Preparation and characterization of Polymersomes for application in cancer therapy

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Master's thesis / Diplomski rad

2016

Degree Grantor / Ustanova koja je dodijelila akademski / stručni stupanj: **University of Zagreb, Faculty of Pharmacy and Biochemistry / Sveučilište u Zagrebu, Farmaceutsko-biokemijski fakultet**

Permanent link / Trajna poveznica: https://urn.nsk.hr/urn:nbn:hr:163:721930

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Nikolina Dakić

Preparation and Characterization of Polymersomes for Application in Cancer Therapy

DIPLOMA THESIS

Submitted to the University of Zagreb, Faculty of Pharmacy and Biochemistry

Zagreb, 2016.

This diploma thesis has been reported at the course Innovative drug delivery systems, at the University of Zagreb, Faculty of Pharmacy and Biochemistry under the supervision of Associate Professor Jasmina Lovrić, Ph.D. The experimental work was carried out in the Division of Pharmaceutical Technology, Faculty of Pharmacy, University of Helsinki under the expert guidance of Assistant Professor Hélder A. Santos, Ph.D., and Vimalkumar Balasubramanian, Ph.D.

Hvala izvanrednoj profesorici Jasmini Lovrić na velikoj pomoći i stručnom vodstvu u pisanju ovog diplomskog rada i što je s velikim entuzijazmom prihvatila ovaj projekt!

Hvala mojoj obitelji, mami, tati i sestri što su samnom prolazili kroz dobre i loše trenutke tijekom studija jer bez njih nebih bila ovdje gdje sam danas!

Hvala mojim prijateljima i kolegama što su mi uljepšali studentske dane i što su uvijek bili tu uz mene!

Special gratitude goes to my supervisor in Helsinki, Docent Hélder A. Santos! Thank you for accepting me in your group and providing me the opportunity to work on this interesting project! Thanks goes to whole Nami unit for all the assistance and fun time spent in your lab and beyond! I would also like to thank my Erasmus friends who supported me in Helsinki and made my time there one of the best periods of my life.

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1. Introduction

Cancer is, by definition, an abnormal growth of cells which tend to proliferate in an uncontrolled way and, in some cases, to metasize. It includes a group of more than 100 distinctive diseases (http://www.medicinenet.com). One of the most common types of cancer among women is breast cancer in particular. Cytotoxic drugs are a diverse class of compounds that treat cancer primarily by being toxic to cells that are rapidly growing and dividing. Despite being used for a long period of time, and the development of numerous new multiple drug regimens for improved clinical success, treatment failure is still frequently encountered (Ho et al. 2007). Currect anticancer drugs have many drawbacks, they often extensively and indiscriminately bind to body tissues and serum and therefore can cause severe side effects, which prevents effective therapy of the disesase (Ratain & Mick n.d.; Ho et al. 2007). However, nanotechnology provides new solutions for the improvement of the effectiveness and safety profile by introducing new drug delivery nanosystems such as polymersomes (Ps).

Ps are nanosystems made out of amphiphilic diblock copolymers which spontaneously self-assemble in water to create vesicular structures with aqueous core, surrounded by the bilayer membrane (Prakash Jain et al. 2011; LoPresti et al. 2009; Lee et al. 2001). Poly(dimethylsiloxane)-block-poly(2-methyloxazoline) (PDMS-b-PMOXA) is a promising material, diblock copolymer, for the formation of these nanosystems. Furthermore, its properties such as mechanical stability and the ability to fine-tune the shape, dimension, solubility and membrane thickness of the vesicles formed, makes this material an interesting one for the research and potential application in anticancer therapy (Jaskiewicz, Makowski, et al. 2012).

In this study, we prepared two different types of Ps, different in the length of the hydrophilic part, using the abovementioned material- PDMS-b-PMOXA. The work was focused on the improvement of the preparation and characterization of the nanosystems and comparison of those two. Furthermore, we tested the stability of potential drug delivery nanosystems in different buffers and throughout some time period and we carried out some *in vitro* experiments to determine the effect of Ps prepared on the breast cancer cell line.

The following literature review describes, more closely, properties of different nanosystems among which are Ps, and properties of the used diblock copolymers as well. Moreover, we

introduce different preparation and characterization methods for the development of these nanosystems . Subsequently, materials and methods section is presented, and in the end we presented and discussed the obtained results and offered the conclusion based on this study.

2.1. Nanosystems

2.1.1. Application of nanoparticles in cancer therapy

Nanotechnology provides a wide range of nanoscale tools for application in medicine, especially in the fields of targeted cancer therapy, where there is currently a limited range of delivery systems and possibilities in treatment (Conti et al., 2006.).

When it comes to targeted cancer therapy, the emphasis is on nanoparticles as innovative drug delivery systems. Nanoparticles are considered colloidal-sized particles with diameters ranging from 1 to 1000 nm. These particles are of a great interest when it comes to encapsulation of drugs and their distribution within the body. Introduction of nanoparticles into therapy has a purpose to enhance the therapeutic efficacy of the drugs, making them more directed to the specific tissue of interest or to lower the daily dose of the drug needed, hence reducing the drug's side effects. There are many constrains and problems when it comes to conventional drugs in cancer therapy. One of the biggest problems is definitely the unspecific drug distribution through the whole organism, which at the end leads to possible severe side effects, fast drug elimination and fast metabolism (Upadhyay et al. 2009; Satchi-Fainaro & Duncan 2006; Tischlerová & Valenčáková 2015).

An ideal drug delivery system has to possess some certain characteristics. Firstly, the ideal nanoparticle should be able to avoid opsonisation by the opsonin proteins in the bloodstream and further phagocytosis of the drug by the reticuloendothelial system (RES). This specific system consists of monocytes and macrophage cells, which are responsible for the phagocytosis of foreign particles, bacterias or proteins. The process of phagocytosis happens when these cells recognize opsonins (opsonin proteins) on the surface of the nanoparticles (Yan et al. 2005).

Nanoparticles can be divided into three generations depending on their ability to protect themselves from opsonization. The first generation is unprotected and tends to be removed from the blood circulation within short period of time after they enter the systematic circulation. Therefore, this kind of nanoparticles possess very low efficiency due to their fast

removal. Nanoparticles referred to as a second generation nanoparticles, have their surface coated with hydrophilic polymers, mostly poly(ethylene glycol) (PEG). This prevents the macrophage from the RES to recognize foreign nanoparticles. If these nanoparticles have the ability to release their content selectively to the cells with specific receptors on their surface, then they are referred to as the third-generation nanoparticles: active targeting (also known as ligand-based targeting) (Allen & Cullis 2004). This term implies that the nanoparticles have some biological entities (*e.g.*, antibody or carrier protein) attached to their surface, which enables target cells to recognize the particle using the receptors (Upadhyay et al. 2009). Secondly, an ideal nanoparticle has to have the ability to protect the encapsulated, dispersed or adsorbed drug from its degradation. A desirable characteristic is most certainly the biocompatibility of the nanocarrier in the human body, thus avoiding undesirable body reactions to a foreign molecule (Hafner, Marković, Ferić, &Filipović-Grčić, 2013).

Some of the most desired characteristics of the drug delivery nanosystems are listed in **Table 1**.

Table 1: *Rational design of an "ideal" nanocarrier for cancer therapy taking into account all the biological barriers and requirements (Upadhyayet al., 2009).*

Biological requirements	Consequences in nanocarrier design
Protect drug from degradation	Encapsulation into a carrier
Intravenous injection	Size < 200 nm
Prevent opsonization (increase circulating half-time)	Coating with hydrophilic polymer (PEG, dextran, poly(L-glutamic) acid, etc.)
Control of biodistribution	Introduction of targeting moieties (antibodies, peptides, carbohydrates)
Control of pharmacokinetics and pharmacodynamics	All previous parameters
Elimination	Use of biocompatible and biodegradable materials

2.2.General characteristics of nanoparticles

2.2.1. Liposomes

Liposomes are vesicular systems, usually between 20 and 1000 nm in diameter, consisting of phospholipids. The most commonly used phospholipid for liposome preparation is lecithin (also known as phosphatidylcholine). This, or other phospholipids, form a bilayer with hydrophilic glycerol-phosphate-alcohol heads facing the surface of the liposome and its core, whereas the hydrophobic chains are orientated towards each other (J.S. Dua, Prof. A. C. Rana 2012).

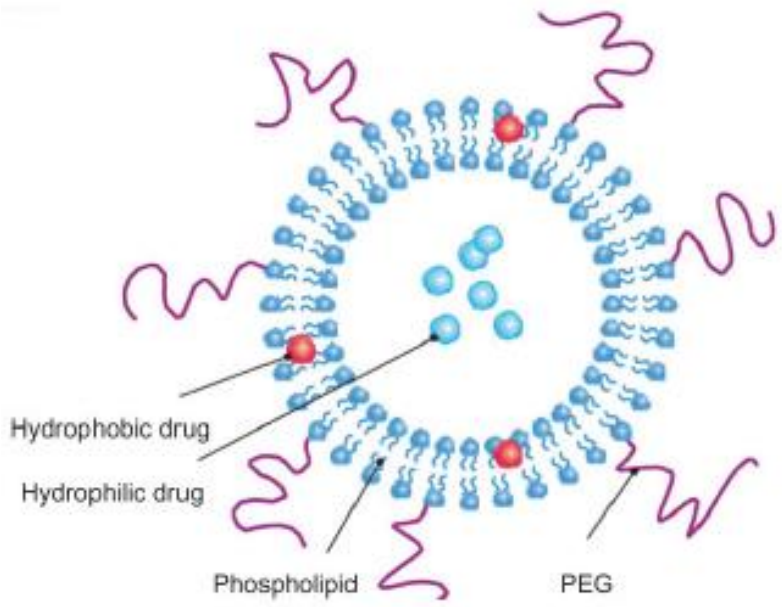


Figure 1. *Schematic of liposomal structure with clearly visible phospholipid bilayer and poly(ethylene glycol) (PEG) molecules on the surface of the vesicle. This image also shows in which particular part of the liposome drugs can be incorporated depending on their different physicochemical properties (Hafner, Lovrić, Lakoš, &Pepić, 2014).*

The preparation process of liposomes consists of dissolving the phospholipids in an organic solvent (usually chloroform or a mixture of chloroform and methanol), which is later evaporated. After the clear lipid film is formed, it is hydrated by the water and also an extrusion process can be applied to customize the size of the liposome. Since their discovery, these vesicles have found their role as drug delivery systems for various drugs and

cosmetically active agents. The great potential of liposomes as drug delivery systems is due to their biocompatibility, their non-toxic, and biodegradable and non-immunogenic characteristics. Furthermore, as shown in **Fig. 1**, liposomes can be used as carriers for various types of drugs, hydrophilic and hydrophobic or even amphipathic, and can also form vesicles with a wide range of sizes and, hence, incorporating both smaller and bigger molecules (Immordino, Dosio, &Cattel, 2006; J.S. Dua, Prof. A. C. Rana, 2012). The liposomes are flexible in their size and they can also be applied in many different forms, such as suspensions, aerosols, gels, creams, lotions, and powders (Berg 2012). There is also an example of poly(lactic-co-glycolic acid) (PLGA) based nanoparticles in use for anticancer treatment (Danhier et al. 2012). This kind of system has been tested as a cancer vaccine by encapsulating tumor associated antigens (TAAs), either independently (Hamdy et al. 2008) or along with different adjuvants, such as Toll-like receptor ligands (TLR) (Hanlon et al. 2011). Furthermore, many small anticancer drugs, such as doxorubicin, were encapsulated into PLGA nanoparticles and PEGylated particles, and have been proved to be even more potent when it comes to targeted drug delivery. Moreover, compared to liposomal formulation of doxorubicin (Doxil), this formulation showed high decrease in cardiomyopathies (Park et al. 2009).

Despite of the numerous advantages abovementioned, there are many constrains and disadvantages of this type of drug delivery systems. The biggest problems are, by far, the poor stability (due to oxidation and hydrolysis of the phospholipids) and prompt uptake by the RES, particularly the Kupffer cells in the liver. When the liposomal phospholipids are exposed to oxygen they undergo oxidation of the fatty acyl groups, which can finally lead to the production of toxic compounds. This unwanted process of oxidation can be minimalized by introducing an inert atmosphere (nitrogen or argon) (Immordino et al. 2006). The physical instability is due to thin lipid membrane (3–5 nm) formed from phospholipids with low molecular weight, which leads to higher leak agerates than anticipated, and also due to the problems with drug entrapment efficacy (Riaz 1995).

2.2.2. Copolymer micelles

Micelles are described as colloidal-sized nanoparticles with a size range from 10 to 100 nm. Copolymer micelles are spontaneously formed by amphiphilic block copolymers when they

are diluted in the water with a concentration above the certain value referred to as a critical micelle concentration (CMC). The process itself happens because of the nature of the block copolymers, more precisely, their hydrophobic part. When the concentration of the amphiphile copolymer is below the CMC value, the polymers exist in the solution as dispersed unimers adsorbed on the surface of the solution/water with their hydrophobic parts oriented towards air, hence lowering the free energy of the system. As the concentration of the polymer rises, there is also the increase in the free energy, which is undesirable, and therefore the molecules spontaneously aggregate to achieve the state of minimum free energy (Lavasanifar, Samuel, & Kwon, 2002; Letchford& Burt, 2007; "Martin's Physical Pharmacy 6th.ed 2011 Dr.murtadhaAlshareifi," n.d.).

Copolymer micelles are usually prepared depending on the solubility of copolymers used for their preparation. If the copolymer is watersoluble, there are two possible methods that can be applied for the preparation. Firstly, a direct dissolution method can be used, which implies that the copolymer is simply dissolved in water or some other aqueous medium with a concentration higher than the CMC (Letchford& Burt, 2007). This method is usually used with highly soluble polymers, such as poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) triblock copolymers, and also can be prepared in higher temperatures to ensure micellization. Secondly, the film casting method can be applied for copolymers with good water solubility properties. It involves making a polymer film by dissolving the copolymer and the drug of interest in a solvent, which is later evaporated only to leave a thin polymer film on the bottom of the flask. The polymer film is afterwards hydrated by water or some other aqueous buffer solution. If a relatively water insoluble copolymer for the micelle formation is used, the choice of two methods to use can be either by dialysis or oil-in-water (o/w) emulsion procedure (Letchford& Burt, 2007; Sant, Smith, &Leroux, 2004). In the dialysis method, water-miscible organic solvent is used in order to dissolve both the copolymer and the drug, and subsequently adding water or adding the mixture (drug/copolymer/solvent) to the water. The process is then continued by dialysis against the aqueous media to remove the organic solvent used at the beginning (Liu et al. 2004; Kim et al. 1998). The o/w emulsion method consists of preparing a micelle aqueous solution in which the added drug is previously dissolved and adding it in the organic solvent which is non-water miscible. By using this procedure, an o/w emulsion is obtained from which it is later left to evaporate the organic solvent, leaving formed micelles (Sant, Smith, & Leroux, 2004).

