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Eudragit® Polymeric Nanoparticles by Nanoprecipitation: Influence of basic variables

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Abstract:	From a pharmaceutical point of view, the size (S) of polymeric nanoparticles (PN) is a critical parameter in their interaction with biological systems. The main objective of this study was to establish the influence of the organic phase (OP) and aqueous phase (AP) volumes as well as the polymer amount (PA) on the size of the PN and the polydispersity index (PDI) prepared by the nanoprecipitation method and also to evaluate the cytotoxic activity of the polymers. PN was formed from preformed polymers derived from methacrylic acid with polyvinyl alcohol (PVA) as a surfactant. The nanoprecipitation technique allowed to obtain particles with sizes smaller than 200 nm and polydispersity index lower than 0.2 and the significant impact ($p < 0.05$) of the three variables in the methodology was established. The cytotoxic potential of the polymers was evaluated in human erythrocytes, where none of the polymers exhibited significant cytotoxic activity ($p < 0.001$). In conclusion by the nanoprecipitation technique, it is possible to prepare PN based on Eudragit ® polymers, with defined and homogeneous sizes.
Keywords:	Eudragit; Nanoparticle; Nano-emulsion; Nanoprecipitation; Polydispersity; Solvent Displacement.
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Eudragit® Polymeric Nanoparticles by Nanoprecipitation: Influence of basic variables

Running title: Nanoprecipitation: Influence of variables

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Abstract

From a pharmaceutical point of view, the size (S) of polymeric nanoparticles (PN) is a critical parameter in their interaction with biological systems. The main objective of this study was to establish the influence of the organic phase (OP) and aqueous phase (AP) volumes as well as the polymer amount (PA) on the size of the PN and the polydispersity index (PDI) prepared by the nanoprecipitation method and also to evaluate the cytotoxic activity of the polymers. For this purpose, PN was formed from preformed polymers derived from methacrylic acid with polyvinyl alcohol (PVA) as a surfactant. The nanoprecipitation technique allowed to obtain particles with sizes smaller than 200 nm and polydispersity index lower than 0.2 and the significant impact ($p < 0.05$) of the three variables in the methodology was established. The cytotoxic potential of the polymers was evaluated in human erythrocytes, where none of the polymers exhibited significant cytotoxic activity ($p < 0.001$). In conclusion by the nanoprecipitation technique, it is possible to prepare PN based on Eudragit® polymers, with defined and homogeneous sizes.

Article Highlights

- The nanoprecipitation technique allows to quickly obtain nanoparticles of defined size.
- It is possible to vary the index of size and polydispersity by increasing or decreasing the organic phase, aqueous phase, and polymer mass variants.
- Eudragit polymers are not toxic against human erythrocytes.

Keywords Eudragit; Nanoparticle; Nano-emulsion; Nanoprecipitation; Polydispersity; Solvent Displacement.

List of abbreviations

PN: Polymeric nanoparticles

S: Size

OP: Organic phase ratio

AP: Aqueous phase

PA: Polymer amount

nm: Nanometers

PDI: Polydispersity index

1 Introduction

Polymeric nanoparticules (PN) or nanocarriers, have been extensively studied in the pharmaceutical field as active ingredients delivery systems with promising future. This is because this type of carrier, improves drug stability, bioavailability, and targeting due to its characteristics such as the particle size (S), which is between 100 and 500 nm. This range of particle size helps absorption and improves the biological subtract interaction giving the delivery system an advantage such as increased surface of contact, capacity of bio-targeting to specific organs/tissues, drug absorption in target tissue, reducing adverse effects of the drug due to reduction of dosages, and easy passage into the cell, bacteria, or protozoan [1]. These types of pharmaceutical carriers, are generally made of natural or synthetic biodegradable polymers and form structures with a diverse chemical nature, where the drug can be encapsulated, adhered or absorbed [2].

There is research of PN formulations made with polymers derived from methacrylic acid, where different types of drugs are encapsulated. These formulations have shown to incorporate a wide variety of active ingredients with an encapsulation efficiency of between 60 and 90% for intravenous or respiratory applications showing efficacy in both, in vitro and in vivo studies, showing low toxicity, compared with the non-encapsulated form. Therefore, this type of drug carrier and nano-formulations represent an excellent option for the treatment of different diseases [3, 4].

