Polycaprolactone nanoparticles as carriers for delivery of enzymes into cells

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ABSTRACT The present thesis aimed at the development of polymeric nanoparticles as a vehicle for the delivery of enzymes into cells. The nanoparticles were prepared by nanoprecipitation of polycaprolactone (PCL) modified with propargyl 3-methyl pentanoate oxide. Superoxide dismutase and alkaline phosphatase were encapsulated in PCL nanoparticles and delivered into HeLa cells. Modulation of cell mechanisms, such as oxidative stress and apoptosis, with PCL nanoparticles, was also attempted.

The uptake (by endocytosis) of polycaprolactone-enzymatic nanoparticles by HeLa cells was followed using a fluorescent red dye, rhodamine. Rhodamine allowed analysis and measurements by fluorescence microscopy and flow cytometry.

Enzymatic and cellular assays were carried out to measure superoxide dismutase (SOD) activity in HeLa cells, after being delivered by polycaprolactone nanoparticles. Detection of intracellular hydrogen peroxide was performed with a cell-permeable green fluorescent dye in the presence of 30 µg/ml of lipopolysaccharide (LPS). However, the results obtained were not conclusive due to bacteria contamination during nanoparticle synthesis. Flow cytometry analysis and cell viability assays were also performed. The results indicated that PCLSOD nanosystems protect HeLa cells from oxidative stress, increasing cell viability.

For alkaline phosphatase (ALP), the encapsulation in polycaprolactone polymeric nanoparticles protected enzyme activity in solution. Analysis by fluorescence microscopy, using the fluorescent substrate Naphthol AS-MX disodium salt, revealed that PCLALP nanoparticles are successfully absorbed by HeLa cells.

Index Terms— polycaprolactone nanoparticles, nanoprecipitation, superoxide dismutase, alkaline phosphatase, oxidative stress, apoptosis.

INTRODUCTION

What is the focus of the project?

Enzyme replacement therapy (ERT) is a medical treatment which consists of the replacing of the defective enzymes in the human organism, by giving the patient a solution containing the enzyme. One of the first development of these type of enzymes was carried out for lysosomal storage diseases and was first proposed by Christian de Duve in 1964[1].

This therapy can replace a defective or absence enzyme in the human system and allows to understand better the correlation between endocytosis and lysosomes, enabling the treatment of many diseases, but it has many hurdles to be overcome such as poor solubility, poor stability of the enzyme in gastrointestinal tract (GIT), in case of oral administration[1].

There are plenty of advantages to use enzymes as drugs to treats many types of diseases, such as the fact that enzymes can bind and act on their targets with great affinity and specificity[1]. Despite many beneficial outcomes of ERT, several limitations such as the high cost of the treatment and various inadvertent side effects (e.g., immunological responses) against the infused enzyme and development of resistance to enzymes persist[2].

What is the developed solution?

Medicine and technology have been evolving to give a better response to these perturbations using more simple, easy access and smaller systems that can act in our body with high efficiency. An area that leads with these types of systems, is the so-called nanotechnology area[3,4].

Cellular processes, such as adhesion, migration, and proliferation are influenced by the chemical composition and morphology of the extracellular environment through interactions between cells and physical stimuli. The design of nanosystems for imaging and therapeutic applications requires an understanding of the interactions between nanoparticles (NPs) and those biological systems[3,5].

Nanostructures can passively interact with cells and mediate cell functions. They are recognized as objects

that have a dimension range that enhances their activities in the living organism, due to the fact that some of the nanomaterials can mask the action of the immune system[4]. Nanomaterials have increased surface to volume ratio compared with their bulk materials, and this will increase mechanical strength, to enter through cell barriers[5].

Polymeric nanoparticles can be made with natural polymers, that are good carriers for drug delivery systems because of their wide sources, better stability, low toxicity, simple mild preparation methods, and versatile routes of administration[6].

Who are the main players?

Superoxide dismutase (SOD) is an enzyme ubiquitous among aerobic organisms, that protects the living being from oxidative stress, produced by reactive oxygen species, which specifically catalyzes the dismutation of the superoxide anion[7]. The SOD families are defined by the metal ions utilized for stability and catalysis, dividing SOD into four different families, Cu/Zn, Ni, Mn, and Fe SODs, in which Cu/Zn SOD is generally homodimeric and is present in diverse locations of different organisms[7,8]. The SOD used in the present study was Cu/Zn SOD from bovine erythrocytes, which is a metalloprotein with a molecular weight of 32 kDa. It is a homodimer enzyme with one copper (responsible for enzymatic activity) and one zinc ion (responsible for structural stability) per subunit and has an antiparallel "greek-key" β barrel fold[8]

Reactive oxygen species (ROS) are extremely reactive oxygen-containing molecules produced during oxygen metabolism within the cells, but sometimes, they can cause oxidative stress when reacting with and damaging intracellular targets, such as lipids, proteins and DNA[7]. In eukaryotic cells, ROS are generated in metabolic processes during mitochondrial respiration. At physiological levels, ROS are important modulators of many cellular functions from metabolism, signal transduction to stress responses, and their levels are controlled by the antioxidant system of the body[7]. The enzyme SOD is one of the major proteins of this system, by converting $O_2^{\cdot -}$ into H_2O_2 and O_2, which is represented in the following chemical reaction:

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \qquad \text{(Eq. 1)}$$

Two major cell signals will activate the ROS-generation mitochondria pathway: when the mitochondria are not making ATP and consequently has a high protonmotive force, and when there is a high NADPH/NADP+ ratio, that will activate the enzyme NADPH oxidase enzyme, the one responsible for producing of superoxide anion[9].