This type of nanoparticle system has its advantages when it comes to drug delivery applications, especially compared to surfactant micelles. Because of the longer hydrophobic part of the molecule, block copolymers have bigger tendency to form micelles than low molecular surfactants. Because of the lower CMC values, they tend to be more thermodynamically stable, and their dissociation after dilution is slower, meaning that they are more kinetically stable. This allows the drug to stay within the nanosystem longer and, hence, to be accumulated in the targeted tissue (Kataoka et al. 2001). These kind of systems also have a higher solubility capacity as a result of the higher number of micelles formed and/or the bigger hydrophobic core. Moreover, as stated above, micelles range from 10 to 100 nm (usually several tens of nanometers), and this narrow size range contributes to the stability of the system after its parental application (Kataoka et al. 2001). To be able to accomplish targeted drug delivery, these nanoparticle systems possess certain important properties, such as flexible polymer brush, which contributes to the biocompatibility and steric stabilization, as well as many other properties, as shown in **Fig. 2**.

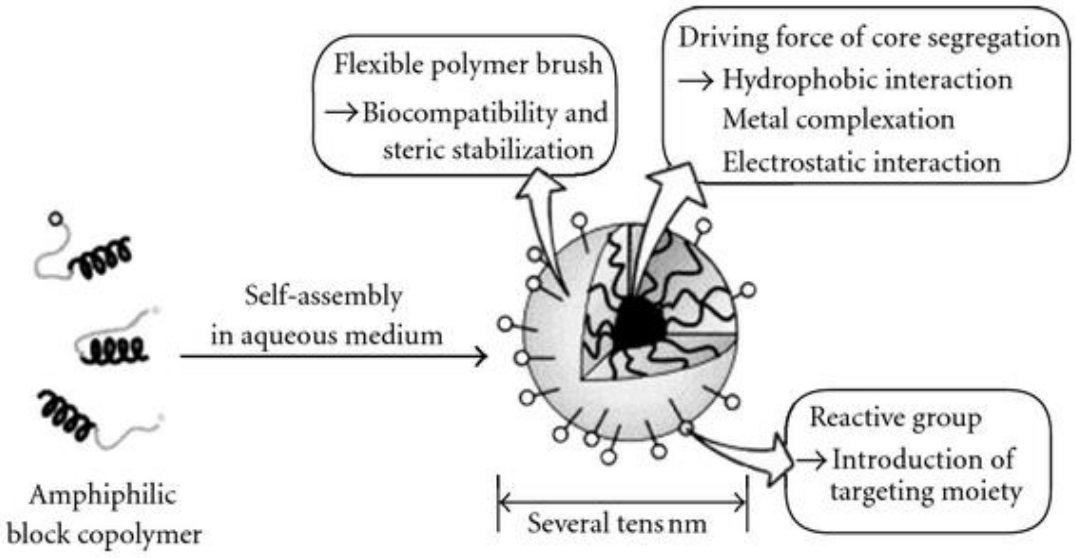


Figure 2. *Important properties of copolymer micelles which makes them desirable nanoparticles for targeted drug delivery applications (Kataoka et al. 2001).*

2.2.3. Nanospheres

Polymeric nanospheres are defined as 10–1000 nm sized solid spherical particles with homogeneous structure (Hafner et al. 2014). Drugs can be incorporated into nanospheres as

dissolved, entrapped, encapsulated or attached to the matrix of the polymer (Singh et al. 2010), as shown in the **Fig.3**.

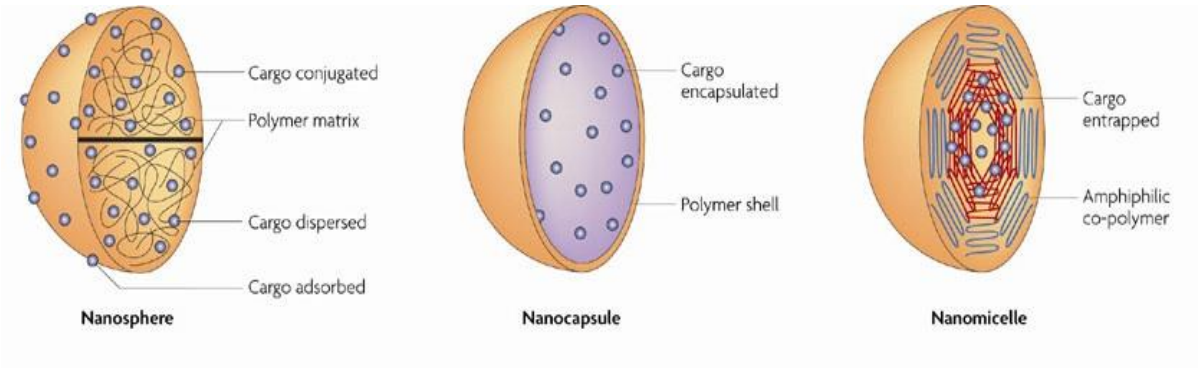


Figure 3. *Illustration shows the schematic transaction of the polymeric nanoparticles emphasising the difference between three types of nanosystems. They include: nanospheres, in which the cargo is dissolved, adsorbed or dispersed throughout the matrix, attached to the surface or attached to the polymer matrix; nanocapsules, in which the cargo is in solution and surrounded by a shell-like wall; and nanomicelles, in which amphiphilic copolymers with hydrophobic and hydrophilic blocks self-assemble to entrap the cargo (Griffiths, Nyström, Sable, &Khuller, 2010).*

These kind of nanoparticles can be divided in two larger groups: biodegradable (*e.g.*, albumin nanospheres, gelatine nanospheres, and modified starch nanospheres) and non-biodegradable particles (polylacticacid) (Singh et al. 2010). Even though nanospheres are larger in diameter size than the previously described micelles, their size is small enough to be considered as an advantage. These two type of nanoparticles are both formed from diblock copolymers, but it can be said that with the growth of the hydrophobic chain of the copolymer nanoparticles tends to be more solid-like, and therefore, resembles more nanospheres than micelles. Due to the small nanosphere diameter they can be injected directly into the systemic circulation, enabling them to pass through the vessels without the fear of blocking the bloodflow (Taylor et al. 2010).

Furthermore, their nanometer size range also contributes to their poor recognition by the RES. The principle of the RES uptake is based on the opsonin recognition and because of the small size and, hence, high curvature of the nanospheres, the uptake is minimized. This enables drugs to circulate longer in the bloodstream and be delivered to the tissue of interest.

Other than the direct injection into the blood vessels, these types of nanoparticles can also be applied through oral or nasal route (T.M. Allen, 1994; Illum& Davis, 1984).

Despite the advantages stated above, nanospheres have also many drawbacks. Due to their small size and large surface area, the chances of particle aggregation are high and also the drug loading is limited. Furthermore, problems with the physical handling of nanospheres may also occur (Singh et al. 2010)**.**

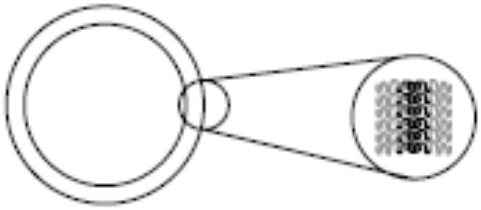
As for the preparation methods, there are two main methods for preparation of this kind of particles. The first method is polymerization. It can be achieved either by the emulsification or interfacial polymerization. In this type of preparation technique the monomers are to be polymerized to form nanospheres in an aqueous medium. The potential drug can be loaded either before the nanospheres are formed (by adding the drug into the polymerization medium), or when the particles are already formed.

Other preparation method, which is also the most popular and commonly used method, is the solvent displacement method (Galindo-rodriguez, Alle, Fessi, &Doelker, 2004). It consists of few steps. First, the polymer is dissolved in the organic solvent which is miscible with water and then the whole solution is added into the aqueous phase (with or without the surfactant). The addition to the aqueous phase enables the organic solvent to immediately diffuse and cause the precipitation of the polymer and formation of nanospheres. Other methods are the phase inversion temperature method and also the solvent evaporation technique (Chiannilkulchai, Ammoury, Caillou, Devissaguet, &Couvreur, 1990; Galindo-Rodriguez, Allémann, Fessi, &Doelker, 2004).

The types of nanoparticles obtained have a wide range of possible applications. They are used for tumor targeting or when there is a need for the long circulation of the drug (in this case they are coated with hydrophilic polymers to protect them from the uptake from RES). There is also the possibility of using this type of systems for the oral or brain delivery (Singh et al. 2010; Moghimi et al. 2001; Chen et al. 2004; Anon 2015)**.**

2.2.4. Polymersomes

Polymersomes (Ps) are small, colloidal-sized particles with amphiphilic block copolymers forming a bilayer membrane around the aqueous core (**Figs. 4** and **5**).



polymersome

Characteristics

Size : 5nm - 5µm

Copolymer unimers assembled in a bilayer around an aqueous reservoir

Figure 4. *The main characteristics of polymersomes including their average size and composition (Letchford& Burt, 2007).*

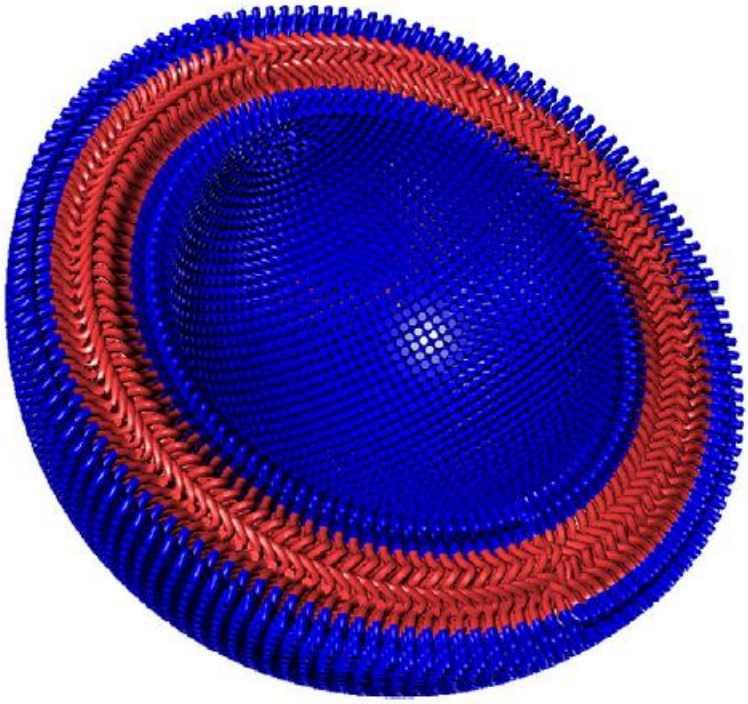


Figure 5. *3D-image of a cross section of polymersome with an aqueous core (blue) and lipid bilayer membrane (red) made from amphiphilic block copolymers. The membrane consists of hydrated hydrophilic coronas both on the inside and the outside of the hydrophobic middle part (Kowalczuk et al. 2014; Lee & Feijen 2012).*

This type of nanoparticles can be described as similar to the liposomes and find their utility in the encapsulation of water-soluble drugs within the aqueous core of the vesicle or hydrophobic drugs in the hydrophobic middle part of the bilayer. However, they possess better properties than liposomes themselves, such as longer circulation times due to the

smaller uptake by the RES. Ps also have more stable membrane (thick and robust) due to better mechanical properties. The membrane thickness is an important issue that determines the stability and utility of Ps. The stability depends on few factors among which are polymer size and ratio of the hydrophilic and hydrophobic parts. Ps show stability within the range of the temperature of interest (biologically relevant temperatures) (Prakash Jain, YenetAyen, & Kumar, 2011; Upadhyay et al., 2009). Compared to previously mentioned liposomes, Ps have lower lateral diffusivity and their membranes show higher viscosity compared to liposomes. The viscosity increases by the increase of the molecular weight of the hydrophobic blocks of the polymers building the membrane. There is also the question of permeability of the bilayer Ps membrane which is known to be very small even though the thickness of the membrane is only few nanometers. This fact can be taken as an advantage when it comes to the protection of the encapsulated drugs from the agents in its surrounding, which may trigger its degradation.

In addition, low permeability may be a serious drawback in the application of these systems as nanoparticles. **Fig. 6** shows the dependence of the permeability of the membrane on the molecular weight of the hydrophobic and hydrophilic block. As it can be seen, the permeability decreases with the enlargement of these structures. However, this is not the only parameter determining the permeability of the Ps membrane. There is also a matter of membrane thickness, chemical composition and aqueous solubility of the block copolymer, diffusion coefficient of the entrapped drug inside the core through the membrane. An important role in the permeability is the nature of the drug, its behaviour towards water (hydrophilicity or hydrophobicity), and also the charge on the surface of the molecule (Kowalczuk et al. 2014).