Drug encapsulation could increase the stability of active substances and protect sensitive substances from chemical degradation induced by pH or ultraviolet light. Furthermore, this improves the efficacy, specificity, and tolerability of the drug [5]. Eudragit® is a versatile range of polymers for drug delivery that is used to improve the stability and bioavailability of various substances, these anionic copolymers are made of methacrylic acid and methyl methacrylate and are widely used for various PN formulations, They are small particles that vary from 1 to 100 nm [6]. These Eudragit® family polymers can protect the drug that breaks down to an acidic pH (stomach), allowing its release only at a pH above 6.0, with its absorption in the intestine. These polymers have been successfully used to increase the therapeutic effects and bioavailability of different substances, such as curcumin, insulin, and pantoprazole, among others [5–7].

Obtaining PN with defined size and polydispersity index (PDI) is achieved by modifying different experimental variables of the method used to prepare PN. There are different PN manufacturing techniques, including evaporation of the emulsion, diffusion of the emulsion, and solvent displacement to produce nanoparticles from preformed polymers. Of these methods, solvent displacement, also called nanoprecipitation, is a

one-step manufacturing process, making it the first-choice method as it is also reproducible, fast, and economical for the preparation of monodisperse nanoparticles in a size range of approximately 50 to 300 nm [8]. However, although this technique has great benefits, the influence of variables that are implicit in the formation of nanoparticles has not been fully addressed [9].

The present study is focused on the evaluation of the main parameters influencing the size and PDI of PN performed by methacrylic acid-derived polymers by the nanoprecipitation technique (Fig. 1). Three variables were evaluated: the effect of the organic phase ratio (OP), the effect of the aqueous phase (AP) and the effect of polymer amount (PA). Finally, the cytotoxic activity of the formulations at different concentrations was evaluated.

2 Materials and Methods

2.1 Chemicals used

Polyvinyl alcohol (PVA, Mowiol R°4-88) donated by Clariant México, polymers derived from methacrylate Eudragit® EPO, E100, L100 and L100-55 donated by Helm México were used in this study. The rest of the solvents and reagents used were analytical grade.

2.2 Polymeric nanoparticles

PN was prepared by the nanoprecipitation technique [10], for this, an organic phase (OP) was used which contained the polymer dissolved and was miscible with water. This polymer solution was injected into an aqueous phase (AP) containing 2% w/w PVA under constant magnetic stirring (250 rpm). Different Eudragit® polymers were

used (Fig. 2): EPO, E100, L100 that was dissolved in absolute methanol (MeOH), and L100-55 that was dissolved in acetone. The diffusion of the OP was carried out by injection into the AP, which favored the aggregation of the nanoparticle-forming polymer. The PN suspension obtained was evaporated under reduced pressure with a Laborota-4003 rotary evaporator (Heidolph, Germany) for the removal of the solvents and finally, the physical characterization of the NPs was continued. The variables evaluated in this study were the following: variation of the volume of OP over AP and constant amount of polymer (PA) (12 mL + 50 mg, respectively), variation of the volume of AP over OP and constant PA (12 mL + 50 mg, respectively) and variation of the PA over constant OP and AP volumes (12 mL each). The amounts tested were as follows: for OP and AP the volumes of 5, 8, 10, 12, 15, 18, and 20 mL and in the case of the PA, were 5, 10, 25, 50, 75, and 100 mg of each polymer. Each batch was made in triplicate in at least 3 different experiments. The size and PDI of PN formulations were determined by photonic correlation spectroscopy in a Zetasizer Nano-ZS90 (Malvern Instruments, USA), from an aliquot of each batch diluted in Milli-Q water.

2.3 Cytotoxicity assay

The cytotoxicity was determined by hemolysis of a suspension of human blood erythrocytes. Human blood was obtained from healthy donors and allowed to stand at room temperature for 20 minutes. After removing the serum, the cell pack obtained was washed and centrifuged four times in phosphate buffer (PBS 10 mM/pH 7.4) with supernatant removal [11]. The erythrocytes obtained were then used to prepare a red cell suspension for the tests at 5% v/v in PBS. For the evaluation of the cytotoxicity, the previously prepared red cell suspension was incubated with different concentrations of the polymers (100 to 1,000 μg/mL) in Eppendorf® tubes (Eppendorf®, Germany), for 30

