Z-L-lysine). Notably, the DS variation was more pronounced when changing the monomer percentage from 25 to 40% w/w compared to the change from 40 to 55% w/w. This result may be associated with steric hindrance effects that limit the grafting process using higher monomer quantity. Thus, this result indicates that possibly there is an optimum grafting efficiency of close to 40% w/w of poly(Z-L-lysine) for the studied system.

To confirm the grafting of PZLL segments in the PULL-DEAE backbone, ^{13}C NMR was performed. Fig. 4a presents the lysine-based monomer used for the grafting copolymerization, with several characteristic resonances at δ (ppm) such as 171 ($-\text{C}(\text{O})\text{NH}-$, “k”), 155 ($-\text{NH}(\text{C}=\text{O})\text{O}$, “j”), 151 ($-\text{C}(\text{C}=\text{O})\text{O}$, “i”), 137 (anomeric carbon from an aromatic ring, “h”), 128–127 (carbon from the aromatic ring), 65 ($-\text{CH}_2\text{O}$, “b”) 57 (CH_2CNH , “g”), 39 ($-\text{CH}_2\text{CH}_2\text{NH}$, “c”), 31 ($-\text{CH}_2\text{CH}_2\text{NH}$, “d”), and 29 ($-\text{CCH}_2\text{CH}_2-$, “f”), 21 ($-\text{CCH}_2\text{CH}_2-$, “e”) as previously reported [45]. The grafted PULL-DEAE-g-PZLL copolymers spectra with varied PZLL content are observed in Fig. 4b-d, and it is possible to notice the presence of the 137, 155, and 171 ppm resonance peaks, indicating that the grafting process was successfully achieved. It is also possible to

Table 1

Mn, Mw, and dispersity values of PULL, PULL-DEAE, PULL-DEAE-g-PZLL 25%, PULL-DEAE-g-PZLL 40%, and PULL-DEAE-g-PZLL 55%.

Sample	M_n (Da)	M_w (Da)	\bar{D}
PULL	98,393	231,943	2.36
PULL-DEAE	90,271	194,001	2.15
PULL-DEAE-g-PZLL-25%	54,680	216,940	3.97
PULL-DEAE-g-PZLL-40%	62,070	218,413	3.52
PULL-DEAE-g-PZLL-55%	34,596	134,547	3.89

observe the disappearance of the resonance at 151 ppm. This result can be explained by the release of CO_2 gas from the N-carboxyanhydride ring after ring-opening polymerization, as previously illustrated in Scheme 2. Another modification noticed in the ^{13}C NMR spectra of PULL-DEAE-g-PZLL is related to the carbons from methylene, which shifted from 57 ppm (“g”, in Fig. 4a) to 52 ppm (“c”, Fig. 4b-d). No significant changes could be evidenced in the ^{13}C NMR spectra, even with the increase in the proportion of PZLL segments grafted into the pullulan backbone. These results evidence the effectiveness of grafting PZLL into PULL-DEAE and are corroborated by the work of Izunobi and Higginbotham.

3.2. Gel permeation chromatography (GPC)

Chromatograms indicated a high dispersity of the molar masses for all polymers, presenting bimodal and trimodal curves, as shown in Fig. 5a. The deconvolution of the chromatograms (Fig. 5b-f) demonstrated three molar mass populations for PULL at elution time values

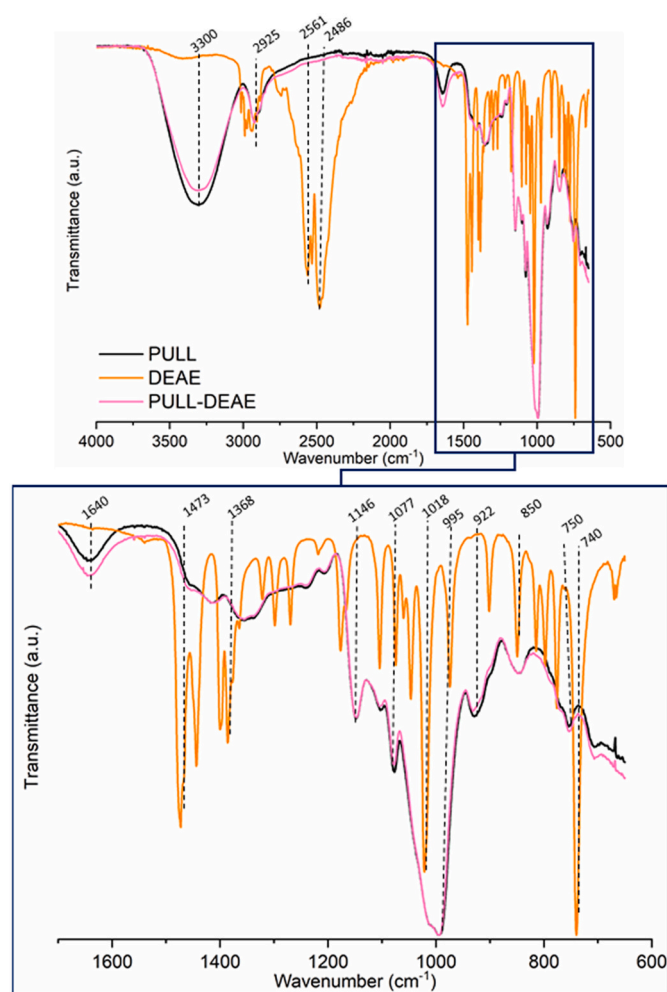


Fig. 6. FTIR spectra of PULL, DEAE, and PULL-DEAE (a) full spectra, (b) zoom between 1700 and 600 cm^{-1} .