Besides its natural roles, SOD has important action in several human diseases. It is known that the mutations in the SOD gene, can have an impact in amyotrophic lateral sclerosis (ALS) and respiratory diseases[7]. ROS-induced oxidative stress has been an implicated pathophysiology in several conditions, such as aging, infertility, cardiovascular diseases, and neurological disorders[10].

SOD has attracted wide research interest because the electron transfer mechanism between the substrate and the enzyme's active site is considered to have reached perfection and because the enzyme shows unusual stability to urea, high temperatures, and prolonged refrigeration[7]. Although SOD is a potent antioxidant, there have been several attempts to further improve its activity, because it is limited by diffusion. To overcome the barriers of antigenicity, stability, and pharmacokinetics, SODs can be functionalized with nanocarriers such as liposomes, gold nanoparticles, or polymeric nanoparticles[11].

Alkaline phosphatase (ALP) is a homodimeric enzyme and a membrane-bound glycoprotein that catalyzes the hydrolysis of phosphate monoesters. It has a molecular weight of 86 kDa and the active site region contains three metal ions, two zinc and one magnesium ions, that are fundamental for the enzymatic activity[12]. In nature, ALP is found in both eukaryotes and prokaryotes organisms. It is present in bacteria, fungi, and mammals[13]. In *Homo Sapiens*, ALP is present in tissues that have the role of transport nutrients and in secretory organs, and it is divided into three tissue-specific isoforms, the placental ALP, the germ cell, and the intestinal ALP. And in one non-tissue specific isoform, the one present in the liver or kidney, for example[12,13].

The knowledge of the biological functions of ALP was carried out by Robinson, which demonstrated that this enzyme plays an important role in ossification since during this event there is an increase in the concentration of inorganic phosphate thus to deposition of bone salt[14].

Also, ALP plays an important role in the regulation of protein phosphorylation, cell growth, cellular migration during embryonic development, and in apoptosis[15].

Polycaprolactone (PCL) is a semi-crystalline biodegradable, biocompatible, and hydrophobic polyester with low glass transition temperature and melting point[16]. It is also non-toxic and non-mutagenic[16,17]. It has the lowest degradation rate of all the common polymers which makes PCL suitable for delivering drugs for chronic therapy[16]. The biodegradation property of PCL is affected *in vitro* by the preparation method of the delivery system, by polymer properties such as initial molecular weight and by physical and chemical parameters like temperature, pH, and ionic strength[18].

METHODOLOGIES

The enzymes used in the present project were the Cu/Zn SOD, from bovine erythrocytes, was supplied by

Sigma Aldrich (3000 U/mg protein), and ALP from bovine calf intestine (1000U/ml), supplied by Promega in a solution containing 10mM Tris-HCl (pH 8.0), 1mM MgCl2, 0.1mM ZnCl2, 50mM KCl and 50% (v/v) glycerol.

The cell linage used in the present study was HeLa cells. The cells were incubated in T25-flasks in the presence of *Dulbecco's Modified Eagle Medium* (DMEM) with 10% of fetal bovine serum (FBS), in an incubator at 37ºC with 5% (v/v) of CO2.

Enzymatic Assays:

Quantification of protein concentration-BCA Protein Assay

The bicinchoninic acid (BCA) assay was performed to quantify the total protein encapsulated in the nanoformulations synthesized by the nanoprecipitation method. The method of BCA is based on the chelation of Cu+ ions by two molecules of bicinchoninic acid[19]. Two molecules of bicinchoninic acid chelate with each Cu+ ion, forming a purple-colored complex that strongly absorbs light at a wavelength of 562 nm[19].

Intracellular Hydrogen Peroxide detection assay

Hydrogen peroxide is a reactive oxygen species produced through the metabolism of molecular oxygen, which serves as both an intracellular signaling messenger and a source of oxidative stress. The compound is generated in cells via multiple mechanisms such as the NOX-mediated ROS production by neutrophils and macrophages or by the dismutase of superoxide anion. This assay was performed to verify the SOD activity (qualitative assessment) after being encapsulated and delivered into HeLa cells.

The kit used to test the presence of H_2O_2 was from Sigma Aldrich [20] and provides a simple method to detect hydrogen peroxide in living cells. The kit utilizes a cell-permeable sensor that generates a fluorescent product (ex/em=490nm/520nm) after reaction with H_2O_2 intracellular.

The assay was performed by seeding 75000 cells/well in a 48-well microplate in the presence of 30 µg/mL of LPS. The plate was incubated overnight in an incubator at 37ºC with 5% (v/v) of CO_2. After incubation, the medium was replaced by DMEM without phenol red, and the nanoformulations: 50 µl PCL, 50 µl PCLSOD into 200 µl of DMEM without phenol red, and free 0.2 mg/mL of SOD were added into each well, following incubation of 4h, at 37 ºC. The results were measured in a microplate reader, BioTek synergy h1.

Alkaline phosphatase assay in solution: p-nitrophenyl phosphate assay

ALP activity can be measured using the substrate p-nitrophenyl phosphate (*p*NPP) that after hydrolyzed by ALP is converted into a water-soluble yellow product (p-nitrophenol), with strong absorption at 405 nm[21].