min at 37 °C protected from light, these were labeled as treatments (Tr). As a Blank, a solution of erythrocytes without treatment was used, the positive control (C+) consisted of erythrocytes without treatment with sterile distilled water to produce osmotic hemolysis [12]. Once the incubation time has elapsed, all treatments are centrifuged at 12,000 rpm (3 min / 4 °C). 200 µL of supernatant was taken and placed in a microplate (Costar®, USA) of 96 flat-bottomed wells. The degree of hemolysis was determined by spectrophotometric readings at 540 nm, the wavelength of maximum absorption of the hemoglobin released in the supernatant [13], by an ELISA reader (BioTek-ELX800). The readings were recorded as the absorbance (Abs) obtained by each treatment (TrAbs) and finally, the percentage of hemolysis was calculated by the formula: Hemolysis % = [(TrAbs - BlankAbs) / (C+Abs- BlankAbs)] × 100

2.4 Statistical analysis

For the statistical evaluation of the S and PDI of PN in the different combinations, an analysis of 2-way variance (2-way ANOVA) was applied and for the evaluation of the cytotoxic activity of the formulants, the analysis of 1-way variance (1-way ANOVA) was performed, followed by Tukey's post hoc HSD test (Honestly-significant-difference) to determine the statistical difference between the treatments, with a $p \leq 0.05$, using SPSS software, version 24.0 (IBM Inc. USA), all trials were performed in triplicate in at least three different experiments.

3 Results

3.1 Polymeric nanoparticles preparation

The particle size obtained by the solvent displacement technique was measured for four different polymers for pharmaceutical use derived from methacrylic acid with different

preparation conditions. In the first stage, the effect of the volume of the organic phase used was kept keeping constant, both the volume of the aqueous phase and the amount of polymer (12 mL of AP + 50 mg of PA, respectively). Fig. 3a shows that regardless of the polymer used in the first increments of organic phase volume (from 5 mL to 18 mL), the particle size decreases and then has little or no influence When the influence of this variable on the PDI of the EPO and E100 polymers is observed, there is a slight decrease as the volume of organic phase used increases. For the other two polymers, the trend is not very evident and there seems to be a minimum of dispersion in size at the center of the range evaluated with increasing extremes, that is, at very small or large volumes of the organic phase ($p \leq 0.05$).

Subsequently, the volume of the organic phase was fixed at 12 mL to evaluate the effect of the volume of the aqueous phase, while also maintaining the polymer mass at 50 mg. In fig. 3b only in the first increments, this variable has a slight influence on E100, L100, and L100-55. It was observed that increasing the volume of the aqueous phase decreases the particle size. The Influence was also shown for the EPO polymer, but only in the first increases in the volume of the aqueous phase, although with a greater effect on the decrease in size. The influence of this variable on the homogeneity of the particle size is not very clear except for the EPO polymer, which exhibits a decrease in the depression index as the volume of the aqueous phase increases.

Finally, the amount of polymer from 5 to 100 mg was evaluated using constant volumes of 12 mL for each of the phases. Fig. 4c shows that E100 and L100-55 polymers tend to form larger particles as the amount of polymer mass increases. The other two polymers evaluated EPO and L100 show behavior of a slight decrease in size at the beginning ($p \leq 0.001$), later, as the mass of polymer used increases, the particle

size also increases ($p \leq 0.001$). Fig. 4c shows the influence of this variable on the homogeneity of size in the populations of particles in each formulation ($p \leq 0.001$). It is shown that there is only a clear relationship between these two variables for EPO and L100-55 polymers, although inversely. As the polymer mass increases in the EPO formulations, the polydispersity index decreases ($p \leq 0.001$), while for the L100-55 polymer it increases.

3.2 Determination of the cytotoxicity of the PN

The cytotoxicity of PN based on the different Eudragit® polymer polymers was evaluated by the human erythrocyte hemolysis test. The formulations were mixed with PBS until obtaining the different concentrations to be evaluated, the erythrocytes were treated with the different concentrations of the polymers and the absorbance of the supernatant was measured at 540 nm. It was observed that none of the four polymers and in the different concentrations analyzed showed to be significantly ($p \leq 0.001$) cytotoxic. The positive control consisting of distilled water showed 100% hemolysis. Polymer-induced cytotoxicity in erythrocytes is expressed as a percentage of hemolysis (Table 1).