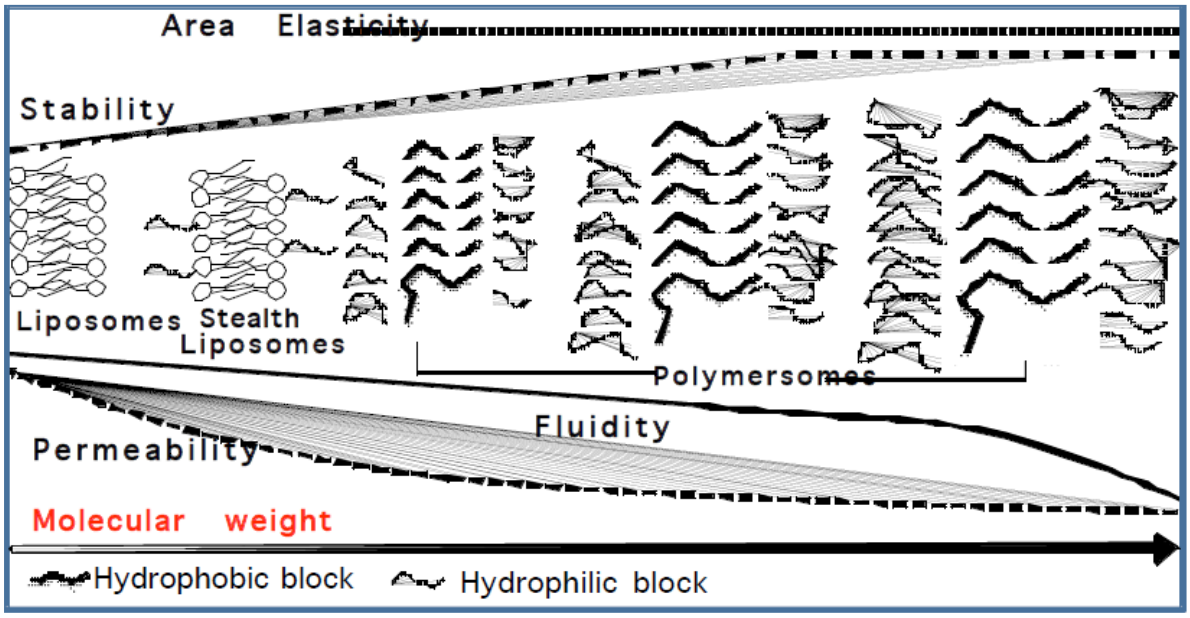


Figure 6. *Change in physicochemical properties of nanoparticles depending on the change of the molecular weight of the polymersomes (Prakash Jain et al. 2011).*

The change in the release profile of the drug encapsulated can also be made by applying different types of block copolymers for the formation of these hollow vesicles. Ps can be prepared by using several different techniques, where each of them bases on the self-assembling of block copolymers. The most commonly used methods for the preparation of Ps can be divided in two groups: solvent-switching methods and polymer rehydration techniques (Jung &Theato, 2012a; J. C. Lee et al., 2001).

2.2.4.1. Application of polymersomes in cancer therapy

Chemotherapy plays a major role in the treatment of cancer despite its many drawbacks. Conventional anticancer drugs are, in general, not specific for the targeted cells, but they also affect healthy cells, and therefore, exert toxic effects (Prakash Jain et al. 2011). Another problem, which often is critical is the limited distribution of the drugs to the targeted tissue (limited drug distribution) (Wartenberg et al. 1998).

Most of the toxicity and inefficiency problems, regarding cancer therapy, are related to the pharmaceutical formulation of a drug. Therefore, an ideal drug delivery system must show high drug loading capacity and encapsulation efficiency stability in the blood stream, long circulation time, selective accumulation in the targeted site, biocompatibility, and appropriate drug release profile (Prakash Jain et al. 2011).

Ps possess many adequate properties which can solve some of the problems of conventional drug molecules and chemotherapy. These delivery systems are stable for a long time and also have a good encapsulation efficiency. For these reasons, Ps find many applications in anticancer therapy as drug delivery systems. Photos *et al.* showed (by injecting polymersomes into rats) that the circulation time of these delivery systems was two times longer than the circulation time of PEG-coated liposomes (Photos, Bacakova, Discher, Bates, &Discher, 2003). Ps in this experiment were composed from poly(ethylene oxide)-b-poly(butadiene) (PEO-b-PBD) and poly(ethylene oxide)-b-poly(ethyl ethylene) (PEE) copolymers.

In addition to the already mentioned applications of Ps for the encapsulation of anticancer drugs, there are cases of encapsulation of more than one drug inside the aqueous core of Ps. For example, doxorubicin and paclitaxel were encapsulated into non-biodegradable PEO-PBD and biodegradable poly(ethylene oxide)-b-poly(lactic acid) (PEO-PLA) polymersomes, and the experiments were conducted both *in vitro* and *in vivo* (Ahmed &Discher, 2004; Ahmed et al., 2006). The results of the experiments on animal models showed selective accumulation of polymer vesicles in the cancer tissue and increased the tumor drug exposure (Prakash Jain et al. 2011). Furthermore, the combination of these two drugs induced 2.5-fold higher apoptosis in the tumor after 2 days and showed higher maximum tolerated doses along with the increased tumor shrinkage and maintenance compared to free drugs (Ahmed et al. 2006).

Moreover, block copolymer assemblies have been used to encapsulate also peptides, proteins and nucleic acids by several different research groups (Arifin& Palmer; J. C. Lee et al., 2001; Hannah Lomas et al., 2007;Rameez, Alosta, & Palmer, 2008).

All of the abovementioned examples show the potential of Ps as drug delivery systems in cancer treatment and with the improvement of the physicochemical properties of the vesicle itself, they can have even bigger role in this area of nanomedicine.

2.3. Self-assembling of polymersomes

2.3.1. Block copolymers

Copolymers consist in general of smaller units (monomers) which can be arranged in a different order. Depending on their different group, there are four types of copolymers (**Fig.7**). Random copolymers consisting of two or more monomers which could follow in any order, whereas alternating copolymers have their monomer units arranged in alternating fashion. There are also graft copolymers which consist of chains of one polymer grafted to chains of another copolymer. Finally, the copolymers of interest in this work are block

copolymers which can be described as two homopolymers combined together. There is no alternation of monomer units, but all of them are combined together and then the other type of monomer is combined together.

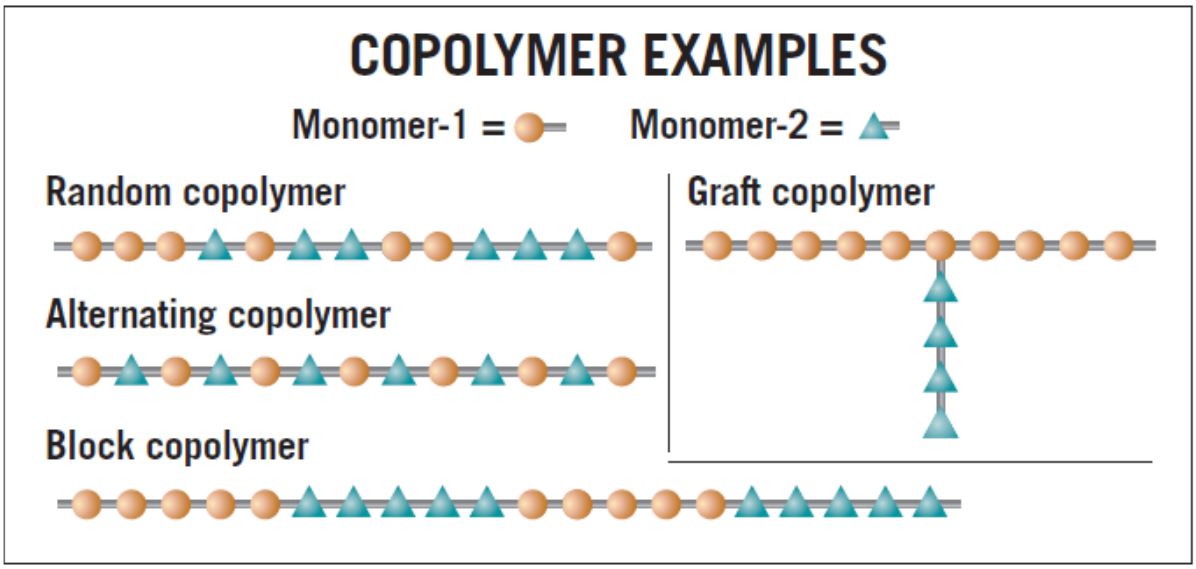


Figure7. *Different types of copolymers and their theoretical composition (Brief 2011)* .

Block copolymers are defined as a combination of two or more polymer blocks, often insoluble in water, in which every block has different properties (*i.e.*, water solubility), contributing to their amphiphilic character. Polymer blocks are bound to one another by the strong covalent bonds which allows them not to segregate completely despite the repulsion of the two blocks. Due to this fact, the possibility of phase separation is rather small (Förster&Antonietti, 1998; Leibler, 1980; Letchford& Burt, 2007). Block copolymers find applications in nanotechnology due to their many advantages, but mainly due to the incompatibility of its constituents (Leibler 1980). There is also a possibility to alternate chemical properties and kinetic stability of the structures formed by block copolymers by changing the molecular weight and the length of the copolymer chain (Förster&Antonietti, 1998). Long segments in copolymer molecules enable them to be used as surfactants and emulsifiers. Furthermore, in comparison to graft copolymers, which they share many similarities with, block copolymers show far better morphological properties that ultimately lead to better physical properties (Noshay& McGrath, 2013).

Regarding different types of block copolymers, they mainly differ in the number of monomer blocks (**Fig. 8**). When consisting of two types of monomer blocks (for example A and B) they

are referred to as diblock copolymers, for example, poly(dimethylsiloxane)-block-poly(2-methyloxazolyne) (PDMS-b-PMOXA) used in this research and described in the next chapter. There is also a possibility of triblock copolymers when the structure consists of two A blocks surrounding by B block (ABA). In the case of triblock copolymers, there is a possibility to have three different types of monomer blocks combined and covalently attached to each other.

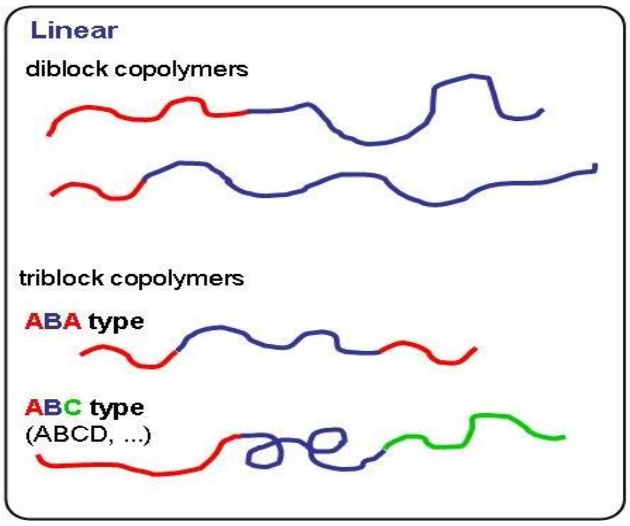


Figure 8. *Examples of various block copolymer types ranging from diblock to triblock copolymers. Diblock copolymers can differ in the size of each part, whereas triblock copolymers can have a different composition depending on how many different types of polymers they incorporate in the structure (http://www.cmu.edu/maty/materials/Synthesis_of_well_defined_macromolecules/block-copolymers.html).*

2.3.1.1. PDMS-PMOXA diblock copolymer

One of the most commonly used combination of copolymers for Ps preparation and further usage in medical and other applications is poly(2-methyloxazolyne) and poly(dimethylsiloxane) either as a triblock or diblock copolymers (**Fig. 9**). These two polymers self-assemble into vesicles (Ps) consisting of hydrophobic cores, outer and inner hydrophilic coronas and, finally, aqueous cores in the middle (Jaskiewicz, Makowski, Kappl, Landfester, &Kroeger, 2012).

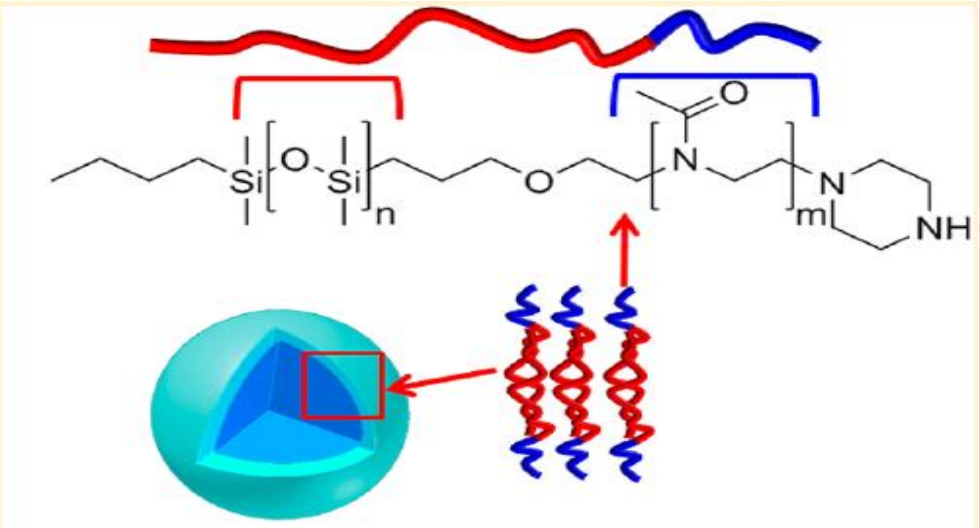


Figure9. *Schematic showing the primary structure of diblock copolymers with hydrophobic poly(dimethylsiloxane) part (red) and poly(2-methyloxazoline) hydrophilic part (blue). They self-assemble into nanosize vesicles (Ps) (Jaskiewicz, Makowski, et al. 2012).*

As abovementioned, these systems are of a great interest in field of nanotechnology and biomedical research (Egli, Martin G. Nussbaumer, et al. 2011; Balasubramanian et al. 2010). They are often utilized as model systems for biomembranes due to the fact that they share many similar properties with biomembranes (Jaskiewicz, Makowski, et al. 2012; Taubert et al. 2004). In order to be utilized in drug delivery they must possess some essential features. Firstly, their hydrophobic membranes must be as much as possible impermeable for hydrophilic substances (potential encapsulated drugs) in order to minimize unwanted loss of the cargo from the core (Litvinchuk et al. 2009). Secondly, ligands must be well attached to the nanoparticle in order to specifically target to the receptors of the cells (Broz et al. 2005). Furthermore, as for every delivery system circulating through blood vessels, adhesion of proteins onto the surface of the Ps must be reduced, in order to minimize the elimination and increase the circulation time, thus improving the drug's efficiency (Egli, Martin G. Nussbaumer, et al. 2011). PDMS-b-PMOXA Ps possess all of the abovementioned listed features, and therefore, are utilized in cell uptake, nanoparticle studies, and ligand-interactions (Egli, Martin G. Nussbaumer, et al. 2011)(Broz et al. 2005; J. Kowal et al. 2014). Moreover, these systems have lower glass temperature (Tg = −124 °C) (then, for example, polystyrene-b-poly(acrylic acid), PS-b-PAA) Ps, often characterized in the literature) (Azzam& Eisenberg, 2006; Q. Chen, Schönherr, &Vancso, 2009; Discher& Eisenberg, 2002), which ensures them

more flexible structures (provided by the poly(dimethylsiloxane) hydrophobic block). While the hydrophobic part of the Ps ensures flexibility of the structure, the poly(2-methyloxazoline) block acts as a buffer in order to prevent protein interactions with the solid substrate (J. T. Kowal et al. 2014). One of the advantages of this Ps is also their mechanical stability which is the result of the slow exchange of polymer chains between aggregates (Jaskiewicz, Larsen, et al. 2012).

