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Synergic Influence of Parameters Involved in the Polymeric Nanoparticle Preparation on the Efficacy of Photodynamic Therapy

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Abstract

The challenge was always great for lipophilic photosensitizer use in the photodynamic therapy (PDT) for treatment of internal body diseases. Photosensitizer metabolism in liver, incompatibility of the molecules in the gastric acid, aggregation in the bloodstream, opsonization of molecules and phagocytosing process hamper the application of the free lipophilic photosensitizer in disease treatment using PDT. This problem has been partially resolved using the drug delivery system to encapsulate the photosensitizer. Many studies have been reported using polymeric nanoparticles to encapsulate the lipophilic photosensitizer showing excellent results for PDT, but few nanoparticulate formulations are available at the pharmacies. The absence of deep knowledge about the influence of synergic effect of parameters used in the nanoparticle preparation on its properties, the photobleaching process of encapsulated photosensitizer and the molecule aggregation into the nanoparticle can decrease the photodynamic efficacy for the lipophilic photosensitizer. Our research group has studied the influence of many parameters on the nanoparticulate properties of several encapsulated phthalocyanines and porphyrin using factorial design, evaluating the free and encapsulated compound aggregation, efficacy to reduce the viability of cancer cells, the photooxidation of the biomolecules and the influence of photobleaching. This work shows the most important results to be considered in the optimization of the polymeric nanoparticle.

Keywords: polymeric nanoparticle, factorial design, phthalocyanine, porphyrin, photodynamic therapy, photooxidation, cancer cells, photobleaching

1. Introduction

Photodynamic therapy (PDT) is an important therapeutic modality used in the treatment of cancer and several non-malignant diseases, including infections and dental treatments [1–5]. It is characterized by the administration of a photosensitizer (PS), a light source to activate it and oxygen molecules (**Figure 1**) [6]. After administration of the photosensitizer, the diseased tissue is irradiated with

Figure 1.

PDT mechanism involving the combination of a photosensitizer, a light source and oxygen molecules. After excitation to a higher energy state (1), the photosensitizer may suffer rotovibrational decays to the excited state S_1 (2), from which the photosensitizer can suffer energy decay to the fundamental state S_0 via fluorescence (3), intersystem crossing (4) or phosphorescence (5).

visible light, causing the excitation of the PS to a singlet electronic state (S_1), which can be deactivated to the fundamental state (S_0) through radiative processes (fluorescence or phosphorescence) or non-radioactive (internal conversion, intersystem crossing or vibrational relaxation). Among these processes, intersystem crossing is essential for PDT. It consists of a prohibited transition by spin from the excited singlet electronic state (S_1) to the excited triplet state (T_1). In this state, the PS can interact with oxygen molecules or other biomolecules that are present in the irradiated tissue generating reactive oxygen species (ROS) that can cause damage to diseased tissues [7, 8]. These ROS can be generated by two mechanisms, [9] involving energy transfer (type II mechanism) or electron transfer (type I mechanism) (**Figure 1**).

The success of the treatment, fundamentally, depends on the photochemical, photobiological and pharmacokinetic properties of the photosensitizer. New porphyrin and phthalocyanine derivatives have been synthesized and encapsulated because their photochemical properties are suitable for PDT [10–12]. In general, hydrophobic photosensitizers tend to form aggregates in aqueous medium, affecting their bioavailability and their ability to generate reactive oxygen species, [13] and consequently, reducing their efficacy in treatment by PDT. In addition, lipophilic molecules hamper the administration of photosensitizer by parenteral via [14]. The nanocarrier systems has proven to be quite effective to solve this problem since they facilitate the administration of the hydrophobic photosensitizer, protect the photosensitizer from aggressive environments and decreasing its aggregation state [15].

Many studies show prominent results with polymeric nanoparticles as carriers of lipophilic photosensitizers due to the benefits associated with their application in PDT for cancer treatment [16–18] such as effectively increase in the amount of PS in the target tissue due to a greater volume/area ratio; prevent the premature release of the photosensitizer, avoiding its accumulation in healthy tissues; maintaining drug concentration at therapeutically appropriate intervals in blood circulation and tissues; greater ability to penetrate the target tissue due to its size; in addition to protecting drugs from liver inactivation and enzymatic degradation [15].

Another advantage of polymeric nanoparticles is their biocompatibility and biodegradability, once it is degraded by enzymatic processes that generates non-toxic and biocompatible products, being removed from the body by physiological pathways. An example is the nanoparticle of poly(lactide-co-glycolide) (PLGA), a polymer approved by the Food and Drug Administration (FDA) and that we used in our research [15]. However, it also be reported that the use of nanomaterials in contemporary clinical practice need to be monitored because of the unpredicted effects of the cumulative exposes of non-biodegradable nanoparticle in the human body [19].