4 Discussion

Solvent displacement technique also commonly known as nanoprecipitation allows the formation of both nanocapsules and nanospheres [10]. To carry out this method, two miscible solvents are required, generally one organic and the other aqueous, and that both the polymer and the drug involved are soluble in only one of them, commonly in the organic system (OP), while in the second, water or aqueous solution both are

insoluble (AP) [14]. When the organic solution of the polymer is added to the non-solvent, generally by injection into the aqueous phase, the polymer undergoes rapid desolvation, which causes its precipitation after the organic solvent diffuses into the medium of dispersion, causing immediate entrapment of the drug [15].

The apparently simple process of preparing PN by the nanoprecipitation technique can involve complex interfacial hydrodynamic phenomena since the origin of the PN formation mechanism could be explained in terms of interface turbulence or spontaneous agitation of the interface between two unbalanced liquid phases, which involve flow, diffusion and surface processes [16]. This process, in which the rapid formation of PN is caused, is linked to the Marangoni effect, which establishes that the turbulences that occur at the interface between the solvent and the non-solvent are due to various accumulated phenomena that include diffusion variations, movement at an interface and longitudinal variations in interfacial tension [17]. Interfacial turbulence can be promoted by several factors that, although identified, have not been widely described [18].

This study evaluated the influence of the main variables of the nanoprecipitation method on particle size and degree of homogeneity in size (Polydispersity Index, PDI) in the preparation of nanoparticles based on preformed polymers derived from methacrylic acid (Eudragit®). As observed in Fig. 3a, where the OP increases were related to the decrease in particle size, and then had little or no influence. As the amount of polymer was kept constant, this produced a decrease in concentration. When this solution is injected into the AP, after the solvent diffuses, tiny droplets are formed that finally lead to the formation of PN. This decrease in size may be because each drop

formed in dilute solutions presented fewer polymer chains for particle formation. However, it is observed that these variable stops influencing when the OP volume increases, that is, with slightly more diluted solutions, the sizes obtained even significantly increases ($p \leq 0.05$). This implies that the sizes of the droplets formed must have increased. The droplet sizes are directly related to the ease of diffusion of the OP in the AP. Because diffusion is a phenomenon related to the concentration gradient, AP and OP meet at the beginning of the process, allowing easy diffusion, but as the process progresses, the amount of OP present in the AP increases, leading to a lower concentration gradient of the OP molecules, leading to less efficient diffusion that will possibly form larger droplets. In Fig. 4a, it is shown that with the increases in the volume of OP tested in three polymers, they showed an increase in the PDI for higher volumes, possibly due to the fact that diffusion has been hindered as mentioned, which possibly increases turbulences in the system.

When the PA volume is varied (Fig. 3b), it was observed that only in the first increments of this variable, the particle size decreases. Particle sizes produced by emulsion-based, or pseudo-emulsion-based methods such as nanoprecipitation, are directly related to droplet size [19]. Thus, it is possible that the increase in AP volume led to a decrease in droplet size, possibly because diffusion is slightly facilitated longer because of the concentration gradient, which normally decreases as OP it is incorporated into the AP, because there is a higher volume of AP, which translates into a dilution of the OP in the AP. It can also be seen that for the volumes tested for three of the polymers, the influence of this variable quickly ended, indicating that possibly the volume portion of the diffusion zone (Marangoni effect zone) presents a defined volume for each system at disseminate and cannot be expanded despite having more AP [17].

On the other hand, the influence of this variable on the homogeneity of the particle size is not very clear.

When evaluating the amount of polymer (Fig. 3c) polymers tend to form larger particles as the PA increases. This behavior indicates that the small droplets formed during the turbulence given by the diffusion of the OP in the AP maintain a greater number of polymer chains than the particles formed possibly have a greater mass and therefore a larger size. In fig. 4c there is no clear trend between the increase in the amount of polymer used and the homogeneity of the size. This shows that this variable does not present such a great influence that it exceeds the combinations of the implicit phenomena during the formation of the NP, which allows a priori to suggest conditions to obtain a homogeneous size. On the contrary, it seems to indicate that the changes caused by increases in the amount of polymer mass (PA), are unique to each system to be disseminated.