In addition to all the abovementioned advantages, there is also the ability to fine-tune properties of the Ps in order to use them in further studies. For example, by using the extrusion technique, their size can be optimized, and therefore, the polydispersity lowered, which finally will lead to higher possibility to control the size of the system (Jaskiewicz, Larsen, et al., 2012; Olson, Hunt, Szoka, Vail, &Papahadjopoulos, 1979). For characterization of these systems a combination of dynamic light scattering (DLS), cryogenic transmission electron microscopy (cryo-TEM) and atomic force microscopy (AFM) is applied (Jaskiewicz, Makowski, et al. 2012; Egli, Martin G. Nussbaumer, et al. 2011). DLS studies give information about the size of the vesicles formed and their polydispersity index (PDI), which are then comparable to cryo-TEM data. Atomic force microscopy, provides information about the conformation of the Ps analysed when they are adsorbed to a flat substrate (Jaskiewicz, Makowski, et al. 2012). These techniques have by now confirmed the existence of many other phenomena in Ps, such as for example, the existence of so called "pregnant" Ps (smaller vesicles inside larger ones) (**Fig.10**). These kind of unusual structures are expected with the higher concentrations of polymer (> 5 g/L) (Jaskiewicz, Makowski, et al. 2012).

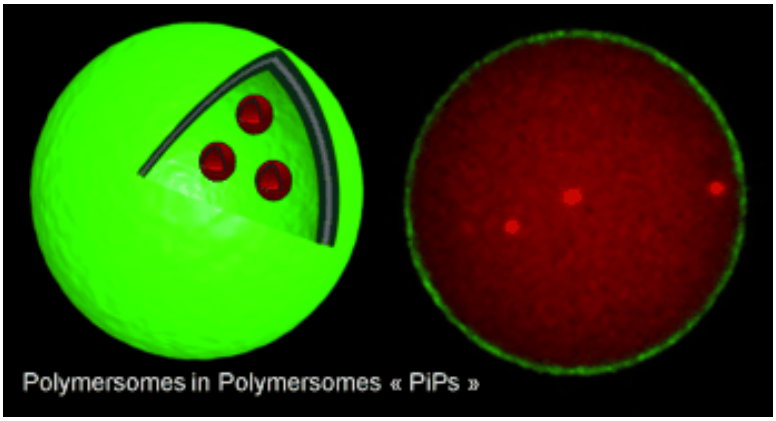


Figure 10.*"Pregnant"Ps structures characterized as smaller Ps entrapped within large vesicles (Marguet, Edembe, &Lecommandoux, 2012).*

Many of the abovementioned features of PDMS-*b*-PMOXA copolymer vesicles make them good candidates as model membranes, as well as potential nanoparticles for medical research. With further improvements and optimization of some of their features they can become one of the most important drug carriers in modern medicine.

2.4. Polymersomes' preparation methods

2.4.1. Solvent-switching techniques

Solvent-switching techniques (also known as phase inversion) consist of few steps to prepare Ps. Using this kind of preparation method, before the self-assembling process, block copolymers are dissolved in an organic solvent in which both parts (hydrophilic and hydrophobic) are proven to be soluble. After the dissolution in an organic solvent the newly formed solution is to be hydrated. This step can be performed either by adding water into the organic polymer solution or by injecting the solution into the water phase. By this procedure hydrophobic blocks become insoluble, which enables them to self-assemble into Ps because of the increase of the interfacial tension between the hydrophobic blocks and water (Jung &Theato, 2012b; J. S. Lee &Feijen, 2012; LoPresti, Lomas, Massignani, Smart, &Battaglia, 2009; Zhang & Eisenberg, 1995). There is also a possibility to remove the organic cosolvent after preparation of the Ps, which can be achieved by dialysis (Du & O'Reilly 2009).

This preparation method is commonly used because of the fact that not all copolymers are water soluble, and therefore, following these procedure they are dissolved in an organic solvent before mixing with an aqueous phase (Du & O'Reilly, 2009; Jung &Theato, 2012b).

2.4.2. Polymer film rehydration techniques

When it comes to the film hydration preparation method, the procedure implies dissolution of the amphiphilic block copolymer in the organic solvent and its mixture to ensure a homogenous, transparent sample. The next step is the evaporation of the organic solvent and addition of water. Evaporation of the smaller volumes of organic solvent can be achieved using dry nitrogen or argon. However, the evaporation of bigger volumes of the organic solvent has to be done by rotary evaporation. The film is then left overnight in a vacuum oven in order to remove any possible organic solvent left. Hydration of the film is simple and

consists of adding the water into the vial or flask with dried polymer film. This method will in most cases produce Ps with wider range of sizes. Therefore, it is necessary to apply extrusion (LoPresti et al. 2009) through filters of defined pore sizes to reduce the size of the vesicles in order to improve their size properties (J.S. Dua, Prof. A. C. Rana, 2012; Jung &Theato, 2012b; J. S. Lee &Feijen, 2012).

2.5. Characterization and purification methods

In this section, it will be described some of the most commonly used characterization techniques for determination of the physical and mechanical properties of small vesicles, such as Ps.

2.5.1. Characterization

2.5.1.2. Dynamic and static light scattering

When it comes to investigation of Ps in aqueous solution, one of the most popular tool used is light scattering, especially dynamic light scattering, DLS (J. S. Lee &Feijen, 2012). These experiments are based on the fact that monocromatic laser light passes through the sample cell and while most of the light passes through the sample, some beams of light are scattered (**Fig. 11**). The intensity of scattered beams is then detected and measured.

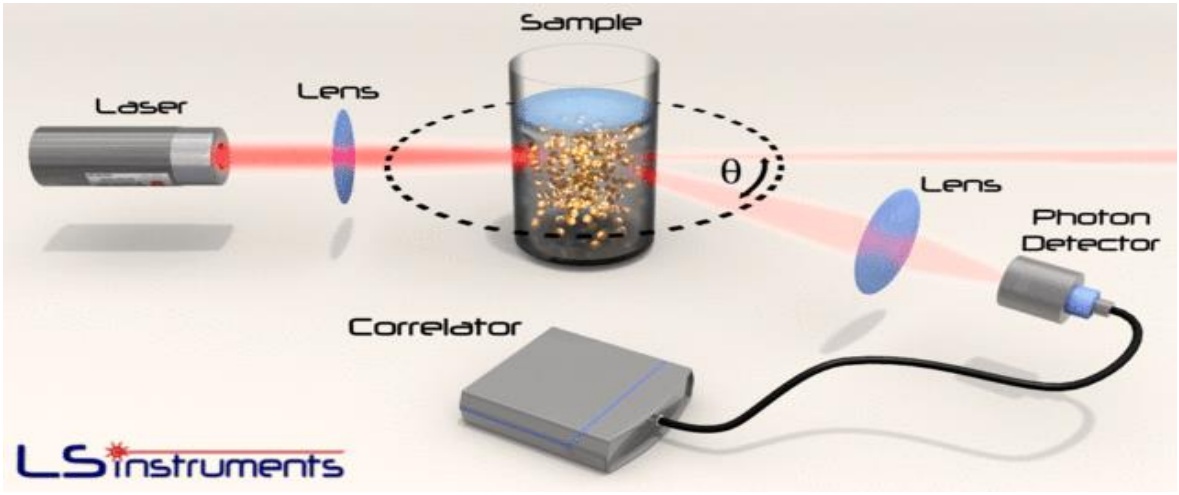


Figure11. *Visualisation of the DLS method and principle of laser beam of light passing through the sample and scattered light being detected and analysed (http://www.lsinstruments.ch/technology/dynamic_light_scattering_dls/).*

Under the term static light scattering, it is consider time-averaged intensity of scattered light, whereas DLS measures the fluctuations of intensity of scattered light in correlation with time (Goldburg 1999). Static light scattering methods can provide information about the average molecular weight, particle shape, as well as their size and even particle–particle or particle–solvent interactions (Kita-Tokarczyk et al. 2005). Scattering experiments are especially compatible for detection of aggregates, because of their ability to scatter light even when present in low concentrations. Moreover, light scattering techniques have found their place in experiments for determination of size polydispersity of vesicles formed, the effect of intravesicular polymerization, critical aggregation concentration, and for determination of vesicular morphology (Kita-Tokarczyk et al. 2005). These studies are related particularly to PMOXA-PDMS-PMOXA triblock copolymers, but there are more examples of nanosize vesicles, where this method is used in determination of size and shape (Otto & Prendergast 2014).

Light scattering are non-invasive and non-destructive techniques which require small volumes of sample and at the same time can determine the size of the vesicles 2–500 nm. Furthermore, this characterization technique when, used for determination of molecular weight, possess a wide range of sensitivity to determine the molecular weight of molecules (from 1 kDa to 10 MDa) (Summit n.d.).

The advantages of static light scattering are fast and accurate determination of molar masses with high accuracy of ± 5 %. Moreover, this technique is easy to implement, fully automated, and highly reproducible (Summit n.d.). For DLS, an important feature is the capability to analyse aggregates fast and accurate, and this technique has been proved to be good for analysis of aggregation of nanostructures.

2.5.1.3. Microscope techniques

Microscope techniques, along with the light scattering techniques, are often used for Ps characterization and determination of their physical properties, such as size, morphology and particle homogeneity. Therefore, in this section the most important features for this field of interest will be described, such as light, fluorescence, and electron microscopy. All of them have both advantages and disadvantages, but are undoubtedly suitable for analysis of polymeric vesicles.

2.5.1.3.1. Optical microscopy

Light microscopy is frequently used as a characterization technique in studies for properties determination of Ps. It provides instant visualization of vesicle without need of drying or staining them. However, there is a major drawback regarding limitations in resolution which requires vesicles of bigger size than needed for electron microscopy, for example.

In order to visualize Ps using transmission light microscopy, an important feature is the contrast; there has to be enough contrast in an image in order to be able to clearly see Ps. Unfortunately, Ps do not absorb light, and therefore, cannot create much needed contrast or it is so poor that they are practically invisible in optical microscope, and the contrast has to be enhanced using other techniques (Kita-Tokarczyk et al., 2005; J. S. Lee &Feijen, 2012).

As a solution to the abovementioned inability to make contrast to visualize Ps in optical microscope, a solution can be by using *phase contrast microscopy.* This technique enhances the contrast and is particularly suitable for samples which absorb very little or show no absorption in a visible part of the spectrum.

This provides the ability to see specimen in their physiological surrounding without drying or staining the samples, but with improved resolution. Therefore, it is often utilized to explore dynamic processes in living cells (Kita-Tokarczyk et al. 2005).

2.5.1.3.2. Fluorescence microscopy

When it comes to fluorescence microscopy techniques, there are several different variations of this technique; however, all are based on the same principle. Compared to transmission light microscopy, where the problem is the inability to create sufficient contrast, in fluorescence microscopy only emitted light from the specimen is detected, and therefore, creates a much needed contrast (Kita-Tokarczyk et al. 2005) (**Fig.12**). Other advantages of fluorescence over transmission microscopy are the most specific ability to recognize fluorescent molecules (which possess fluorochromes) from non-fluorescent molecules. Furthermore, the possibility to stain the sample with different probes enables the characterization and determination of the presence of specific target molecules (Kita-Tokarczyk et al. 2005).

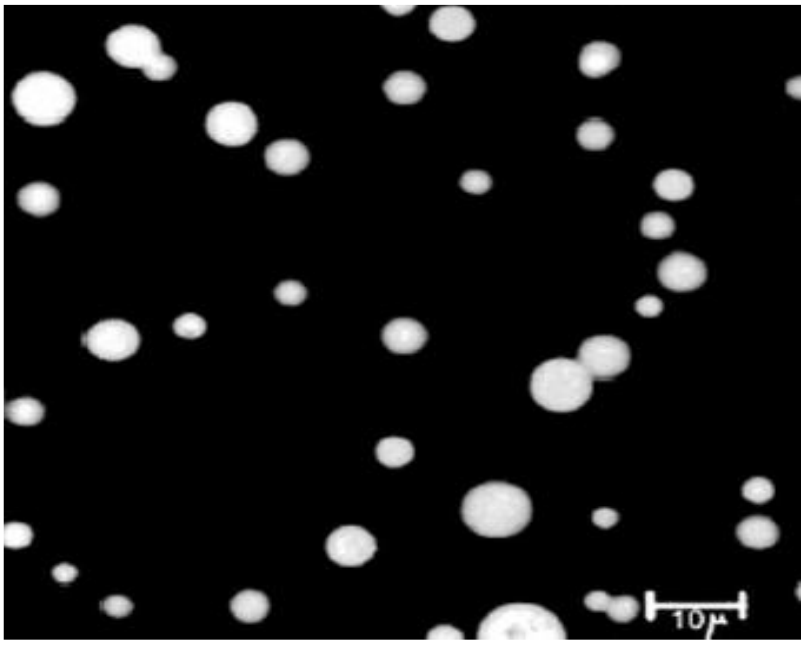


Figure12. *Ps from oligoanhydrides-PEG block copolymer under the fluorescence microscope; scale bar =10 μm (Kita-Tokarczyk et al., 2005; Najafi&Sarbolouki, 2003).*

When it comes to fluorescence visualization of polymeric vesicles built out of amphiphilic copolymers, they usually do not exhibit intrinsic fluorescence, therefore staining is required. Staining can occur either during the encapsulation process where hydrophilic dye is then encapsulated into the aqueous core during the formation of vesicle followed by the exclusion of dye from Psin later stages of the process. Other possibility is to include fluorescent dyes into the Psmembrane (Kita-Tokarczyk et al. 2005).

Fluorescence microscopy allows us to visualize different structures, but also to explore dynamic processes of macromolecules (*e.g.*, diffusion, binding constants, enzymatic reactions, and rotational mobility). Moreover, studies have been made using fluorescent probes to determine the intracellular pH (Mulkey et al. 2004), monitor the local concentrations of certain ions (Yip & Kurtz 2002) and some other cellular functions (Morgan & Mitchell 1996) (Kita-Tokarczyk et al. 2005).

