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**Formulation and characterization of oxime loaded PLGA nanoparticles**

Diploma Thesis

Hradec Králové 2021

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I declare that this diploma thesis is my original work in which I have prepared it independently. All sources and literatures which are used to create this work have been listed in reference list in which I duly cited them.

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## **ABSTRACT**

Thesis title: Formulation and characterization of oxime loaded PLGA nanoparticles

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The diploma thesis was focused on PLGA nanoparticles (NPs) which could be loaded with oximes, prepared by a double emulsion technique, and characterised by size, polydispersity and zeta potential. The theoretical part deals with the most common methods of the NPs preparation, the polymers and stabilizers employed, and drug delivery to brain. In the experimental part the effect of various formulation factors on NP characteristics were studied: linear or branched PLGA derivative, the concentrations of polymer, the volumes of primary emulsion. Dichloromethane (DCM) or Dimethyl sulfoxide (DMSO) as solvent for polymers were used and Poloxamer 407 or Didodecyldimethylammonium bromide (DDAB) as an outer phase stabilizer were employed. By comparison among the collected results, it seemed 1% A2 in DMSO and stabilization with poloxamer 407 could be best candidate for the oxime loaded drug delivery systems as it was possible to produce nanoparticles with size from 152 to 168 nm with PDI of below 0.15. Electrostatic stability in case of using DDAB was resulted excellent and above 70.3 mV.

**Keywords:** nanoparticles, obidoxime, double emulsion technique, particles size, polydispersity, zeta potential, stability.

## **AIM OF THE DIPLOMA THESIS**

The main goal of this thesis was to evaluate possible resources for the creation of oximes loaded delivery systems into CNS, in which to test various features of linear or branched configuration of poly(lactic-co-glycolic acid) including their solvents and stabilizers by applying double emulsion method.

The assignments could be summarized into:

- Testing of formulation of various concentration of linear PLGA 70:30 and branched PLGA with oxime loaded or blank primary emulsions.
- Testing of solubilization of PLGA with dimethyl sulfoxide and dichloromethane.
- Testing of stabilization of nanoparticles with poloxamer 407 and didodecyldimethylammonium bromide.
- To determine the most suitable formulations for the oximes loaded delivery systems by analyzing the collected values from the sizes, polydispersities and zeta potentials of produced nanoparticles.

## INTRODUCTION

To overcome the defects and limitations of common drug delivery systems (DDS), targeted and controlled drug delivery strategy was established. In which the era for the innovative modes of drug delivery systems were exceedingly requested [1]. Nowadays, the use of nanocarriers to delivery of therapeutic agents such as drugs, genes, vaccine antigens and other therapeutic compounds have received great attention in which a significant number of this delivery systems have been emerged [2]. In addition to the drug and therapeutic compounds delivery, nanocarriers can be used as diagnostic and imaging agent delivery systems [3, 4].

In last decades, drug delivery has accomplished vast improvement, but drug entry into the human organs such as CNS stays complicated mission to carry on. Despite the advance in research for the nano-drug delivery vehicles to improve targeted drug delivery into the brain is ongoing and progress has become to contribute a realistic foundation to regulate drug transportation across blood brain barrier in which these transporter systems are generally used for brain cell nutrition [5]. These days Nanocarriers are going under research for many illnesses such as Alzheimer, diabetes, oncology, neurodegenerative diseases, psoriasis, central nervous system targeting, tuberculosis [6].

In an overview, nanoparticles are colloidal molecule with unique properties, and they can provide site-specific delivery of drugs. One of many types of nanoparticles could be mentioned as polymeric nanoparticles (PNPs) in which they provide very functional system and it could be in different forms such as lipid-based nanoparticles, PEGylated, etc. [1].



## LIST OF ABBREVIATIONS

A2	PLGA branched on Poly(Acrylic Acid)
DCM	Dichloromethane
DDAB	Didodecyldimethylammonium bromide
DMSO	Dimethyl sulfoxide
DSC	Differential scanning calorimetry
F127	Pluronic <sup>®</sup> F-127 (Poloxamer 407)
HI-6	Asoxime chloride
LC	loading capacity
NP	Nanoparticles
OBD	Obidoxime
PDI	Polydispersity index
PEG	Polyethylene glycol
PLA	Poly(lactic acid)
PLGA	Poly(D,L-Lactic- <i>co</i> -Glycolic Acid)
WFI	Water for Injection
ZP	Zeta potential

# 1 THEORETICAL SECTION

## 1.1 Polymeric nanoparticles as colloidal DDSs

Study and exploration on polymeric nano drug delivery has gained tremendous advancement since 1980's [5]. Between the available nanocarriers, polymeric nanoparticles as solid colloidal structures because of their benefits such as improved medical efficiency, prolonged clearance time, reduced dose - toxicity and related side effects, easy preparation method and appropriate drug protection by having the most applications, can be deliberated as the structural backbone [1, 2]. In addition to those mentioned advantages, polymeric nanoparticles can be considered as smart polymer in which they are able to deliver the drug to active site at specific rate and also they can achieve an impressive high drug loading capacity easily [1].

Polymeric nanocapsules and polymeric nanospheres are the main types of polymeric nanoparticles [8].

Generally based on the preparation method, excipients and active ingredients can be encapsulated, attached or dissolve into the matrix. These types of nanostructures can be administered via different ways such as oral, intra-ocular, nasal, parenteral, etc. [1].

Nowadays, the use of polymeric nanocarriers for the transfer of various drugs is very significant. However, due to their form, size, and other constitutional chemical or biophysical features, these carriers have shown good performance in the delivery of hydrophilic drugs, and drugs with low solubility [9, 10]. Moreover polymeric nanoparticles due to their structure and capacity can be used as ideal candidates for the transfer of important therapeutic agents such as vaccines, antibiotics, anti-tumor particles in which they gain great accomplishment in cancer therapy [11].

A variety of nanocarriers for drug delivery was shown in Figure 1.

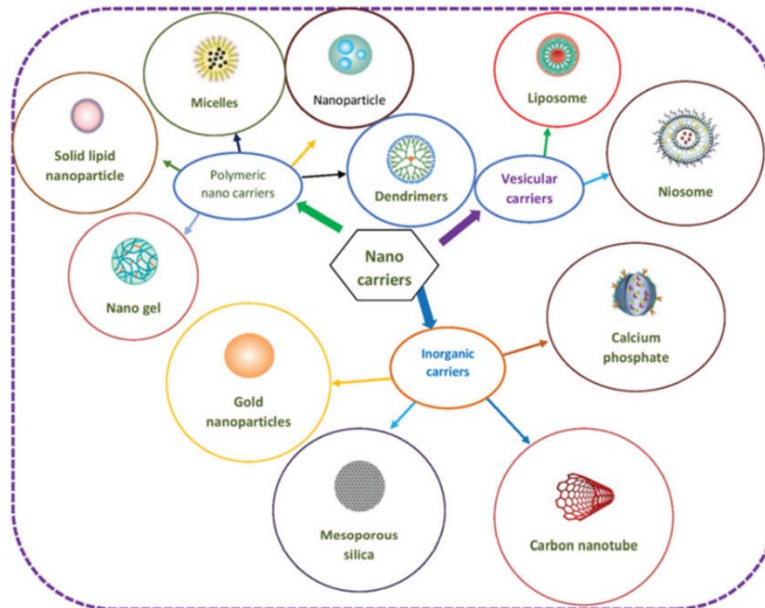


Fig. 1: Classification of most commonly used nanocarriers for drug delivery [7]

### 1.1.1 Benefits of polymeric nanoparticles:

- Polymeric nanoparticles have moderately an easy preparation
- They can be used as targeted drug delivery
- It could be the best candidate for the tolerance against changing of humidity
- Have most acceptable bioavailability
- Having acceptable control related to particles size and its distribution
- It has a great therapeutic efficacy
- Clearance time for these types are quite long
- Prolonged concentration of medication at the active region [11].

While there are a variety of methods for preparing these vehicles, adequate knowledge of polymers is needed in which choosing the suitable method based on physical and chemical properties of the polymer to act as an active ingredient. Stability and compatibility problems of excipients and active ingredients with polymers is another important issue during the preparation [1].

Encapsulation of medications in the form of nanospheres, nanocapsules and adhesion of them onto nanoparticles are the main common technique of creation of polymeric nanoparticles drug carriers and has been one of the most exploited systems for drug delivery.

Main benefits of drug encapsulation:

- (i) Conservation against physiological (in-vivo) degeneration factors,
- (ii) Decreasing harmful side-effect,
- (iii) Achieving the more desirable and reliable pharmacokinetic specs,
- (iv) By preventing recurrent small injections or keeping on using perfusion methods, they are able to increase the compliance in various patients [12].

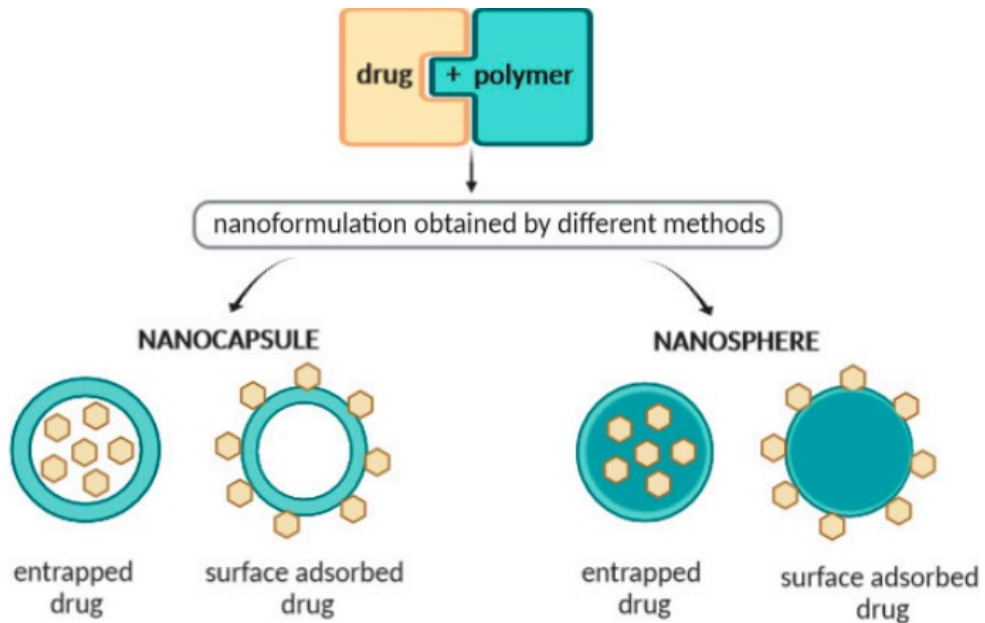


Fig. 2: Different possibilities of the drug association with nanospheres and nanocapsules [13]

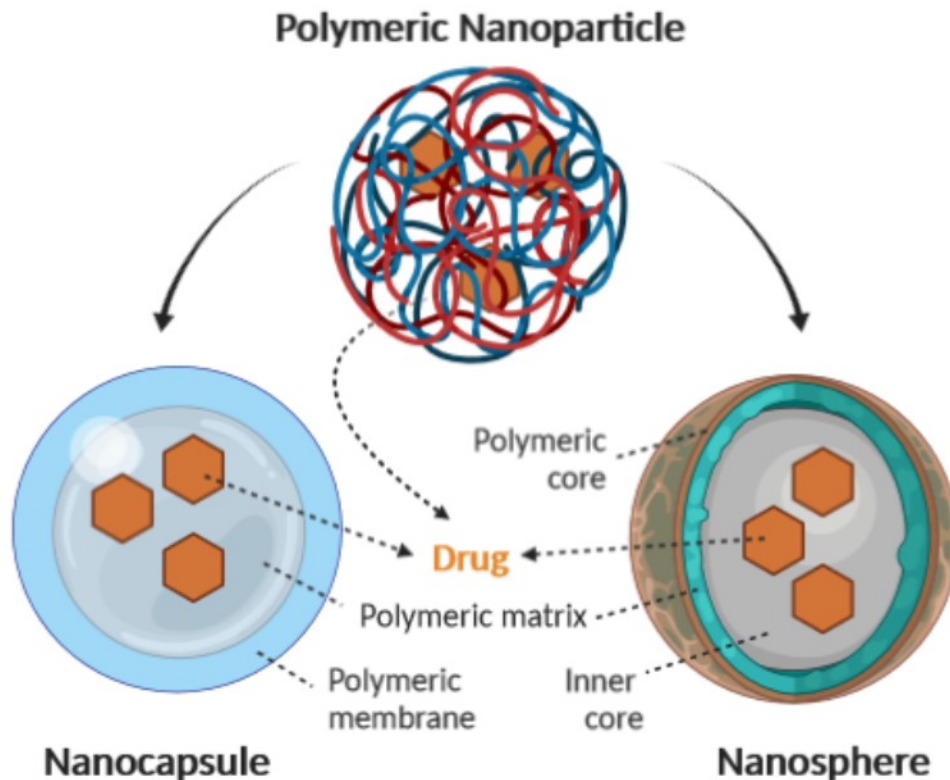


Fig. 3: Schematic representation of the structure of nanocapsules and nanosphere [13]

Effective elements on nanoencapsulation course of action and final resulted products:

- Using organic (carbon-based) solvents, capable of dissolving more substances
- Constitution and solubility of active ingredients
- Ratio difference of polymer to drug
- Amount and type of used stabilizer
- Viscosity and amount of the dispersed and sustained phases
- Mixing velocity and level heating degree in case emulsifying
- Class and molecular weight and concentration ratio of polymer [14].

Through employing the polymeric nanoparticles on drugs, reaching to different sites like inflammation, tumors and intracellular areas have become passively possible. And it has been facilitated due to increased permeability and retention effect (EPR). Physicochemical properties include size, surface charge and administration routes of the colloidal particles are impressive factors which could affect the biodistribution. polymeric nanoparticles surface modification with

various hydrophilic polymers like PEG and polysorbate 80 can reduce the opsonization and improve availability of drugs [15].

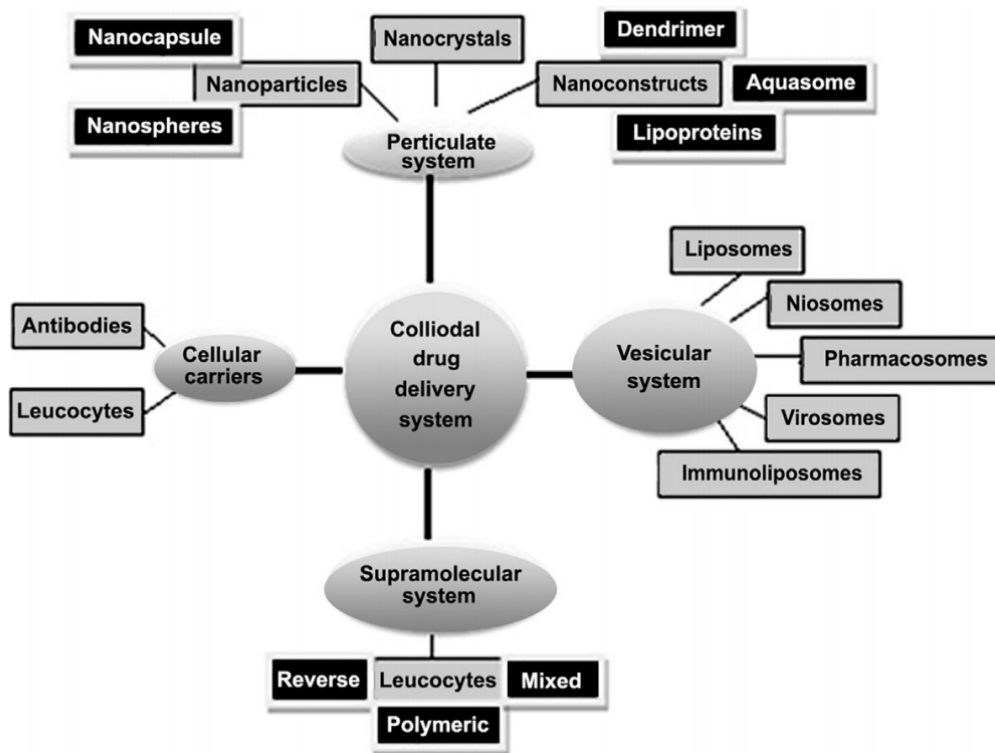


Fig. 4: Types of colloidal drug delivery systems [15]

## 1.2 Polymeric Nanoparticle Fabrication Methods

Choosing the right method to prepare polymeric nanoparticles with desired properties is very significant. Physicochemical features of the polymer and the drug is so important factor in selecting suitable preparation method [6].

Nanoparticles which created unsatisfactory and incompetent to be absorbed at the site of action for the cell will be absorbed by the reticuloendothelial system (RES) of cells. It is important to note, organs like lungs, spleen and liver very quickly absorbs nanoparticles with a hydrophobic surface, and in other hand those with hydrophilic structure exhibits longer circulation time in the body. Therefore, for targeting improvement and circulation time in the bloodstream, applying hydrophilic small nanocarriers (100 nm or less) are suggested [16].

The harmony of the polymer with the human body is very serious factor to decide how the polymer will react to their environment and it should be: non-toxic, biodegradable, biocompatible.

Mainly polymeric nanoparticles can be formulated by using several different methods. Moreover, two general methods for creating of polymeric nanoparticles are:

- physical method based on dispersion and dissolving of preconstructed polymers
- chemical method based on creation of polymers from each mono-particles [15].