The buffer used in this assay was 50mM Tris-HCl pH 8.0, with and without the addition of 1mM of $MgCl_2$ and 0.1mM of $ZnCl_2$ metal ions. The assay was performed in a 96-well microplate by preparing 5mM of *p*NPP and mixed with 10 µl of ALP (free ALP and PCLALP NPs). The assay was performed under two different temperatures, at 25 ºC and at 37 ºC, for 60 minutes. The absorbance was measured in a microplate reader, Biotek synergy h1. And the activity of ALP was measured by performing the ratio between the absorbance of the *p*-nitrophenol (405 nm) and *p*-nitrophenyl phosphate (370nm)

$$\frac{A405}{A370} = \frac{Abs(405nm)}{Abs(370nm)} \qquad \text{(Eq 2)}$$

Alkaline phosphatase assay in cells: Naphthol AS-MX disodium salt assay

The assay with naphthol AS-MX disodium salt was performed to verify the activity of ALP in HeLa cells, after being delivered by PCL NPs. The naphthol phosphate derivate, like the one that was used, is a non-fluorescent compound, with a molecular weight of 415.29 Da, that after hydrolysis by ALP becomes red fluorescent with an emission/excitation wavelength of 388nm/512 nm, between pH 8.0 and pH 9.0[22,23].

The assay was carried out in a 48 well-microplate by seeding 75000 cells/well, with an overnight incubation at 37 ºC with 5% (v/v) CO_2 incubator. In the day after, the cells were washed x1 with phosphate-buffered saline (PBS) and the medium was replaced by a new DMEM. The nanoformulations PCLALP, with different amounts of ALP, were incubated for 4 hours at 37 ºC. After incubation, a concentration of 5mM of naphthol AS-MX disodium salt (dissolved in DMEM without phenol red) was added into each well and was incubated for 45 min at 37 ºC. The results were measured in a microplate reader, BioTek synergy h1.

Synthesis and Characterization of Polymeric nanoparticles:

Nanoprecipitation

Nanoprecipitation is a technique based on the interfacial deposition of a polymer after the displacement of the organic solvent from a lipophilic solution to the

aqueous phase. It is a one-step procedure for polymeric nanoparticle synthesis, which means that is a method that does not need an emulsification step before the generation of NPs[24]. The polymer that was used in this thesis was polycaprolactone (with propargyl 3-methyl pentanoate oxide, PMPO) 2.5 mg/ml dissolved in acetone. PCL is dissolved in a water-miscible solvent (acetone) and this solution is added into a stirred aqueous solution in one shot. As the solvent diffuses out from the nanodroplets, the polymer precipitates in the form of nanocapsules or nanospheres[24]. After dissolving 2.5 mg/ml of PCL in 1ml of acetone, it was added the same volume of milli-Q water (1ml) in one-shot to synthesize the nanoparticle PCL NP. To synthesize the PCLALP and PCLSOD, ALP, and SOD enzymes were added in different amounts to the aqueous phase (Milli-Q water 1ml). These enzyme solutions were then added to the organic phase (2.5 mg/ml of PCL in 1 ml of acetone), also in the one-shot procedure.

Dynamic Light Scattering

Dynamic light scattering (DLS) was performed to verify the hydrodynamic diameter of the nanoformulations and see their stability by polydispersity index values and continuous measurements over time. The hydrodynamic diameter was measured in DelsaNano C particle analyzer from Beckman Coulter.

Cellular assays:

Flow Cytometry

Flow cytometry was performed to verify the relative fluorescence intensity in HeLa cells, after treated with fluorescent dyes, for both enzymes: SOD and ALP. The samples need to be prepared before performing the assay, by removing the medium and washing the cells with PBS. After washing the cells, it was required the addition of trypsin to generate a cell suspension. Then, it was added three times the amount of trypsin of DMEM without phenol red and the samples were collected into Eppendorf tubes, to be centrifugated. The samples were centrifugated at 1.2 rpm for 5 minutes. After centrifugation, it was necessary to remove the supernatant and add 200 µl of PBS.

The flowing cytometer software, FlowingSoftware 2.5.1, was used to measure the relative fluorescence intensity, all fluorescent channels were used (FL1 to FL10), with main focus on green channel (in case of SOD enzyme) and on the red channel (in case of ALP enzyme and nanoparticles that encapsulates rhodamine dye).

Fluorescence Microscopy

In the present study, the acquisition of fluorescence images of HeLa cells, after the treatment with nanoformulations: PCL and PCLALP, was performed in a ZEISS microscope (ZEISS Axiovert 200M Fluorescence/Live cell imaging) with the ZEISS lite software (ZEN 3.1 Lite blue edition). The exposure time was 41.2 ms and it was used the channel HF594 (red channel) with a bandgap between 570-590nm.

RESULTS AND DISCUSSION

Intracellular Hydrogen Peroxide detection assay

All the nanoformulations of PCL-enzymes, will be designated as PCLSOD(X), where (X) is the different concentrations of SOD in mg/ml. And PCLALP(Y), where (Y) is the different concentrations of ALP in µg/ml.