There are different variations of fluorescence microscopy, such as *wide-range epifluorescence microscopy* and *total internal reflection microscopy*, but for the characterization of Ps the most important technique has proven to be *confocal fluorescence microscopy*, more precisely *laser scanning confocal microscopy (LSCM).* Using this technique, optical slices of specimen

are visualized in *x*,*y* and *z* direction, and they can be combined together in order to create a 3D image of the vesicle (Kita-Tokarczyk et al., 2005; J. S. Lee &Feijen, 2012; Patel & McGhee, 2007; Wilson & Sheppard, 1984). Compared to transmission light microscopy, LSCM possesses higher resolution (enhanced by $\sqrt{2}$) (Bleyl 1989), and compared to wide-range techniques there is a reduction in the background fluorescence, as well as an improved signal-to-noise ratio (Kita-Tokarczyk et al. 2005). Even though epifluorescence microscopy provides high level of sensitivity, using this type of fluorescence microscopy, sensitivity can still be further improved.

Considering all of the abovementioned advantages, it can be stated that confocal fluorescence microscopy serves as an extremely useful tool for the characterization of polymeric vesicles. However, there is still an issue of high costs of equipment, which is stopping this technique from becoming a standard tool in the characterization process.

2.5.1.3.3. Transmission electron microscopy (TEM)

Electron microscopy as a characterization technique for polymeric vesicles ensures hundred thousand times better resolution (in theory) than that of light microscope. In addition to that, there is 100-fold increase in a depth of field compared to light microscopy (Kita-Tokarczyk et al. 2005). This technique is based on the irradiation of specimen with an electron beam of uniform current density (Reimer & Kohl 2008) and an aperture consisting of an illumination system, a specimen stage, an objective lens system (consisting of three to eight lenses and providing image of the intensity distribution on the fluorescent screen behind specimen (Reimer & Kohl 2008), the magnification system, the data recording system, and the chemical analysis system (Wang 2000). The most important part of an illumination system is the electron gun which provides coherent beam of electrons. Furthermore, the condenser lenses are important part of this system in order to ensure a fine electron probe (Wang 2000). The most important part for carrying out structure analysis is specimen stage, providing the possibility to characterize the physical properties of nanostructures. The objective lens determines the limit of stage resolution while the intermediate and projection lenses (magnification system) give magnification up to 1.5 milion (Wang 2000). The data recording system is usually digital and the chemical analysis system consists of energy-dispersive X-ray spectroscopy (EDS) and electron energy loss spectroscopy (EELS), both used to quantify the chemical composition of the specimen (Wang 2000).

Despite many advantages of the electron microscope, there are however also few disadvantages. For example, the electrons, being high-energy particles, easily interact with atoms by elastic or inelastic scattering. These interactions result in the emission of all the lower forms of energy, which prevents electrons from penetrating deeper into the specimen (Wang 2000; Reimer & Kohl 2008). Furthermore, the sample for analysis by this specific technique must be dried and stained (to enhance the contrast) (Clarke 1973; Ward. 1964), which is not optimal for biological samples. However, Ps in a hydrated state can be studied using cryogenic-TEM (Kuntsche, Horst, &Bunjes, 2011), which is usually the method of choice when it comes to biological systems for TEM imaging. (J. S. Lee &Feijen, 2012). This is achieved by freezing the specimen and viewing it in vitreous ice. These conditions are as close as possible to the natural state of the vesicles (Kita-Tokarczyk et al. 2005).

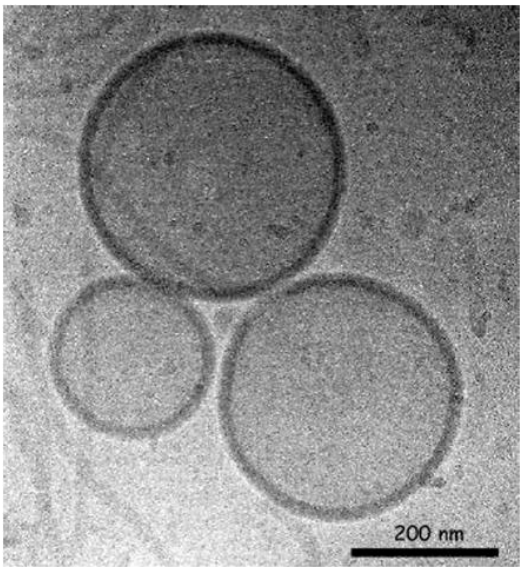


Figure 13. *Cryogenic image of PMOXA-b-PDMS-b-PMOXA vesicles prepared by film swelling in water ; scale bar =200 nm (Kita-Tokarczyk et al. 2005).*

Using cryo-TEM, the structure of the vesicle is preserved if it is perfectly frozen, because dehydration is avoided. Furthermore, this characterization technique allows the observation of phase behaviour of macromolecules which comes as a result of the self-assembling process in water. Such processes are: micellar polymorphism (Won et al. 2003), spontaneous formation of vesicles (Won et al. 2002), and their transition to lamellar structures (Kita-Tokarczyk et al. 2005).

Considering the fact that electrons cannot penetrate deeply into the specimen, because of the previously mentioned reasons, TEM is mainly used to explore the surface and sub-surface of the specimen and with such purpose has been used in the field of Ps (**Fig. 13**) (Arifin& Palmer; Góźdź, 2004; Kita-Tokarczyk et al., 2005; Meng, Engbers, &Feijen, 2005; Napoli et al., 2004; Prakash Jain et al., 2011).

2.5.2. Purification techniques

As it is well-known that in a process of Ps preparation there may be some impurities, such as free drug, free polymer or other unwanted molecules left, and thus, purification techniques must be applied in order to obtain a sample as clean as possible. Therefore, in this chapter the two most popular and commonly used methods for purification of Ps will be described.

2.5.2.1. Dialysis

Dialysis is a purification method and separation technique which leads to the removal of small, unwanted molecules (impurities) using selective and passive diffusion through semi-permeable membrane dependable on the size of the molecules (**Fig.14**).

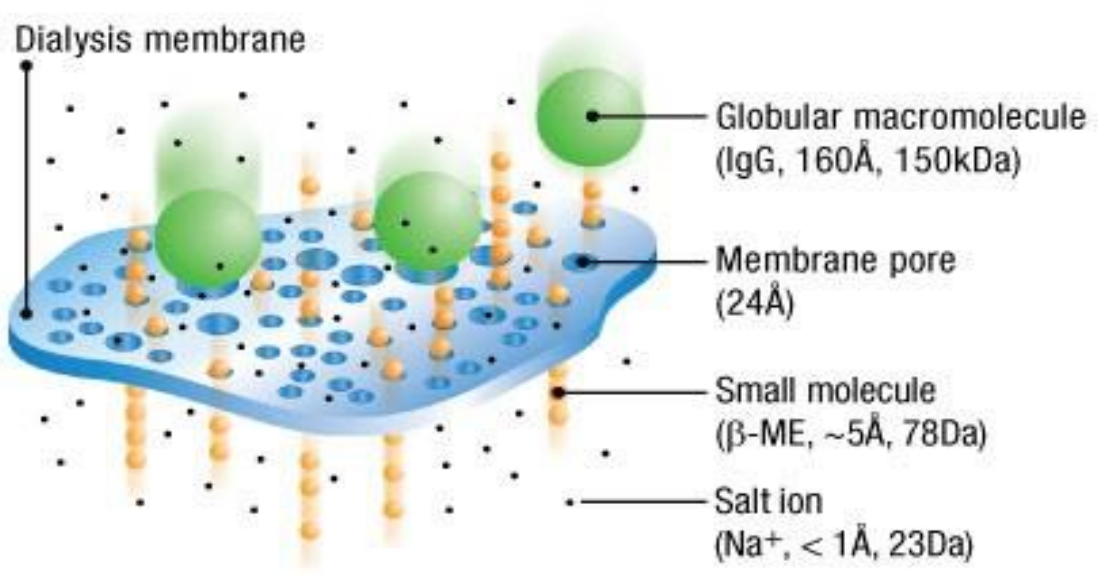


Figure 14. *Image showing the principle on which dialysis membrane works. It is semi-permeable cellulose film with pores of a certain size. Molecules bigger than the size of the pores cannot pass through the membrane, whereas smaller molecules pass freely through the pores (https://www.thermofisher.com/hr/en/home/life-science/protein-biology/protein-biology-learning-center/protein-biology-resource-library/protein-biology-application-notes/separation-characteristics-dialysis-membranes.html).*

Dialysis bags are conventionally used for removal of low-molecular-weight solute, which is undesirable, from the sample and replacement with buffer used for sample preparation. This bags are membranes with pores ranging from 15 to 20 kDa, allowing only low molecular weight molecules to diffuse outside, into the buffer (**Fig.15**) (Scopes 1994). The volume of the buffer is recommended to be fifty times the volume of the bag. However, even with that amount of buffer, after certain amount of time the concentrations of the buffer on the both sides of the membrane equalizes. Therefore, it is required to change dialysis medium (buffer) after 2 or 3 h, at least once during the process. Furthermore, mixing of the buffer and the dialysis bags themselves can help speed-up the process. This separation/purification procedure consists of different steps. First, the dialysis bags can be cut directly from the dry role and appropriately used for the volume of the sample. After wetting the bag, it is closed on one side (using appropriate clamp) and on the other side the sample is injected and it is closed in the same way, very carefully, in order not to lose any of the sample volume. Bags are then placed into the buffer and left stirring (Scopes 1994).

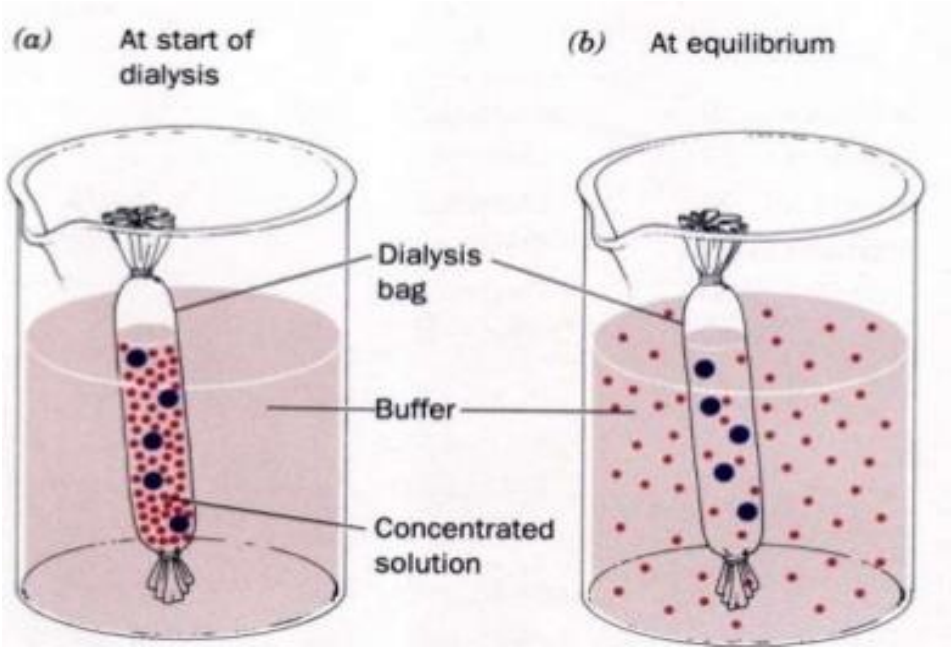


Figure15. *Visualisation of the dialysis process. After certain amount of time the concentrations inside and outside of the dialysis bag are the same (equilibrium) and the buffer has to be changed (http://www.slideshare.net/jaspreetmaan/protein-fractionation).*

2.5.2.2. Size exclusion chromatography (SEC)

The SEC method enables the determination of the molecular weight of a polymer, but at the same time can be used as a method of separation based on the molecular weight of a molecule or compound (Mori & Barth 1999). It is based on the principle that smaller molecules need more time to pass through a matrix and their longer path is due to the fact that they have to pass through pores of the beads, whereas bigger molecules pass in between the beads (**Fig. 16**). This technique is primarily applied for separation of water-soluble polymers. This purification technique system is also called gel filtration (for separation of biopolymers) or gel permeation chromatography (for separation of organosoluble polymers), and the matrix for filtration usually consists of beads made of cellulose or agarose (Mori & Barth 1999; Chromatography n.d.).

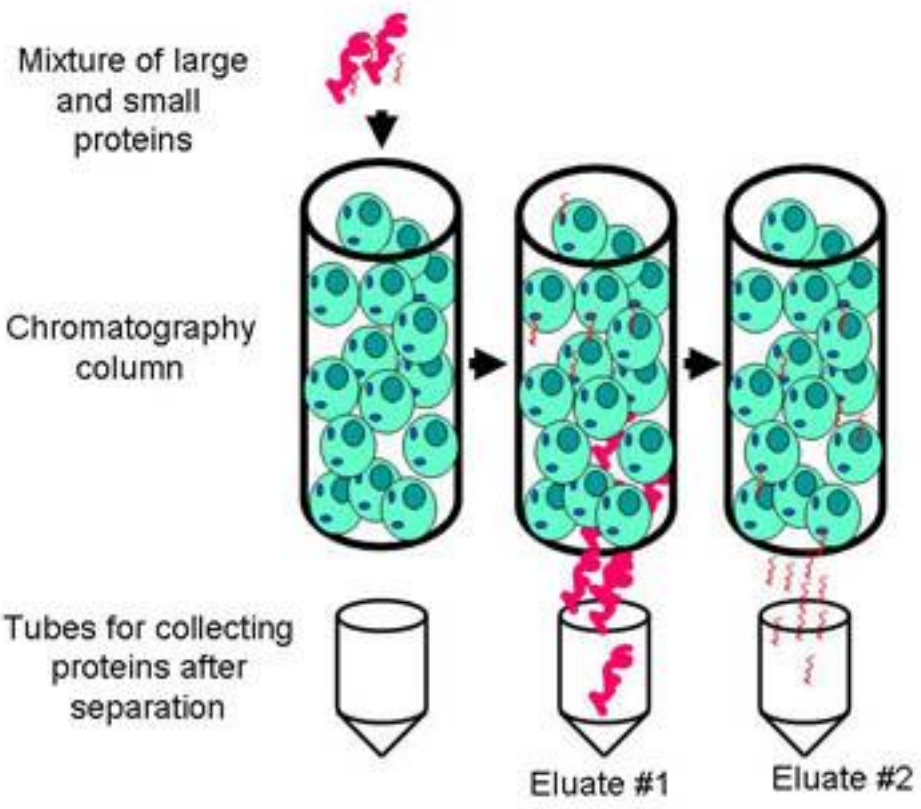


Figure 16. *Gel permeation chromatography column and its principle of separation. While larger molecules pass only between the beads, smaller ones go into the pores and therefore come out of the column later (* [*http://labicenter.org/picsowc/gel-permeation-chromatography-columns.html*](http://labicenter.org/picsowc/gel-permeation-chromatography-columns.html) *).*

This method takes around 30 min for the separation (time of the separation is dependable on the sample), using high-performance liquid chromatography (HPLC), to have all the information about the polymer molecular weight. The column is packed with small gel particles which have pores of different and variable sizes. The solvent used for this procedure fills both the space between beads, as well as the space inside pores of the beads. The sample is dissolved, introduced into the column, and eluted to pass through the column. All of these steps are done using the same solvent (Action et al. 1964). There are three possibilities for particles/molecules to behave. If particles are smaller than the size of the pores in the beads, they tend to go through the pores, and therefore, it takes them longer to pass through the column. If, however, the molecules are too big to even enter the column they get excluded in the very beginning. The third possibility is for the molecules to be small enough to enter the column, but not small enough to enter the pores of the beads. In that case they pass in between gel beads and appear in the end of the column earlier than small molecules (Action et al. 1964).