around 22, 24, and 26 min. After modification with DEAE, the elution time values changed to 24, and two populations around 26; this effect was noticed in the M_n value, as demonstrated in Table 1, M_n reduced from 98,393 Da to 90,271 Da after the DEAE modification. Among the possible reasons for reducing the molecular weight of PULL-DEAE, alkaline hydrolysis corresponds to one of them, since the presence of NaOH in the aqueous medium could result in polysaccharide degradation, as aforementioned. Besides that, incorporating the DEAE in the pullulan chain reduced the amount of hydroxyl groups and the presence of an amine group, which consequently changed the intermolecular and hydrogen bond interactions between the molecules. Further, the presence of amine could contribute to a slight interaction between the molecule and the GPC column, increasing the retention time in the GPC. Thus, the PULL-DEAE displays a smaller molecular weight than the unmodified PULL.

Comparing PULL-DEAE chromatograms with PULL-DEAE-PZLL copolymers chromatograms, they are noticeably different. The copolymers based on PULL-DEAE-g-PZLL evidenced a new population at low elution time between 20 and 24 min, demonstrating higher apparent molar masses occurred by the copolymerization into the PULL-DEAE backbone. On the other hand, new populations were also noticed in higher elution time, around 30 min, evidencing smaller hydrodynamic volumes of polymer, which decreased M_n and increased D of the copolymers (as demonstrated in Table 1). This fact could be attributed to the intramolecular and hydrogen bond interactions between PZLL hydrophobic grafted, leading to strongly compacted structures, as Hadrich and co-

workers mentioned before [47]. Another significant event influencing the decrease of M_n is the homopolymerization of PZLL. Since the amount of PZLL increased in the copolymer, M_n decreased, and the population moved to a lower elution time. This fact demonstrated that the monomer reaction reached a limit of graft copolymerization. The amphiphilicity of the copolymers resulted in a steric hindrance and spatial configuration that reduced the collisions among the reactive sites on the macromolecules, playing an important role in the graft copolymerization amount, promoting lower M_n values for PULL-DEAE-g-PZLL copolymers. This result is corroborated by the previous work reported by Madau and co-workers [48].

3.3. FTIR

The FTIR spectra for PULL, DEAE, and the product PULL-DEAE are presented in Fig. 6 and Fig. S2. Pullulan displays characteristic bands at 3300 and 2925 cm^{-1} associated with hydroxyl and C—H stretching, respectively. By extending the spectrum from 2000 to 600 cm^{-1} , as presented in Fig. 6b, it can be observed the stretching of the O—C—O, C—O—C, and C—O bonds of PUL at 1640, 1146, and 1077 cm^{-1} , respectively. The stretching at 995, 922, and 750 cm^{-1} are characteristic of the α -(1,4) and α -(1,6) glycosidic bonds of PULL (AN et al., 2017). The spectrum of DEAE shows stretching at 2561 and 2486 cm^{-1} , characteristic of the C—N axial stretching from a salt of tertiary amines. Besides that, the DEAE spectrum also shows peaks at 1473 and 740 cm^{-1} , characteristic of the symmetric and asymmetric angular deformation of the C—H bonds, and peaks at 1386 and 1018 cm^{-1} , referring to the symmetric and asymmetric axial deformation of the C—H bonds, respectively. The axial deformation of the C—Cl bond of the salt is shown at 850 cm^{-1} . The spectrum PULL-DEAE reveals the presence of the bands referring to the main O—H bonds, originally from PULL, but with reduced intensities, suggesting some groups' consumption for the modification process. After the functionalization of pullulan with DEAE, characteristic peaks of DEAE at 2925, 2561, and 2486 cm^{-1} were not observed, and pullulan peaks may overlap the other peaks. This result indicates that the pullulan backbone structure was preserved and, combined with low DS observed by NMR analysis, indicates that the reaction promoted only a surface modification at a few available sites.