Tab. 1: Different method for polymeric nanoparticle preparation [13]

<b>Polymeric Nanoparticles</b>	<b>Production Methods</b>
Nanospheres	Solvent evaporation Emulsification/solvent diffusion Nanoprecipitation Emulsification/reverse salting-out
Nanocapsules	Nanoprecipitation

### 1.3 Most common preparation methods:

#### 1.3.1 Solvent evaporation

This is the most general and first polymeric nanoparticles preparation method, developed by Vanderhoff et al. In this approach, at first a preformed polymer should be dissolved in a volatile organic solvent consists of oil-phase and aqueous-phase including stabilizer/emulsifier in water. Mixing the oil phase and aqueous phase with high speed homogenization lead to make an emulsion. In the next step, evaporation of the solvent followed by obtain a suspension containing of nanoparticles. Finally, by applying ultracentrifugation, nanoparticles can be collected.

In this method there are two main classes of emulsions which commonly used:

1. Single emulsion evaporation; oil-in-water (o/w) or water-in-oil (w/o)
2. Double emulsion evaporation; water-in-oil-in-water (w/o/w) or oil-in-water-in-oil (o/w/o).

Based on the dispersity and solubility of the polymer particles in water or organic phase, each of these methods can be employed [15].

First method which is used to create the polymeric NPs from preassembled polymer was Solvent evaporation. To create an oil-in-water (o/w) emulsion is needed, in which to produce nanospheres. As it shown in figure 5, for starter we create organic phase, it includes organic polar solvent have

dissolved the polymer and the drug either dissolved or dispersed. Formally owing to the harmful results caused by using chloroform and dichloromethane, their popularity decreased for the biological applicants and it altered by the ethyl acetate. Surfactants such as Poly Vinyl Acetate in which they help the polymers to have better solubility in water have been employed frequently. To create a dispersion of nano particles, at start by adding surfactant to organic solution it emulsified in water phase, and usually by using homogenization or ultrasonication created a dispersion of nanodrops in water. And in continue by evaporation of polymer solvent, it will create a suspension of nanoparticles through the continues phase. Evaporation of solvent usually done by persisted magnetic stirring at normal room temperature for polar solvents and if it is less polar it could be done through decreasing pressure. And then the precipitated nano drops washed and accumulated after solvent evaporation by using centrifugation and freeze-drying to save it for extended periods of time. This is how to produce the nanospheres [13].

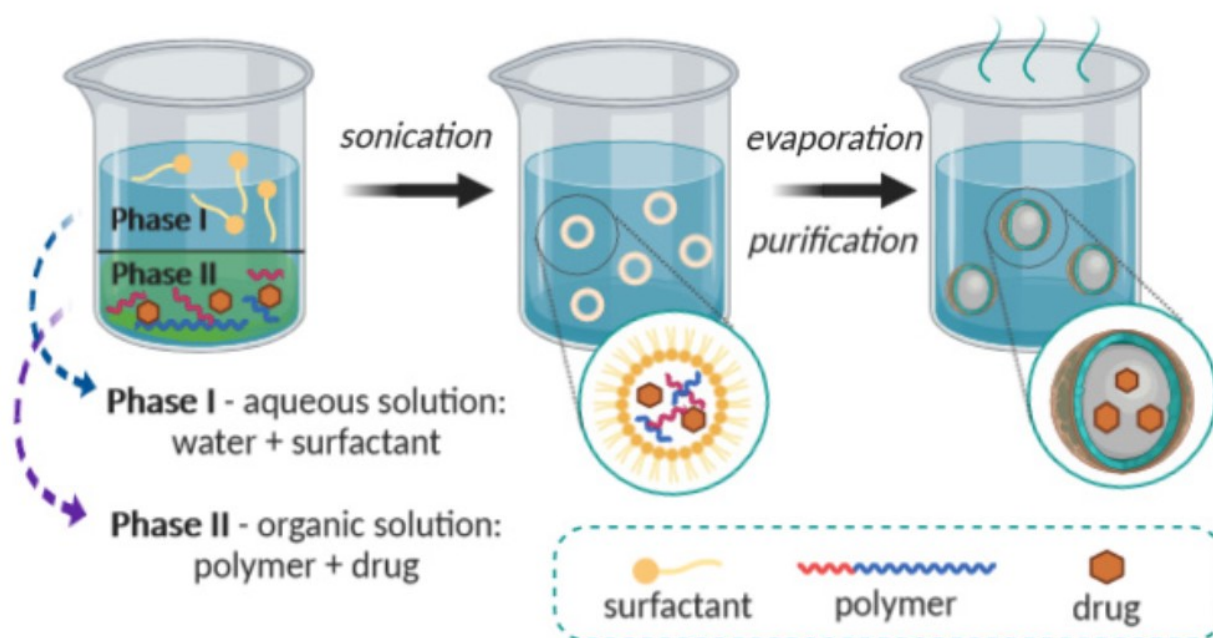


Fig. 5: Illustrative description of the solvent evaporation method [13]

### 1.3.2 Single emulsion evaporation

This method of emulsion evaporation includes two levels. The step one involves the dissolving the drug and polymer in organic solvents which could evaporate. The preparation is based on two parts in which emulsion of oil in water phase created by mixing water and some stabilizer to oil phase



of organic polymer solution. To speed up the creation of nanoparticles or nano-drops, homogenization or ultrasonication are recommended. And in step two organic solvent evaporated till the creation of precipitation of nanospheres in diameters around few hundred nanometer and thus it will be collected. It usually continued by restoring the particles through ultracentrifugation and washing them by distilled water. End of process has to be passing the nano-particles with drugs through the lyophilization in order to remove the water and extent the stocking time for the particles [14].

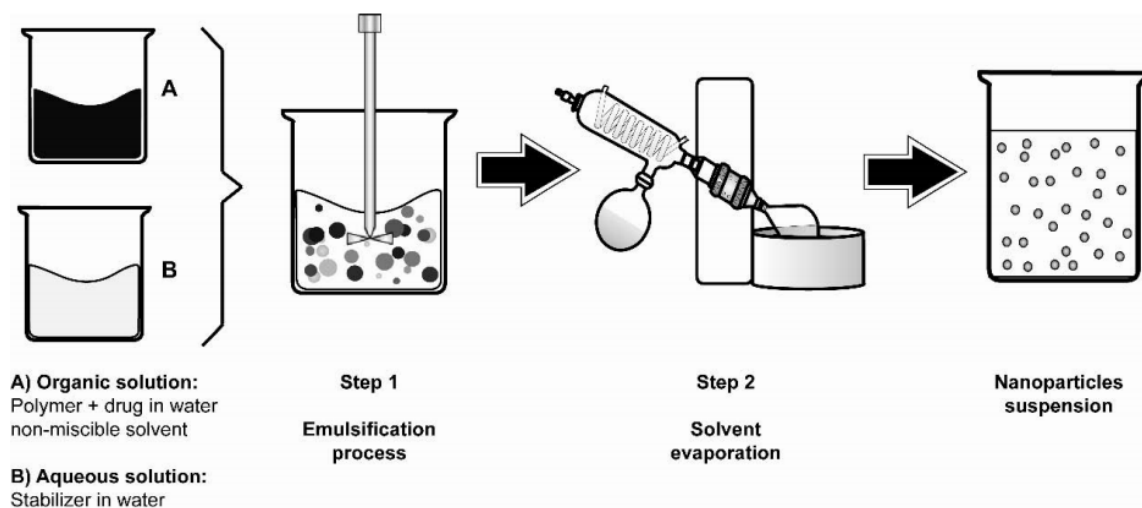


Fig. 6: Illustrative description of single emulsion-solvent evaporation method [14]

### 1.3.3 Double emulsion evaporation

High encapsulation efficacy of hydrophilic drugs is important advantage of double emulsion evaporation method. Also, preparing of nanoparticles encapsulated drug by this strategy supplies small particle size, high encapsulation product and reduced size distribution.

Commonly the process of double emulsion includes two levels, and it done to create water/oil/water double emulsion. Step one has done to create inner phase by scattering the aqueous phase (W1) in oil phase in which lipophilic emulsifier will be produced and in step two process will continue by scattering W1/O phase into an outer phase (W2) in which it includes hydrophilic emulsifier. W1/O phase is produced by scattering hydrophilic drug in aqueous medium poured under fast mixing into vehicle of continues phase including the polymer in the hydrophobic solvent. And in next step to create W1/O/W2 double emulsion, primary emulsion of W1/O phase under fast mixing added to aqueous phase of outer phase W2. It's common to have surfactant in

W2 vehicles phase to help the emulsification. Precipitation of and solidification of polymer will be done by the process of organic solvent evaporation and thus nanoparticles will go through the fast speed ultracentrifugation, which will lyophilized to increase stocking periods for the products [17].

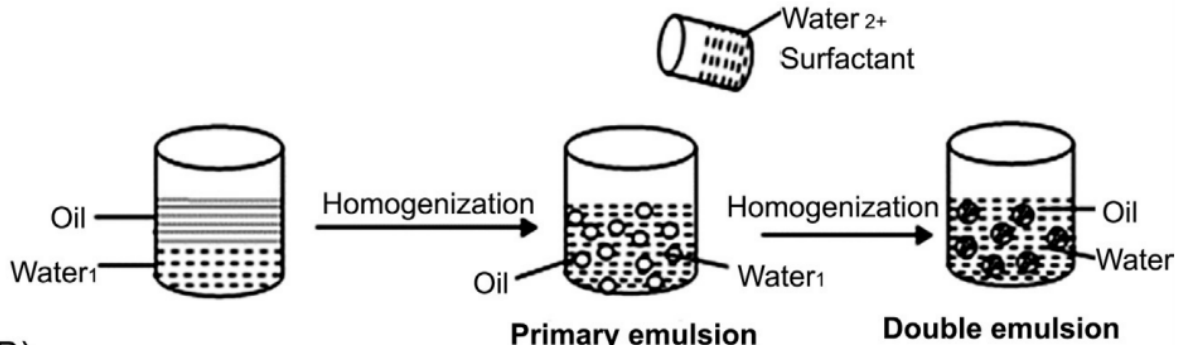


Fig. 7: Illustrative description of production of double emulsion evaporation [15]

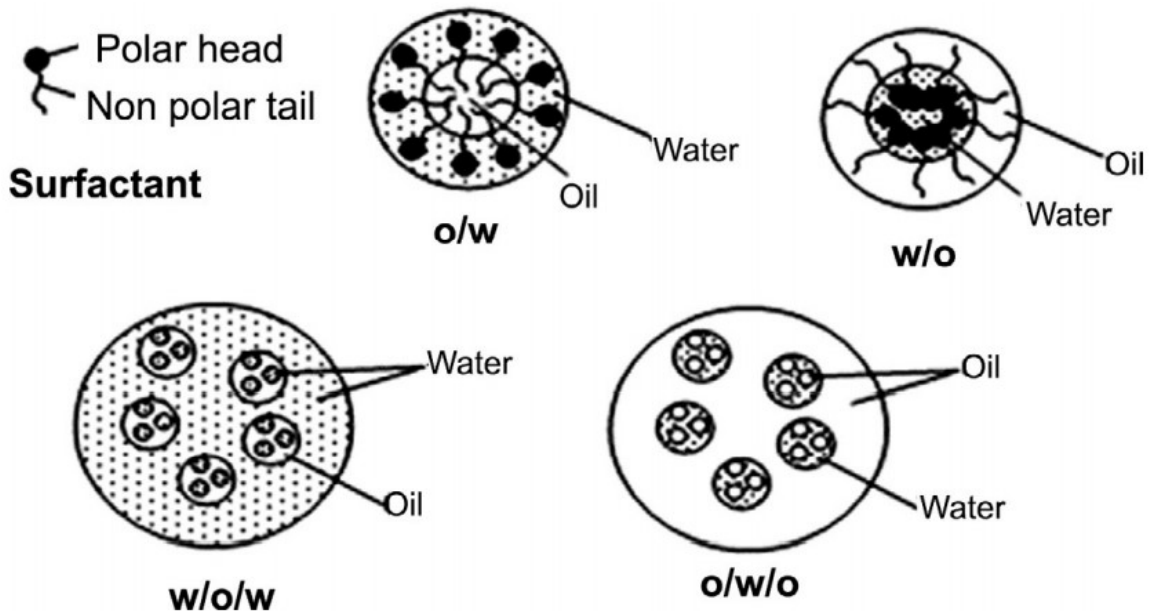


Fig. 8: Illustrative description of single emulsions (o/w and w/o) & double emulsions (w/o/w and o/w/o) [15]

### 1.3.4 Nanoprecipitation

Nanoprecipitation is a method that is also known as interfacial deposition or solvent displacement approach [1] and especially is useful for the synthesis of nanoparticles with size below 100 nm. This method is established on precipitation technique and widely applied in the pharmaceutical and agricultural studies as drug carrier preparations for both hydrophobic, hydrophilic and or even natural compounds. Generally, this technique exhibits a suitable reproducibility and especially is important for the industrial high scale production of nanoparticles [18]. To create the nanocapsules with oil-based carrier in its center which could exhibit better loading efficiency for lipophilic drugs, generally nanoprecipitation methods are used. In this procedure at first step, prepared polymer should be dissolved in organic solvents like acetone, ethanol, etc. The presence of surfactant is optional. At the next step, polymer solution mixed with aqueous phase and thus by continuing the agitation nanoprecipitation will be created at the mixing pod in which it's happening by fast diffusion of the organic solvent in aqueous environment [15].

This technique can be recalled as solvent replacement method which includes two miscible solvents. Organic solvents which do not dissolve in water like acetone or acetonitrile will be mixed with a polymer to create the inner phase. As the result of hydrophobicity solvents could be easily evaporated and eliminated. Main theory and rules behind this method is based on interfacial deposition of polymers following the replacement of organic solvent from a lipophilic solution into water phase. In next step the polymer is mixed with medium polarity solvent which is miscible in aqueous environment, and the solution is in stepwise manner by the dropwise technique in specific controlled speed have been added to a water solution while being stirred continuously. As the result of high speed impulsive diffusion of the polymer solution to the water phase, the nanoparticles are created immediately due to not willing to mix with water molecules. Once the solvent spread out from nanoparticles, nanospheres or nanocapsules will be created. In overall, commonly the organic phase mixed into the water phase, but this procedure could be done in another way around by not happening any change in final product of nanoparticle creation. Commonly the surfactant can help the creation of nanoparticles in the way the colloidal suspension stabilized although their existence isn't essential to create the final product and whole procedure can be done with or without their help. Created nanoparticles are normally known with their distinct size and a confined size distribution compared to solvent evaporation methods. Nanoprecipitation is commonly employed for the creation of nanoparticles with dimension size around 170 nm, but it

could be required to obtain the nanocapsules or nanospheres. Nanocapsules can be achieved once the drug already mixed with an oil, and thus it will be emulsified in the organic polymeric solution in which prior the internal phase will be scattered in an external phase. Nanospheres are achieved once the active ingredients are scattered or disintegrated in the polymeric solution [13].

Generally in the nanoprecipitation procedure, formation of nanoparticles has done in single step by employing the miscible solvents which comes with benefits of having an absence of complication, fine regeneration, least consumption of energy [19].

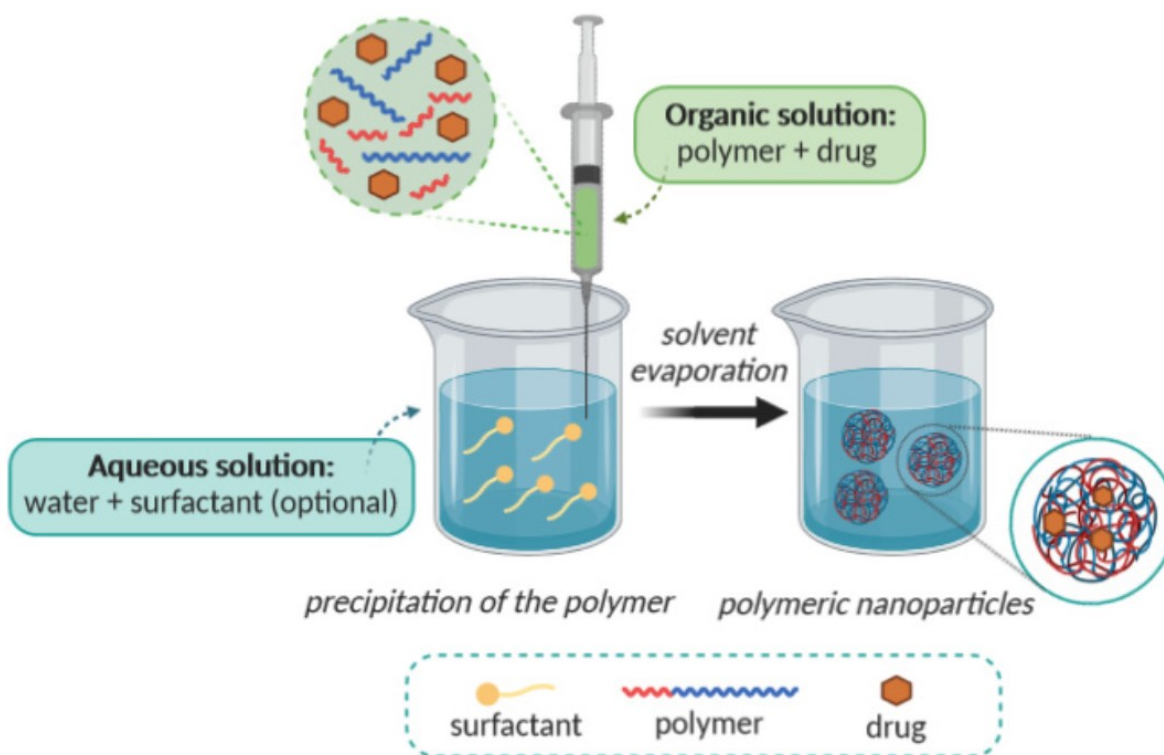


Fig. 9: Illustrative description of the nanoprecipitation method [13]

## 1.4 Polymers used for polymeric nanoparticles preparation

In order to prepare polymeric nanoparticles, a lot of ingredients and constituents have to be mixed with each other to create aimed polymeric nanoparticles with specific characteristics in which main constituents is polymers. Based on the main point of supply of polymer, they can be divided to two natural and synthetic polymers to be involved in preparation. The most widely used natural polymers are gelatin, sodium alginate, chitosan [20], hyaluronic acid and protamine sulfate (PS) [21]. In addition, common synthetic polymers used for polymeric nanoparticles formulation are

poly (lactide co-glycolides) (PLGA), polyglutamic acid (PGA), polylactic acid (PLA), poly (N-vinyl pyrrolidone) (PVP), poly (vinyl alcohol) (PVA), polyethylene glycol (PEG), etc. [20]. some common synthetic and natural polymers was shown in fig 9.