In relation to the polymer used, we can distinguish that two structurally related groups were tested (Fig. 2). One group would be those made up of the Eudragit® E100 and EPO polymers that have the same monomer and with molecular weights around 47,000 g/mol, and the other, the one formed by the structurally very similar L100 and L00-55 polymers with molecular weights of approximately 125,000 g/mol [6, 20]. The behavior of these materials in the different variables tested showed indeed a similar performance per polymer group. Regardless of the variable analyzed, the Eudragit® E100 and EPO polymers formed the largest particles. This indicates that the molecular size is not the main factor as these polymers have lower molecular weight than the L100 and L00-55 polymers. Nevertheless, their polymeric chains have larger ramifications, resulting in a greater steric effect when compacted by aggregation during nanoparticle

formation [21]. The challenge of nanoprecipitation is the choice of key parameters or variables for the system to allow a functional diffusion zone generally called the Ouzo region, in which nanoparticles will form and which other authors have also identified [22]. However, successful PN production is restricted to a close condition of the Ouzo region, beyond which microparticles or polymer aggregates are produced [23].

There are some examples described in the literature where the basic parameters are sufficient for the preparation of particles with desired characteristics [24]. However, there are situations where these variables are not sufficient to have this functional diffusion zone. In addition to the critical variables, the authors have had to increase the study of other parameters, for example, the preparation of docetaxel-charged PN, which were achieved by modifying the polymer [25]. Another example is found in the encapsulation of procaine hydrochloride, where the diffusion of the drug was decreased [26]. Although nanoprecipitation remains the first choice particle preparation technique for its simplicity, research in the literature shows that it is not exempt from evaluations of its critical parameters, as these are linked to the diffusion zone and which is not yet exhausted in such studies [27].

For a pharmaceutical drug to be useful, it must possess bioactive properties and exhibit a non-cytotoxic profile [28]. Erythrocytes have been used as a model system by several researchers to determine the interaction of drugs with mammalian membranes, the erythrocyte model has been commonly used in the elaboration of toxicity profiles since it provides a direct indication of the toxicity of formulated either injectable or administered by another route such as oral [29]. Hemolysis is the result of the destruction of the erythrocyte caused by lysis of the lipid bilayer of the membrane, the lysis of erythrocytes can cause anemia, an increase in plasma hemoglobin that causes

nephrotoxicity and vasomotor instability [30]. In a hemolytic assay, carried out with a suspension of erythrocytes and Drabkin's reagent, which is used for the quantitative colorimetric determination of blood hemoglobin, $Fe2^{+}$ of hemoglobin molecules was oxidized by potassium ferricyanide to $Fe3^{+}$, this, resulted in the formation of methemoglobin which combined with the cyanide ions to form cyanometahemoglobin, a stable compound color pigment that is read calorimetrically at 590 nm [31]. The four polymers did not show significant ($p \leq 0.001$) cytotoxicity, the hemolytic activity was less than 0.1% (Table 1) in all the formulations. Therefore, the hemolytic activity less than 1% obtained for the four polymers is an indicator of non-toxicity for the red cell membrane, which therefore favors the subsequent study with these polymers.

5 Conclusion

The results of the nanoparticle formation of Eudragit® polymers by the solvent displacement technique confirmed that size and homogeneity are directly related to the fundamental variables of the technique. This comparative study allows to choose the right combination for the formulation of NP with a defined particle size in the range of 70 to 230 nm in a simple way and to reproduce it, through polymers derived from methacrylic acid with populations of particles with a high homogeneity size.

Compliance with ethical standards

Conflict of interest The authors report no conflicts of interest.

Ethical statement All procedures were approved by the institutional research ethics committee and performed in accordance with the recommendations of the Declaration

of Helsinki on biomedical research involving human subjects. The study with human erythrocytes was carried out under the approval of the ethics committee of the Autonomous University of Nuevo León., College of Medicine (Reg.No.HI11002) and the consent of healthy donors, following the provisions of the Official Mexican Technical Standard NOM-253-SSA1-2012

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Table 1. Cytotoxic activity by different Eudragit® polymers at different concentrations