Gel permeation chromatography can be used for determining the molecular weight of a certain polymer, for fractionation of polymers or oligomers, or finally for separation of larger molecules from smaller ones (Gamble et al. 1965; WALLENIUS 1954).

2.6. Drug loading and drug release

2.6.1. Drug loading

Considering the fact that polymersomes show greater stability in blood circulation than the nanoparticles similar to them (liposomes), they are attractive target for drug encapsulation and also used as nanocarriers.

There is a possibility of loading both hydrophilic and hydrophobic drugs within the vesicles, but the methods differ from each other and can also be done in different phases of the vesicle formation. There is also a possibility to load amphiphilic type of molecules. This last type of drug molecules along with the hydrophobic ones are incorporated into the membrane of the vesicle. This can be compared to the incorporation of proteins and cholesterol into the cell membrane. There are few different possibilities how to incorporate these drugs into the nanoparticle. It can be done by dissolving or dispersing the molecules of the drug along with the block copolymers forming the polymer film into the organic solvent (Ghoroghchian et al.

2006). After this step, the solution which is formed is added to the aqueous phase. For example, the anticancer drugs doxorubicin and paclitaxel have been incorporated into Ps using this method (Kowalczuk et al., 2014; J. S. Lee &Feijen, 2012). Furthermore , there is a possibility to load hydrophobic payloads into the Ps during an emulsion process (Beaune et al. 2007), or by using a diffusion method (Ahmed et al. 2006)(Kowalczuk et al. 2014). When it comes to the incorporation of the hydrophobic drugs into the membrane, some works have shown that there is a possibility to incorporate hydrophobic molecules into the bilayer membrane when their molecular length is up to ½ of the core thickness of their bilayer membrane without endangering the stability of the Ps itself (Ghoroghchian et al. 2006).

The aqueous core of the hollow sphere can be utilized for encapsulation of the hydrophilic drugs and many other molecules, such as peptides, siRNA and DNA molecules (Christian et al., 2010; Hannah Lomas et al., 2007; Pangburn, Petersen, Waybrant, Adil, &Kokkoli, 2009). There are several methods currently being used for the drug encapsulation into this type of particles which include direct encapsulation during the formation of the nanoparticle (Hannah Lomas et al. 2007), diffusion over the membrane in relation with pH (Choucair et al. 2005) and salt gradient (Ahmed et al. 2006). There is also a possibility to encapsulate hydrophilic drugs into the Ps by dissolving it in the organic solvent along with the copolymers used for the formation of the nanoparticle.

The first method abovementioned consists of dissolving the polymer film in the aqueous phase in which the payload was previously dissolved. Changing the pH as a preparation method can be used if the Ps formed is pH responsive and with the change of pH or even temperature, the formulation stays in the aqueous phase the whole time and it enables DNA molecules and proteins (water soluble molecules) to be entrapped within the vesicle.

2.6.2. Drug release

When it comes to drug release from Ps, there is a wide range of possibilities to control the time and the place for the release of the encapsulated drug by changing the pH (pH responsive systems) (W. Chen, Meng, Cheng, &Zhong, 2010; Ulbrich et al., 2004), temperature (temperature sensitive systems) (Xu, Meng, &Zhong, 2009), or by using some other stimuli that can cause dissolution of the Ps membrane or diffusion of the drug through the membrane and its release (M.-H. Li & Keller, 2009; Meng, Zhong, &Feijen, 2009; Rijcken, Soga,

Hennink, & Nostrum, 2007). This stimuli-responsive drug release leads to the improvement in targeted delivery and reduction of possible side effects.

In principle, it could be said that the release from Ps is due to the diffusion of the drug through the membrane and the driving force is the concentration gradient between the drug encapsulated and its surrounding medium (J. S. Lee &Feijen, 2012; Saylor, Kim, Patwardhan, & Warren, 2007).

Furthermore, there are few parameters which could play significant role in the drug release rate from these kind of systems. Size distribution can, for example, affect the drug release, and therefore, some adjustments can be made to improve, optimise and predict the drug release kinetics (Siepmann et al. 2004). In addition, the drug release can be adjusted only to the certain level due to the constrains related to block copolymers building a thick polymersome membrane (Mecke et al. 2006).

As abovementioned, the change of the conditions in the Ps environment or some external stimuli can easily change the chemical or physical properties of the Ps membrane, which consequently changes the drug release and the possiblity to enhance the drug delivery (J. S. Lee &Feijen, 2012). Some of the most popular external stimuli used for this purpose are changes in pH, temperature, redox conditions, light or magnetic field. For example, change in pH is a particularly appealing external stimuli to deal with, especially when the designed Ps respond to acidic pH, characteristic for the tumor tissue, inflammatory tissue, as well as for the endosomes and lysosomes (Chen et al. 2010; Ulbrich et al. 2004). There have been many cases of successful application of pH responsive polymer vesicles in cancer therapy. One of the examples are Ps based on poly(2-(methacryloyloxy) ethyl phosphorylcholine)-b-poly(2-(diisopropylamino) ethyl methacrylate applied for the controlled release of doxorubicin (Jianzhong Du et al. 2005) and delivery of DNA (H. Lomas et al. 2007; Chen et al. 2010). Another example of pH sensitive Ps are the ones prepared from diblock copolymer of PEG and an acid labile polycarbonate, poly(2,4,6-trimethoxybenzylidene-pentaerythritol carbonate) (PTMBPEC), as demonstrated in the study by Chen *et al.* (Chen et al. 2010). These Ps showed the ability to encapsulate both paclitaxel and doxorubicin hydrochloride, but were prone to hydrolysis at acidic pH of 4.0 to 5.0 (Prakash Jain et al. 2011).

Furthermore, temperature has also been used as a stimulus for controlled drug delivery in the vesicles which are thermo-sensitive. Block copolymers based on poly(N-isopropylacrylamide) (PNIPAAm) have been used in several different studies for preparation of thermo-sensitive Ps

(X. Chen, Ding, Zheng, & Peng, 2006; Hales, Barner-Kowollik, Davis, &Stenzel, 2004; Y. Li, Lokitz, & McCormick, 2006; Qin, Geng, Discher, & Yang, 2006). This system is based on the fact that due to the change in the conformation of PNIPAAm below certain temperature (lower critical solution temperature), it becomes soluble in an aqueous environment (J. S. Lee &Feijen, 2012).

In addition to these methods, which include the presence of some external stimuli, there are also novel approaches to control the release of drugs from Ps. Some of the methods use biodegradable polymers to form a membrane and other cause change in the composition of Ps (J. S. Lee &Feijen, 2012).

3. Aims of the study

The main purpose and objective of this research was to prepare and characterize two types of PDMS-b-PMOXA Ps with a different length of hydrophilic part for application in cancer therapy. Therefore, after the preparation, the nanoparticles were tested on two different cancer cell lines (MCF-7 and 231 breast cancer cells) in order to explore the effectiveness and characteristics of this kind of nanosystems in targeted anti-cancer drug delivery and to evaluate the toxicity and stability of the system itself.

In detail, the specific aims of this research were:

- To prepare two kinds of PDMS-b-PMOXA Ps with different length of hydrophilic chain.
- To compare the properties of these two Ps systems depending on different polymerization number of the hydrophilic part.
- To characterize the size distribution, shape, and stability in different buffers of the prepared nanosystems.
- To test the *in vitro* viability studies on two different cell lines (MDA-MB 231 and MCF-7).

4. Materials, Instruments and Methods

4.1. Materials

4.1.1. Poly(dimethylsiloxane)-block-poly(2-methyloxazoline) polymersomes (Ps)

$PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$ diblock copolymers were provided by the group of Professor Wolfgang Meier, University of Basel (Switzerland), and used for the preparation of Ps. The degree of polymerization of hydrophilic and hydrophobic chains is described by the numbers next to the acronyms. Furthermore, the difference between two types of Ps prepared is in the length (degree of polymerization) of the hydrophilic part of the molecule. Because of the cationic ring-opening polymerization of the hydrophilic PMOXA block from an activated PDMS the amphiphilic block copolymer is formed (Dieu, Wu, Palivan, Balasubramanian, &Huwyler, 2014). After the formation of block copolymers, self-assembling process leads to the formation of polymeric vesicles (Ps) with the membrane consisting of hydrated hydrophilic coronas both on the inside and the outside of the hydrophobic middle part.

4.1.2. Cell lines

For the *in vitro* part of the study we used two types of breast cancer cell lines- MCF-7 and MDA-MB-231 breast cancer cell lines. The cell lines were obtained from the American Type Culture Collection and incubated in 75 cm^2 culture flasks (Corning Inc. Life Sciences, USA) at 37 ºC, at an atmosphere of 95% humidity and 5% CO_2 (BB 16 gas incubator, Heraeus Instruments GmbH, Germany). MCF-7 and MDA-MB-231 were used for the in vitro studies with passage numbers 8-25 and 5-30, respectively.

4.2. Instruments

4.2.1. Extruder

The extrusion is a preparation method used in a production process for liposomes and Ps to increase encapsulation of target macromolecules and form vesicles of uniform size. Particle suspension is pressed through a polycarbonate membrane with a defined pore size, thus resulting in a suspension with the diameter of particles being similar to the ones of the pores of the extrusion filter (Rameez et al. 2010). This preparation method was used on both Ps suspensions (AB-10 and AB-14) in order to reduce the size of the particles to up to 200 nm using the *Avanti* Mini-Extruder (**Fig. 17**).

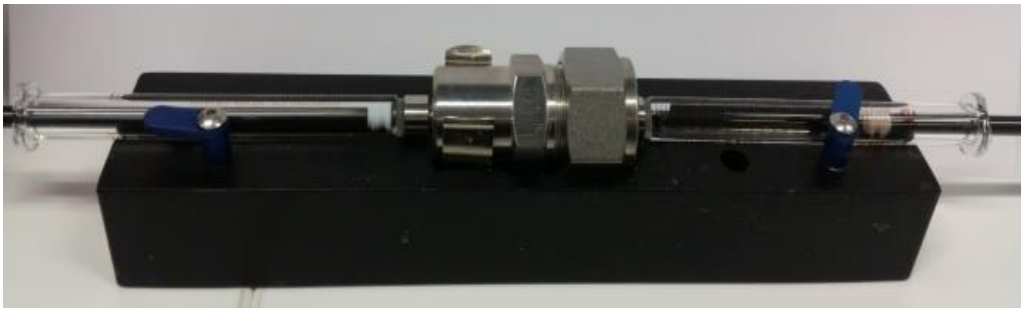


Figure 17. *Avanti Mini-Extruder used for production of Ps with main size around 200 nm (http://www.sapphirebioscience.com).*

The instrument kit contains:

- Two syringes of 1000 µL;
- Polycarbonate membrane;
- Filter supports;
- Two internal membrane supports;
- Extruder outer casing; and,
- Retainer nut.

Before using the extruder, all its parts were washed with deionized water and left to dry on the clean paper. Syringes were washed several times between each usage and polycarbonate membranes and filter supports were used for preparation of one sample exclusively.

4.2.2. Dynamic light scattering (DLS)

DLS was used in this experiments in order to define size of the Ps in an aqueous solution, as well as the PDI of vesicles formed using the sophisticated instrumentation. Besides determination of size and its polydispersity, this method, as already mentioned in chapter **2.5.1.1.**, is generally used to study the effect of intravesicular polymerization, critical aggregation concentration, and for determin ation of the vesicular morphology (Kita-Tokarczyk et al. 2005).

The sample of the particles in an aqueous solution was put into the disposable polystryrene cuvette and the laser beam possessed through the cuvette until the photon counting device and finally to the digital signal processor. The main principle of this method lies in the fact that the particles of the sample in the solution, Ps, undergo Brownian motion defined as a random movement of particles caused by the collision of the particles with the molecules of the solvent. DLS technique measures the speed of particles undergoing the Brownian motion,

which is influenced by the viscosity of the sample, particle size and the temperature. Therefore, the smaller the particle is, the more rapid the motion becomes, and it also rises with the rise of the temperature in the system. The velocity of the Brownian movement is defined by the transational diffusion coefficient (*D*), which can be easily converted into the size of the particle using Stokes-Einstein equation (**Eq.1**):

$$r = \frac{kT}{6\pi\eta D} \qquad \textbf{(Eq. 1)}$$

where *r* stands for the radius of the particle, *k* represents the Boltzmann's constant, *T* is the temperature, and the symbol η is the viscosity of the solvent used in the preparation of the solution.

With the DLS technique being used, we measure the hydrodynamic diameter of the particle, defined as the diameter of a hard sphere that diffuses at the same speed as the particle or the molecule being measured. This diameter depends on the size of the "core" of the particle, as well as the size of any surface structures, concentration and type of the ions in the medium (Goldburg 1999).