Few nanoparticulate formulations are on the shelves of pharmacies due to the lack of knowledge of the combinatorial influence of the parameters used in the preparation of the nanoparticles on the fundamental properties for maximum therapeutic potential, [5] a fact that hampers the scale up process for the production of nanoparticulate formulations. Besides, the poor batch-to batch reproducibility to prepare polymeric nanoparticle, the low solubility of some polymers in water that requires the use of organic solvent to synthesize the nanoparticle, the low glass transition temperature of some polymers that limit the use of them to prepare the nanoparticulate formulation and the high cost of biodegradable polymers are drawback that hamper the development of nanoparticulate pharmaceutical formulation for using in PDT. For these reasons, we have studied the influence of the parameters involved in the preparation of polymeric nanoparticle loaded with several porphyrin and phthalocyanine derivatives (**Figures 2-4**) that have different physicochemical properties (**Table 1**).

Given these considerations, we present an overview of the main results obtained by our research group on the influence of several preparation parameters on the final properties of polymeric nanoparticles loaded with photosensitizers for

Figure 2.
Molecular structures optimized by Avogadro and MOPAC software for (A) gallium phthalocyanine chloride (GaPc), (B) indium phthalocyanine chloride (InPc) and (C) chloro(5,10,15,20-tetraphenylporphyrinato)indium (III) (InTPP).

Figure 3. Molecular structures optimized by Avogadro and MOPAC software for (A) 5-hexyl-10,20-bis(3-hydroxyphenyl)porphyrin (hex-m-bisHPP), (B) 5-hexyl-10,15,20-tris(3-hydroxyphenyl)porphyrin (hex-m-trisHPP) and (C) 5,10,15,20-tetra(3-hydroxyphenyl)porphyrin (m-THPP).

Figure 4. Molecular structures optimized by Avogadro and MOPAC software for 1,4-(tetrakis[4-(benzyloxy)phenoxy]phthalocyaninato)indium(III) chloride (InTBPPc).

application in PDT, besides regarding the effect of encapsulation in reducing the photobleaching process of photosensitizer and on the efficiency of nanoparticles containing photosensitive compounds in reducing the viability of cancer cells or in biomolecules photooxidation.

Compound	GaPc	InPc	InTPP	m-THPP	Hex-m-BisHPP	Hex-m-TrisHPP	InTBPPc
Heat of Formation / kj/mol [§]	867.59016	866.26780	912.86350	418.11873	569.44802	412.25908	545.82102
Volume / Å ³ [§]	596.57	606.78	782.35	781.06	691.12	799.51	1555.32
Ionization Potential / EV [§]	7.304876	7.272500	7.860061	8.012104	7.779898	7.811042	7.160890
Energy Difference HOMO-LUMO / EV [§]	4.862	4.903	5.965	6.185	5.969	6.106	4.922
Dipole Moment / D [§]	7.00151	7.40825	4.43138	3.91867	2.74405	1.76290	8.27066
Log P*	4.52	1.67	2.33	6.82	6.78	7.57	9.17
Log S*	−4.04	−4.59	−6.12	−5.23	−5.10	−5.33	−6.52
Polarizability**	66.75	67.90	90.90	87.68	75.43	87.05	157.27
HLB**	4.20	4.50	3.40	3.22	3.20	3.05	7.63

*Values were calculated from ALOGPS 2.1 Program <http://www.vclab.org/lab/alogps/>
**Values were calculated from MarvinSketch 20.16 Program.
[§]Values were calculated from MOPAC 2016 Program.

Table 1.
Physicochemical properties of the studied photosensitizers.

2. Influence of nanoparticles preparation parameters on their final properties

The development of carrier systems involves a lot of study of the variables used in the formulations preparation and their influences on the nanoparticulate properties that reflect on the cellular uptake of nanoparticles, its bioavailability and, consequently, the photodynamic efficiency. The approach to analyze the individual effect and the combinatorial effect (synergistic or antagonistic) of the parameters is usually done by factorial design of experiments, which all levels (experimental domains) of a factor are combined with all levels of the other factors of the experiment [16].

The influence of factors on the characteristics of nanoparticles is intrinsically linked to the production process [16, 20, 21]. Many works have shown the individual effects of some parameters involved in the nanoparticles preparation stage on their properties. However, the influence of a parameter used in a polymeric nanoparticles formulation will not always produce the same response for similar formulations [5].

There are significant challenges to consolidate polymeric nanoformulations in the pharmaceutical market since small changes in the composition of the formulation, for example, the encapsulated drug, can influence the nanoparticles properties, such as the particle size, the surface charge, the residual amount of emulsifier on the surface of the particles, and encapsulation and recovery efficiencies of the nanoparticles [5].