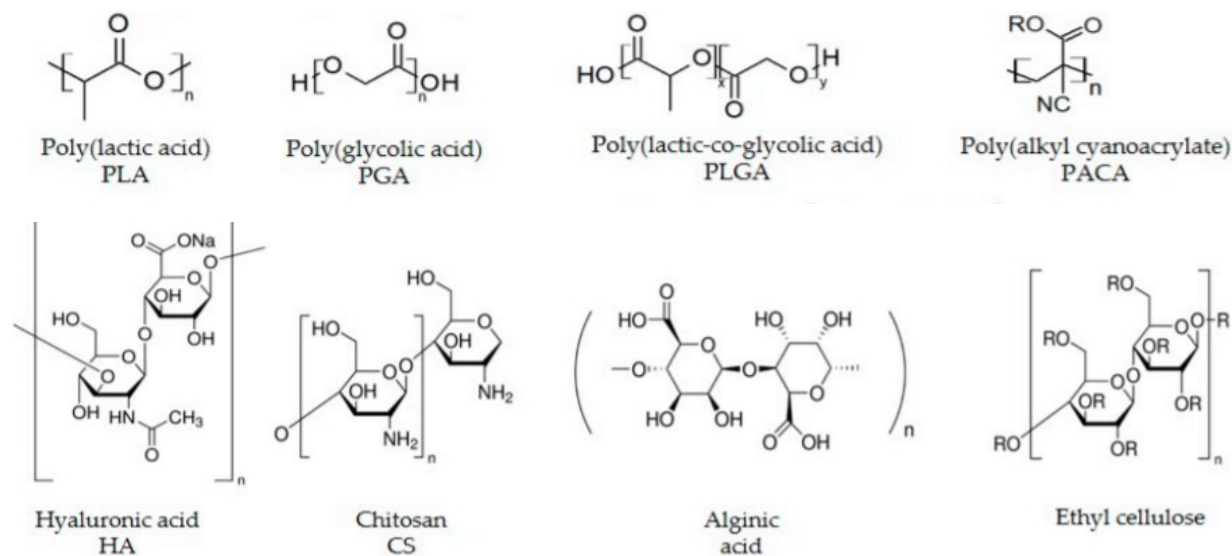


Fig. 10: Most frequent polymeric nanocarrier used to produce polymeric nanoparticles [21]

### 1.4.1 PLGA

The most widely used polymer to preparation of polymeric nanoparticles is poly-DL-lactic- co-glycolic acid (PLGA) as a biodegradable and biocompatible synthetic polymer [19, 22]

Based on previous researches, it has shown that the administration of PLGA nanoparticles basically served to cancer, neurological disorders and vaccine [23].

The desired physiological elimination and deep clinical knowledge features and achievability to create sustained drug delivery are the most favorable characteristics of PLGA. It could be most known possibilities to make the for drug delivery by having the consideration to structure and performance [22].

By employing tin (II) alkoxide and 2-ethyl hexanoate tin (II) as enzyme, PLGA is manufactured by ring-opening polymerization and creation of ester bond with various fraction of the lactic and glycolic acid. Adjustability and flexibility of the PLGA is without the doubt as the result of various intrinsic characters of it, in which they can affect the crystallinity of polymer. For instance, PLA is in chiral form and it can be found in different arrangement like Poly L-lactic acid, Poly D-lactic

acid, and Poly D-L-lactic acid. Although the Poly L-lactic acid has found in extreme crystalline forms, Poly D-lactic acid is completely amorphous, and Poly D-L-lactic acid is amorphous. In opposition, PGA does not have any chirality while being remarkably crystalline. although PLGA copolymers are being produced from PGA and PLLA, created polymer shows a crystalline feature, and if it produced from PGA and PDLA, the amorphous features are being distinct. While mixture of PGA and PDLLA has amorphous final feature. The feature of crystallin can have main effect on the degradation, for an e.g., an amorphous structured polymer can disintegrate easier at a physiological environment with less energy compare to those have better crystalline rearrangement which in need of higher energy. This result can be due to truth that further usage of Poly-D, L-lactic-glycolic acid, can be named PLGA, of better improved amorphous feature related to PDLLA and it used for biomedical administration. It should be realized that lactic acid is way more hydrophobic than glycolic acid and that's reason in which the polymers which having more hydrophobic characteristic have more lactic acid than the glycolic acid, and it will be eliminated from body at the lower speed due to having less water inside. Thus, polymer with more capability of holding water and wise versa or if it's having higher ratio of glycolic to lactic, it will be hydrophilic. As the result of having glass transition degree above 37 °C for the PLGA copolymers, they could be suitable for to be used for a physiological environment. By bringing the copolymer lactic acid ratio and molecular weight down, we can manage to cut down the glass transition temperature and thus in hardness of polymers. Molecular weight can have crucial impact on size of particles and another features. For example, if exists particle size and molecular weight in smaller scale, or bigger scale the PGA concentration in PLGA (bigger the content of hydrophilicity) will result in bigger scale the elimination rate and maximum concentration available in blood and thus less store up in cells. In general, physical features like constitution, size, glass transition temperature, molecular weight, level of crystalline can have impact on mechanical firmness and elimination rate and how long it will stay stable [21].

So far, in numerous studies, the potency of this polymer to transfer of various drugs especially hydrophilic and water soluble ones has been investigated [24, 25]. Beside that many researches were concentrated on toxicological aspect of PLGA [1]. The use of PLGA for medical applications has been approved by the US Food and Drug Administration (FDA) [19].

Tetrahydrofuran, chlorinated solvents ethyl acetate or acetone are common solvents for PLGA [1].

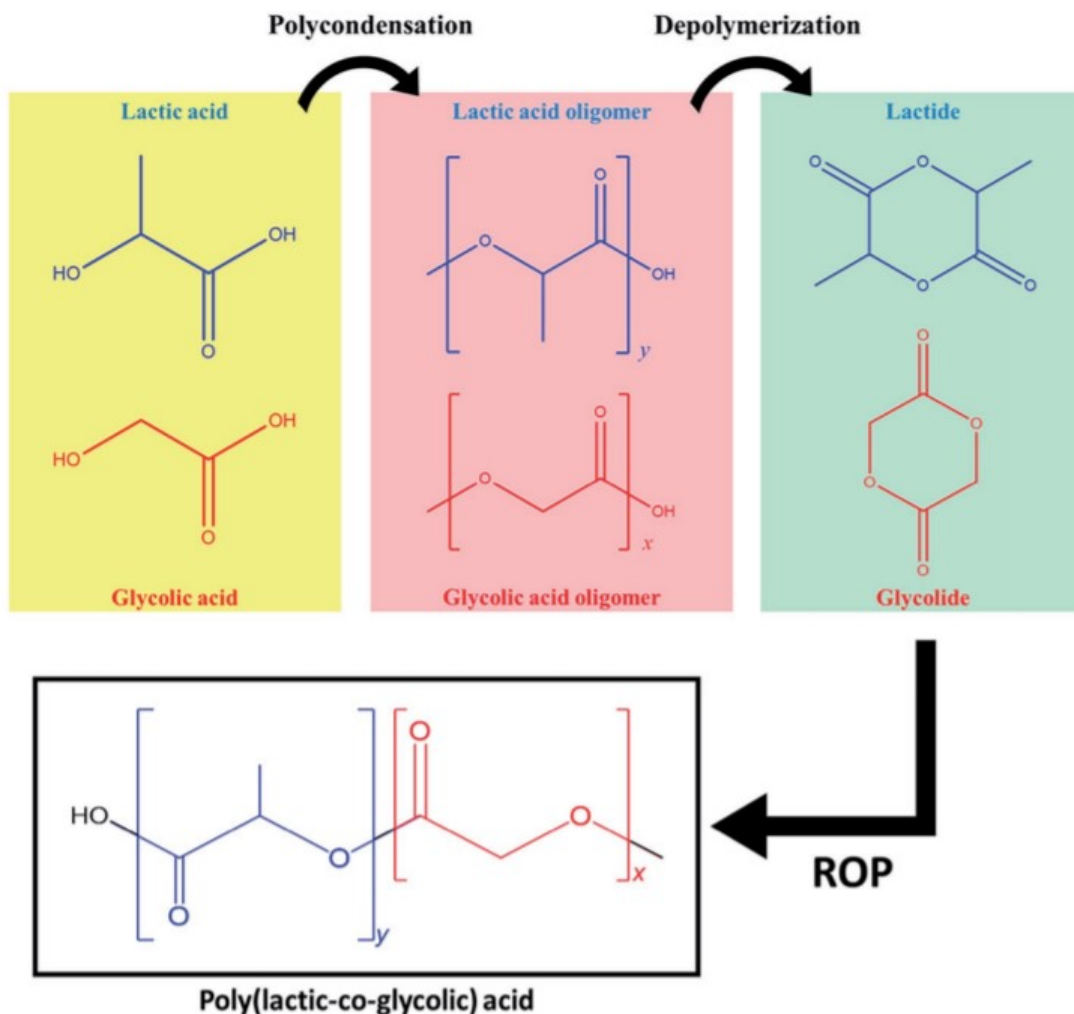


Fig. 11: Manufacturing of the PLGA copolymer by using the ROP methodology [26].

Based on research by Salama et al, it appeared that when lower glycolic acid subsection in polymers have seen, zeta potential and particle size and encapsulation efficacy scales for PLGA will decrease too [27].

#### PLGA types

By using different lactic: glycolic acid ratio, various forms of PLGA like PLGA 50%:50%, 65%:35%, 75%:25%, 70%:30%, 85%:15% can be produced [28, 29].

Based on previous studies, composition of the polymer is the major parameter of determining the elimination speed of nanoparticles from body thereafter drug release could be modified considerably by changing the concentration of polymer [28, 30].

Based on researched results, it shows that by adding in amount of glycolic acid ratio in oligomers, speed degradation will increase. Thus, PLGA PLA50%: PGA50% can have higher degradation rate than PLGA 65%:35% as result of containing capability of absorbing more water due to having more percentage of glycolic acid. And same concept can be valid in cases of PLGA 85%:15% shows lower degradation rate than PLGA 75%:25% than PLGA 65%:35% [22].

At the base, PLGA lactide 50%: glycolic 50% copolymers have shortest half-life in which it's around 50-60 days to be eliminated from the body and it has been used most commonly in nano-compounding, while PLGA copolymer with 65%:35%, 75%:25%, and 85%:15% of lactide/glycolic have prolonged elimination rate in physiological environment. The half-life of the polymers could be changed due to co-mixing with more hydrophobic or hydrophilic ingredients like polycaprolactone or polyethylene glycol [31].

In another word, the amount of glycolic acid is a key factor in controlling the matrix hydrophilicity, degradation, and drug release rate.

Through lowering the level of Lactic acid in the polymer of PLGA, Glass transition temperature of it will decreased as result of this modification and it causes to decrease in their molecular weight [31].

Also, in several studies, effect of polymer composition on zeta potential were determined and at the conclusion it seems PLGA constitution have shown very little effect on zeta potential numbers [30, 32].

Tab. 2: Physical properties of different PLGA nanoparticles [31]

<b>Polymer (lactide to glycolide ratio)</b>	<b>Inherent viscosity (dL/g)</b>	<b>Physical state</b>	<b>Glass transition temp (°C)</b>	<b>Solvent solubility*</b>	<b>Approx. degradation time (months)</b>
PLGA (50:50)	0.55-0.75	Amorphous	45-50	1,2,3,4,5,6	1-2
PLGA (65:35)	0.55-0.75	Amorphous	45-50	1,2,3,4,5,6	3-4
PLGA (75:25)	0.55-0.75	Amorphous	50-55	1,2,3,4,5,6	4-5
PLGA (85:15)	0.55-0.75	Amorphous	50-55	1,2,3,4,5,6	5-6

\* 1 = acetone, 2 = tetrahydrofuran, 3 = hexafluoroisopropanol, 4 = chloroform, 5 = ethylacetate, and 6 = dichloromethane



## 1.5 Stabilizers

Stabilizers are anionic or cationic surfactants, hydrophilic polymers and due to providing chemical stability during synthesis of nanoparticles considered as one of the substantial components of polymeric nanoparticles. Generally, in solution media, nanoparticles are less stable system which have tendency to decrease their high surface energy through coagulation, aggregation/agglomeration. The best proposed solution to reduce energy levels and to promote the stability of nanoparticles is using stabilizing agent over the nanoparticles surface which can help to separates nanoparticles from each other [14, 33].

In addition to the effect on final product characteristics and physicochemical properties such as particle size and zeta potential, the type and concentration of the stabilizer can influence drug payload of nanoparticles, biodistribution, release profile of drug, therapeutic efficacy *in vivo*, cellular uptake, etc. [34, 35].

One of the main benefits of using different surfactants such as polysaccharides, poloxamers or polysorbates in nanoparticle preparation is related to BBB penetration improvement that led to enhancing their uptake by tumor cells [36].

Frequently employed stabilizers could be such as poly(vinyl alcohol) (PVA), poloxamer 127, poloxamer 188 and polysorbate 80 [14].

### 1.5.1 Poloxamer 407

From poloxamer or Pluronic specific types are Poloxamer 407 or Pluronic F127 (F127) are amphiphilic, by having no charge they are non-ionic triblock copolymers surfactant consisting of hydrophobic polyoxypropylene (PPO) and hydrophilic polyoxyethylene (PEO) domains with a molecular weight of around 12,500. Some physiological properties such as minimalized toxic and side effect, minimalized provoking immune systems and significant biocompatibility lead to use this stabilizer in many pharmaceutical applications especially for preparation of polymeric nanoparticle such as PLGA as drug delivery carrier. Heat-reversible gelation and capacity to mix with hydrophobic solution of F127 has created great value and attention in controlled-release administration. The most frequently uses of F127 or Poloxamer 407 can be associated to the surfactant features. Also, after application as a solution it can create an inelastic semisolid gelatin net by an extra heating. This could be featured as such depot-like prolonged release in situ gelatin

in which in parenteral use it can help to manage the speed of drug release from nanoparticles [37-40].

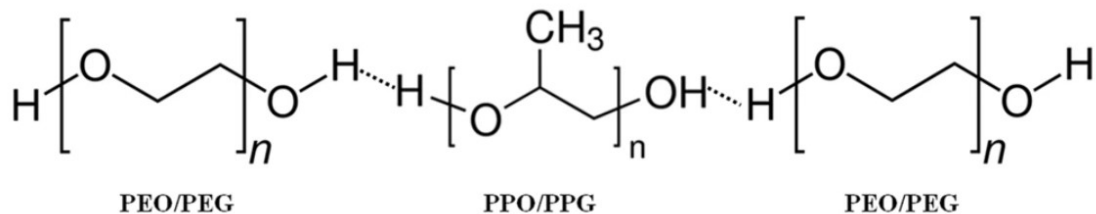


Fig. 12: Schematic structure of PF127; it consists of 67 units of hydrophobic Polyoxypropylene oxide (PPO) winged by 98 units of hydrophilic polyethylene oxide (PEO) from each ends [38]

### 1.5.2 Didodecyldimethylammonium bromide

Didodecyldimethylammonium bromide (DDAB) is a cationic surfactant that can be applied in various concentration in which it is capable to change the surface charge of desired structure in which it can affect the zeta potential [41].

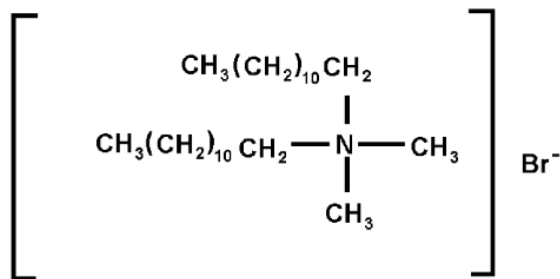


Fig. 13: Chemical structure of DDAB [42]

So far, in several studies, DDAB has been used to cover on the PLGA nanoparticle surface. Based on observed results, some factors such as high homogenizing stirring rate and DDAB weight percentage leads to decrease the DDAB/PLGA nanoparticles size. Also, zeta potential of DDAB on PLGA nanoparticles will be reduced when DDAB level in microemulsion decreased or homogenizing stirring rate increased. Furthermore, the surface coverage of DDAB on PLGA nanoparticles may be reduced by adding in the homogenizing stirring rate, but by adding the weight percentage of DDAB, it become improved the surface coverage of DDAB on PLGA. The

abundance in number of DDAB which found on the surface of the DDAB/PLGA nanoparticles could considerably promote the characteristics of them [43].