The FTIR for Lys(Z)-NCA and grafted pullulan is presented in Fig. 7 and Fig. S3 (Supplementary Material). Lys(Z)-NCA spectrum, presented in Fig. 7b., shows the symmetric and antisymmetric C=O stretching modes of the carboxyanhydride (O=C—O—C=O) at 1857, 1804, 1772, and 1745 cm^{-1} . It can be noticed in Fig. 7b. that an amide C=O stretching vibrations from benzyloxycarbonyl group at around 1692 cm^{-1} and N—H bending vibration frequency (amide II) from carboxyanhydride group at around 1529 cm^{-1} . The absorption band from N—C—O stretching and C—O stretching can be observed in Fig. 7b-c., on the frequency at 1260 and 945 cm^{-1} , respectively. Evaluating the grafted copolymer, Fig. 7a., they presented a similar spectrum to pullulan with slight intensity variations, such as at 3300 and 2925 cm^{-1} , indicating that the polysaccharide structure was preserved. In Fig. 7b., Lys(Z)-NCA peaks at 1858, 1805, 1775, and 1747 cm^{-1} were not observed for the grafted pullulan since in the polymerization process by ROP, there is the release of carbon dioxide, which corroborates the success of the grafting process. Moreover, the grafted materials present a new amide I stretching frequency at 1623 cm^{-1} , and an increasing intensity for higher monomer content was observed, indicating a higher grafting degree of pullulan structure and corroborating the NMR and DS results. Additionally, in Fig. 7b., the peak at 1686 cm^{-1} was observed with a little shift to 1692 cm^{-1} in the grafted pullulan. Similar behavior was observed for the 1529 cm^{-1} band that shifted to 1538 cm^{-1} for PULL-DEAE-g-PZLL. Moreover, the bands 1454, 1260, and 1246 cm^{-1} from PZLL were observed in the grafted materials, and the intensity was proportional to the amount of Lys(Z)-NCA used during grafting. These results indicate that the higher monomer content was successfully grafted into the pullulan structure and corroborates the NMR results.

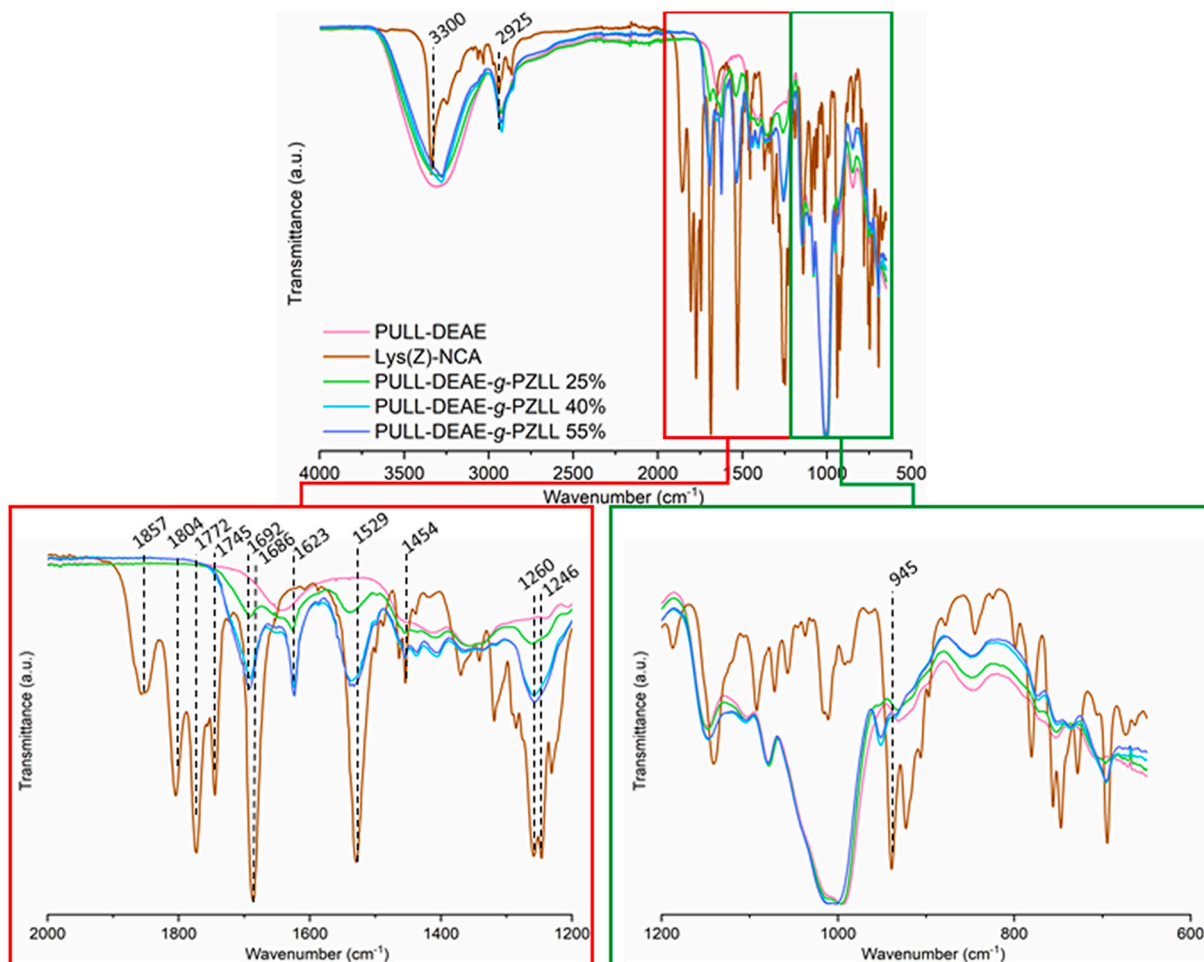


Fig. 7. FTIR spectra of Lys(Z)-NCA, and PULL-DEAE-g-PZLL 25, 40 e 55 wt%, (a) full spectra, (b) zoom between 2000 and 1200 cm^{-1} , (c) zoom between 1200 and 600 cm^{-1} .