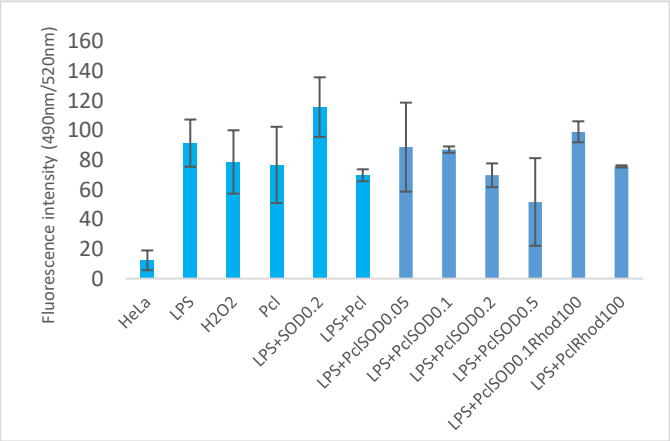


Figure 1 - Intracellular hydrogen peroxide detection assay by 1st batch of nanoformulations PCLSOD with different concentrations of SOD 0.05, 0.1, 0.2, 0.5, and PCL NPs with rhodamine (Sigma Aldrich) 100 µl encapsulated. The test samples are represented by dark blue bars. In all the test samples, the HeLa cells were subjected to 30 µg/ml of LPS. The control samples are represented by the light blue bars: 75000 cells/well HeLa cells, 30µg/ml LPS; 5mM H2O2; 30µg/ml LPS with 0.2mg/ml of SOD; PCL and 30µg/ml LPS with PCL. The incubation time of 4 h at 37ºC. The assay was performed in quadruplicates. Results were obtained using microplate reader Biotek synergy h1.

As it is shown in Figure 1, the control sample (HeLa cells subjected to 30 µg/ml of LPS) responded with a high fluorescence intensity signal. This is according to the literature since LPS, even in a small amount (6 µg/ml), can increase the fluorescence intensity signal of hydrogen peroxide detection in HeLa cells, at the same incubation time, up to 50%[25]. In the present study, the signal increased up to 79% when compared to the control HeLa cells in DMEM.

One important result that is demonstrated in Figure 1 is the signal that is generated by the adding of PCL NP into the HeLa cells. Why is PCL NP generating a signal that comes from the presence of intracellular hydrogen peroxide if this well was not subjected to the stimuli of LPS? It is possible to answer this question in two ways: i) PCL NP is capable of leading to the production of H_2O_2

when enters in the cell through endocytosis mechanism, maybe because it generates some cell stress, or ii) the nanoformulations are contaminated by some bacteria, and that contamination leads to the formation of hydrogen peroxide.

In terms of possibility i), the literature does not show, until now, studies about PCL inducing oxidative stress or production of hydrogen peroxide when entering in HeLa cells, only if the cells are already stimulated by another compound. But some cell lineages can suffer from transitory oxidative stress, after polycaprolactone enters by endocytosis mechanism, as L929 fibroblast[26].

To verify this hypothesis, it is important to test the possibility ii), in which is said that the nanoformulations could have been contaminated by bacteria. This may happen during polymer synthesis or nanoparticle synthesis. In fact, during nanoprecipitation, as it is done with organic solvents, they can accumulate some residues that will degrade the encapsulated enzyme[56], or contamination can occur during the test and due to long storage time. To verify this hypothesis, the different PCL and PCLSOD nanoformulations were incubated in LB-Agar, a bacteria growth medium, and they showed contamination, after 4 days of incubation, at room temperature.

Cellular viability assay-MTS reagent

The cellular viability assay was performed based on the reduction of the reagent MTS tetrazolium by viable mammalian cells that will generate a colored formazan dye that is soluble in cell culture media. This conversion is thought to be carried out by NADPH-dependent dehydrogenase enzymes in metabolically active cells. The product strongly absorbs at 496 nm.

The HeLa cells were subjected to a concentration of 125 µM H_2O_2. This concentration was selected based on literature[27], in which it is said that this concentration of hydrogen peroxide can promote apoptosis of HeLa cells by mitochondria pathway, which leads to uncontrolled oxidative stress of the cells. It inhibits cell growth of about 70%[27].

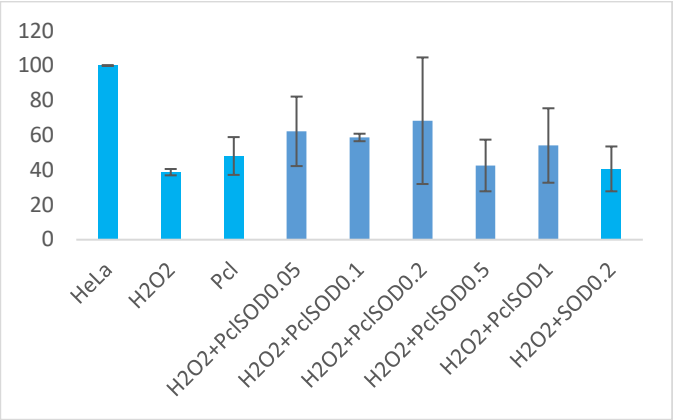


Figure 2 - HeLa cells viability assay carried out by MTS reagent. A concentration of 15000 cells/well was subjected to the nanoformulations PCLSOD with different concentrations of SOD 0.05, 0.1, 0.2, 0.5, and 1 mg/ml. The test samples are represented by dark blue bars. In all the test samples, the HeLa cells were subjected to 125 µM of H_2O_2. The control samples are represented by the light blue bars: 15000 cells/well HeLa cells,125µM H2O2, 125µM with PCL, and 125µM with 0.2mg/ml of SOD. The cells were incubated for 4 h at 37ºC. The assay was performed in triplicates and the absorbance was read at 496nm. Results were obtained using microplate reader Biotek synergy h1.

As it is possible to see in Figure 2 the treatment of HeLa cells with 125 µM H_2O_2 reduced cell viability of about 40%. When the cells were treated with the nanoformulations this viability increased, for example, the nanoformulations PCLSOD0.05 and PCLSOD0.2 could increase the cell viability up to about 60%. The control sample HeLa cells that after the treatment with hydrogen peroxide were treated with 0.2mg/ml SOD enzyme, showed almost the same cell viability as the ones treated with only hydrogen peroxide, this can answer the question, does 0.2mg/ml of SOD is more effective than the enzyme encapsulated into PCL NPs?