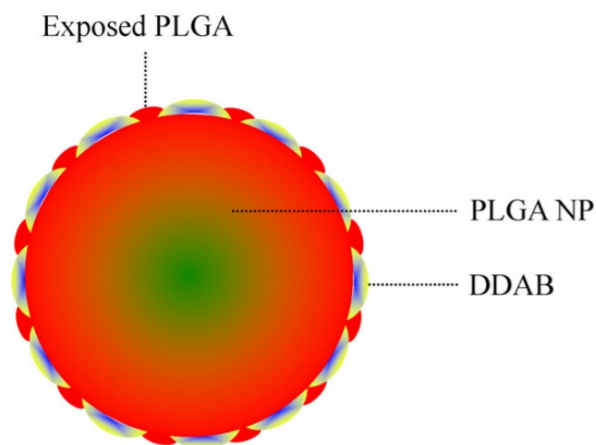


Fig. 14: Schematic representative structure of DDAB/PLGA NP [43]

### 1.5.3 Dichloromethane (DCM)

Dichloromethane,  $\text{CH}_2\text{Cl}_2$  (DCM), also known as methylene chloride, is an aliphatic hydrocarbon compound. And it been manufactured due to its adjustable low toxic solvent to be mixed with different organic ingredients in many production processes [44]. It been realized that employing dichloromethane (DCM) as a solvent will normally create bigger nanoparticles with a higher distribution size [45]. Exceptionally higher DCM ratio by having less water will result in bigger particles size [46].

Ability to dissolve with water for the DCM is little, but vapor pressure is noticeably high, thus it will be dispersed very quickly in water and will evaporate very quickly creating the swift precipitation of polymer with not letting the drug particles to be dispersed into water phase, resulting in high capturing with increasing the DCM volume [47].

### 1.5.4 DMSO

Dimethyl sulfoxide (DMSO) has lack acidic proton and polar in which it called an aprotic solvent with capability to dissolve any type of polarity ingredients such as hydrophilic PLGA. By decreasing the intermolecular statics within the PLGA particles due to enhanced mobility even at low temperature as result of using DMSO as solvent for the polymers. Also, DMSO as the result of being aprotic polar solvent have capacity to protect the ester bond of polymer from being hydrolyzed even in very low amount and protect from the degradation which make it more suitable

in comparison to water. In addition, It been researched that the DMSO can be manufactured for controlled release in micro/nanospheres medication [48].

Primary solvent has huge effect on the size of PLGA nano-compound and amount of drugs ingredient to be contained [49].

## **1.6 Chemotherapeutic agents and transport across the brain**

Regardless of advancement for the new and innovative chemotherapeutic candidates to treat the brain tumor, accomplishing the adequate drug delivery into the tumor cells still remains a major challenge as it been always the blood brain barrier (BBB) the greatest limitation to overcome [50]. Frequently chemotherapeutic agents are hydrophilic with bulky size and electronic charges on it, in which passing the blood brain barrier with high limitation is not easy task to overcome and it have been a challenge to reach therapeutic dosage at the site of action. Also passed medication would remain at the site of action and it can diffuse back immediately, thus creating another difficulty to keep the constant concentration of drug to be supplied [36].

### **1.6.1 Blood Brain Barrier**

The multilayered structure of blood brain barrier has duty to have electro-chemical surrounding on the balance, and thus it can cause a considerable difficulty for the drug delivery systems to central nervous systems and to be effective on the CNS illnesses. Most effective limitation of various chemical substances to be crossed the blood brain barrier can be size of particles, hydrophilicity, being affected by polarity, substrate specificity, and active efflux pumps.

Various physiochemical criterion can be associate with capability of medication to cross the blood brain barrier and thus stay at the site of action in brain cells:

- molecular weight
- lipid-solubility (low lipid-soluble molecules do not simply penetrate to the brain)
- charge and ionization at physiological pH
- interactions with plasma proteins
- interactions with efflux pumps and transporters [51-54]

Consequently, only small (molecular mass under 400–500 Da) and lipophilic compounds can facilely diffuse across the BBB. Which means almost no drug compounds with non-lipophilic and having large molecular size cannot cross of this barrier [55].

## 1.7 Drug delivery to the brain cells

Medication can reach the aimed places in the brain either by going around the blood brain barrier and bypass it or cross-pass the BBB [36].

Six basic routes can be determined to cross-pass the BBB:

- passive transcellular diffusion,
- paracellular transport,
- receptor-mediated transcytosis (RMT),
- carrier-mediated transport (CMT),
- adsorptive-mediated transcytosis (AMT),
- cell-mediated transport.

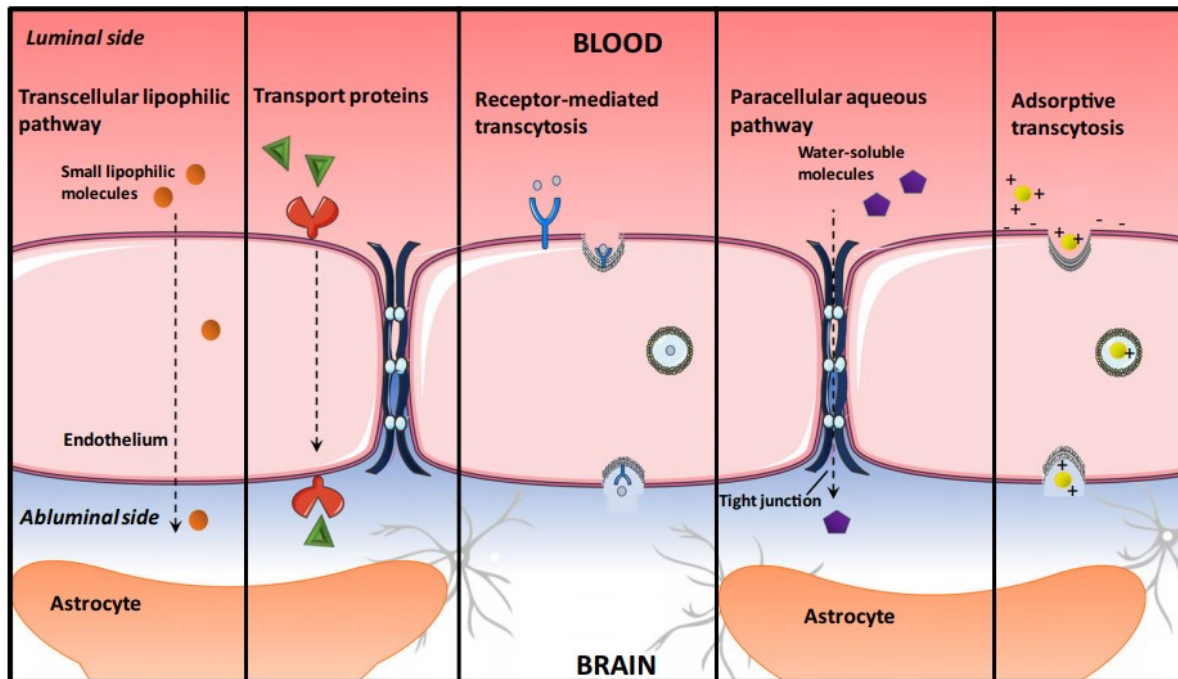


Fig. 15: Short schematic summary of transport processes across the blood brain barrier (BBB) [56]

BBB disruption and direct intracerebral injection are the classic drug delivery technique but discovering the safer and less invasive paths which can improve and upgrade the brain drug delivery system is one of the most noteworthy areas in drug delivery research. Nowadays, employing the nanomedicine methods to cross-pass the BBB and convey the medication to the

areas of CNS and to cure the related illnesses are thus becoming more critical and necessary to gain [56].

### **1.7.1 Nanomedicine for CNS drug delivery**

Lately, employing a range of different nanoparticle formulations as a carrier for drug delivery to CNS has received significant attention [52, 57]. The greater part of these preparations included the appropriate polymers which follows the accurate requirement demanded to be admit for the biological relevance [36].

Nanoparticles are considerate as assuring structures which can be applied to improve the uptake of drugs across the BBB. Nanoparticles are capable to be filled with various medicaments chemical agents and act on the various ligands which enable to cross-pass the BBB. And also they are capable to diffuse into the cracked vasculature of cancer tissue through increasing the retention effect (EPR) and permeability [36].

Advantages of nanoparticles as drug delivery carries [57, 58]

- drug circulation improvement
- payloads enhancement
- protection of the drugs against degradation
- the potency to ‘solubilize’ drugs
- easy manipulation of shape, size, chemistry structure, surface charge and hydrophobicity
- controlled release of therapeutic agents
- small size

Generally, nanoparticles approaching routes are consider as non-invasive strategies. Being that, these approaches are able to enhance drugs solubility and improve the delivery to CNS and keep it at the therapeutic concentration at areas of need in CNS [52]. Therefore, brain delivery of drugs by nanoparticles is a promising strategy that could bring up new approaches for the chemotherapy of brain tumors and development of novel systems to treat brain disorders is one of the biggest confronting and high priced merchandize compartment for pharmaceutical companies [59].

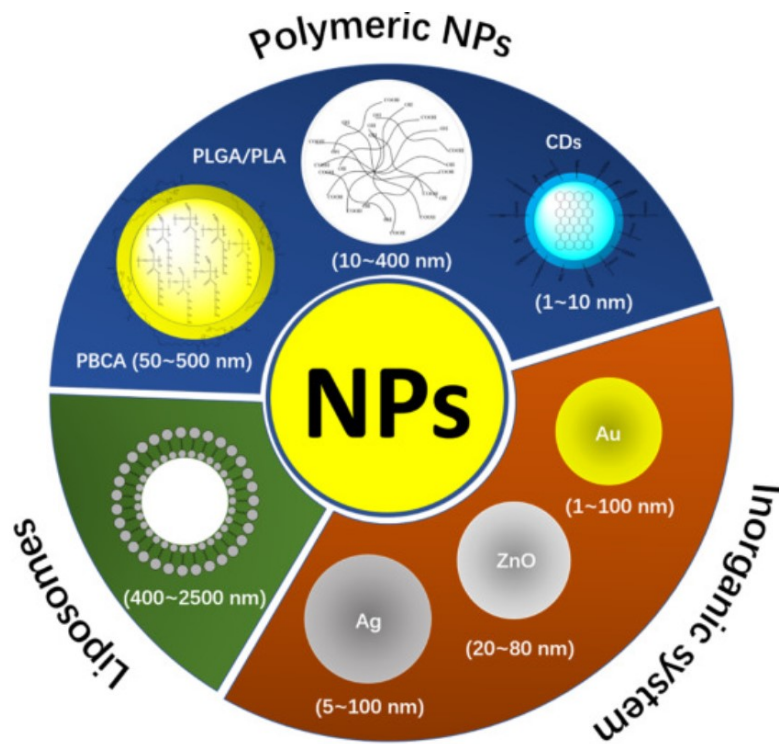


Fig. 16: Various types of NPs came into exist within the past decade helping drugs across the BBB [60]

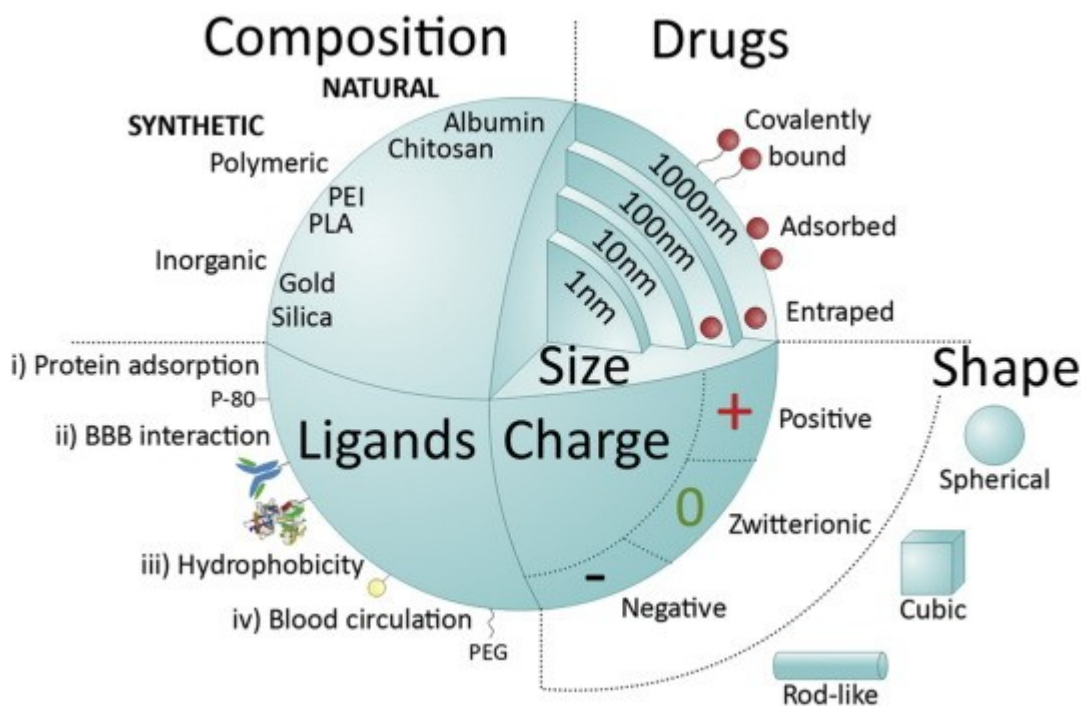


Fig. 17: Common nanoparticle characteristics affecting systemic delivery and BBB passes [59]

### 1.7.2 Polymeric Nanoparticles for Brain Delivery

Polymeric nanoparticles have exhibited to be reassuring vehicles for CNS drug delivery, due to their capacity in encapsulating preparations, therefore preventing them from being metabolized and eliminated from body systems, and in distribution pharmacochemical particles cross-passing the blood brain barrier with no imposing any threats to the protection of barrier [61].

Based on various in vitro/in vivo studies, some significant properties such as cytotoxic effects, cellular uptake and brain penetration with drugs bound to polymeric nanoparticles have been improved. So far various chemotherapeutic compounds such as temozolomide (TMZ), doxorubicin, methotrexate, etc. by applying polymeric nanoparticles have been delivered to the brain [62-65].

Pioneer in polymeric NPs which administered for the drug delivery system cross-pass the BBB known to be PBCA (poly butyl-cyanoacrylate) nanoparticles [36].

As result of assurance in secure experiences, PGA, PLA, and the copolymer PLGA been researched for CNS administrations. For an example in one scientific report done by Chang et al, Transferrin-coated PLGA nanoparticles which resulted in a 20 times enhancement in targeting of an in-vitro BBB example as in contrast to non-coated nanoparticles [52].

Tab. 3: Various cases of PLGA polymer nanoparticles as targeted drug delivery system used in therapy of CNS cancers [36]

Polymer Type	Size (nm)	Drug Loaded
PLGA	90	Dil
PLGA	100	Loperamide
PLGA	120	Doxorubicin
PLGA	230–255	Paclitaxel
PEG-PLGA	19	TMZ
PEG-PLGA	125	Coumarin6



There are three main routes of delivery for nanoparticles designed to medication CNS tumors:

- (1) direct administration to CNS
- (2) direct systemic administration to CNS
- (3) indirect systemic administration to CNS

Direct Administration to CNS presents a technique of bypassing the BBB by straight injection of the nano-polymers into CNS. The main disadvantages of this method are the infection risk, the necessity of controlling crucial physiological elements such as osmolarity and pH which could harm the brain cells.

In Direct systemic administration nanoparticles are straightly delivered in blood circulation through the carotid artery in which it been eliminated to be streamed back to the heart and thus through entire body.

The Indirect systemic administration includes the conveying the nanoparticles to the systemic circulation through the path which necessity to be absorbed like topical, nasal, oral, and peritoneal delivery. The main benefits of oral delivery are accessibility, patient compliance, and not being invasive [36].

Various methods to prevail over the blood brain barrier:

- (a) Opening tight junctions (TJs) between endothelial cells or enhancing toxic effects at local areas that causes localized increasing in membrane permeability which letting to diffuse the drug in mixture with the NPs or without it in released free form.
- (b) cytopempsis or transcytosis within the endothelial cells
- (c) endocytosis: NPs are moved within endothelial cells by endocytosis, their loads are released within the cell endocytosis and then exocytosis in the endothelium luminal
- (d) mixture of these methods

Many various elements can affect the nanoparticles passing the BBB like shape, size and also for specific conveying the nanoparticles changing ligand structure [66].