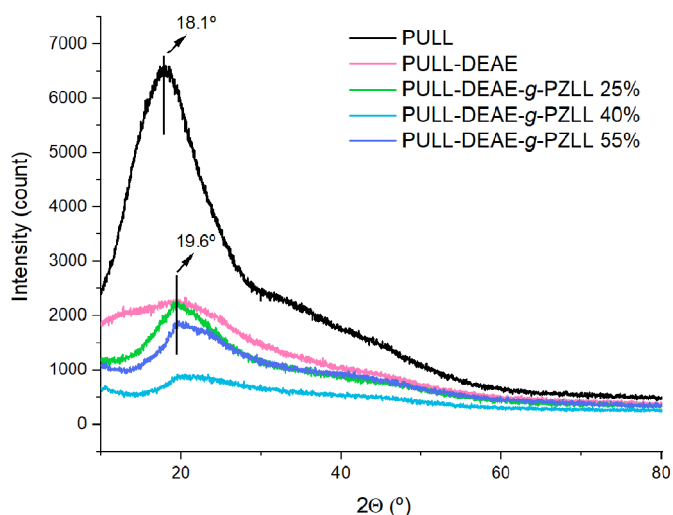


Fig. 8. XRD pattern of PULL, PULL-DEAE, PULL-DEAE-g-PZLL 25%, PULL-DEAE-g-PZLL 40%, and PULL-DEAE-g-PZLL 55%.

3.4. XRD

The XRD patterns for PULL, PULL-DEAE, and the grafted pullulan are presented in Fig. 8. The absence of a sharp signal indicates that pullulan presents an amorphous structure, presenting only a broad peak around

18.1° that is related to the compact arrangement of pullulan molecules through hydrogen bonds. Similar results were observed by Singh and co-workers and Haghghatpanah and co-workers [49,50]. During modification, a shift was observed from the broad peak that pullulan possesses from 18.1° to 19.6° and an intensity reduction, which suggests that the modification and grafting procedures impacted the hydrogen bonding from pullulan, possibly reducing these interactions and increasing the amorphous state. Moreover, in Fig. 8 is possible to observe a change in the peak diffraction intensity, where the unmodified pullulan showed the highest intensity, while for the PULL-DEAE it was observed a considerable reduction of the peak's intensity, and this effect was enhanced for the grafted samples. These observations highlight the success in modifying the surface properties of pullulan without compromising its change in the diffraction intensities observed in Fig. 8, where the unmodified pullulan showed the highest intensity. With the modification with DEAE, the sample showed a considerable reduction in diffraction intensity, which was enhanced with the copolymerization process, highlighting the success of the pulp structure modification process.

3.5. Thermal analysis

Fig. 9 evidence the thermograms of pullulan and pullulan modified thermally analyzed by DSC and TGA techniques, and the data can be observed in Table 2.

Analyzing the thermograms of pullulan (PULL) and chemically modified pullulan (PULL-DEAE and PULL-DEAE-g-PZLL) evidenced a