	POLYMERS			
µg/mL	**EPO**	**E100**	**L100**	**L100-55**
C+	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0	100.0 ± 0.0
100	0.013 ± 0.002^{a}	0.013 ± 0.004^{a}	0.029 ± 0.007^{a}	0.012 ± 0.002^{a}
200	0.016 ± 0.002^{ab}	0.014 ± 0.002^{a}	0.041 ± 0.012^{b}	0.016 ± 0.002^{a}
400	0.018 ± 0.001^{ab}	0.017 ± 0.002^{a}	0.042 ± 0.010^{b}	0.017 ± 0.004^{a}
600	0.019 ± 0.002^{ab}	0.017 ± 0.003^{a}	0.043 ± 0.007^{b}	0.019 ± 0.001^{a}
800	0.020 ± 0.004^{ab}	0.018 ± 0.001^{a}	0.045 ± 0.005^{b}	0.020 ± 0.009^{ab}
1,000	0.025 ± 0.003^{b}	0.021 ± 0.005^{ab}	0.048 ± 0.005^{b}	0.023 ± 0.006^{ab}
SE	0.002	0.001	0.003	0.002
***p* ANOVA**	< 0.001	< 0.001	< 0.001	< 0.001
F ANOVA	17.235	8.795	4.387	0.933

Values are shown as the mean ± SD (n=3) ($p < 0.05$) of the % of cytotoxicity, and the standard error (SE). Different letters within the same column are significantly different analyzed via the Tukey test.

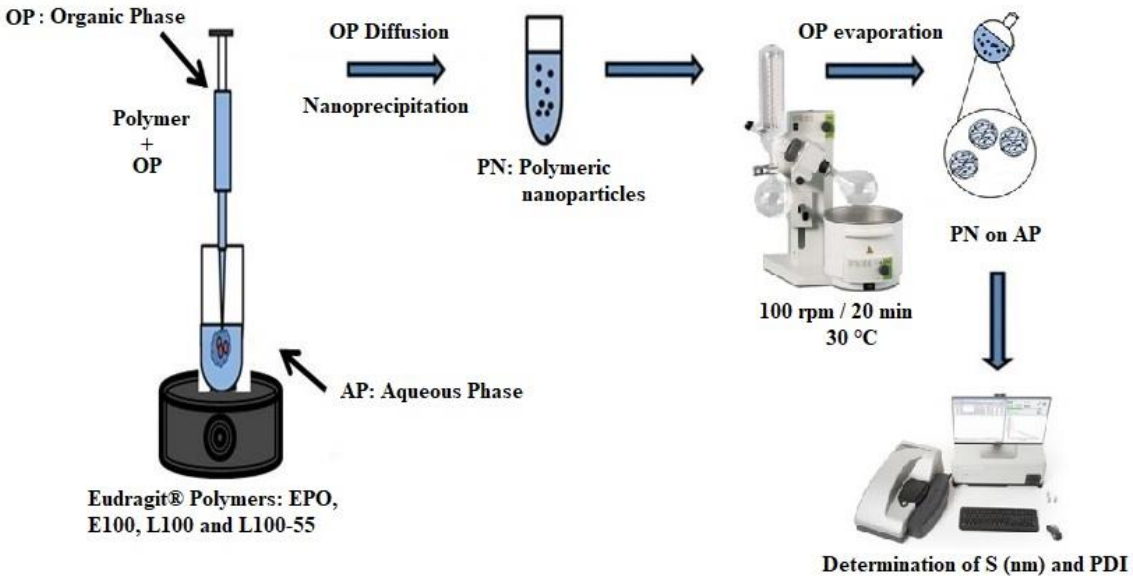


Fig. 1. Diagram of the nanoprecipitation technique. OP: organic phase, AP: aqueous phase, PN: polymeric nanoparticles, PDI: Polydispersity, S: Size.