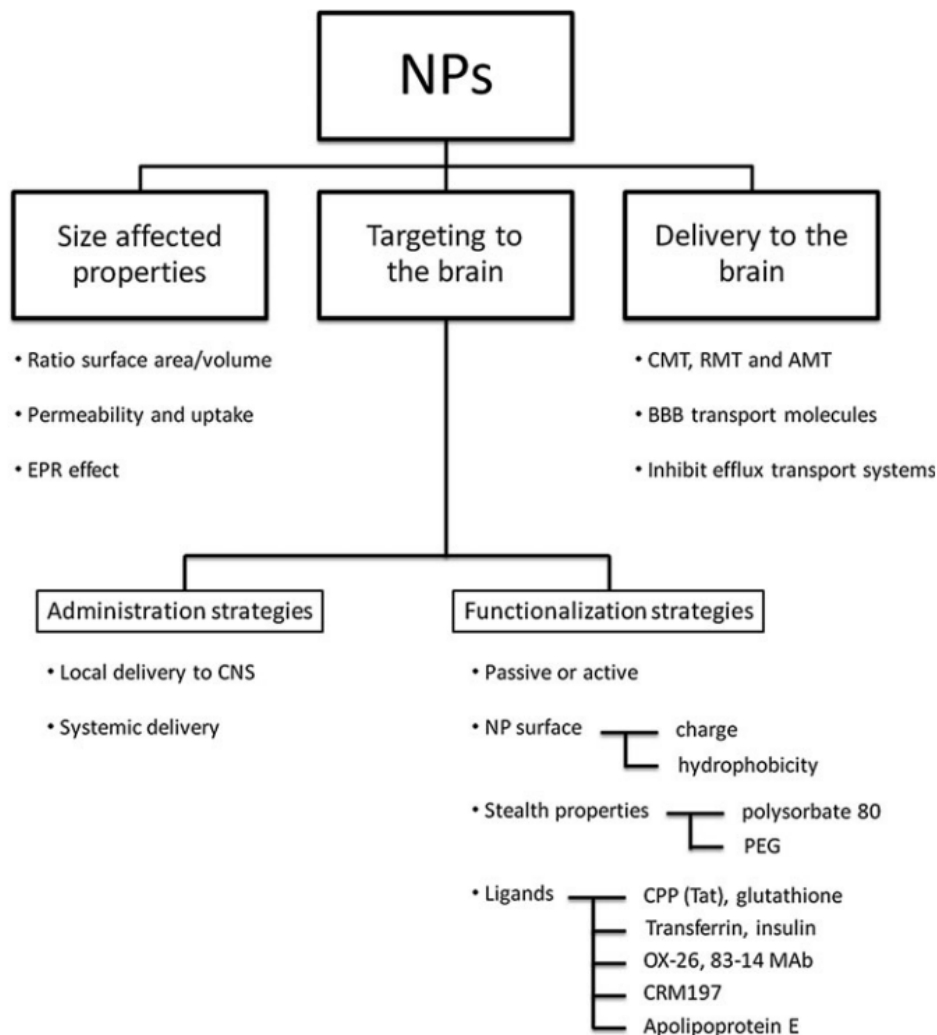


Fig. 18: Nanoparticles' characteristics and the ways for drug delivery to the brain [67]

## 1.8 Physicochemical Properties of Nanoparticles

The various physicochemical features in NPs such as particle size and polydispersity, the surface charge or zeta potential could affect destination of aimed methods. In the other hand, they can influence the cell membrane-nanoparticles interaction and modify progress in passage through biological barriers.

These are substantial elements, capability of nanoparticles to cross-pass the BBB is not relied on the structure of the drug, which is not simple to be changed, but it based on changes done on the physicochemical features of the nanoparticles [36].

Generally, information to realize the effectiveness of the features such as size, zeta potential, and surfactant could be how much helpful to create the nanoparticles and to carry the drugs across the blood–brain barrier (BBB) are insufficient [68].

### **1.8.1 Particle size and polydispersity**

Size is a significant determinant that have an influence on cytotoxicity, clearance, biodistribution and transport of nanoparticles in the blood stream and thus transport of nanoparticles to the tumor site. Particles with size of 10 to 200 nm can facilely trans-pass by the leaky blood vessels of the tumor, or normal tissues [36, 69]. As the result, improving the size of nanoparticles can enhance their activity and availability at the tumor tissues [36].

In addition, latest research have exhibited that size of nanoparticle have an important function in many biological activities varying through surface adjusting, molecular attaching, adhesion of nanoparticles-cell, entering into cell, and cell-internal transports, excreting from cell, vascular dispersion, and dispersion through different tissues, and by that controlling the delivery effectiveness [70].

Currently, small nanoparticle (less than 230 nm) with a polydispersity of 0.10 suggested as drug brain delivery carrier [71].

Transmission electron microscopy (TEM), atomic force microscopy (AFM), dynamic light scattering (DLS), analytical ultracentrifugation and fluorescence correlation spectroscopy are common technique for measurement of nanoparticle size and polydispersity [70]

Nanoparticles have to be sufficiently large to avoid accelerated inclusion to blood circulation and sufficiently small to avoid getting disposed to the immune system [19].

### **1.8.2 Drug release profile**

Fick low of diffusion within the matrix of the polymeric nanoparticles and degradation speed can explain the releasing profile of nanoparticle drugs.

Many varieties can affect the complicated process of drug release, in which these factors may able to forecast the profile:

- Degradation speed of polymer
- molecular weight and polydispersity of block copolymer
- molecular weight and load of PEG
- water which taken up by nanoparticles

- drug-polymer solubility
- interactions between drug-polymer
- in vivo and vitro drug solubility

In various study, in biological medium at the beginning happens the blast of drug near or on the surface of polymer in which continues with a sustained release [72].

several mechanisms of drug release including:

- Release of the adsorbed drug from the bounded surface
- breaking down into small particles
- diffusion within the matrix particles
- when the drug is enclosed in the core polymer, it can diffuse within the wall
- main mass and surface breaking down into small pieces
- degradation/diffusion combined methods [47].

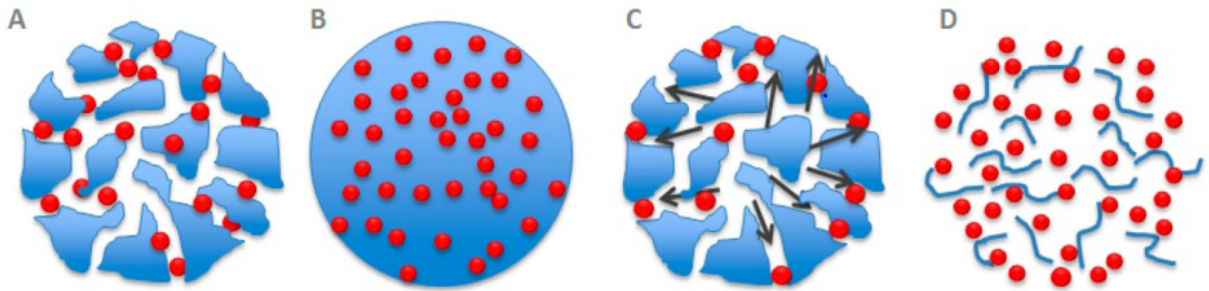


Fig. 19: Drug release processes from nanoparticles: (A) diffusion within aqua filled spaces, (B) diffusion within the polymer matrix, (C) osmotic pressured transports, and (D) abrasion and erosion [73]

It is expected PLGA copolymer degradation is based on mixture of mechanisms including surface diffusion, surface abrasion and content diffusion, content abrasion.

Due to the existence of numerous factors which can affect the process of biodegradation such as polymer level of crystallinity, molecular weight (Mw), molar ratio of the lactic and glycolic acids in the composition of polymer chain, and glass transient temperature of the polymer, therefore predict of drug release rate from PLGA matrix will be complex [22].

### 1.8.3 Zeta-potential (ZP)

The zeta potential is an important index for identifying the stability and especially nanoparticles surface charge [74]. It can also have significant effect on the cross-pass of nanoparticles within the BBB [59]. Surface charge considered as a key factor and responsible for some biological effect of nanoparticles such as: toxicity, release profile, cellular uptake.

Another descriptive name have been used for ZP is *electrokinetic potential*, in which it means that potential caused by the slipping/shear plane of a nanoparticles at the motion supported by the electrical background [74].

In zeta potential measurement, zetasizer measures electrophoretic movement which is follows the rule of Henry's equation. Electric double layer which is around the particles are exist in two sets. In one is exist solid layer which has no charge and acts as an inert in which ions can firmly bind to it and another is diffuse part in which electronic charged ions are less firmly bound to it. Due to movement of ions with charged particles, potential will be created within charged particles and their surrounding and it is recalled as zeta potential [80].

There are some reported that explain nanoparticles with large positive charged zeta potential can result in fast toxic effect to the BBB. Thus, many of the NP description have explained in the different articles for the drug delivery to the CNS have mentioned negative charged potential of balanced from  $-1$  to  $-15$  mV or high from  $-15$  to  $-45$  mV. Also, few of them can be positive charged on the balanced form up to  $15$  mV or high above  $15$  mV, were have capability to cross-pass the BBB and effective drug delivery in CNS [59].

pH, nanoparticles concentration and ionic strength are parameters which influencing ZP.

ZP and nanoparticles colloid stability:

ZP values which is used as instruction for the categorization of nanoparticles-diffusion,

- highly unstable ( $\pm 0-10$  mV)
- relatively stable ( $\pm 10-20$  mV)
- moderately stable ( $\pm 20-30$  mV)
- highly stable ( $> \pm 30$  mV) [74]

## 1.9 Oximes

Oximes are bioactive molecules with biological activity including anticancer, antioxidant, anti-inflammatory and antimicrobial activities that widely used in the medical sciences. Some types of

oximes are found in nature in plants, animals and are also produced using chemical synthesis methods.

Two oximes are already applied in therapy. Due to these capacity, novel oxime structures have been synthesized, and their biological activity has been confirmed.

Latest projects have demonstrated that new oxime compounds are able to be potential next-generation drugs and adjuvant therapy for pathogenic diseases, various types of cancer and inflammation. Also, oximes are able to mixed with metals and as therapeutic agents can be used as metalloenzymes inhibitors [75].

One of most important application of oxime has been therapy of organophosphorus (OP) nerve agent poisoning, in which it acts as an acetylcholinesterase (AChE) inhibitor.

The advancement in the research filed, showed that diffusion in the BBB for various drugs like oximes by having constant cationic charge is quite narrow. Restrains such as hydrophilicity, size of particles, substrate specificity, polarity, and efflux channels. Although these features are missing in oxime's structures. Thus, creating an adequate scope to interact with issue of being effective in OP poisoning for oxime by diffusing into BBB is yet challenges to be faced.

Recently drug delivery system has achieved huge progress in figuring out the physical and structural body BBB regarding pathophysiological circumstances, which it makes possible to oxime and many other drugs to be effective on CNS illnesses [76].

### **1.9.1 Methods to enhance blood brain barrier diffusion for Oximes:**

- (a) Through direct transferring the medication to the brain. which is done commonly with direct injections or having the degradable delivery mechanisms such as nanoparticles, etc.
- (b) Regulation and promoting delivery mechanism along an unharmed BBB using non-specialized and specialized techniques like modifying the lipid solubility or receptor and transporting tools like Pgp.
- (c) Possibilities of adding concentrations of the medications at the areas of need in CNS following the systemic administration, which involves immunological and magnetic targeting methods and ultrasound technique [76].

The most important oximes are:

- obidoxime (bis-/4-pyridiniumalldoxime-N-methyl/ ether dichloride)

- HI-6 (4-aminocarbonyl-pyridinium-1-methyleneoxy-20-(hydroxyiminomethyl)-10-methylpyridinium dichloride monohydrate)
- pralidoxime (2-pyridiniumalldoxime-N-methylchloride)
- methoxime (N, N0-methylen/4-pyridinium alldoxime/dichloride) [77].

### 1.9.2 Obidoxime

Obidoxime (OBD) is drug of choice for the organophosphorus toxication by being a good known as a bis-pyridinium activators [78].

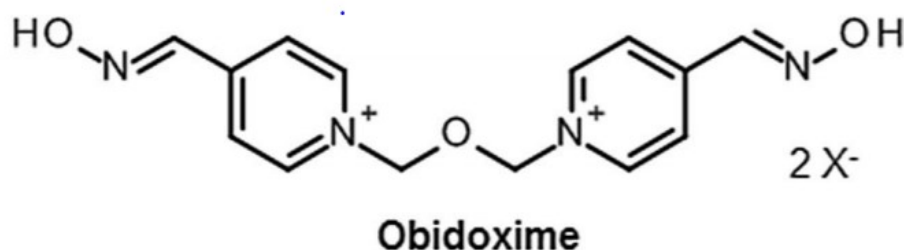


Fig. 20: Chemical structure of Obidoxime [79]

### 1.9.3 HI-6

HI-6 is an bis-pyridinium oxime and it counts as one of the best choice from quaternary oxime to treat OP nerve agent toxicity [76].

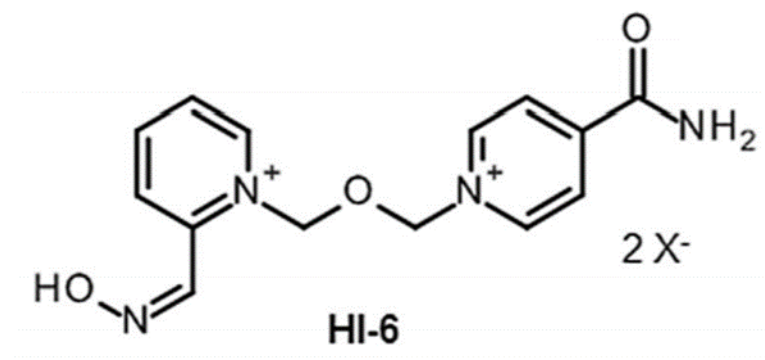


Fig. 21: Chemical structure of HI-6 [79]

The concentration of obidoxime and HI-6 which can cross withing the BBB at concentration around 3-5% and 10%, accordingly [76].

## **2 EXPERIMENTAL SECTION**

### **2.1 Materials**

Asoxime chloride (Faculty hospital Hradec Kralove)

Dichloromethane (Merck)

Didodecyldimethylammonium bromide (Merck)

Dimethyl sulfoxide (Merck)

Obidoxime (Faculty hospital Hradec Kralove)

PLGA branched on Poly(Acrylic Acid) (Faculty of Pharmacy Hradec Kralove)

Poloxamer 407 (Pluronic<sup>®</sup> F-127) (Merck)

Poly(D,L-Lactic-*co*-Glycolic Acid) (Faculty of Pharmacy Hradec Kralove)

Purified Water (Faculty of Pharmacy Hradec Kralove)

Water for Injection (Kulich Pharma)

### **2.2 Instrument**

Bandelin SONOREX<sup>™</sup> Digital 10 P Ultrasonic

Kern analytical balance ABS 220-4 N, [Max]. 220 g, d = 0.1 mg

Kern analytical balance ABS 440 – 53 N, [Max]. 6000 g, d = 1 mg,

Magnetic stirring- IKA - WERKERT, 100 - 1000 rpm

Ultrasonic bath, BANDALIN SONOREX SUPER

Zetasizer Nano ZS, Malvern instrument Ltd. UK

#### **2.2.1 Aids**

Capillary cuvette DTS 1060, Malvern

Cuvette UV High Precision Cell 6030, Hellmy Analytics

Single-channel adjustable volume pipette, 10-1000  $\mu$ l and 1-10 ml, Eppendorf<sup>™</sup> Research plus<sup>™</sup>

Syringe membrane filter 0.45  $\mu$ m



## **2.3 Preparation of nanoparticles**

### **2.3.1 Preparation of the drug solution**

Required amount of HI-6 or OBD was weighed on analytical balance and then transferred into 5 ml beaker and by adding required amount of WFI into the beaker, stock solution of 1% hydrophilic drug in water was prepared. And then solution was brought for the sonication, in which by putting it into ultrasonic water bath for a minute, homogenized solution would be created. And then by putting a dry magnetic bar into the solution beaker, it was let to be stirred by 100-500 rpm on a magnetic stirring for full dissolving of the drug in water. Choice of magnetic stir bar depends on dimension of bottom size of beaker. For the preparation of stock solution and transferring it, single channel pipette was used.

### **2.3.2 Preparation of the polymer solution**

Required amount of PLGA 70:30 or A2 polymer crystal was weighed on analytical balance and transferred into 50 ml beaker and by pouring the required amount of solvent DMSO or DCM, stock solution of 10% PLGA 70:30 or 10% A2 in the desired solvent prepared and then by adding dry magnetic bar, the solution was left on stirring with 100-500 rpm for full dissolving. In various measurement, it was necessary that the stock solution was diluted to 1% or 3% or 5% polymers by using various desired solvents and then it was transferred to 25 ml beaker and left for stirring on the magnetic stirring. For the preparing of the stock solution and transferring the solvents, single channel pipettes were used.

### **2.3.3 Preparation of the stabilizer solution**

Required amount of Poloxamer 407 or DDAB was weighed on analytical balance and transferred into 100 ml beaker and by adding required amount of purified water to the beaker stock solution of 1% Poloxamer 407 or 0.5% DDAB were prepared, and then by adding dry magnetic bar, it was left for the stirring with 100-500 rpm till full dissolving. From the stock solution required amount was transferred into 25 ml beaker and after that by adding dry magnetic bar, it was left for the stirring with 100-500 rpm. Choice of magnetic stirring bar depends on dimension of beaker's bottom. For preparation of the stock solution and transferring the water solution, 10 ml graduated pipette with pump or various size of volumetric flask were used.

### **2.3.4 Nanoparticle formulation by double emulsion method**

Desired amount of internal phase from stock solution was transferred to narrow bottom 25 ml beaker and placed on magnetic stirring with stirring bar inside and left on stirring with rotation 400-600 rpm. For the preparation of primary solution, described amount of drug solution in water drop-wisely added to the inner phase solution. The drop-wise technique was in the way that at beginning, Single channel pipette 100-1000  $\mu$ l which was filled with required volume was sucked and by using both hand on push bottom and slowly pressing it, solution will be inject drop-wisely while having carefully keeping the rate of emptying at the steady state. End of pipette tip should be very close to stirring polymer solution and by releasing slowly push bottom, solution will be injected into the stirring solution. Primary emulsion is step in double emulsion methods which created W1/O, and by drop-wise method PE will be poured into the outer phase. In this step, a beaker which contain desired amount of the outer phase were placed over the stirring magnet and then by putting a dry stir bar inside the beaker, it was left for stirring under speed of 400-600 rpm and then drop-wisely added desired amount of PE into the outer phase and thus it creates the W1/O/W2 solution, in which it would provide the stabilized finalized NP. It's important to inject the prepared PE immediately into outer phase otherwise PE particle will be agglomerate, and size of NP will increase. All process of preparation were done in room temperature.