2.1 Size

The particle size used for the treatment of oncological and non-oncological diseases depends on the administration route and/or the type of diseased tissue. For example, in intravenous administration, the particles must be smaller than 5 μm in order to circulate through the capillaries, however, smaller sizes are necessary for nanoparticles reach the tumor vessels and remain longer in the blood stream [5, 8, 22].

Researches have shown that nanoparticles with sizes smaller than 200 nm have a longer circulation time in the bloodstream due to the reduction of the recognition of the nanoparticle by plasma proteins (opsonin) that signal the reticuloendothelial system to act in the phagocytosis process of the nanoparticles. Remaining longer in the circulatory system, smaller diameter particles could interact more effectively with cell membranes, presenting greater capacity of cellular internalization due to the overexpression of porous in tumor cells membranes, a fact that would result in greater efficiency of nanoparticulate photosensitizers in reducing cell viability through PDT [5, 8, 22].

A highly significant parameter in causing changes in the nanoparticles size was the stirring rate used in the preparation process. The increase of stirring rate leads to smaller sized nanoparticles due to the better dispersion of the organic phase in the aqueous phase, reducing the droplet size of the organic phase and, consequently, the nanoparticle size [20].

Although the stirring rate is considered the main factor responsible for the size reduction of the nanoparticle, in some formulations this parameter is not significant [23]. In the preparation of PLGA-PEG nanoparticles loaded with chloro(5,10,15,20-tetraphenylporphyrinato) indium(III) (InTPP – **Figure 2C**), the ethanol percentage in the aqueous phase was the main parameter responsible for size decrease, not the stirring rate [20].

Our group demonstrated that the individual increasing in the ethanol percentage in the aqueous and organic phases contribute to reduce the nanoparticles size. The ethanol present in the aqueous phase causes an increase in viscosity while the addition of ethanol in the organic phase accelerates the separation of phases from

the PLGA during the dispersion of the organic phase in the aqueous phase. These effects hinder the coalescence of organic droplets dispersed in the aqueous phase, preventing an increase in the nanoparticles size [16, 20].

Analyzing two preparation methods, the PLGA-PEG nanoparticles loaded with gallium phthalocyanine (GaPc - **Figure 2A**) prepared by the Emulsification-Diffusion Method (EDM) were smaller in size than the nanoparticles prepared by the Emulsification-Evaporation Method (EEM). In the EDM method, the organic solvent is dispersed in the aqueous phase generating droplets that are stabilized by colloidal stabilizing agents, however, the rapid efflux of solvent can cause the formation of aggregates and a population with varying sizes [5].

The aqueous phase temperature is another factor that can positively or negatively influence the nanoparticles size. The increase in temperature reduces the viscosity of the mixture between the organic phase and the aqueous phase, favoring the coalescence of organic solvent drops and consequently increasing the particles size, but the increase in temperature also favors the diffusion of the organic solvent into the aqueous phase, favoring the reduction of particle size. In the EDM method the effect of coalescence is more pronounced, causing the size increase, while in the EEM method the diffusion of the organic solvent to the aqueous phase is more pronounced, decreasing the nanoparticle size [5].

Combinatory effects of two parameters can also be significant for nanoparticle size. The binary effect between changing the method from EEM to EDM and increasing the aqueous phase temperature tends to increase the nanoparticles size [5]. Univariate methods do not allow to identify the combinatory effect that could be important for a determinate nanoparticulate property being necessary the use of factorial design.

The ratio between the photosensitizer mass and the polymer mass is also a parameter that can influence the nanoparticle size. In the study of the preparation of PLGA nanoparticles loaded with three porphyrins (Hex-m-bis-HPP, Hex-m-tris-HPP and m-THPP - **Figure 3A-C**, respectively) with different amphiphilicities, [16] the effect of the porphyrin/polymer mass ratio on the nanoparticle size was only significant in m-THPP nanoparticles, which the increase in the mass ratio caused a reduction in size. The low polymer concentration reduces the organic phase viscous resistance against the shear force during the emulsification process, favoring the reduction of the organic phase droplets size dispersed in the aqueous phase and, consequently, reducing the nanoparticles size [16].

Different results have also been reported in the literature, not observing any effect of the photosensitizer mass/polymer mass ratio on the PLGA nanoparticles size loaded with bupivacaine [24], while others have reported that an increase in the proportion decreased the nanoparticles size [25, 26].

Another parameter that can also influence the nanoparticles size is the polyvinyl alcohol (PVA) concentration, which is the most used emulsifier in formulations preparation stage. In some cases, the effect of increasing the PVA concentration on the particle size may be very similar to the effect of the stirring rate. It is known that PVA molecules anchor in the aqueous/organic interface formed during emulsification, causing a decrease in interfacial tension and favoring the mechanical and spatial stabilizations of the organic droplets dispersed in the aqueous phase [27]. In addition, polymeric PVA chains not anchored to the nanosphere surface can increase the aqueous phase viscosity. The reduction in interfacial tension and the increase in viscosity caused by PVA favor a decrease in the nanosphere size [20].