The instrumentation used for the measurement of the size, using the DLS method, was Zetasizer Nano ZS (Malvern Instruments Ltd., UK) and the values of the size were calculated as the average of the three measurements (http://www.malvern.com/en/products/product-range/zetasizer-range/zetasizer-nano-range/zetasizer-nano-zs/default.aspx).

4.2.3. Transmission electron microscope (TEM)

For the characterization of the Ps samples in the experiment we used TEM as an analytical tool for visualization of the specimen in the realms of microspace (1 micron = 10^{-6}m) to nanospace (1 nanometer = 10^{-9}m). Because of the principle of the method (described more in details in **2.5.1.3.3.**) based on the focused beam of high energy electrons passing through the sample, some details of the structure, inaccessible to the light microscope, can be detected (http://www.ammrf.org.au/myscope/).

In this research, we have prepared samples for TEM analysis using 10 µL of the Ps dispersion, which we put on the carbon-coated copper grids previously charged in order for the sample to attach more easily. After leaving it to dry for 1 min, the grid was dried using clean filter paper and negatively stained with 2% uranyl acetate aqueous solution. For visualization of empty Ps (AB-10 and AB-14), we used Jeol JEM-1400 TEM (Jeol Ltd., Tokyo , Japan) (**Fig. 18**).

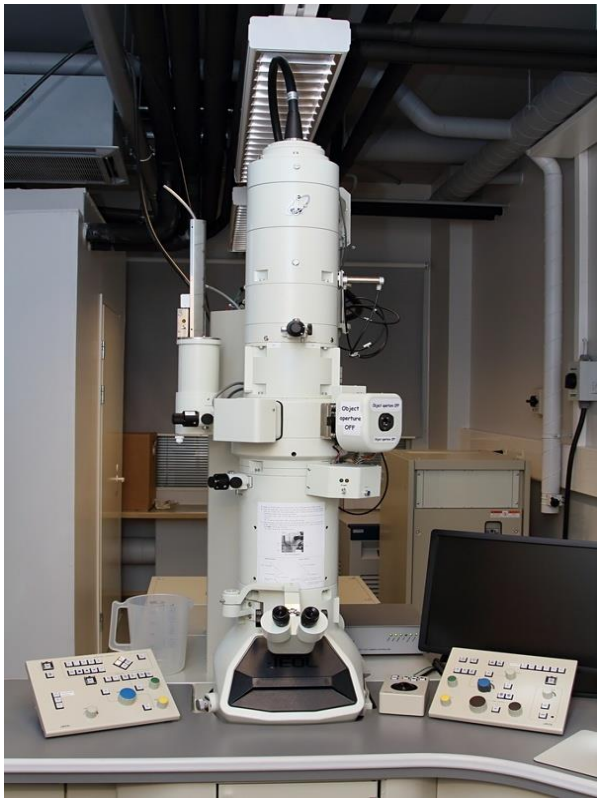


Figure 18. *Jeol JEM-1400 TEM used for the visualization of empty Ps in this experiments (http://www.biocenter.helsinki.fi/bi/em/emu_inst_microscopes_jeol1400.html).*

4.2.4. Luminometer

In an *in vitro* part of the research, we used luminescence detection to investigate an influence of the nanosystems itself on two different cancer cell lines, which we detected using a luminometer. This instrument measures the light and other optical properties of specimens in chemiluminescent and bioluminescent applications. It is commonly used for ATP assays, luciferase assay, immunoassays and proteomics, clinical diagnostics, genomic analysis, various toxicity tests, cell viability tests, etc.

Luminescence is a term used for the emission of light, which does not happen as a result of higher temperatures and it can be considered as a form of the cold body radiation. Therefore, light is emitted from the atom after it has been caused to vibrate by some external stimuli, such as heat, chemical reaction, electronic current, or electromagnetic radiation. Vibration causes an electron to move to unstable, higher energy state while another transition causes it to turn back to the less energetic state and that is when the light emission occurs. Moreover, this light emission can have chemical or biochemical reactions as a cause.

In this study, we measured luminescence for cell viability assay using a spectral scanning multimode reader Varioskan Flash (Thermo Fischer Scientific Inc, USA; **Fig. 19**). The samples were put in the 96-well plates.



Figure 19. *Varioskan Flash spectral scanning multimode reader (Thermo Fischer Scientific Inc, USA) used in this study to measure luminescence in cell viability assays (http://www.thermoscientific.com/en/product/varioskan-flash-multimode-reader.html).*

4.3. Methods

4.3.1. $PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$ preparation

In this study, we used film rehydration technique as a preparation method for the Ps' suspensions (Reeves &Dowben, 1969). This method was previously described in **2.4.2.** In order to get the thin polymer film in the vial, which was later to be rehydrated, we first had to dissolve $PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$ diblock copolymers in organic solvent (in our case ethanol; EtOH). The organic solvent was later evaporated under the stream of air, while the vial was being kept on the stirring plate in order to get the uniform and this polymer film. After the formation of film, the vial was left to dry in the vacuum oven overnight. In order to get the concentration of Ps solution 1 mg/mL we added 1 mL of phosphate buffer saline (PBS) + 100 mM NaCl solution in the vial with the polymer film, which was previously prepared using 1 mg of diblock copolymer and 200 µL of EtOH. The vial was then left stirring overnight at the room temperature at 600 rpm. Since this preparation method usually gives a wide range of sizes of the nanosystems to obtain the mean diameter of

200 nm, we used an extruder with the polycarbonate filters of 200 nm. The Ps suspension was forced to pass through the filter 20 times in order to ensure the uniform size of the particles in the solution.

4.3.2. Stability studies

The stability of Ps was tested in the 100 mM PBS + 100 mM NaCl, as well as in the Hank's balanced salt solution– 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer (HBSS–HEPES) at pH 7.4 for both $PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$ Ps solutions using DLS with a Zetasizer Nano ZS (Malvern Instruments Ltd., UK). The size of the Ps was measured before the stirring and after 5, 15, 30, 60, 90 and 120 min of stirring at the speed of 300 rpm. The measurement was done by putting 1 mL of the sample into the disposable polystyrene cuvette and the size was recorded as the average of the three measurements. When the stability was investigated in the HBSS–HEPES 1 mL of particle suspension was added into the 9 mL of the buffer, thus giving a 10× dilution, whereas for the PBS there was no dilution.

4.3.3. Cell culturing

4.3.4.1. Cell culture solutions

MCF-7 and MDA-MB-231 breast cancer cell lines were cultured in Dulbecco's modified eagle medium (DMEM) (HyClone®, Thermo Scientific, USA) and Roswell Park Memorial Institute (RPMI) 1640 medium (HyClone®, Thermo Scientific, USA), respectively, both supplemented with 10% (v/v) fetal serum bovine (FBS), 1% non-essential amino acids (NEAA), 1% L-glutamine, and 1% streptomycin-penicillin (100 IU/mL) all purchased from HyClone®, Thermo Scientific, USA, as well . Cells were cultured in 25 and 75 cm^2 flasks obtained from Nunc™, Corning Inc, USA.

DMEM, RPMI, NEAA and FBS solutions were stored at +4°C in the refrigerator while L-glutamine and streptomycin-penicilin were stored in the freezer at -22°C. The final medium used for the cells was prepared in the sterilized 500 ml Schott® bottle in aseptic conditions

and kept at +4°C but before the usage of the medium on the cells it was prewarmed up to 37°C.

The cell passaging for both cell lines was performed when the cells reached approximately 80% of the confluence in the flask with the usage of PBS-ethylendiaminetetraacetic acid (EDTA) and PBS-EDTA-trypsin solutions. First above mentioned solution was used to rinse the cells and chelate the calcium ions helping the detachment of the cells from the walls of the flask. The solution was acquired by mixing 2.5 mL of 0.1 M EDTA solution, 50 mL of 10x PBS (Gibco®, Invitrogen, USA) and sterile MiliQ-water up to 500 mL in a sterilized Schott® bottle. The solution containing trypsin was used to detach the cells during the passaging and 30 mL of the solution is prepared by using 3 mL of a 2.5% trypsin solution added to 27 mL of PBS-EDTA solution, prepared as previously described, in a 50 mL Falcon® tube.

For the preparation of 20mL volume of 0.1 M EDTA, 0.76 g of EDTA sodium salt (Sigma-Aldrich, Finland Oy) was weighed and added into 20 mL of MilliQ-water in a volumetric flask. Furthermore, the solution was filtered in the laminar flow cabinet into a sterilized Falcon® tube using a sterile syringe and a disposable 0.22 µm pore size filter. Trypsin solution for the cells was stored in the freezer at -22°C while EDTA and PBS solutions did not require any special storage treatement. The final solutions were stored at +4°C and warmed up to +37°C before applying them on the cells.

In the cell viability assay, the 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffered HBSS-HEPES (pH 7.4) was applied. To prepare 1M buffering solution, 5.6 g of HEPES sodium salt (Sigma-Aldrich, Finland Oy) was weighed and dissolved in sterile MilliQ-water in the volumetric flask. From the volumetric flask the solution was filtered under the sterile air into a sterile Falcon® tube using the syringe and previously mentioned sterile filters with the pore size of 0.22 µm. 5ml of prepared 1M buffering HEPES solution was mixed with 50 mL of 10x HBSS (Gibco®, Invitrogen, USA) and sterile MilliQ-water was added up to the volume of 500 mL. The pH was adjusted to 7.4 and the final solution was filtered in the sterilized 500 mL Schott® bottle using again a 0.22 µm pore size filter. The solution were stored at the room temperature.

All the preparations were done in an aseptic conditions using the laminar flow cabinet (Hera Safe, Heraeus Instrument GmbH, Germany). Prior to use, media and buffers were pre-warmed (if necessary) up to + 37°C in a water bath (Thermo Haake C10 Heating Circulating Water Bath, Thermo hake, USA).

4.3.4.2. Cell culture maintenance

MDA-MB-231 and MCF-7 cells were maintained at + 37°C in an incubator (HERA cell 240, Heraeus Instrument GmbH, Germany) at an atmosphere of 5% CO_2 and 95% of relative humidity.

The change of the medium for both cell lines was done, as recommended, three times per week, usually every second day. The old medium was removed from the flask by using the vacuum pump (KNF Neuberger Mini Labport Diaphgram Vacuum Pump, KNF Neuberger GmbH, Germany) and then 13 mL of fresh medium was added in case of the 75 cm^2 flask or in case of the 25 cm^2 flask we added 7 mL of fresh medium (depending on the cell line we added either DMEM or RPMI). Before the addition of the medium into the flask containing cells it had to be pre-warmed up to + 37°C in a water bath (Thermo Haake C10 Heating Circulating Water Bath, Thermo hake, USA).

Cell passaging for both cell lines used in the study was done when the 80% of cell confluence was reached in the culture flask (determined under the light microscope - Olympus IMT-2 Inverted light microscope, Olypmpus, Japan). To passage the cells we first had to remove the old medium from the flask using the vacuum pump after which we added 10 mL of PBS-EDTA solution used to chelate the calcium ions present in the cultures. After the addition of the PBS-EDTA solution we waited for 4 minutes after which we removed the solution using again the vacuum pump and added 0.5 mL of PBS-EDTA-trypsin for the detachment of the cells from the wall of the flask. The flask with the added solution was placed gently into the incubator (+ 37°C) for 5 minutes. Subsequntly, 10 mL of fresh medium was put into the flask to stop the action of trypsin. Then, the solution was mixed using the pipette by aspirating and dispensing the whole volume in order to get the homogeneous suspension of cells collected into 50 mL Falcon® tube and centrifuged at 800 rpm for 4 min (Heraeus Labofuge Centrifuges, Heraeus Instrument GmbH, Germany). After the centrifuge the cells were attached to the bottom of the Falcon® tube while the supernatant containing previously added trypsin was easily removed using the vacuum pump. Finally, the volume of fresh medium required to reach the desired concentration of cells was added in the tube and the cells were homogeneously suspended in that volume by using the pipette. The obtained cell suspension was then removed to new flask for further cell culturing.

In order to determine the correct volume to obtain the desired cell concentration , for example in case of cell viability assay, we used haemocytometer counter (Bürker counting chamber,

Marienfeld, Germany) and a light microscope (Olympus IMT-2 Inverted light microscope, Olypmpus, Japan). The counting of the cells was done using the suspension of the cells before the centrifuge. According to the procedure we took 50 µL of the suspension and placed it onto the haemocytometer counter. The volume was calculated using the following **Equation 3**:

$$\frac{\frac{cells}{squares} \times 10000 \times A}{cells\ concentration} = Volume$$

(Eq.2)

In the equation above *Volume* represents mL of fresh medium added to achieve the desired concentration of cells in the flask, *Cells* is the number of viable cells counted using the haemocytometer counter, *Squares* means the number of counted squares in the haemocytometer counter, *10000* represents the correction factor for the sample chamber volume (0.1 µL), *A* is a total volume of the suspension of cells from the pipette scale (mL) and finally *Cells concentration* represents the desired concentration of cell suspension which has to be seeded.

4.3.4. Cell viability assay

In this study we used two type of Ps with the purpose of application in anticancer therapy. Since the Ps nanosystems are meant to be applied to the human body we have to test the toxicity of the nanosystems studied and for that we used cell viability assay, in particular, the CellTiterGlo® Luminescent Cell Viability Assay (Promega Corporation, USA). The viability assay performed in this study enables us to count the number of viable cells able to maintain or recover their vitality, based on the quantification of Adenosine triphosphate (ATP) present in the culture, produced only by the metabolically active (viable) cells. The quantification of ATP is possible because of the mono-oxygenation reaction between the oxygen produced by the cells and luciferin. The reaction is catalised by the luciferase enzyme in the presence of Mg^{2+} and the ATP which leads to generation of luminescent signal. According to the protocol, a single reagent (CellTiterGlo® Reagent, Promega Corporation, USA) is directly added to the cells, causing the cell lysis and hence allowing the release of ATP, meanwhile stoping the endogenous ATPases from destroying ATP and at the same time producing luciferin, luciferase and all of the other molecules needed

to generate luminescent signal proportional to the amount of ATP present, which is directly proportional to the number of living cells in the culture (http://worldwide.promega.com/products/cell-health-and-metabolism/cell-viability-assays/).