### **2.3.5 Measurement of size and polydispersity of nanoparticles**

These measurements were performed by instrument Zetasizer Nano ZS. Before starting with machine, it needed to be calibrated. For that reason, machine was turned on and left on working for 30 min to be tuned and reach the steady state temperature 25 °C. there were two types of cuvette available for the size measurement by the normal and for the zeta potential by DTS 1060. By using 1 ml syringe, cuvettes are filled from the polymer solution in beaker. To avoid bubble and dust we usually eject the sample from middle and inside of beaker. Samples were tested by three cycles; each cycle runs 10-15 times and results were collected by each three cycles and by creating the table of mean and standard deviation from them.

When particles within suspension carrying bigger value of similar charges, they tend to repel, while they are having smaller similar charges, the particles tend to agglomerate or flocculate. In general particles with zeta potential greater than +30 mV or less than -30mV are stable.

Polydispersity value expresses the level of uniformity of size of nanoparticles and created crucial data for accepting the produced particles. PDI below 0.05 is monodispersed standards, and results of above 0.7 is high polydisperse particles. In overall, particles with value of less than 0.2 PDI are acceptable and desired quality production.

### **3 RESULTS AND DISCUSSION**

To produce oxime nanoparticles with desired features which already explained in theoretical part, double emulsion methods were applied. Factors which were influenced and were experimented including size, polydispersity, zeta potential, polymers of 1%, 3%, 5% of PLGA 70-30 and 1%, 3% of A2, solvents of DMSO, DCM and outer phase Poloxamer 407, DDAB were subjects of the experiment. By applying the dropwise technique in process of NP production, I was managed to avoid the creation of clump and agglomeration among them and produce small sized particles with lower polydispersity. By decreasing time interval between the processes of creation of PE (W1/O) and injecting it into outer phase (W2), I was managed to create smaller sized NP with less clumps among them. These factors of nanoparticles causing such an impact on the motion and activity of these particles in the circulation systems, having communication with cell structures, in entering the cell and then reaching the area of action and getting released from its formulation there [81]. The size of PLGA nanoparticles can vary within various periods of preparation to their application due to swelling, degradation, and especially by the formation of clumps. The size of NPs will passively determine the aimed area in cells and thus particles in range of 100 nm - 200 nm are the best choice for having most efficient in circulation periods and ability to cross-pass the physiological membrane in different area of body, particularly in CNS blood brain barrier. Nanoparticles larger than 200 nm are efficiently phagocytosed and distributed mainly to the liver and spleen [82].

All PLGA based nanoparticles formulated in the presented diploma thesis have done through double emulsion methods of W1/O/W2. W1/O was mainly Obidoxime dissolved in water to be injected as W1 phase and polymers dissolved in DMSO or DCM as an outer organic (oil) phase. W2 was water solution of stabilizer either Poloxamer 407 or DDAB as an outer phase. The linear PLGA 7:3 or branched A2 were used as polymer carriers.

#### **3.1 Nanoparticles based on linear PLGA**

The linear PLGA 7:3 was employed in concentrations of 1%, 3% or 5% and DMSO was used for solubilization of the polymer. Various volume 250  $\mu$ l, 500  $\mu$ l, 1000  $\mu$ l, 1500  $\mu$ l of inner phase (primary emulsion) for blank and drug loaded sample were employed. Stabilization with Pluronic 1% were used as an outer phase.

The examples of scans from the measurement of the NPs size are presented in [Figures 22 – 26].

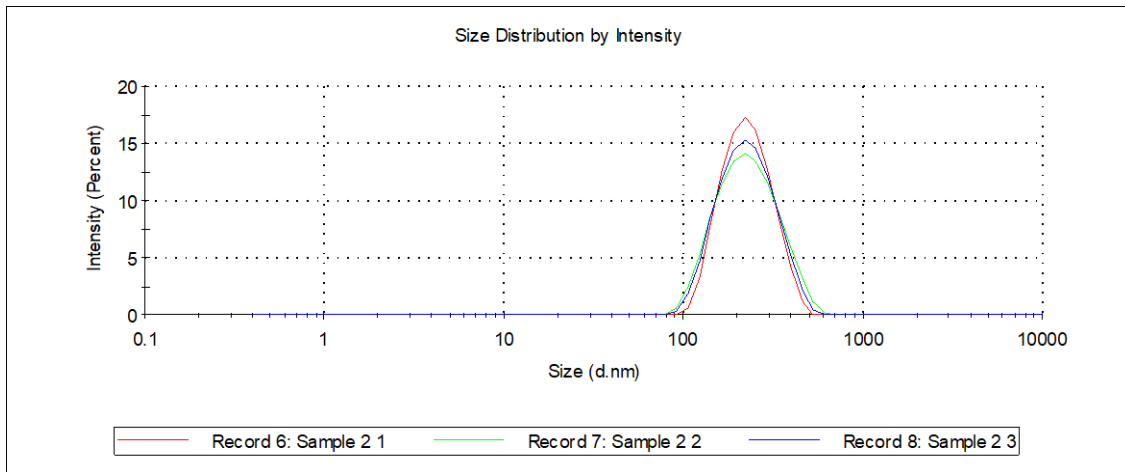


Fig. 22: Particle size distribution (nm) to the intensity of scattered beam 5% PLGA 7:3 (250 µl PE)

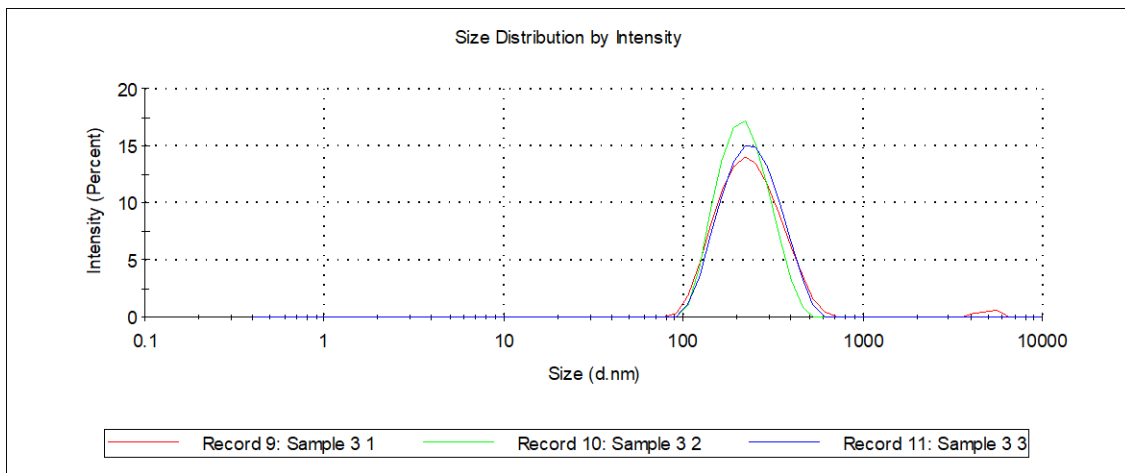


Fig. 23: Particle size distribution (nm) to the intensity of scattered beam 5% PLGA 7:3 (500 µl PE)

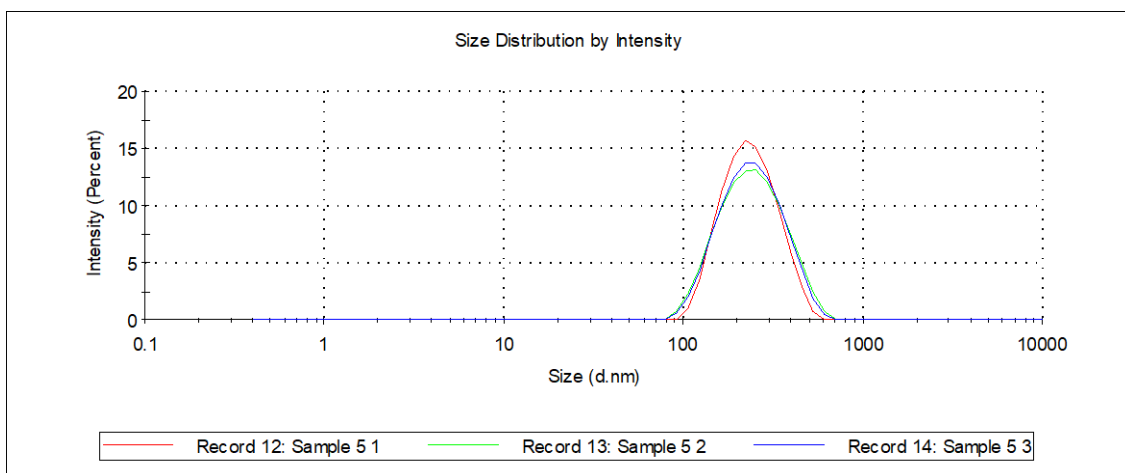


Fig. 24: Particle size distribution (nm) to the intensity of scattered beam 5% PLGA 7:3 (1000 µl PE)

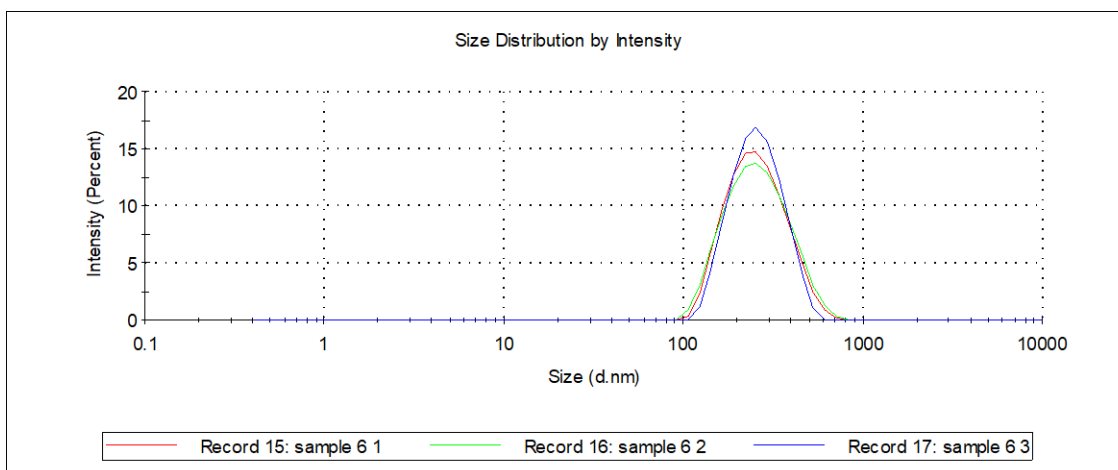


Fig. 25: Particle size distribution (nm) to the intensity of scattered beam 5% PLGA 7:3 (1500 µl PE)

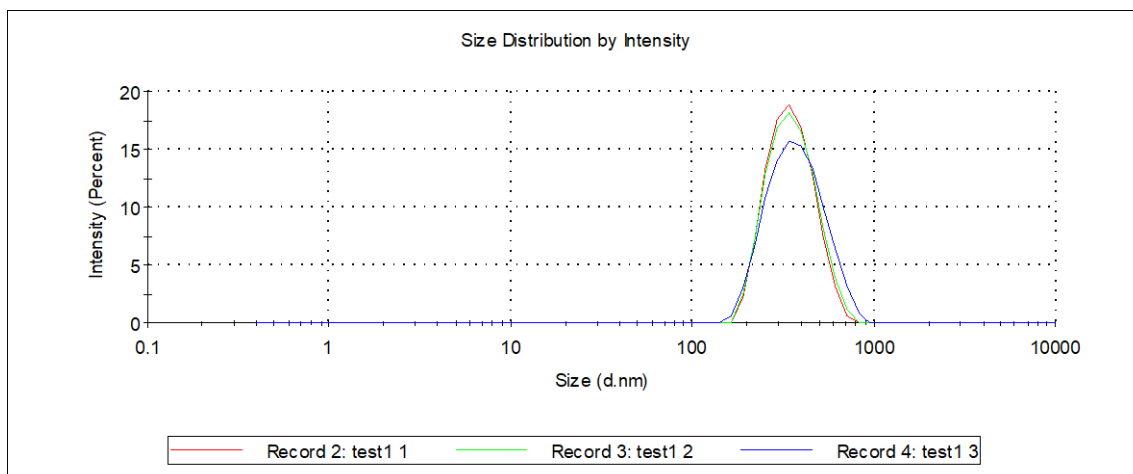


Fig. 26: Particle size distribution (nm) to the intensity of scattered beam 5% PLGA 7:3 (Blank PE)

The values of size and polydispersity index of NPs made of various concentrations of PLGA 7:3 dissolved in DMSO and stabilizer with Poloxamer 407 are presented in [Tables 5-7].

Tab. 5: Size and Polydispersity index of NP made of 1% PLGA 7:3 (DMSO & Pluronic 1%)

Size of nanoparticles (nm)				
primary emulsion	blank 500 µL	500 µL	blank 1000 µl	1000 µl
1. measurement	254.1	169.4	313	73.38
2. measurement	245.9	125.9	305.1	55.07
3. measurement	242.9	100.8	301.8	88.01
<b>NPs size mean value (nm)</b>	<b>247.6</b>	<b>132</b>	<b>306.6</b>	<b>72.15</b>
<b>SD</b>	<b>5.8</b>	<b>34.7</b>	<b>5.8</b>	<b>16.5</b>
Polydispersity index (-)				
primary emulsion	blank 500 µL	500 µL	blank 1000 µl	1000 µl
1. measurement	0.013	0.28	0.086	0.162
2. measurement	0.003	0.265	0.026	0.184
3. measurement	0.104	0.315	0.072	0.188
<b>NPs PDI value (-)</b>	<b>0.04</b>	<b>0.287</b>	<b>0.061</b>	<b>0.178</b>
<b>SD</b>	<b>0.056</b>	<b>0.026</b>	<b>0.031</b>	<b>0.014</b>

Tab. 6: Size and Polydispersity index of NP made of 3% PLGA 7:3 (DMSO & Pluronic 1%)

Size of nanoparticles (nm)				
Primary emulsion	blank 500 µL	500 µL	blank 1000 µl	1000 µl
1. measurement	574.8	233.7	543.7	224.7
2. measurement	588.9	235.6	533.7	226.9
3. measurement	576.6	233.6	536.5	224.1
<b>NPs size mean value (nm)</b>	<b>580.1</b>	<b>234.3</b>	<b>538</b>	<b>225.2</b>
<b>SD</b>	<b>7.7</b>	<b>1.1</b>	<b>5.2</b>	<b>1.5</b>
Polydispersity index (-)				
Primary emulsion	blank 500 µL	500 µL	blank 1000 µl	1000 µl
1. measurement	0.267	0.169	0.291	0.057
2. measurement	0.265	0.157	0.279	0.093
3. measurement	0.259	0.121	0.294	0.076
<b>NPs PDI value (-)</b>	<b>0.264</b>	<b>0.149</b>	<b>0.288</b>	<b>0.075</b>
<b>SD</b>	<b>0.004</b>	<b>0.025</b>	<b>0.008</b>	<b>0.018</b>

Tab. 7: Size and Polydispersity index of NP made of 5% PLGA 7:3 (DMSO & Pluronic 1%)

Size of nanoparticles (nm)					
Primary emulsion	blank	250 $\mu$ l	500 $\mu$ l	1000 $\mu$ l	1500 $\mu$ l
1. measurement	342.6	209.4	219.9	218.6	240.2
2. measurement	338.5	207.7	218.4	220.3	236.6
3. measurement	346.5	206.5	218.7	219.6	239.6
<b>NPs size mean value</b>	<b>342.5</b>	<b>207.9</b>	<b>219.0</b>	<b>219.5</b>	<b>238.9</b>
<b>SD</b>	<b>4.0</b>	<b>1.5</b>	<b>0.8</b>	<b>0.9</b>	<b>1.8</b>
Polydispersity index (-)					
Primary emulsion	blank	250 $\mu$ l	500 $\mu$ l	1000 $\mu$ l	1500 $\mu$ l
1. measurement	0.16	0.10	0.18	0.11	0.13
2. measurement	0.11	0.13	0.20	0.14	0.13
3. measurement	0.11	0.12	0.12	0.13	0.09
<b>NPs PDI value</b>	<b>0.13</b>	<b>0.12</b>	<b>0.17</b>	<b>0.13</b>	<b>0.13</b>
<b>SD</b>	<b>0.03</b>	<b>0.01</b>	<b>0.04</b>	<b>0.01</b>	<b>0.02</b>

First experiment working with 5% PLGA 70:30, an excellent result was achieved. In which nanoparticles sizes of 208-239 nm and with PDI around 0.13 [Tab. 7]. By increasing in volume of primary emulsion, size of nanoparticles slightly increased while having perfect polydispersity [Fig. 27].