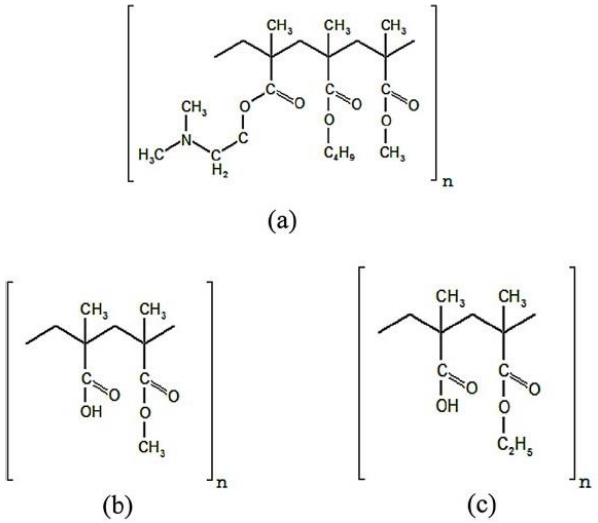
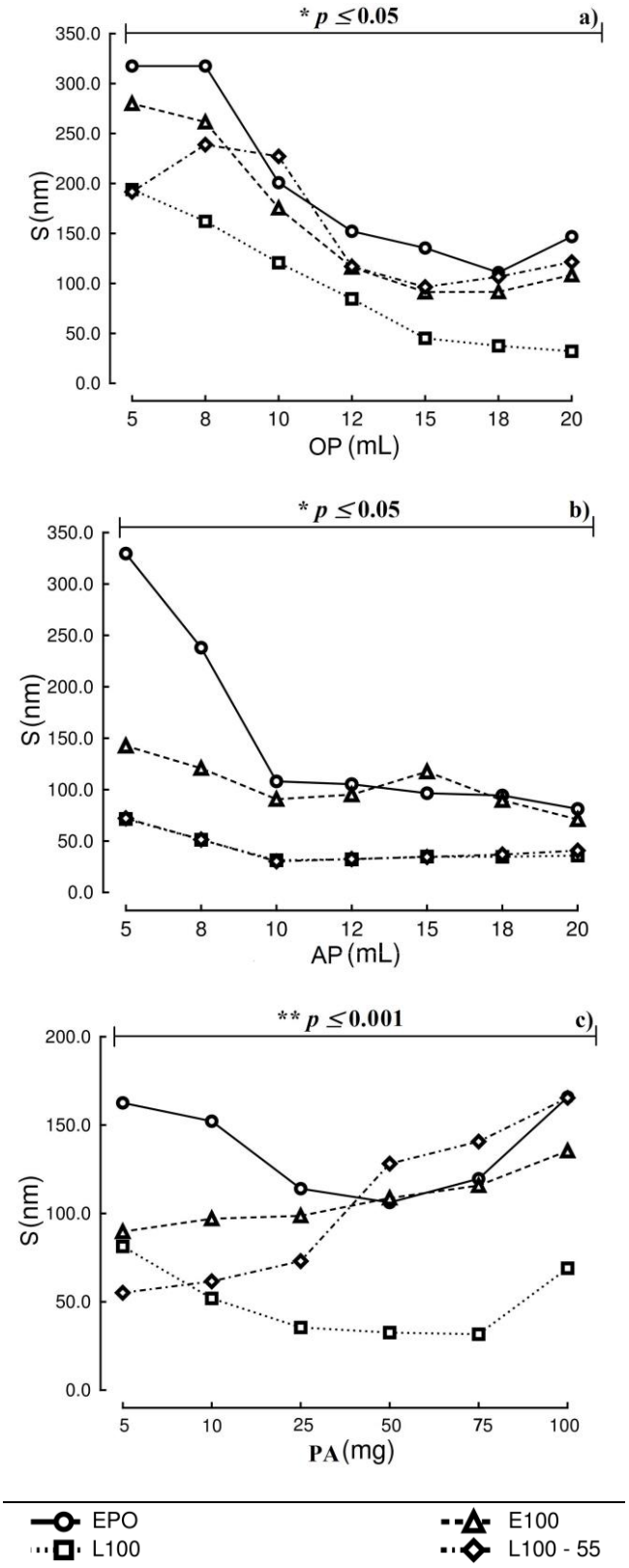


Fig. 2. Chemical structures of Eudragit® polymers: **a):** EPO, **b):** E100, **c):** L100 and **d):** L100-55. [6, 20].