Emulsification time is a factor that may or may not be significant on size. In the preparation of PLGA-PEG nanoparticles containing gallium phthalocyanine, this parameter did not significantly influence the nanoparticles size [5], however some studies have already shown that it can increase or decrease the size [28]. As stated

earlier, each parameter behaves in a particular way according to the parameters used in the preparation of nanoparticles. All these influences on size were summarized in **Figure 5**.

Besides the preparation parameters, the physicochemical properties of photosensitizers can also influence nanoparticles size. The polymeric PLGA-PEG nanoparticles loaded with GaPc or InTBPPc had different results for size distribution. The average diameter of the InTBPPc nanoparticles was 1.3 times greater than for nanoparticles with GaPc. According to the optimized structures designed by the molecular modeling program (**Figure 2A** and **4**), it was shown that InTBPPc has a volume of 1555.32 Å³ (**Table 1**), that is 2.6 times greater than GaPc. This result suggests that the molecular size of the encapsulated phthalocyanine may influence the increase in the size distribution and the nanoparticles diameter [29].

The storage of lyophilized samples at certain temperatures is another factor that can influence the particles size. Studies were conducted with the PLGA-PEG nanoparticles loaded with GaPc (**Figure 6**) to evaluate the influence of the formulation storage at different temperatures on the nanoparticles size. The experiments were carried out with a formulation characterized by presenting 88.9% of the nanoparticles with a diameter smaller than 199.9 nm, an important outcome since particles smaller than 200 nm remain longer in the circulatory system [5]. The experiments suggest that the temperature of 20°C is more suitable for storage purposes of the formulations for 4 weeks, due to the results of less variation in the average diameter of the particles. Even considering the statistical variation of the measurements, there are changes in the PLGA-PEG nanoparticles size that may be associated with the aggregation of the particles during the storage period and the difficulty of disintegrating them during the process of redispersion in water. Such average size variations were greater for lower or higher temperatures than 20°C.

Similar analysis was performed with lyophilized formulations of PLGA-PEG nanoparticulate loaded with InTBPPc (**Figure 7**) for only 12 days at temperatures of 5°C and 35°C. Before the storage process, the formulation was characterized with a population of 98.9% of the particles with an average diameter smaller than 199.9 nm. In the short storage period, the generation of small and large aggregates

Figure 5. Effects of some parameters involved in the nanoparticles preparation stage [(A) stirring rate, (B) PVA concentration, (C) ethanol concentration in the aqueous phase, (D) ethanol concentration in the organic phase, (E) emulsification time, (F) changing the preparation method from EEM to EDM, (G) aqueous phase temperature, (H) photosensitizer mass/polymer mass ratio] over different nanoparticulate properties [(RC) residual chloroform, (EE) entrapment efficiency, (RE) recovery efficiency, (PVAr) residual PVA, size, (ZP) zeta potential].

Figure 6.
Average diameter of the PLGA-PEG nanoparticle loaded with GaPc after storage for 1–4 weeks in different temperatures.

Figure 7.
Average diameter of the PLGA-PEG nanoparticle loaded with InTBPPc after storage for 12 days in different temperatures.

was observed at temperatures of 5°C and 35°C, with the size variation being greater for the temperature of 35°C in the first 8 days. This temperature is above the PLGA-PEG glass transition temperature (T_g at 30°C), [30] a fact that favors particle aggregation. Therefore, it can be concluded that storage temperatures, whether low or high, can influence the formation of aggregates, a fact that could reduce the photosensitizer efficiency during PDT.

2.2 Zeta potential

The zeta potential is a property related to the particles physical stability that can be used to measure the magnitude of the repulsion or attraction. The maximization of the repulsive forces between the nanoparticles, minimizes the interactions responsible for colloidal instability, consequently interfering in the photodynamic efficacy of an encapsulated photosensitizer [5, 20].

Nanoparticles coated with amphiphilic polymers, such as PEG, usually have a higher zeta potential due to the increase in the contact surface and, consequently, to the shielding of the nanoparticle surface charge [31–34]. Therefore, the greater surface area (ratio of surface area/volume) of the nanoparticle, the greater is the residual PVA percentage at the nanoparticle interface and, consequently, the greater is the zeta potential value [5].

Thus, it is expected the parameters that influence the nanoparticles size will also be significant on the zeta potential values. As the stirring rate is one of the main factors in causing the decrease of the nanoparticles size, it will also be expected that this factor is able to affect the zeta potential, increasing its value [20].

GaPc-loaded PLGA-PEG nanoparticles presented higher zeta potential values when prepared by EDM than those prepared by the EEM. This fact corroborates with the results obtained from the nanoparticle prepared by the EEM, which presented greater sizes and smaller values of zeta potential, suggesting that they are more stable from an electrostatic point of view [5].