Alkaline phosphatase assay in solution: *p*-nitrophenyl phosphate assay

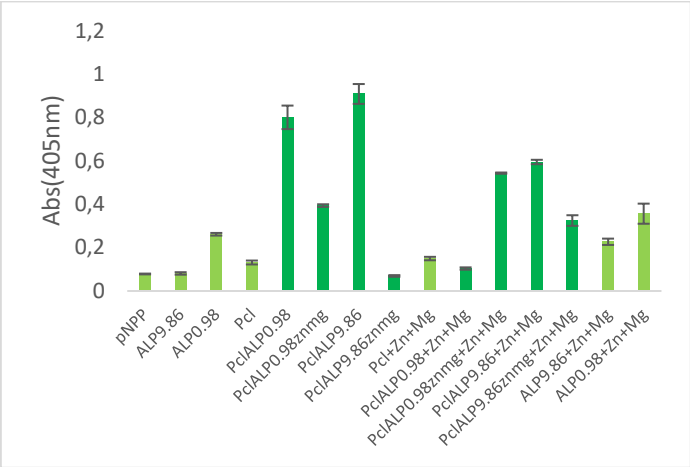


Figure 3 - Absorbance ratio between *p*NPP absorbance at 405 nm and *p*-nitrophenyl phosphate absorbance at 370 nm. All the samples were treated with 5mM of *p*NPP, mixed with 10 µl of ALP (free in solution and encapsulated in PCL NPs) in 40 µl of Tris-Hcl 50mM pH 8, in a final well volume of 100 µl. The control samples are represented by light green bars and are divided between the ones without the addition of 0.1mM of Zn and 1mM of Mg, and with the addition of the meta ions. The test samples are represented by dark green bars and are divided between the ones without the addition of 0.1mM of Zn and 1mM of Mg, and with the addition of the metal ions. The experiment was carried at 25ºC for 60 minutes

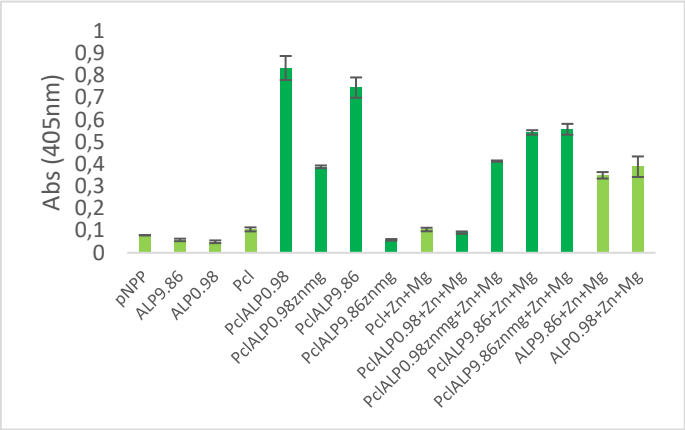


Figure 4 - Absorbance ratio between *p*NPP absorbance at 405 nm and *p*-nitrophenyl phosphate absorbance at 370 nm. All the samples were treated with 5mM of pNPP, mixed with 10 µl of ALP (free in solution and encapsulated in PCL NPs) in 40 µl of Tris-Hcl 50mM pH 8, in a final well volume of 100 µl. The control samples are represented by light green bars and are divided between the ones without the addition of 0.1mM of Zn and 1mM of Mg, and with the addition of the meta ions. The test samples are represented by dark green bars and are divided between the ones without the addition of 0.1mM of Zn and 1mM of Mg, and with the addition of the metal ions. The experiment was carried at 37 ºC for 60 minutes.

The assay at 37 ºC is demonstrated in Figure 4 and the results are closely related to the ones that are obtained at 25 ºC (Figure 3). As it is possible to see, the nanoformulations PCLALP0.09, PCLALP9.06 are the ones that show higher pNPP conversion (higher absorbance ratio), 0.83 and 0.74, respectively. The samples that were not synthesized in the presence of magnesium and zinc ions showed a higher conversion than the ones that were synthesized with magnesium and zinc ions. These samples also showed a higher substrate conversion ratio than the control samples, 5mM of *p*NPP, ALP 90.6 mg/ml free in solution, ALP 0.09 mg/ml free in solution and 5mM *p*NPP with PCL NP. This result indicates that the enzyme ALP has a higher activity in PCL NPs than free in solution.

When it was added 0.1mM of ZnCl2 and 1mM of MgCl2 into the wells of the 96-well microplate, it was verified that the test sample PCLALP0.09 lowered its capability of substrate conversion, since the ratio only reach the value of 0.09. This lowering on substrate conversion is due to the interference of metal ions concentration in ALP activity, which if they are not in their proper concentration value, they can inhibit enzyme activity[21]. The test sample PCLALP9.06$_{zn0.1mg1}$ demonstrated an increase in its absorbance conversion ration up to 0.56.