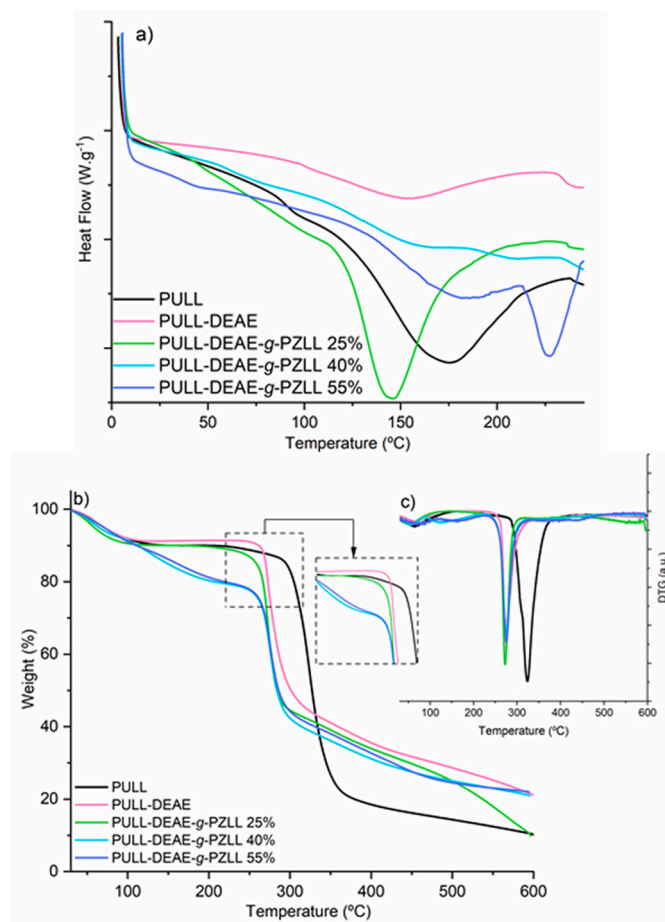


Fig. 9. a) DSC, b) TGA, and c) DTG curves of PULL, PULL-DEAE, PULL-DEAE-g-PZLL 25%, PULL-DEAE-g-PZLL 40%, and PULL-DEAE-g-PZLL 55%.

Table 2

TGA and DSC thermal parameters, such as glass transition temperature (T_g), the temperature of starting degradation (T_{onset}), and temperature of maximum degradation rate (T_{max}) for PULL, PULL-DEAE, PULL-DEAE-g-PZLL 25%, PULL-DEAE-g-PZLL 40%, and PULL-DEAE-g-PZLL 55%.

Sample	T_g (°C)	T_{onset} (°C)	T_{max} (°C)
PULL	91.3	299	323
PULL-DEAE	100.0	266	272
PULL-DEAE-g-PZLL-25%	46.7	259	272
PULL-DEAE-g-PZLL-40%	61.2	262	274
PULL-DEAE-g-PZLL-55%	38.3	263	275

different thermal behavior. The thermograms of pullulan (PULL) and pullulan modified (Fig. 9a) presented a different glass transition temperature (T_g). The copolymerization with PZLL resulted in a considerable reduction of the T_g value, reaching 38.3 °C for the PULL-DEAE-g-PZLL 55%. This reduction is probably associated with the disruption in the molecular arrangement promoted by Lys(Z)-NCA during grafting, which reduced the intermolecular interactions between pullulan chains, such as hydrogen bonds. These results may also approximate the T_g of the PZLL homopolymer. Izunobi and Higginbotham reported similar behavior at approximately 32 °C, which can corroborate the success of the grafting process.

Fig. 9b and c show the thermogravimetric curves (TGA) and their respective derivatives (DTG) of PULL, PULL-DEAE, PULL-DEAE-g-PZLL 25%, PULL-DEAE-g-PZLL 40%, and PULL-DEAE-g-PZLL 55%. All samples showed a multi-step weight loss during thermal decomposition. The

first weight loss occurred between 30 and 100 °C related to the materials' water [51,52]. The second and main weight loss occurred between 200 and 280 °C, corresponding mainly to glycerol and biopolymer degradation, except for pristine pullulan [53]. The final weight loss occurred between 290 and 400 °C due to the decomposition of the biopolymers [54].

Based on the results, PULL has a step of degradation (T_{onset}) at 299 °C and a temperature of maximum degradation (T_{max}) at 323 °C, which were higher than the modified pullulan. After modification with DEAE, the T_{onset} and T_{max} were reduced to 266 °C and 272 °C, respectively, presenting lower thermal stability than PULL. For the grafted copolymers, similar temperatures of PULL-DEAE were observed, indicating little influence of the Lys(Z)-NCA on the materials' thermal stability. It was only observed that for the PULL-DEAE-g-PZLL 25% sample, the weight loss was similar to PULL, while the grafted materials with higher PZLL content (40 and 55% w/w) presented weight loss close to PULL-DEAE. Molero and co-workers also observed similar behavior when evaluating glandless cottonseed meal/pullulan fine fiber mats [55]. Omar-Aziz and co-workers evidenced similar behavior when chemically modifying pullulan exopolysaccharide by octenyl succinic anhydride [56].

3.6. Critical aggregation concentration

PULL is a well-known hydrophilic polysaccharide, which makes it incapable of self-aggregation in aqueous solutions [57]. Through chemical modification, PULL can present new physical-chemical properties that contribute to its application in drug delivery systems. Therefore, the CAC analyses were only done for PULL-DEAE and PULL-DEAE-g-PZLL copolymers, evaluating the amphiphilic properties of the modified polysaccharide. Since PULL-DEAE presented a low degree of substitution, it kept the solubility similar to that of the starting material, PULL. Therefore, Fig. 10a. did not show any drastic modification of intensity, even with the increase in polymer concentration; this fact was expected and is justified by the hydrophilic nature of PULL-DEAE, which presents high solubility in water. PULL-DEAE-g-PZLL 25% (Fig. 10b.), on the other hand, showed a drastic change in the intensity value, reflecting the amphiphilic property of the copolymer. Thus, the CAC value was determined from the crossover point between the low and high concentrations range, resulting in 2.0 mg L⁻¹ for PULL-DEAE-g-PZLL 25% (Fig. 10b), evidencing the nanoparticle forming ability of the copolymer. With the increase in PZLL, the hydrophobicity also increases and decreases the critical aggregation concentration [58]. For PULL-DEAE-g-PZLL 40% and 55% (Fig. 10c and d.), a change in the intensity value as a function of concentration was observed, and the CAC value obtained for PULL-DEAE-g-PZLL 40% and PULL-DEAE-g-PZLL 55% was 1.6 mg L⁻¹ and 0.8 mg L⁻¹, respectively. Therefore, the copolymer with a higher percentage of monomer, PULL-DEAE-g-PZLL 55%, showed the lowest CAC. This result corroborates the increase in the hydrophobicity group grafted in the copolymer backbone.