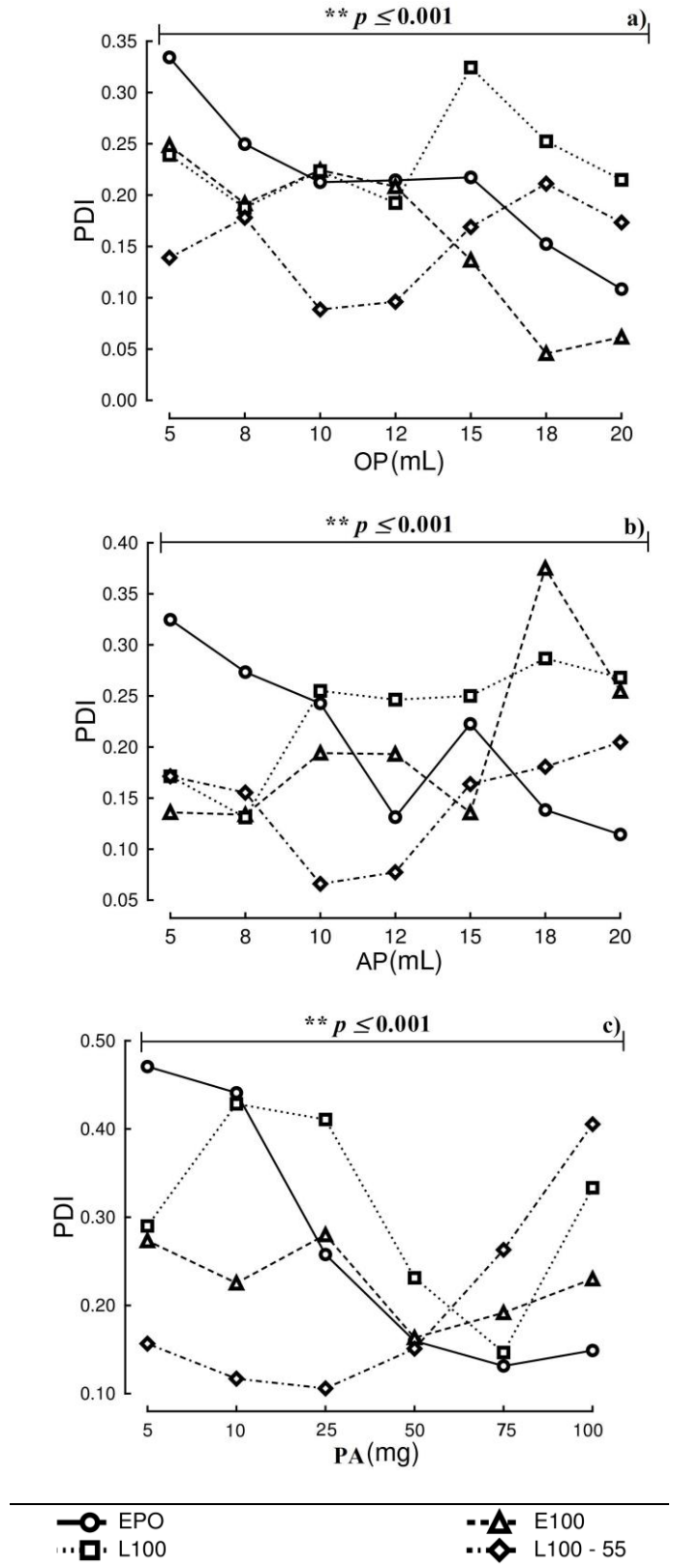


499

500 **Fig. 3.** Effect on S (nm), in the different formulations. **a).** Effect of OP volume on S (50

501 mg MP + 12 mL AP). **b).** Effect of AP volume on S (50 mg PA + 12 mL OP). **c).** Effect

of PA on S (12 mL + OP 12 mL AP). OP: organic phase, AP: aqueous phase, PA: polymer amount. Each point represents the mean of 3 different experiments (n = 6). *Significant difference: $p \leq 0.05$. **Highly significant difference: $p \leq 0.001$.


** p ≤0.001
a)
PDI
OP(mL)
** p ≤0.001
b)
PDI
AP(mL)
** p ≤0.001
c)
PDI
PA(mg)
EPO
L100
E100
L100 - 55

521

522 **Fig. 4**. Effect on the PDI, in the different formulations. **a)**. Effect of OP volume on S
523 (50 mg MP + 12 mL AP). **b)**. Effect of AP volume on S (50 mg PA + 12 mL OP). **c)**.
524 Effect of PA on S (12 mL + OP 12 mL AP). OP: organic phase, AP: aqueous phase, PA:
525 polymer amount. Each point represents the mean of 3 different experiments (n = 6).
526 *Significant difference: $p \leq 0.05$. **Highly significant difference: $p \leq 0.001$.
527