Alkaline phosphatase assay in cells: Naphthol AS-MX disodium salt assay

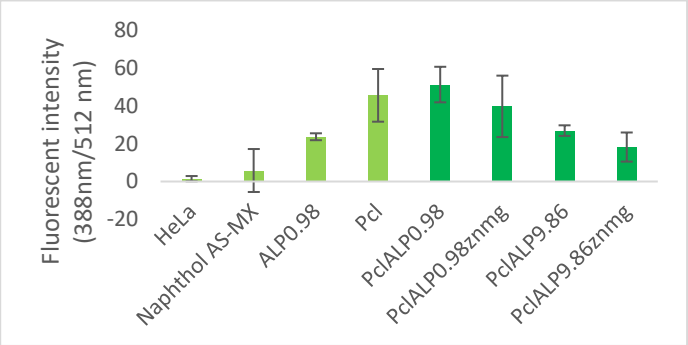
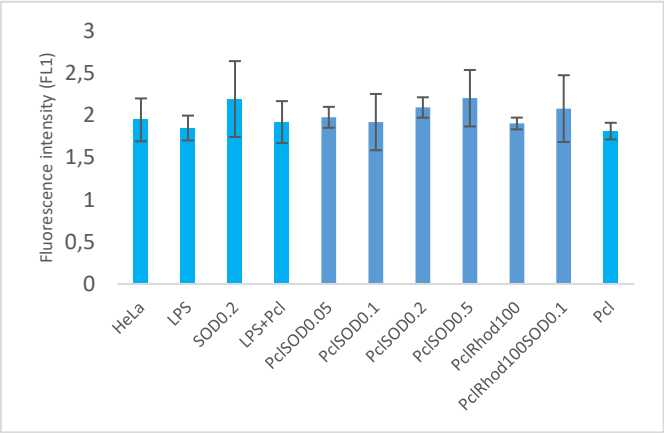


Figure 5 - Fluorescence intensity of Naphthol AS-MX disodium salt (ex/em=388/512nm). The assay was carried out with 5mM of Naphthol AS-MX disodium salt with 10 µl of control samples into 200 µl of DMEM without phenol red. The control samples are represented by light green bars, and the test samples are represented by dark green bars. The test samples are divided between the ones that are synthesized with 0.1mM of Zn and 1mM of Mg, and the ones that are synthesized without the addition of zinc and magnesium ions. The experiment was carried at 37ºC for 45 minutes. The results were performed in quadruplicates.

As it is possible to verify, the control sample, 0.98 µg/ml of ALP free in DMEM with HeLa cells, showed that ALP is capable of hydrolyzing the substrate Naphthol AS-MX

disodium salt since it reaches a fluorescence intensity value higher than the other control, 5mM of Naphthol AS-MX in HeLa cells. This result may be explained by the fact that some of the reaction can be occurring in solution, so, outside from the cell, because the ALP and most of the enzymes have some difficulty to cross by itself the cell membrane without compromising their activity and structure.

The test samples PCLALP0.98 and PCLALP0.98$_{zn0.1mg1}$ showed the highest fluorescence intensity among all samples. And if we compared to the



blank (5mM of Naphthol AS-MX disodium salt), it is possible to verify that encapsulated 0.98 µg/ml ALP is capable of hydrolyzing the substrate after being delivered into cells, and this action is responsible for the exogenous ALP and not the endogenous ones, since the substrate by itself is not fluorescence, it is only fluorescence when it reacts with ALP.

Flow cytometry

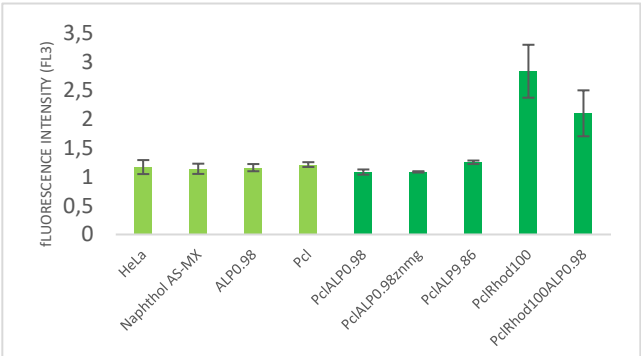


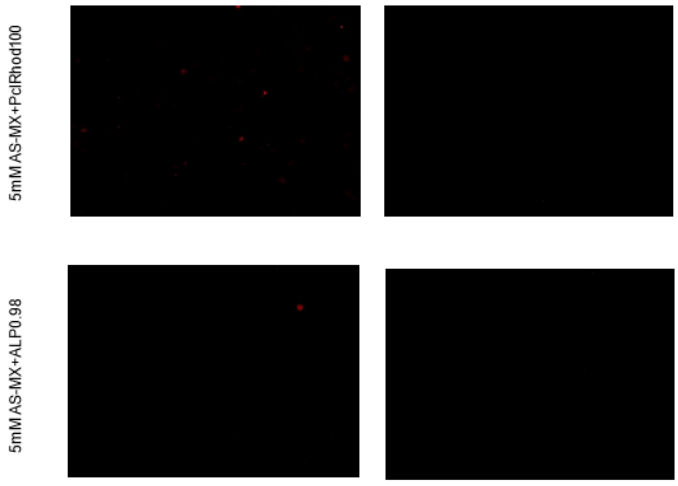
Figure 6 - Fluorescence intensity of Naphthol AS-MX disodium salt (ex/em=388/512nm). The assay was carried out with 5mM of Naphthol AS-MX disodium salt with 10 µl of control samples into 200 µl of DMEM without phenol red. The control samples are represented by light green bars, and the test samples are represented by dark green bars. The test samples are divided between the ones that are synthesized with 0.1mM of Zn and 1mM of Mg, and the ones that are synthesized without the addition of zinc and magnesium ions. The experiment was carried at flow cytometer Gallios Flow cytometer 9 colors, 3 lasers from Beckman Coulter Life Sciences. The results were acquired in triplicates, using the FL3 (red) channel.