3.7. Cytotoxicity test

The average values of the viability of cells obtained from the cytotoxicity test are shown in Fig. 11a. None of the samples were cytotoxic to L929 MEF cells since cell viability values were very close to the viability of negative control (cells grown in the culture medium). Also, all the cell viability values were higher than 70%, which is the lowest cell viability value to consider the material as cytotoxic according to the ISO 10993-5 regarding the in vitro cytotoxicity of medical devices. The viability of cells incubated with the 24 h extract from PULL-DEAE was high and close to 100% (negative control samples) at all concentrations. The values of the viability of cells incubated with the 24 h extract from PULL-DEAE-g-PZLL (25; 40, and 55%) were 98; 97, and 94%, respectively, which showed a slight decrease in cell viability with the increase of PULL-DEAE-g-PZLL content, but the average values were not statistically

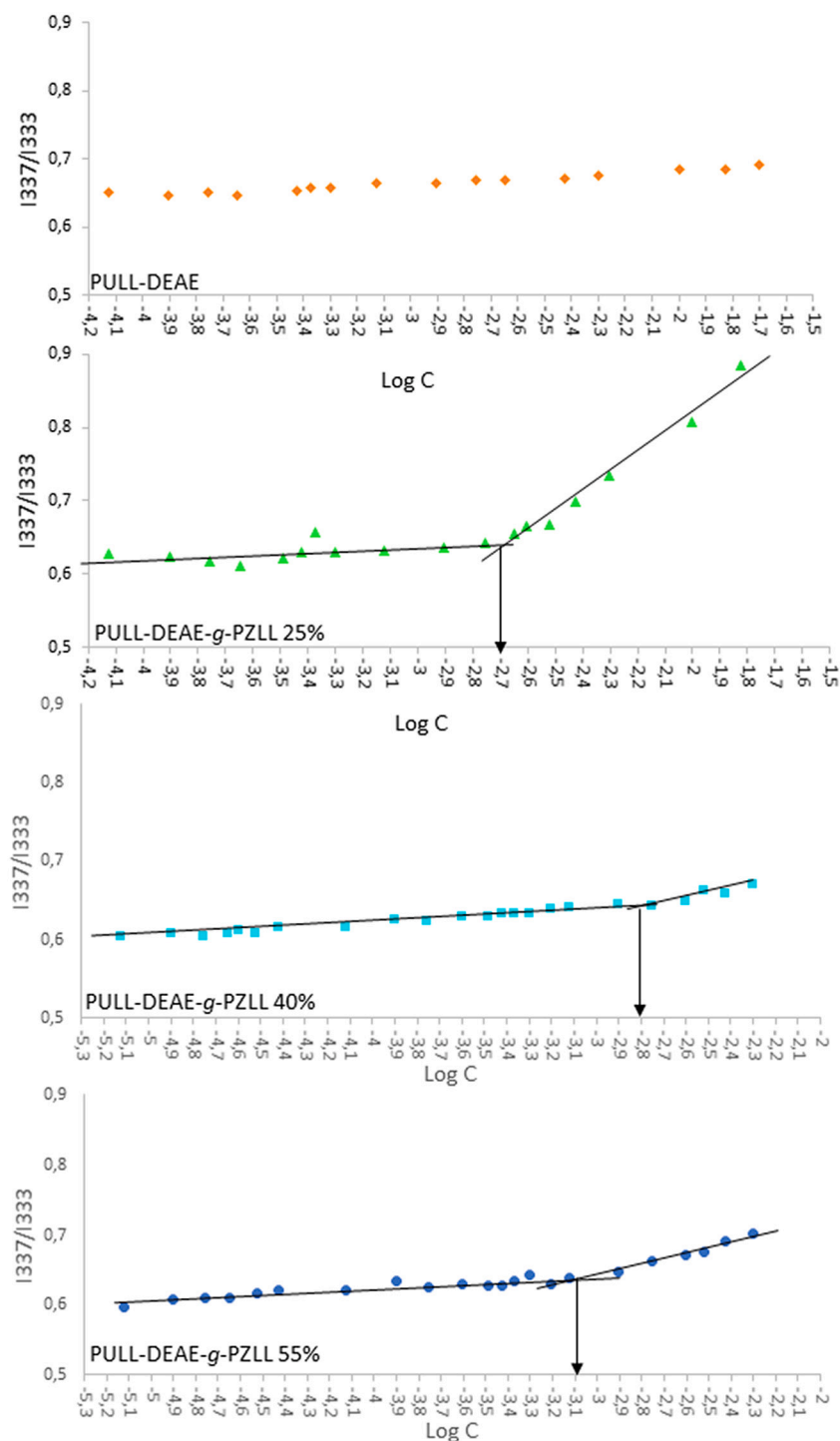


Fig. 10. Critical aggregation concentration of PULL-DEAE, PULL-DEAE-g-PZLL 25%, PULL-DEAE-g-PZLL 40%, and PULL-DEAE-g-PZLL 55%.