The increase in the aqueous phase temperature also caused a significant decrease in the absolute value of the zeta potential, because this factor induced an increase in the nanoparticles size which have less residual PVA adsorbed on their surfaces, resulting in a smaller zeta potential [5].

In the preparation of PLGA nanoparticles containing three porphyrins (m-THPP, Hex-m-bisHPP and Hex-m-trisHPP) with different amphiphilicities, each formulation presented a different response of the preparation parameters related to the zeta potential, with results intrinsically linked to particle size. For Hex-m-bisHPP-loaded nanoparticles, the increase in the ethanol percentage in the aqueous phase caused an increase in the zeta potential due to the decrease of the nanoparticle size. While for nanoparticles containing m-THPP, the porphyrin/polymer mass ratio was the only significant factor that caused an increase in the zeta potential value since this factor decreased the particle size [16]. The summary of all influences on the zeta potential was indicated in **Figure 5**.

2.3 Entrapment efficiency

The entrapment efficiency relates the amount of drug that was effectively encapsulated/adsorbed on the nanoparticle. This property depends on the physicochemical properties and the interaction between the photosensitizer, the carrier matrix and the surrounding environment. Studies have shown that higher entrapment efficiency is associated with better photodynamic efficiencies for a short period of light activation [19, 35].

The diffusion process of the photosensitizer from the organic phase to the aqueous phase has significantly influenced the substance entrapment efficiency during the nanoparticle preparation process. Results have shown that the individual increase in the PVA concentration and the ethanol concentration in the aqueous phase tend to increase the photosensitizer encapsulation. The aqueous phase viscosity increases with the PVA and ethanol concentrations, which favors the formation of smaller sizes nanoparticles, having a specific surface area (area/volume) that

allows a greater number of PVA molecules at the interface of the organic/aqueous phase. This hinders the diffusion of photosensitizers from the organic phase to the aqueous phase, favoring an increase in nanoparticle encapsulation. On the other hand, the combinatory effect caused by the simultaneous increase in the concentration of PVA and ethanol in the aqueous phase decreases the entrapment efficiency of InTPP in PLGA nanoparticles, as experiments showed that PVA and ethanol favor the solubilization of InTPP in aqueous medium [20].

When the method is changed from EEM to EDM, the entrapment efficiency decreases since the EDM method favors the formation of smaller diameter nanoparticles, facilitating the organic solvent diffusion into the aqueous phase and decreasing the entrapment efficiency of the photosensitizer in nanoparticles [5].

The increase of the aqueous phase temperature combine with the change in the preparation method also influences the photosensitizer entrapment efficiency. In the EEM method, the increase in the aqueous phase temperature causes the more effectively evaporation of the organic solvent, leading to fast polymer coacervation and, consequently, the organic/aqueous interface solidification. This increases the photosensitizer entrapment efficiency in the PLGA-PEG nanoparticles prepared by EEM. In the EDM method, the same increase in the aqueous phase temperature favors the solvent diffusion from the organic phase to the aqueous phase that carries the photosensitizer out of the nanoparticle, decreasing the entrapment efficiency [5]. All effects of parameters on entrapment efficiency were registered in **Figure 5**.

In addition to the parameters used in the nanoparticle preparation, the physicochemical properties of the photosensitizer may interfere on the entrapment efficiency. As an example, molecules of greater polarity tend to diffuse more easily from the organic phase to the aqueous phase, decreasing the entrapment efficiency [16]. The theoretical calculations compared to experimental results have suggested that photosensitizers with higher volume tend to be less efficiently encapsulated by nanoparticles. This was observed for InTBPPc molecules and also for Hex-m-TrisHPP molecules (**Table 1**). Molecules that have close volume values have shown similar entrapment efficiency as GaPc and InPc (**Figure 2A, B**, respectively).

2.4 Recovery efficiency

The recovery efficiency calculates the percentage of nanoparticle that has been produced and recovered. It is a property that has economic importance and has great value for the pharmaceutical industries, since they aim to reduce the production costs of the nanoparticulate formulation.

The nanoparticles size influences directly the recovery efficiency. Smaller nanoparticles are expected to be less recovered during the washing step than larger nanoparticles since the sedimentation rate of the particles in a centrifugal field is proportional to the square particle diameter. Thus, the parameters that influence the nanoparticles size tend to influence the recovery efficiency [20].

Parameters that cause a reduction in size, such as stirring rate, the EDM preparation method, the ethanol concentration in the aqueous or organic phase, as well as the emulsification time can favor the decrease of the recovery efficiency [5, 20].

Synergistic effects can be significant for recovery efficiency. For example, increasing the aqueous phase temperature together with the change in the preparation method, or increasing the emulsification time together with the change from the EEM method to EDM, can increase the recovery efficiency [5]. All effects of the parameters used to prepare of nanoparticles on the recovery efficiency are shown in **Figure 5**.