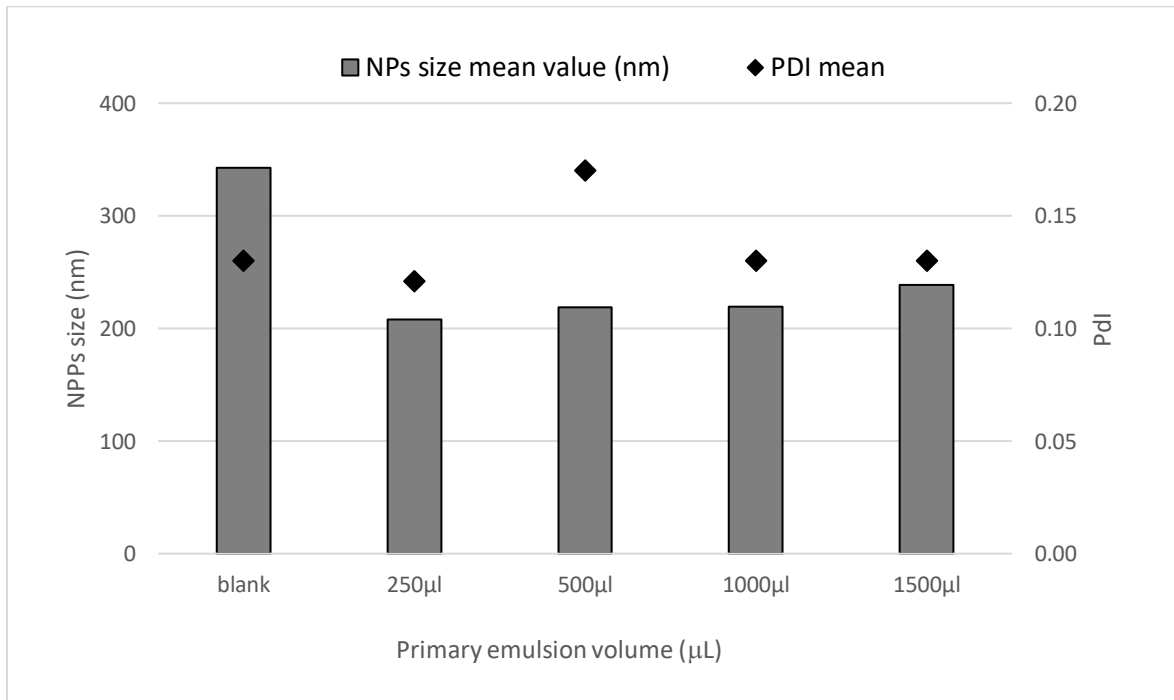


Fig. 27: Size and Polydispersity index of NP made of 5% PLGA 7:3 (DMSO & Pluronic 1%)



Decreasing concentration to 3% PLGA was designed to produce smaller size NPs [Tab. 6]. But by brief comparison, it appears that size of NPs did not decrease. Even though, PDI level have dropped, and it seemingly provided the evidence that the agglomerations are less in lower concentration of polymers. By having NPs mean size within range of 234 to 225 nm, and having excellent mean PDI values, acceptable and usable NPs was produced [Fig. 28].

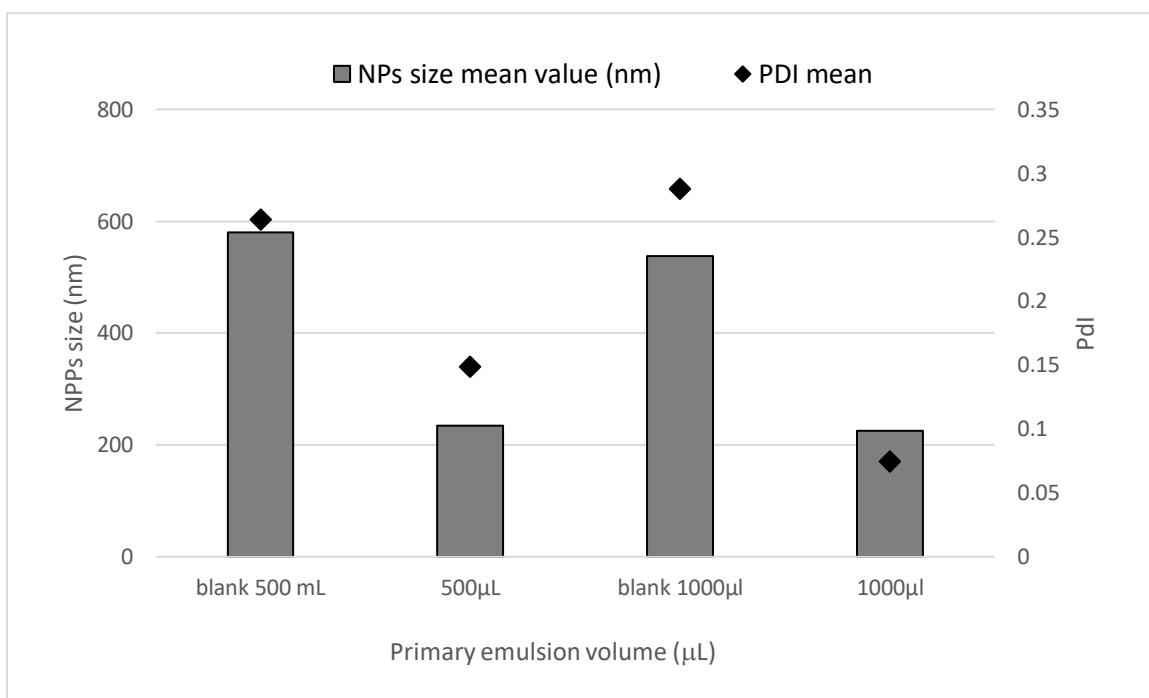


Fig. 28: Size and Polydispersity index of NP made of 3% PLGA 7:3 (DMSO & Pluronic 1%)

Shifting from 5% PLGA 7:3 to 1% PLGA 7:3 was designed to search for the better particle sizes with acceptable PDI [Tab. 1]. By comparison in the different volumes of blank primary emulsions, there were seen an undeniable evidence in which by increasing in the volume of PE, particle sizes will increase as well. PDI levels in blanks are far better than loaded polymers, and yet in both cases PDI levels were resulted excellent and it could provide evidence that tendency to agglomerate in blank is far less than loaded NPs [Fig. 29]. In comparison of 1% PLGA and 5% PLGA, it appeared that the size of NPs, have dropped to be around 100 nm [Fig. 27 & 29].

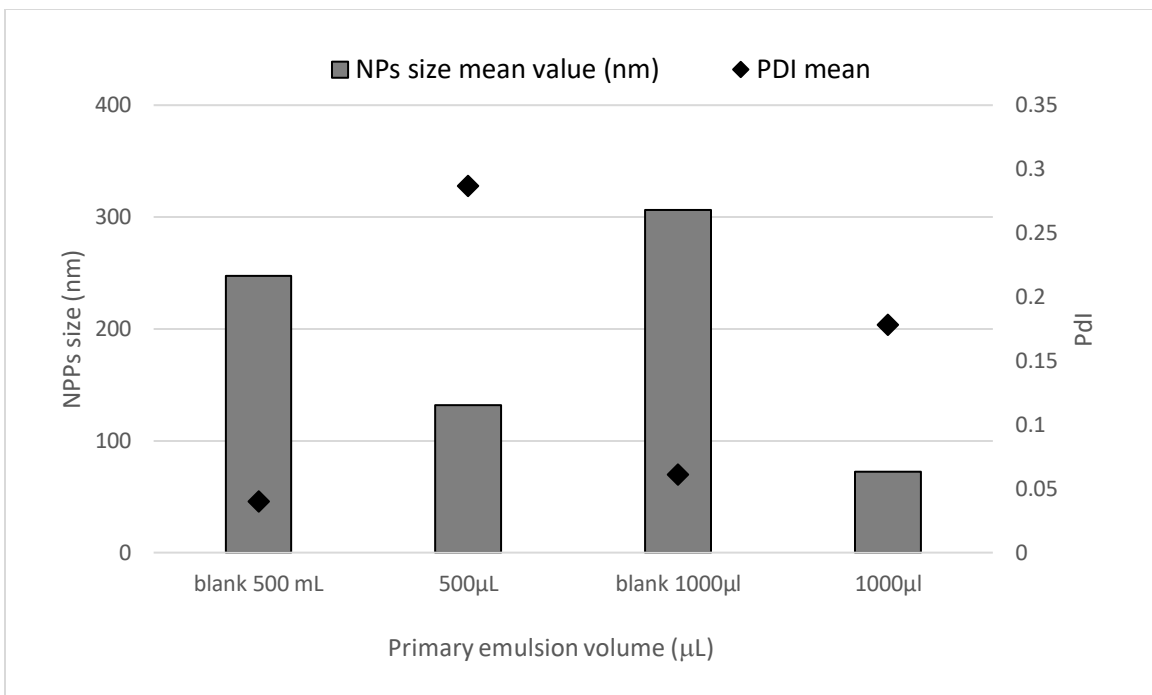


Fig. 29: Size and Polydispersity index of NP made of 1% PLGA 7:3 (DMSO & Pluronic 1%)

### 3.2 Nanoparticles based on branched PLGA

Changing type of polymer was another research criterion, in which A2 was used for production of nanoparticles.

The examples of scans from the measurement of the NPs size are presented in [Fig. 30 – 32].

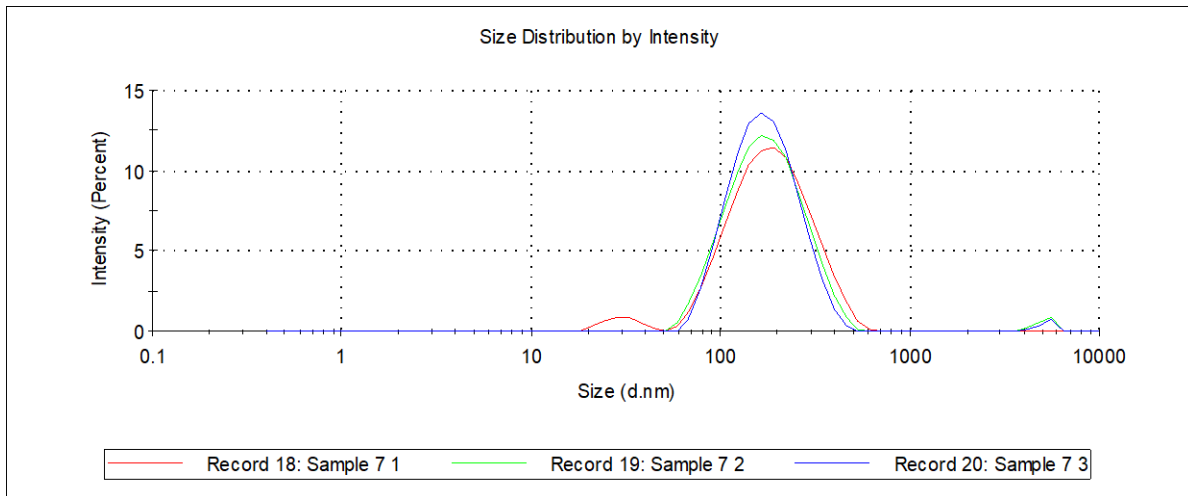


Fig. 30: Particle size distribution to the intensity of scattered beam 1% A2 (250 µl PE)

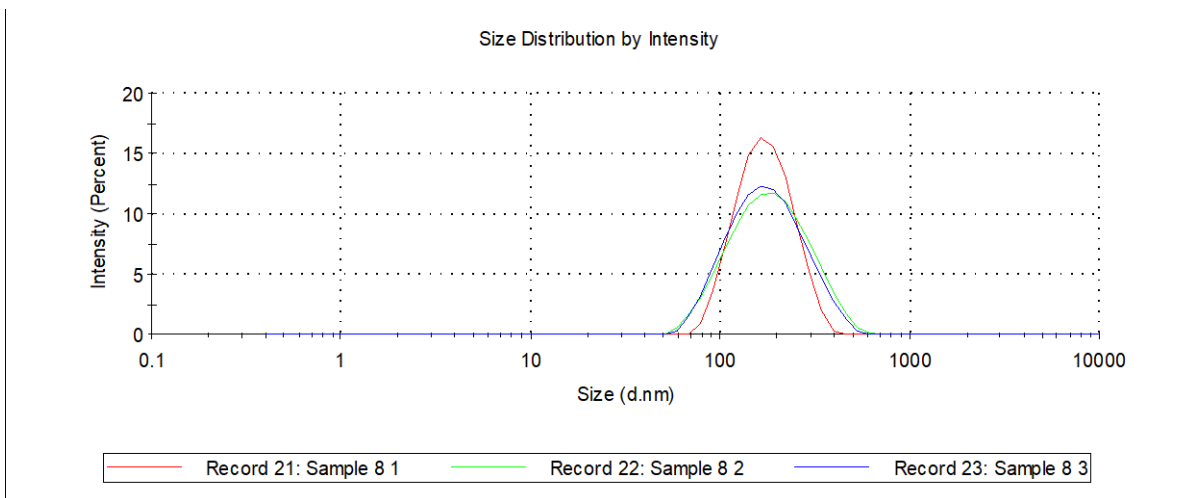


Fig. 31: Particle size distribution to the intensity of scattered beam 1% A2 (500 µl PE)

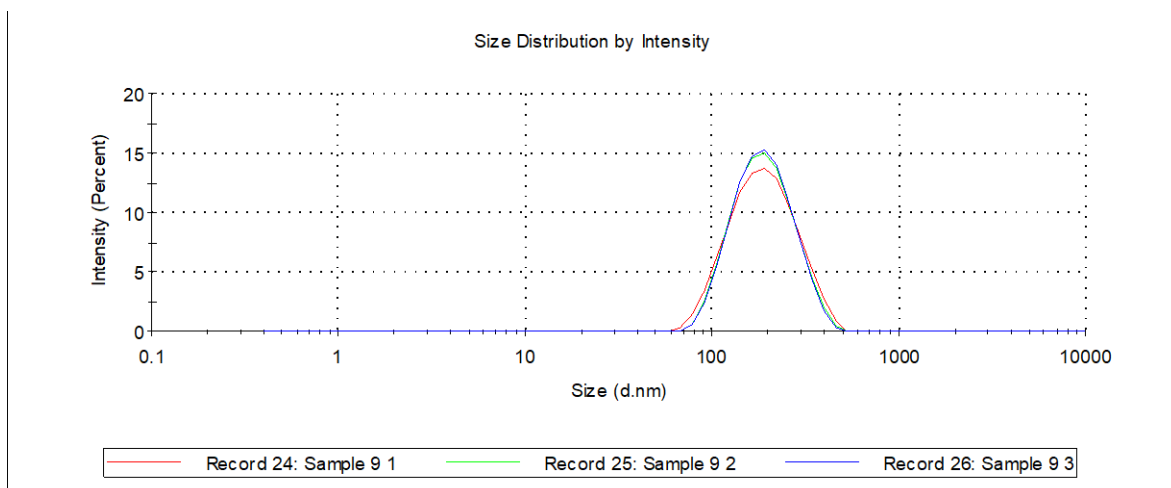


Fig. 32: Particle size distribution to the intensity of scattered beam 1% A2 (1000  $\mu$ l PE)

The values of size and polydispersity index of NPs resulted by 1% & 3% A2 dissolved in DMSO and stabilized by Poloxamer 407 are presented in [Tables 8 & 9].

Tab. 8: Size and Polydispersity index of NP made of 1% A2 (DMSO & Pluronic 1%)

Size of nanoparticles (nm)					
Primary emulsion	blank 500 $\mu$ l	blank 1000 $\mu$ l	250 $\mu$ l	500 $\mu$ l	1000 $\mu$ l
1. measurement	286.2	272.0	151.7	153.9	166.5
2. measurement	283.3	272.6	153.5	153.9	169.1
3. measurement	285.5	271.4	153.1	154.0	169.7
<b>NPs size mean value (nm)</b>	<b>285.0</b>	<b>272.0</b>	<b>152.8</b>	<b>153.9</b>	<b>168.4</b>
<b>SD</b>	<b>1.5</b>	<b>0.6</b>	<b>1.0</b>	<b>0.1</b>	<b>1.7</b>
Polydispersity index (-)					
Primary emulsion	blank 500 $\mu$ l	blank 1000 $\mu$ l	250 $\mu$ l	500 $\mu$ l	1000 $\mu$ l
1. measurement	0.22	0.08	0.27	0.21	0.17
2. measurement	0.21	0.08	0.26	0.22	0.15
3. measurement	0.19	0.11	0.25	0.21	0.14
<b>NPs PDI value (-)</b>	<b>0.21</b>	<b>0.09</b>	<b>0.26</b>	<b>0.21</b>	<b>0.15</b>
<b>SD</b>	<b>0.01</b>	<b>0.02</b>	<b>0.01</b>	<b>0.01</b>	<b>0.01</b>

Tab. 9: Size and Polydispersity index of NP made of 3% A2 (DMSO & Pluronic 1%)

Size of nanoparticles (nm)				
Primary emulsion volume (μL)	blank 500μL	500μL	1000μL	blank 1000μL
1. measurement	394.8	197.8	243.1	439.9
2. measurement	400.7	203.5	243.9	423.1
3. measurement	393.0	198.9	243.4	418.4
<b>NPs size mean value (nm)</b>	<b>396.2</b>	<b>200.1</b>	<b>243.5</b>	<b>427.1</b>
<b>SD</b>	<b>4.0</b>	<b>3.0</b>	<b>0.4</b>	<b>11.3</b>
Polydispersity index (-)				
Primary emulsion volume (μL)	blank 500μl	500μl	1000μl	blank 1000μl
1. measurement	0.28	0.16	0.09	0.30
2. measurement	0.33	0.13	0.08	0.37
3. measurement	0.32	0.14	0.10	0.36
<b>NPs PDI value (-)</b>	<b>0.31</b>	<b>0.15</b>	<b>0.09</b>	<b>0.34</b>
<b>SD</b>	<b>0.03</b>	<b>0.02</b>	<b>0.01</b>	<b>0.04</b>

Starting with 3% A2, it appeared the size of NPs in both blank and loaded samples were slightly decreased in comparison with 3% PLGA while PDI were excellent and similar in both concentrations [Fig. 33]. Mean value of NPs size were around the 200 to 243 and PDI were 0.02 to 0.01 [Tab. 9].