different. Therefore, these results pointed out that the grafting did not compromise the cytocompatibility of the pullulan. Also, since the cell viability did not change with the incubation time of 72 h in the presence of the sample solution at $100 \mu\text{g mL}^{-1}$ (Fig. 11b.), it could be concluded that the polymers were not cytotoxic when in contact with L929 MEF cells, even under prolonged time.

In order to further evaluate the biocompatibility of the samples, cell morphology was analyzed by optical microscopy after 72 h of incubation with sample extracts at 1 mg mL^{-1} . For all samples, the cells showed morphology usually observed in the L929 cell line (Fig. 12). The cells did not show membrane disruption or increased nuclear size, which could

point to cytotoxicity. Therefore, optical microscopy evidenced the absence of cytotoxicity of the investigated polymers.

4. Conclusion

In this work, cationic pullulan derivatives were prepared to evaluate the importance of a hydrophilic or amphiphilic nature for future applications in drug delivery systems. Therefore, PULL was first modified with DEAE to present tertiary amine groups capable of protonating under a low pH medium. The chemical modification was successfully confirmed using ^1H and ^{13}C NMR, revealing a partial substitution of the

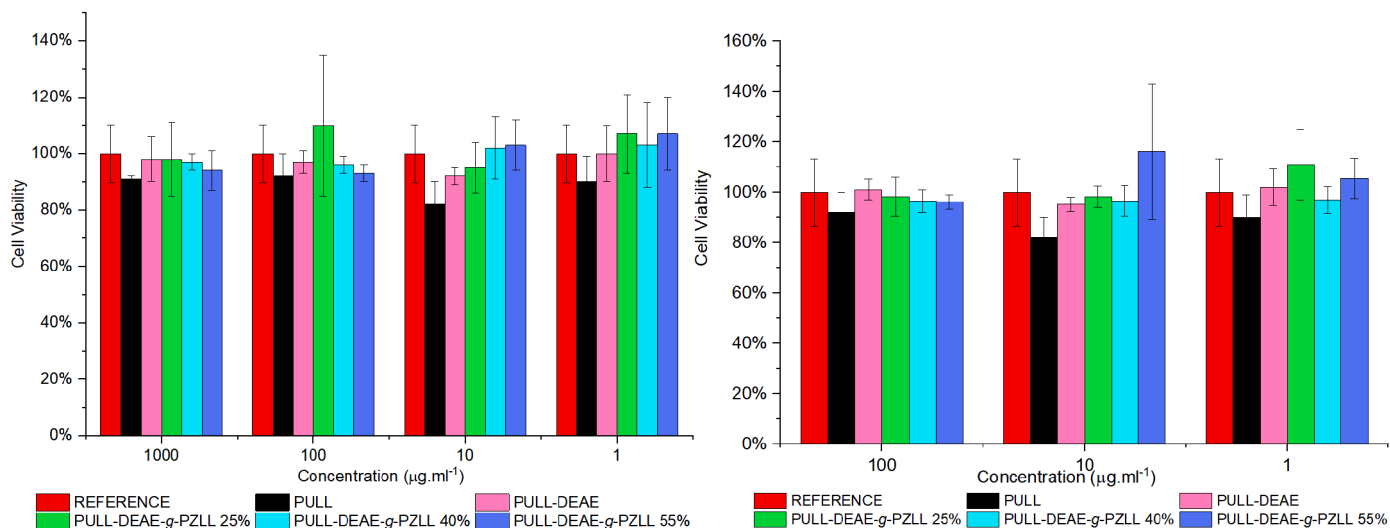


Fig. 11. Cell viability in the presence of PULL, PULL-DEAE, PULL-DEAE-g-PZLL 25%, PULL-DEAE-g-PZLL 40%, and PULL-DEAE-g-PZLL 55% at different sample concentrations with (a) 24 h of incubation and (b) 72 h of incubation.