2.5 Residual polyvinyl alcohol (PVA)

PVA is the emulsifier most commonly used in the polymeric nanoparticles preparation. Even with the washing steps during the process, aiming to reduce the excess of PVA, an amount of these molecules remains adsorbed to the nanoparticle polymeric matrix due to the orientation of the PVA hydrophobic part in the organic phase, keeping molecules attach on the surface of the particle after the coacervation process [31]. This residual PVA on the particles surface can interfere on the nanoparticles physicochemical and biological properties, such as the size, release profiles of encapsulated drugs and intracellular uptake of the nanoparticles.

PVA tends to be adsorbed on the nanoparticle surface through the hydrophobic part of vinyl acetate, which tends to anchor the polymer in the aqueous/organic interface formed during the emulsification process. Smaller sized particles have a greater specific surface area, so it requires a greater amount of PVA to stabilize the emulsion droplets. Thus, these nanoparticles retain a greater amount of PVA adsorbed on its surface. Therefore, parameters that influenced the particle size, tend to affect the percentage of residual PVA [5, 16, 20, 22].

As the ethanol in the aqueous phase and the stirring rate favor the preparation of smaller nanoparticles, it is expected a higher amount of residual PVA on the small nanoparticle surface. However, the relation between the nanoparticles size and residual PVA is not immutable. An example is the PLGA-PEG nanoparticles containing gallium phthalocyanine [20]. It was reported that the aqueous phase temperature increased the nanoparticles size and the residual PVA while the change in the preparation method from EEM to EDM decreased the nanoparticles size and the residual PVA. Therefore, a different situation that it was expected. Probably, the presence of PEG linked to PLGA hindered the interactions of PVA molecules with the organic/aqueous interface [20].

Residual PVA can also be influenced by synergistic effects. For example, changing the preparation method from EEM to EDM, associated with an increase in the aqueous phase temperature can cause an increase in the residual PVA. However, the increase of the emulsification time together with the change of the preparation method can reduce the residual PVA [5]. All influences of the parameters used in the preparation of polymeric nanoparticle were summarized in the **Figure 5**.

It should be noted that the residual PVA values can still vary according to the number of washing steps and the method used to wash the nanoparticle suspension [5, 22].

2.6 Residual chloroform

The organic solvent can be retained by nanoparticles during the preparation of the nanoparticulate formulation, becoming a residual organic impurity. Therefore, the quantification of solvent residual is necessary to eliminate toxicological risks for patients. According to the American Pharmacopeia, the residual chloroform limit is 60 ppm for pharmaceutical formulations. Thus, it is very important to evaluate the influence of the factors involved in the nanoparticle preparation on the residual chloroform concentration [20].

The percentage of residual chloroform, as described for other properties, is also related to the nanoparticle size. Thus, there is a tendency to reduce residual chloroform linked to the reduction in the nanoparticles size.

In the preparation of PLGA-PEG nanoparticles containing chloro(5,10,15,20-tetraphenylporphyrinato) indium(III) (InTPP - **Figure 2C**), the influence of four parameters on the residual chloroform percentage was studied: PVA concentration, stirring rate, ethanol percentage in the aqueous phase and in the organic phase [20].

The stirring rate and the ethanol percentage in the aqueous phase were the factors that significantly influenced the residual solvent, favoring the decrease of residual chloroform. The increase in the stirring rate favors the organic phase dispersion into the aqueous phase, generating small organic droplets that favor the fast solvent diffusion into the aqueous phase. On the other hand, ethanol in the aqueous phase hinders the coalescence of organic droplets dispersed in the aqueous phase, favoring the formation of smaller diameter particles [20].

Although the individual effects of ethanol in the aqueous phase and the increase in stirring rate cause a decrease in residual chloroform, the synergistic effect of the simultaneous increase of these factors caused an increase in residual chloroform. Both factors favored the decrease in the nanoparticles size. Small size nanoparticles tend to present a higher residual PVA percentage on the particles surface, which makes difficult the solvent diffusion from organic droplets into the aqueous phase. This diffusion becomes even more difficult after the solidification of the polymeric matrix surface layer during the process of evaporation of the organic solvent, favoring the residual increase of chloroform in the nanoparticles [20]. All influences on residual chloroform have been reported in **Figure 5**.

3. Photobleaching

Experimental results have shown that the photobleaching process can hinder the photodynamic efficiency of photosensitive compounds [36, 37]. Photodegradation of photosensitizers can reduce the concentration of these photoactive compounds in diseased tissue, decreasing the efficacy of PDT to reduce cell viability, leading to an incomplete treatment. On the other hand, photobleaching can reduce the photosensitivity of healthy tissues after irradiation due to the lower amount of reactive oxygen species generated in the photodynamic process motivated by the destruction of photosensitizer molecules. Considering that phthalocyanines are photosensitizers that tend to suffer photobleaching, [37] as well as to aggregate in aqueous medium, our group evaluated the ability of polymeric nanoparticles to reduce the aggregation of these lipophilic molecules and also the effect of photobleaching [29].