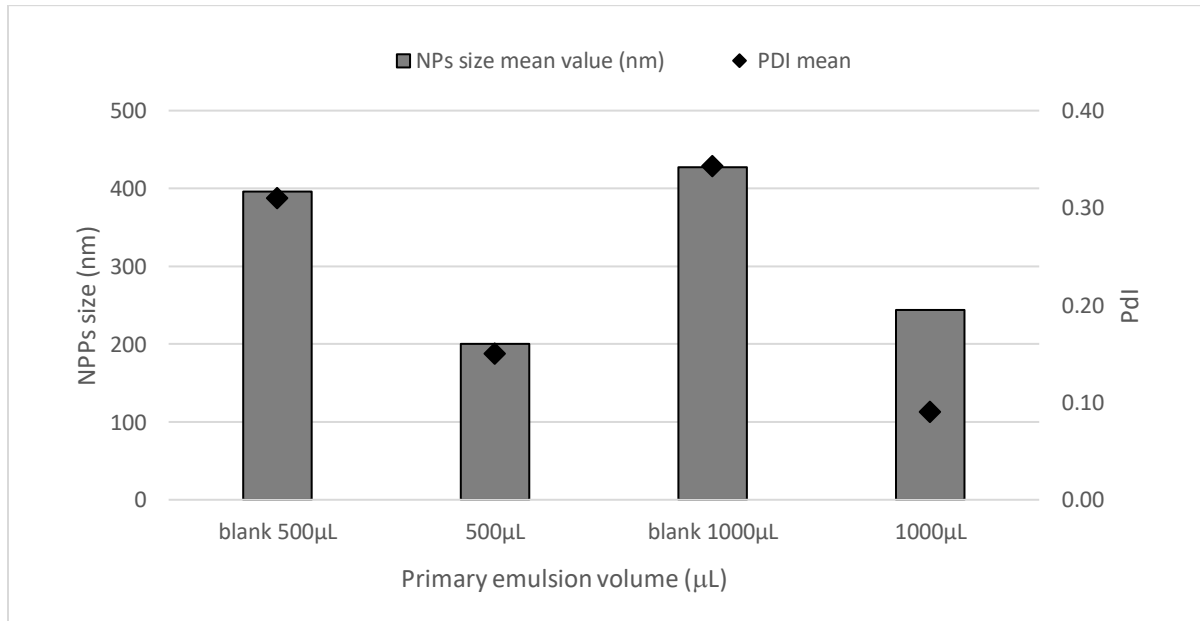


Fig. 33: Size and Polydispersity index of NP made of 3% A2 (DMSO & Pluronic 1%)

Changing from 3% A2 to 1% A2 designed to produce smaller size of NPs. By achieving in mean value for NPs size from 152 to 168 nm, and excellent PDI, it proved that by having lower concentration of polymer A2, it could be possible to gain excellent products of NPs [Tab. 8]. By increasing in amount of PE, size of NPs was slightly increasing while PDI remained the same. Question remains for the size of NPs in blank samples which is being higher in comparison with loaded polymers [Fig. 34].

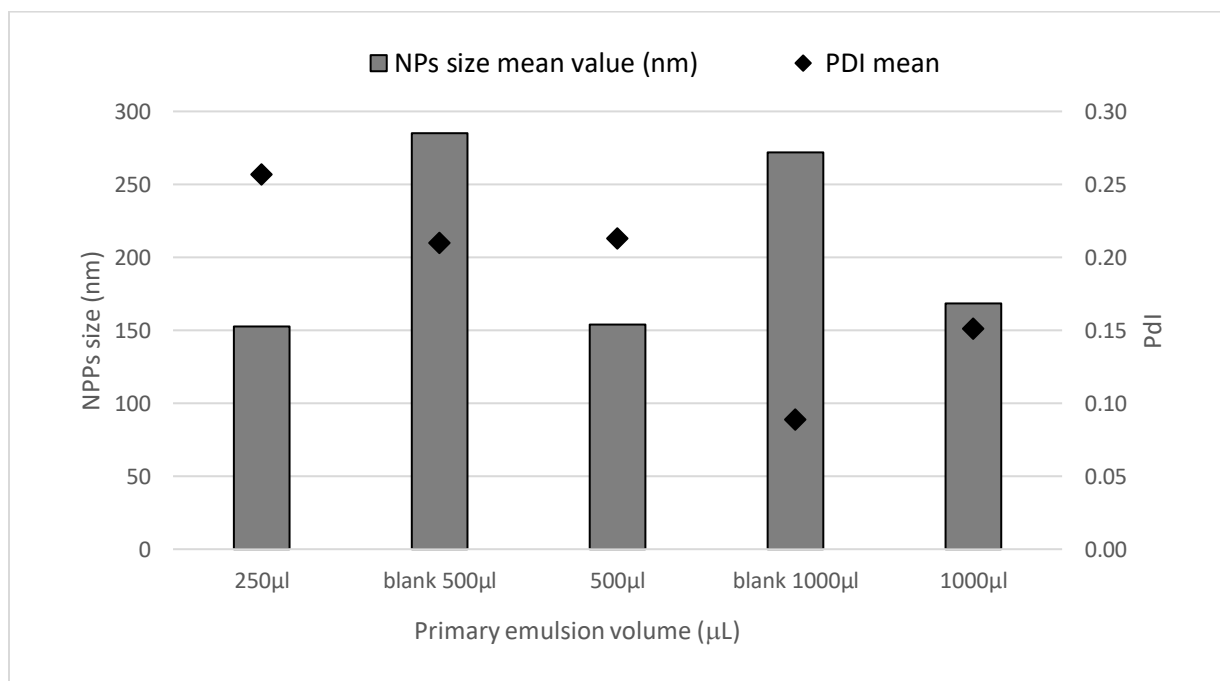


Fig. 34: Size and Polydispersity index of NP made of 1% A2 (DMSO & Pluronic 1%)

### 3.2.1 Nanoparticles based on branched PLGA stabilized with DDAB

In another criteria, was changing polymer solvent and outer phase in which DMSO and 1% Poloxamer 407 was replaced with DCM and 0.5% DDAB [Tab. 10 & 11].

Tab. 10: Characteristics of NP made of 1% A2 (DCM & 0.5% DDAB)

Primary emulsion	NPs size (nm)		Pdl (-)		ZS (mV)	
	500 $\mu$ L	1000 $\mu$ l	500 $\mu$ L	1000 $\mu$ l	500 $\mu$ L	1000 $\mu$ l
1. measurement	366.8	377.8	0.568	0.465	72.2	76.3
2. measurement	365.9	345.6	0.475	0.581	75	81.2
3. measurement	358.2	350.9	0.539	0.581	76.1	82
<b>Mean value</b>	<b>363.6</b>	<b>358.1</b>	<b>0.527</b>	<b>0.542</b>	<b>74.4</b>	<b>79.8</b>
<b>SD</b>	<b>4.7</b>	<b>17.3</b>	<b>0.048</b>	<b>0.067</b>	<b>2.0</b>	<b>3.1</b>

Tab. 11: Characteristics of NP made of 3% A2 (DCM & 0.5% DDAB)

Primary emulsion	NPs size (nm)		Pdl (-)		ZS (mV)	
	500 $\mu$ L	1000 $\mu$ l	500 $\mu$ L	1000 $\mu$ l	500 $\mu$ L	1000 $\mu$ l
1. measurement	260.7	351.8	0.563	0.579	71	67.2
2. measurement	258.5	348.1	0.548	0.551	71.6	70.7
3. measurement	256.3	364.6	0.565	0.514	68.4	73
<b>Mean value</b>	<b>258.5</b>	<b>354.8</b>	<b>0.559</b>	<b>0.548</b>	<b>70.3</b>	<b>70.3</b>
<b>SD</b>	<b>2.2</b>	<b>8.7</b>	<b>0.009</b>	<b>0.033</b>	<b>1.7</b>	<b>2.9</b>

Scans from zeta potential measurements of the NPs were presented in [Fig. 35 – 38].

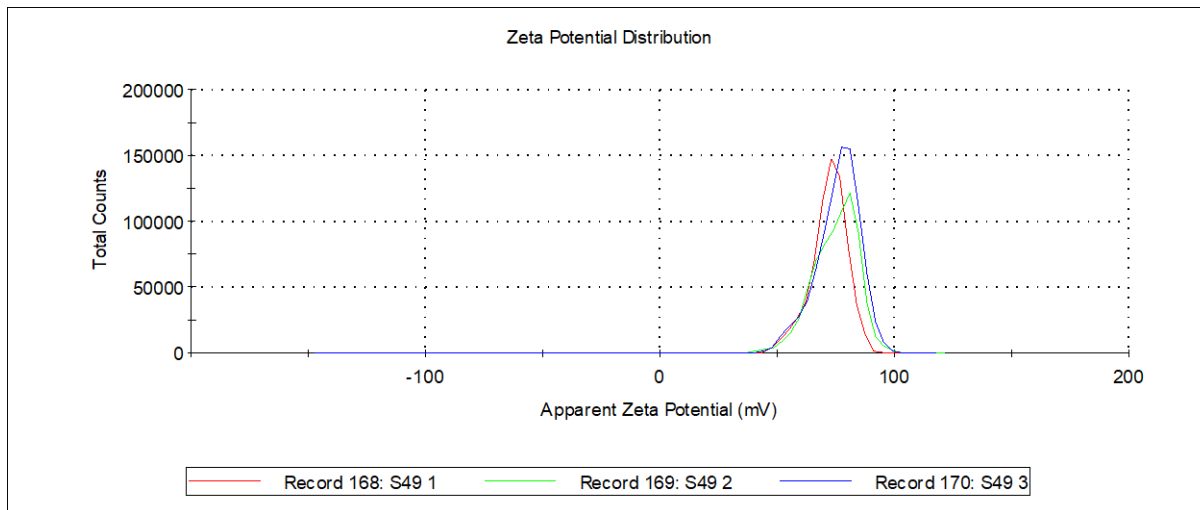


Fig. 35: Zeta potential of 1% A2 (DCM & 0.5% DDAB) 500 µl PE

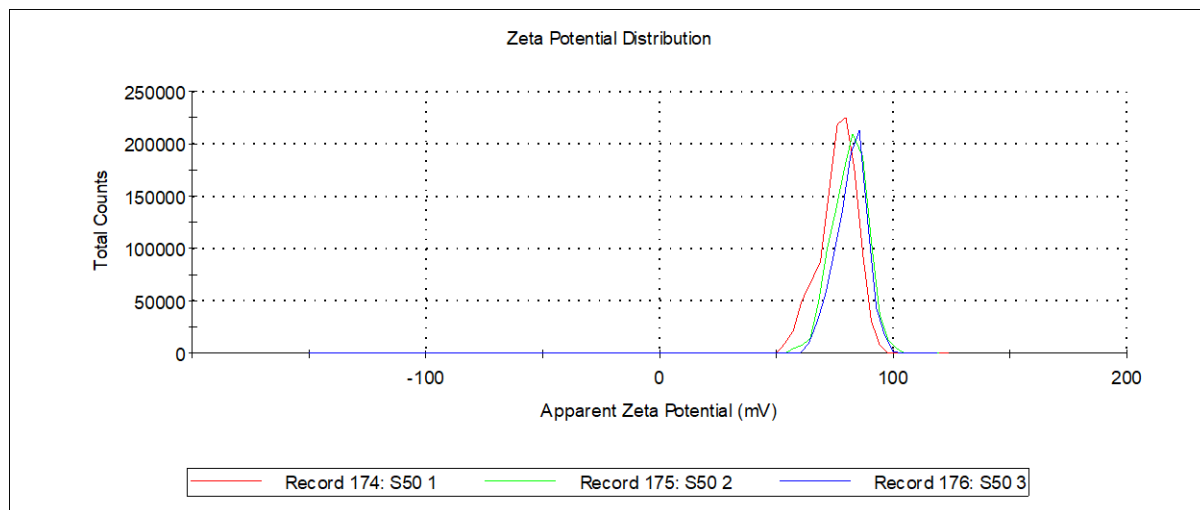


Fig. 36: Zeta potential of 1% A2 (DCM & 0.5% DDAB) 1000 µl PE



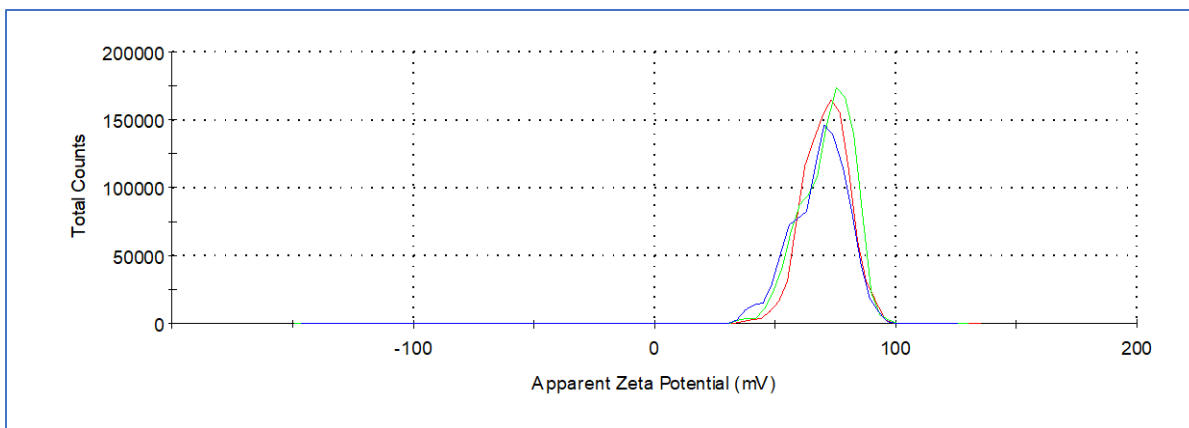


Fig. 37: Zeta potential distribution (mV) of 3% A2 (DCM & 0.5% DDAB) 500 µl PE

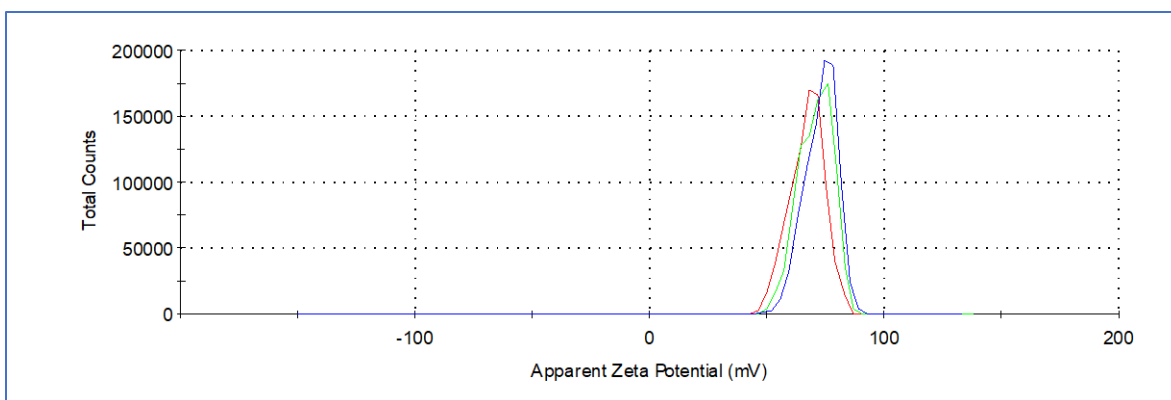


Fig. 38: Zeta potential distribution (mV) of 3% A2 (DCM & 0.5% DDAB) 1000 µl PE

By knowing that poloxamer 407 is non-ionic block of copolymer of ethylene oxide and propylene oxide which is sterically stabilized by forming polymer on the surface of them and having it in mind that DDAB is cationic surfactant which could stabilize NP electrosterically, and it causes an effect on zeta potential between -10- -30 and +10- +30 mV, any range above or below will consider strongly cationic or strongly anionic. And if an absolute value of ZP is above 60 mV, NPs were stabilized excellently. Thus in my samples by having zeta potential values above +70.3 mV, they were accounted as excellent results [Fig. 35 -38].

Size of NPs only in 3% A2 of 500µl PE was in range of 258 nm and in others PE value, NPs size was around 360 nm. PDI value were above 0.5 which is not acceptable [Tab. 10 & 11].

## 4 CONCLUSION

In this diploma thesis, double emulsion method was chosen to produce the nanoparticles from oximes. Its main pathophysiological reason was to be able to cross-pass the blood brain barrier.

By doing the tests in various samples and by decreasing in the concentration of PLGA 7:3, size of NPs also declined. By shifting from PLGA to A2 was designed to achieve lesser in size of NPs and in case of 1% A2 have shown that oxime loaded NPs are having size from 152 to 168 nm with very good PDI of below 0.15, and it could provide information that in lower concentration of polymer A2, it could be possible to gain excellent products of NPs.

In most samples by increasing in volume of PE, size of NPs also was increased, while having no effect on PDI. In most samples, blank samples were having better PDI than loaded samples.

By using DDAB as outer phase, electrostatic stability of A2 was tested and it has shown an excellent result of above 70.3.

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