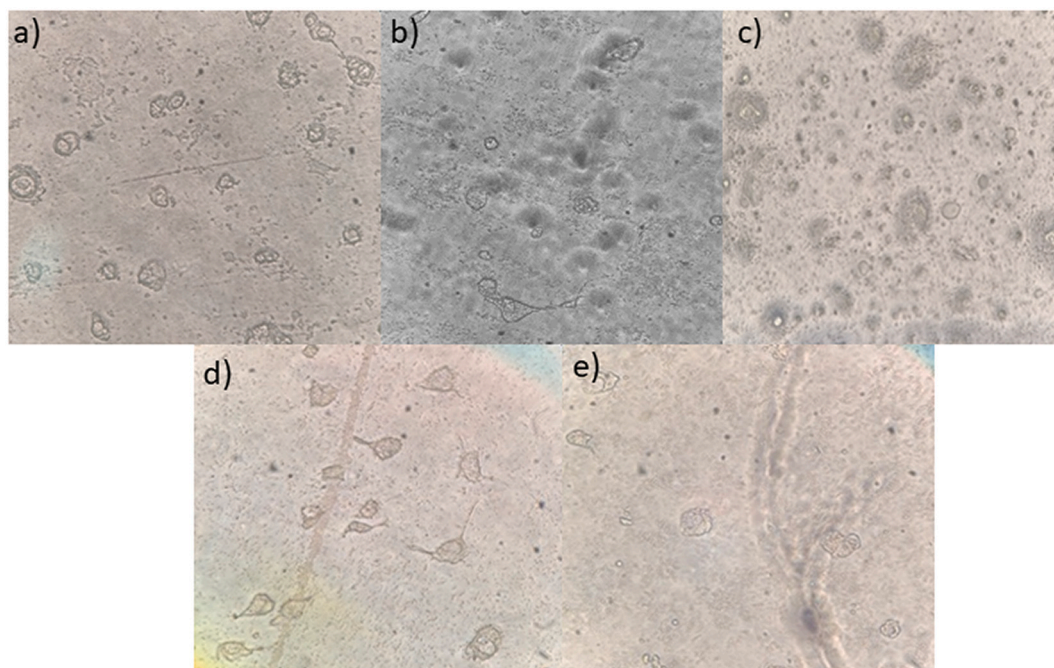


Fig. 12. Optical microscopy images of L929 cells incubated in the presence of (a) PULL, (b) PULL-DEAE, (c) PULL-DEAE-g-PZLL 25%, (d) PULL-DEAE-g-PZLL 40% and (e) PULL-DEAE-g-PZLL 55% extracts at 1 mg mL^{-1} for 72 h.

hydroxyl groups in the pullulan backbone by DEAE, with a substitution degree equivalent to 0.15. This degree of substitution was sufficient to be detected by the analyzes performed and to modify the thermal properties of pullulan while maintaining its hydrophilic behavior. When the modified pullulan was reacted with Lys(Z)-NCA by ring-opening polymerization, grafted PULL-DEAE-g-PZLL copolymers, presenting amphiphilic behavior, were obtained. Different amounts of Lys(Z)-NCA were used during the synthesis, obtaining copolymers with different amphiphilicity, demonstrated by the results of Critical Aggregation Concentration (CAC), which varied from 2.0 mg L^{-1} to 0.8 for the grafted copolymers with 0.13 and 0.63 of substitution, respectively. FTIR analysis showed that the pullulan structure was preserved even under grafted copolymerization. The grafting of different amounts of PZLL influenced the material's final thermal properties, showing a slight

reduction of thermal stability after modification. In vitro cell tests demonstrated the absence of cytotoxicity even under prolonged contact of the polymers with L929 MEF cells, which represents a promising result for the copolymers' future application as drug delivery systems. Finally, the development of cationic PULL-DEAE and PULL-DEAE-g-PZLL by the procedures described in this work represents an important contribution to future research involving the use of hydrophilic and hydrophobic pullulan derivatives for different kinds of controlled drug release devices, such as orodispersible films, solid particles suspension for oral and topical administration and particles in aqueous suspensions for intravenous administration.

Data availability

The raw/processed data required to reproduce these findings cannot be shared at this time as the data also forms part of an ongoing study.

CRedit authorship contribution statement

Layde T. Carvalho: Investigation, Conceptualization, Funding acquisition. **Ana Julia R.M. Teixeira:** Investigation, Validation. **Rodolfo M. Moraes:** Writing – original draft. **Rennan F.S. Barbosa:** Validation, Writing – original draft, Investigation. **Rafaela C. Queiroz:** Investigation, Resources. **Dayane B. Tada:** Investigation, Resources. **Daniella R. Mulinari:** Investigation, Resources, Writing – original draft. **Derval S. Rosa:** Writing – review & editing. **Maria Inês Ré:** Supervision, Writing – review & editing. **Simone F. Medeiros:** Supervision, Writing – review & editing, Conceptualization, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

The authors acknowledge Prof. Dr. Talita Martins Lacerda for the assistance with FTIR analysis and Prof. Dr. Antonio Gilberto Ferreira for NMR analysis; the Coordination for the Improvement of Higher Education Personnel (CAPES) and São Paulo Research Foundation [FAPESP, 2019/04269-0, 2021/04838-5, 2019/12940-4 and 2021/08296-2] for financial support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.reactfunctpolym.2022.105441>.

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