The laser power and the concentration of free phthalocyanine significantly influenced the photobleaching for concentrations in which the molecule is in the monomeric state since the photosensitizer aggregation state tends to decrease the photobleaching process due to the difficulty to produce reactive oxygen species [29].

Works have shown that the encapsulation of photosensitizers decrease the effect of photobleaching in phthalocyanines when compared to free molecules due to the scattering of light caused by the polymeric matrix [5, 7, 8, 29]. More soluble photosensitizers tend to be more susceptible to suffer photobleaching and even encapsulated can be photodegraded according to the laser power and irradiation dose used, limiting its ability to be used as a good photosensitizer in photodynamic therapy [29]. The short storage period at several temperatures did not cause significant influence in the photobleaching behave of the encapsulated phthalocyanines, probably due to the aggregation process of the particles (**Figures 6, 7**) (results not showed).

4. Photooxidation

Phthalocyanines are a class of compounds used as photosensitizers due to their chemical, electronic and spectroscopic properties, [38, 39] in particular, due to the intense absorption of these compounds in the therapeutic window and their ability to generate singlet oxygen in the presence of a light source.

Researchers have shown that the presence of heavy atom in the phthalocyanine structure favors the generation of singlet oxygen due to the increase in spin-orbital coupling and, consequently, the transition of the photosensitizer from an excited singlet state to a triplet state (intersystem crossing) [40–42]. In addition, the literature suggests that photooxidative mechanisms for singlet oxygen are usually more efficient due to their greater diffusibility and higher reaction rate constants with substrates [43, 44]. However, the metallophthalocyanines present limited solubility in certain solvents due to the symmetry of molecular structure, hamper their application in PDT [29].

The chemical structure of phthalocyanines has been modified by introducing substitutes in the peripheral or non-peripheral positions of the phthalocyanine nucleus to reduce the molecule symmetry and consequently increase the polarity and solubility of the phthalocyanines [41, 42]. Besides, studies show that encapsulation improves the photodynamic efficiency of the photosensitizer, as well as decreases side effects such as photosensitivity of the skin after photodynamic treatment, and reduces molecular aggregation compared to phthalocyanines dissolved in aqueous medium [5, 7, 8, 29, 45].

We have studied the photodynamic efficiency of different porphyrins and phthalocyanines encapsulated in polymeric nanoparticles (**Figures 2-4**). As an example, gallium phthalocyanine (GaPc - **Figure 2A**) and 1,4-(tetrakis[4-(benzyloxy)phenoxy] phthalocyaninato) indium(III) chloride (InTBPPc - **Figure 4**) are convenient photosensitizers for PDT. These compounds have high singlet oxygen (0.41 and 0.94, respectively) and triplet (0.69 and 0.97, respectively) quantum yield. However, InTBPPc has more interesting features for use in PDT [29].

The photooxidation of simple molecules (as dimethylantracene (DMA) and tryptophan (Trp)) was used to evaluate the photodynamic efficiency of each free and encapsulated phthalocyanines. It is notable that the asymmetry caused by (benzyloxy)phenoxy group in phthalocyanine seems to increase the photodegradation of InTBPPc, due to the greater solubility of the photosensitizer which favors the reduction of its aggregation state. The decrease in the aggregation state favors the generation of singlet oxygen and consequently, the efficacy of the free photosensitizer in photooxidizing simple molecules such as DMA and Trp, as well as the phthalocyanine photobleaching [29]. Therefore, free InTBPPc was more efficient than free GaPc in photooxidate DMA and Trp molecules, due to its lower aggregation state and the higher capacity of free InTBPPc to generate singlet oxygen. However, the encapsulated GaPc proved to be more efficient than the encapsulated InTBPPc in photooxidate the Trp molecules, corroborating that the encapsulation can enhance the photosensitizer photocytotoxicity and reduce the aggregation state of the free photosensitizer [29].

We have demonstrated that the photocytotoxicity of encapsulated photosensitizers depends on the incubation time, the photosensitizer concentration and the laser power [5, 7, 8, 16, 29]. However, these observations cannot be considered a fact for all free or encapsulated photosensitizers due to their solubility characteristics, their states of aggregation and the influences of the parameters used in the nanoparticles preparation on the nanoparticulate properties. For example, the aggregation state of free InPc decreases the photodynamic efficiency of this photosensitizer, so that the viability of tumor cells is not altered by increasing the concentration and laser power [7].