In Figure 6 it is demonstrated that there is no difference between control and test samples. It is possible to verify that the substrate 5mM naphthol AS-MX disodium salt is not being cleaved by endogenous ALP enzyme, since there is almost no difference between the untreated cells (75000 cells/well HeLa cells) and the cells treated with just 5mM of the substrate. The results got from flow cytometry are not in accordance to the ones got from microplate reader (Figure 5). Figure 6 shows that the nanoformulations could not successful delivery ALP to the HeLa cells without compromising its activity

Figure 7 - Fluorescence intensity of intracellular detecting H_2O_2 green dye (ex/em=490nm/520nm). The 75000 cells/well of HeLa cells were treated with 30 µg/ml of LPS, both control, and test samples. The control samples are represented by light blue bars, and the test samples are represented by dark blue bars. The experiment was carried at flow cytometer Gallios Flow cytometer 9 colors, 3 lasers from Beckman Coulter Life Sciences. The results were acquired in triplicates, using the FL1 (green) channel.

From SOD enzyme flow cytometry results represented in figure 7, it is not possible to see a clear difference between the untreated cells (75000 cells/well HeLa cells), the control samples, and the test samples. For example, the control sample 75000 cells/well HeLa presents a value of fluorescence intensity which is close to the value of the test sample PCLSOD0.1. By flow cytometry results it is possible to consider the hypothesis that the reaction is occurring in the extracellular environment.

3.7 Fluorescence microscopy



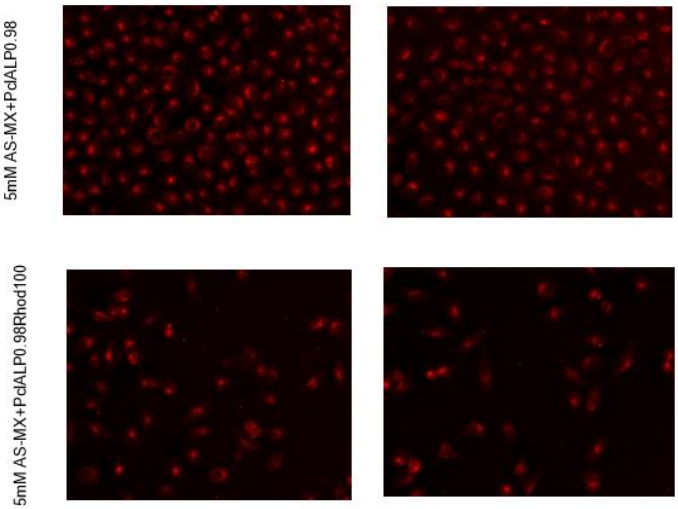


Figure 9 – Fluorescence microscopy images of HeLa cells after treatment with nanoformulations PCLALP0.98; PCLALP0.98Rhod100; PCLRhod100 and the control sample ALP0.98. Images were taken with ZEISS microscope (ZEISS Axiovert 200M Fluorescence/Live cell imaging), red channel HF594 with an exposure time of 41.2 ms, and a band gap between 570-590nm. Images were analyzed with ZEN 3.1 life blue edition.

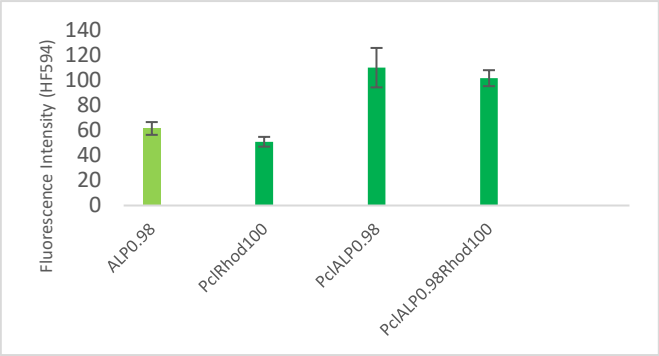


Figure 10 – Fluorescence intensity of HeLa cells after the treatment with PCLALP nanoparticles. All the samples were subjected to 5mM of Naphthol AS-MX, and the assay was carried out with a concentration of 750000 cells/well of HeLa cells. The control sample is represented by a light green bar, and the test samples are represented by dark green bars. The results were analyzed with ZEEIS software, ZEN 3.1 life blue edition.

As it is demonstrated in Figure 9, the fluorescence images showed that the uptake of HeLa cells for PCLALP0.98 was successful. It is the one that showed a higher fluorescence intensity value (110.21), as it is possible to see in Figure 10, in comparison with the control sample ALP0.98, which has a fluorescence intensity value of 61.60.