We have observed that each nanoparticulate formulation should be treated in a particular way, taking care to do generalizations about certain conclusions as susceptible to be applied to all formations. An example was the result observed with InTBPPc, the encapsulation process did not increase its efficiency in the photooxidation process of Trp due to the photobleaching process suffered by the

photosensitizer [29]. In general, the encapsulation process of photosensitizers has not created barriers for the singlet oxygen generation, and has increased the uptake of photosensitizer into the cancer cells, improving the efficiency of the phthalocyanines and porphyrins to reduce the viability of cancer cells [7, 8, 16].

It was also shown that the photocytotoxic activity of nanoparticles loaded with porphyrins did not depend on the different amphiphilic characteristics of the compounds, probably due to the encapsulation process [16]. Even the similarities in photodynamic efficacy are related to the degree of similarity in the internalization of each encapsulated photosensitizer inside tumor cells [16].

5. Future perspectives and challenges

The greatest challenge when it comes to oncology is prepared drugs with high specificity to promote the death of malignant cells without harming healthy ones. PDT has been used with successful to treat several cases of cancer with a lesser side effects than those treated by conventional chemotherapy. New photosensitizers have been synthesized to increase the photodynamic efficiency on the disease tissues and to facilitate the photosensitizer administration during the treatment. However, the hydrophobicity of new compounds should not limit their use in PDT. The use of nanoparticles as carrier has motivated the research in PDT since results had showed very promising advances in reducing the viability of the cancer cells due to the specificity achieved by the targeted or magnetic drug delivery system and due to the increase of the bioavailability of the nanoparticle. But the synthetic control is a very challenger for preparing a targeted polymeric nanoparticle in order to maintain the reproducibility of the nanoparticulate properties and its efficiency in the cancer treatment. Thus, there is much to be studied about the synthetic particularities of the polymeric nanoparticulate formulation for greater clinical application in the cancer treatment by PDT. In this sense, the development of nanoparticulate systems consistently involves a lot of work to study the several variables and its synergistic or antagonistic combinations that influence the properties of nanoparticulate formulations. Besides, the decrease of the entrapment efficiency of the photosensitizer associated to decrease of the nanoparticle size, the influence of combinatory effect of the photobleaching of encapsulated photosensitizer and the aggregation state of the compound into the nanoparticle, the establishment of adequate loading of photosensitizer into the nanoparticle and the uptake of the polymeric nanoparticle into the disease cells should be considered to development nanoparticulate formulation with high photodynamic efficiency to reduce the viability of the cancer cells.

6. Conclusions

In this chapter we demonstrate the most significant parameters in decreasing the nanoparticles size were the increase in stirring rate and PVA concentration. Other factors that also reduced the particles size were the increase in the ethanol percentage in the aqueous phase and in the organic phase, and the increase in the photosensitizer mass/polymer mass ratio. Nanoparticles prepared by the EDM showed smaller sizes than the nanoparticles prepared by the EEM but are less stable. The aqueous phase temperature showed double behavior in relation to the nanoparticles size, increasing or decreasing the size depending on the method used to prepare the nanoparticle. The other properties evaluated, such as zeta potential, entrapment and recovery efficiencies, residual PVA and residual chloroform, are dependent on

the size of the nanoparticle. Therefore, parameters that are significant in relation to size will also influence these properties. Properties as zeta potential, residual PVA and entrapment efficiency presented an inversely proportional relation with nanoparticle size while the recovery efficiency and residual chloroform were directly proportional. In most of the properties some significant binary effects were observed, but their influence was not predominant in the results.

Besides the parameters used in the nanoparticle preparation, the physicochemical properties of the photosensitizer can interfere on the entrapment efficiency, as well as the washing step of the nanoparticulate formulation can influence the residual PVA and the recovery efficiency. The short storage period of the nanoparticulate formulation can affect the characteristics of the particle, favoring the nanoparticle aggregation in different temperature.

We have shown that the encapsulation of photosensitizers reduces the photobleaching effect due to light scattering caused by the polymeric matrix, however more soluble photosensitizers even encapsulated can suffer photobleaching according to the laser power and irradiation dose used in the experiment, limiting their ability to be used in PDT. The aggregation of the photosensitizer also causes a reduction in its photodynamic efficiency because it reduces the singlet oxygen generation, but the encapsulation improves the photosensitizer efficiency since the entrapment can reduce the aggregation of the lipophilic compounds in aqueous medium.

The photocytotoxicity of encapsulated photosensitizers depends on the incubation time, the photosensitizer concentration and the laser power, as well as the uptake of photosensitizer into the cancer cells. Drug delivery systems have improving the efficiency of the phthalocyanines and porphyrins to reduce the cell viability. However, the generalization of the conclusions about preparation of nanoparticulate formulations and photooxidation conditions should be done carefully, each nanoparticulate formulation behaves in a characteristic way and should be treated singularly.

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