Dynamic Light Scattering

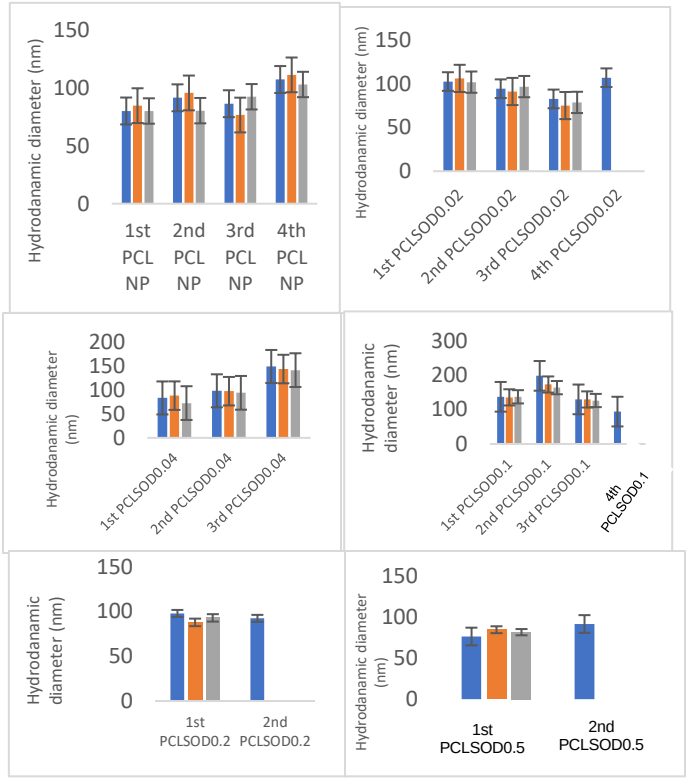


Figure 11 – Hydrodynamic diameter (nm) of nanoformulations PCLSOD measured by dynamic light scattering in the DelsaNano C particle analyzer from Beckman Coulter. Blue bars: hydrodynamic diameter after 1 day of nanoprecipitation; orange bars: hydrodynamic diameter after 1 week of nanoprecipitation and orange bars: hydrodynamic diameter after 1 month of nanoprecipitation.

As it is possible to see in Figures 11A to 11F the nanoformulations PCL and PCLSOD maintained their hydrodynamic diameter mostly constant over time.

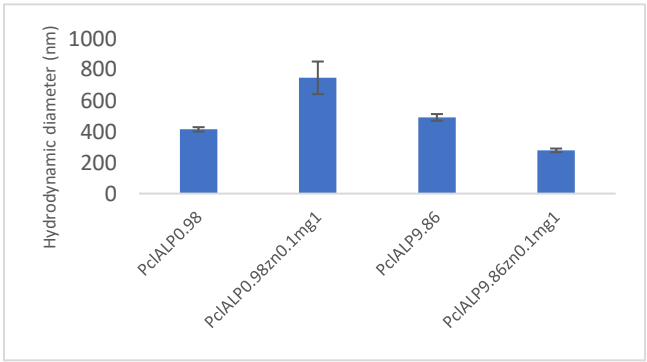


Figure 12 – Hydrodynamic diameter (nm) of nanoformulations PCLALP measured by dynamic light scattering in the DelsaNano C particle analyzer from Beckman Coulter. Blue bars: hydrodynamic diameter after 1 day of nanoprecipitation.

By the values of hydrodynamic diameter of PCLALP represented in Figure 12, it is possible to verify that the values are higher than the ones obtained for PCLSOD. These values could be caused by the presence of the metal ions zinc and magnesium. Besides this factor the size of the ALP enzyme is higher than of the SOD enzyme, 86 kDa compared to 32 kDa.

CONCLUSION

To conclude SOD approach, it was possible to see that in Figure 6 (microplate assay for detection of intracellular H_2O_2 by the green detecting dye) all PCLSOD NPs could not increase the fluorescence intensity signal. The nanoformulation PCL NP gave a higher signal of fluorescence intensity, and this result may be explained due to bacteria contamination. In this set of particles, only the nanoformulation PCLSOD0.1Rhod100 could increased the signal, higher than the signal of control sample HeLa cells treated with 30 µg/ml of LPS. This may be explained by a lower incubation time (1 week at 4ºC), leading to the protection of nanosystem's integrity.

In the flow cytometry analysis, it was not possible to see a clear difference between the control and test samples that were delivered into HeLa cells. This assay was performed with the second set of particles also, and with PCLSOD0.1Rhod100. This last NP was the one that could increase the signal of fluorescence intensity a little bit higher than the control sample (75000 cells/well HeLa, when using the red channel FL3, the red one). This result may indicate that the SOD enzyme could not be successfully delivered to the HeLa cells. This could be due to either loss of activity in the encapsulation step or its activity being compromised by the bacteria contamination.

To conclude the ALP approach, it was possible to see that in the p-nitrophenyl assay in solution (Figures 9 and 10) PCL NPs could protect the enzyme in solution. It could maintain its activity since the nanoformulations PCLALP0.98 and PCLALP9.86, containing 0.98 and 9.86 µg/ml ALP, respectively, gave a higher substrate (p-nitrophenol) conversion ratio than the free enzyme in solution.

In the naphthol AS-MX disodium salt assay in HeLa cells, the nanoformulations PCLALP0.98 and $PCLALP0.98_{zn0.1mg1}$ were the ones that gave a higher fluorescence signal, so the ones that could hydrolyze the substrate better. The interesting part of this result is the fact that the nanoformulation PCLALP0.98 was also the one that gave a higher substrate conversion ratio in the pNPP assay in solution. So, maybe 0.98 µg/ml ALP encapsulated in PCL NP is a concentration that is possible to be added without compromising enzyme activity after being delivered into cells.

In fluorescence microscopy results, it was possible to verify that the nanoformulation PCLALP0.98 was successfully delivered into HeLa cells, without compromising ALP activity. The fluorescence microscopy assay was performed at a different time of the naphthol AS-MX flow cytometry assay, 1 week later.

In terms of future perspectives related to this study, it could be interesting also to see in which endocytosis compartment are occurring these enzymes reactions.

For the SOD approach, it can also be interesting to test the PCL capability of inducing oxidative stress in HeLa cells, since the fact that until our days there are no references in the literature of these assays, and maybe it is important to see if PCL alone can induce some oxidative stress when entering in HeLa cells.

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