In this cell viability assay we tested the toxicity of both Ps nanosystems in five different concentrations (25, 50, 100, 300 and 500 µg/mL). Solutions were prepared by diluting the stock solution of 1 mg/mL with the cell medium (10% FBS- DMEM in case of MCF-7 cell line or 10%FBS- RPMI in case of MDA-MB-231 cancer cell line). Meanwhile, 1% Triton-X solution (Merck KGaA, Germany) was prepared adding 10 mL of HBSS into 100 µL of Triton X-100 and the prepared solution was used in the study as a negative control.

Furthermore, CellTiter-Glo® Reagent was prepared by mixing the CellTiter-Glo® Substrate (closed bottle) with the CellTiter-Glo®Buffer (10 mL), previously equilibrated to room temperature.

MCF-7 and MDA-MB-231 breast cancer cells were suspended in the corresponding cell culture media at a concentration of 1.5 x 10^5 per mL, and approximately 1.5 x 10^4 cells per well were seeded in 96-well plates (Corning Inc. Life Sciences, USA). The cells were allowed to attach overnight at 37 C, after which the cell culture medium was removed and replaced with 100 mL of Ps nanoparticle suspensions ($PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$) at concentrations of 25, 50, 100, 300 and 500 mg/mL , with cell medium and 1% Triton X-100 as positive and negative controls, respectively. After incubating for 6 and 24 h at 37 C, medium was carefully removed without touching the cells and, subsequently, 50 µL of HBSS-HEPES (pH 7.4) and 50 µL of CellTiterGlo® Reagent were added into each well on the plate. The plates were then covered with aluminium foil and mixed using an orital shaker for 2 min to help the cell lysis and finally left at room temperature for 15 min before measuring the luminescence. The number of viable cells was determined by measuring the luminescence from the living cells using a Varioskan Flash Luminometer (Thermo Fisher Scientific Inc., USA). The results presented correspond to the average of at least three independent measurements.

The cell viability percentage was calculated using the **Equation 3**:

$$\frac{treated\ cells}{untreated\ cells}\ x\ 100 = Cell\ viability\ (\%)$$

(Eq. 4)

Where *treated cells* represent the cells in contact with the sample while *untreated cells* represent the cells in cell medium.

5. Results and Discussion

5.1. Characterization of $PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$ Ps

The characterization of both polymeric nanoparticles was done using the DLS technique for determination of size distribution, and TEM for imaging of the Ps.

5.1.1. Size measurements

In this work, we compared the size distribution of two polymeric nanoparticle systems made from the same diblock copolymer, but with a different length of hydrophilic part of the molecule. The measurements were carried out using the DLS technique (see **4.2.2.**) and after the reduction of size by the extruder with filters of the size 200 nm (see **4.2.1.**). The measurement was successfully performed on both type of empty Ps ($PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$).

As shown in the **Fig.21**, the size of the Ps nanoparticles after the extrusion with 200 nm polycarbonate filters was around 200 nm, as expected, with a good PDI below 0.2 in both cases. Sizes of both $PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$ were almost the same, while there was slightly bigger difference in the PDI, being slightly higher for the Ps with 10 hydrophilic units (**Fig. 22**). This size is in the expected range according to the work of Camblin *et al.* whose group used the same preparation method to prepare PDMS-b-PMOXA Ps and extrusion afterwards to fine-tune the size of the system. The results they gained were 205 nm for empty Ps with 14 hydrophilic units (Camblin et al. 2014). The size of the Ps nanosystem we gained in our study was around 205 nm as well while the size of the Ps with 10 hydrophilic units was slightly smaller but not significately (around 203 nm). As it was mentioned in the literature review, Ps give us a possibility to fine-tune this nanosystems in order to improve some of their characteristics (for example, size) and therefore lower the PDI like it was mentioned in the work of Jaskiewicz, Larsen *et al.* In our study we managed to uniform the size of the nanosystem with performing 20 extrusions and therefore to minimize the dispersity of the Ps suspension. While in the work of Camblin *et al.* the measured PDI was 0.075 for the nanosystem with 14 hydrophilic units, in our study we managed to obtaun the PDI not higher then 0.19 for the same nanosystem while for the $PDMS_{65}$-b-$PMOXA_{10}$ the PDI was 0.05.

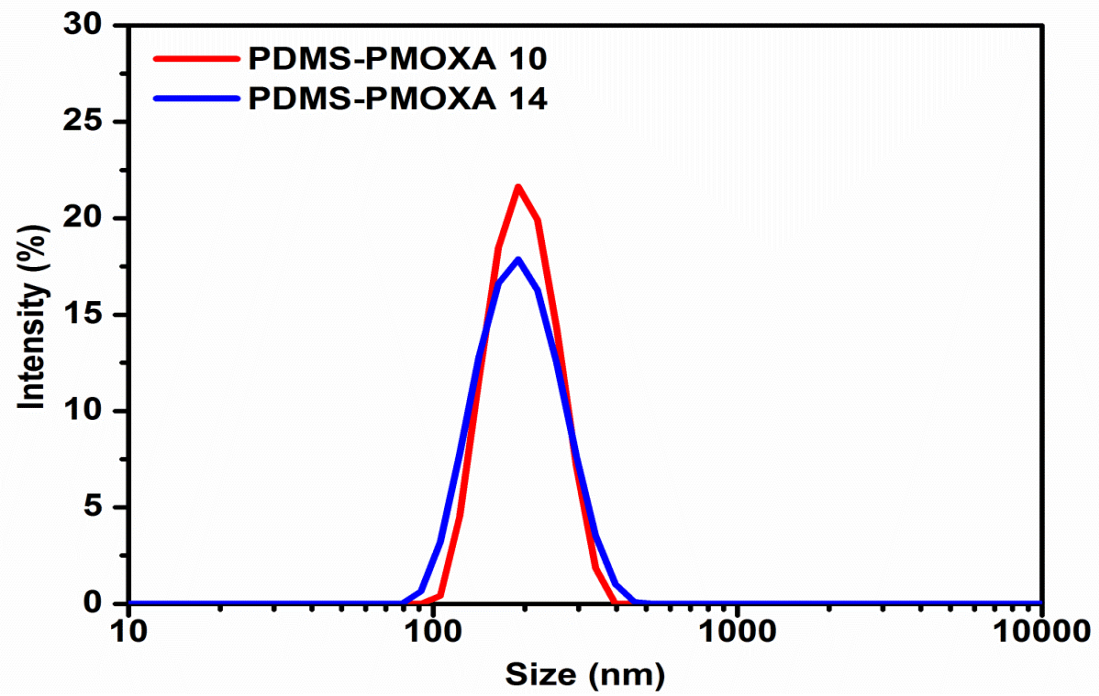


Figure 21. *Intensity based size distribution of $PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$ Ps after the extrusion with 200 nm filters.*

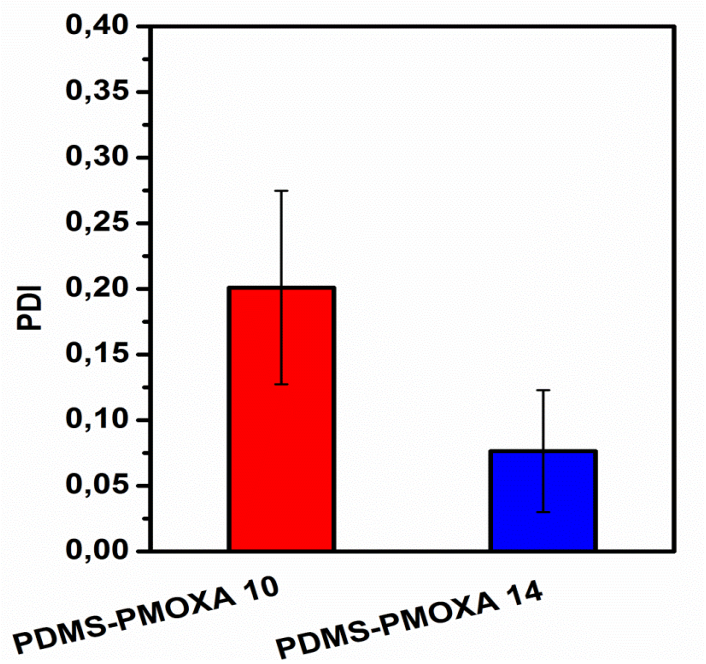


Figure 22. *Polydispersity index for two Ps nanosystems measured.*

5.1.2. Shape and morphology measurements

The shape, morphology and the size of the Ps were more closely examined using TEM imaging. **Fig.23** shows the empty $PDMS_{65}$-b-$PMOXA_{10}$ Ps in two different scales, while **Fig.24** shows also the $PDMS_{65}$-b-$PMOXA_{14}$ Ps in two different magnifications.

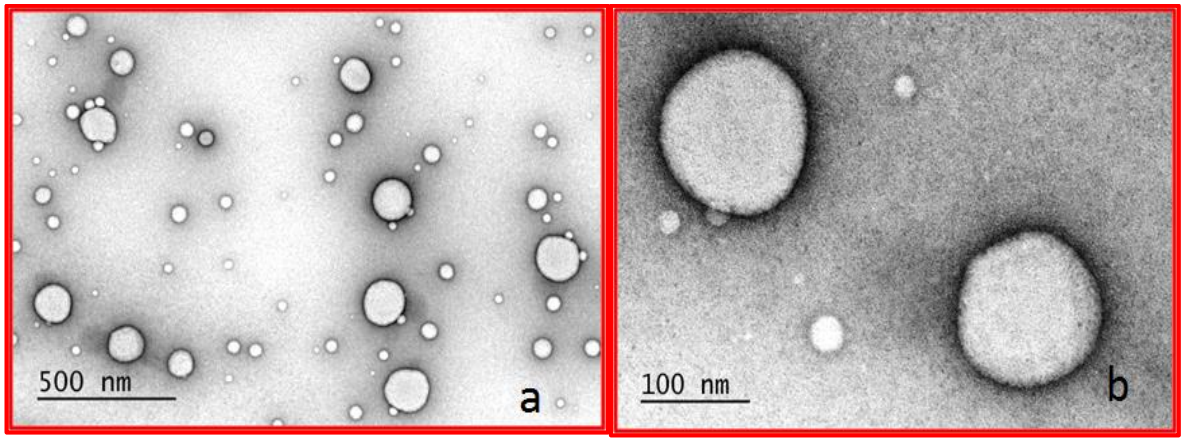


Figure 23. *TEM images of empty $PDMS_{65}$-b-$PMOXA_{10}$ Ps prepared using the film rehydration technique combined with the extrusion for uniformation of the size. Scale bars used are 500 nm and 100 nm.*

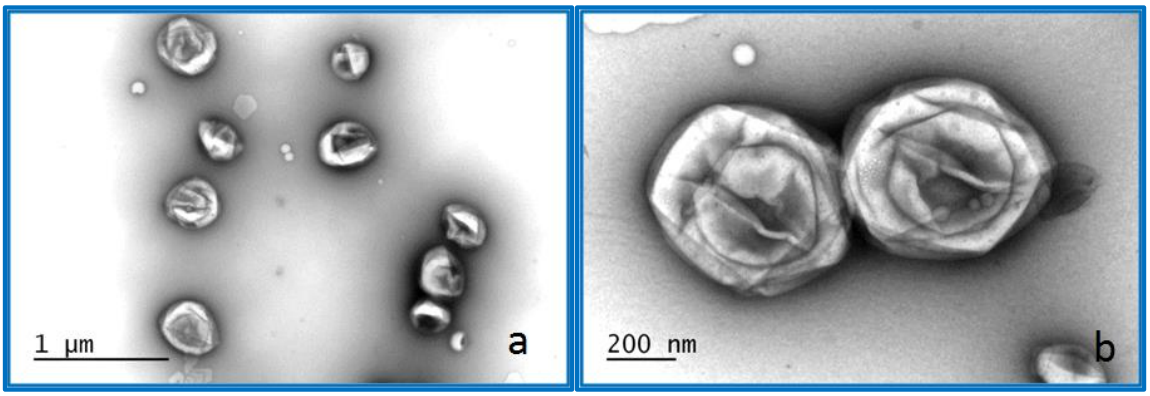


Figure 24.*TEM images of $PDMS_{65}$-b-$PMOXA_{14}$ Ps prepared using the film rehydration technique and extrusion through 200 nm filters. Scale bars used are 1µm and 200 nm.*

The part of the particle characterization carried out by the means of the DLS technique has given good results regarding the PDI of both Ps nanosystems (**Fig.22**), which was now

confirmed using the TEM imaging technique. We took images of the Ps nanosystems after the extrusion procedure using higher (**Fig.23a** and **Fig.24a**) and lower (**Fig. 23b** and **Fig. 24b**) magnification. Lower magnification image for $PDMS_{65}$-b-$PMOXA_{10}$ Ps nanosystems confirmed the results from the DLS measurements and showed that the size of the particles in the suspension was quite uniform while **Fig.23b**, with the scale of 100 nm showed that the size of the Ps, as well as their spherical shape, met the expectations of the research. Light colour of the system itself can be described as a result of the low density of the Ps. Furthermore, for the $PDMS_{65}$-b-$PMOXA_{14}$ Ps, it was evident that the shape and the membrane structure were slightly different than those of the $PDMS_{65}$-b-$PMOXA_{10}$ Ps, which was probably as a result of the collapse of the membrane due the longer hydrophilic part. According to the scale bars, $PDMS_{65}$-b-$PMOXA_{14}$ Ps on these images are slightly bigger than the expected size (ca. 200 nm), which can be explained by the fact that the usage of TEM imaging was to focus more on the shape of the Ps nanosystems rather than on their size, thus the extrusion process might have been skipped with this particular sample. A recent report on self-assembly of a similar molecular composition of PDMS-PMOXA into vesicular structures supports our findings (Egli, Martin G Nussbaumer, et al. 2011) and, again, group of Camblin *et al.* has obtained similar results regarding the TEM imaging of $PDMS_{65}$-b-$PMOXA_{14}$ (Camblin et al. 2014).

5.2. Stability studies

In order to develop and characterize a nanosystem which would have successful application in nanotherapy, we have to test the stability of the Ps in different conditions (buffers and time-points). In this particular work, we tested the stability of PDMS-PMOXA Ps in the HBSS–HEPES at pH 7.4 and in PBS+NaCl. The size of the particles was measured using the DLS technique in different time-points through the time frame of 2h, while the sample was constantly being mixed. The values obtained are shown in **Fig. 25** (for the PBS+NaCl) and **Fig.26** (for the HBSS–HEPES, pH 7.4).

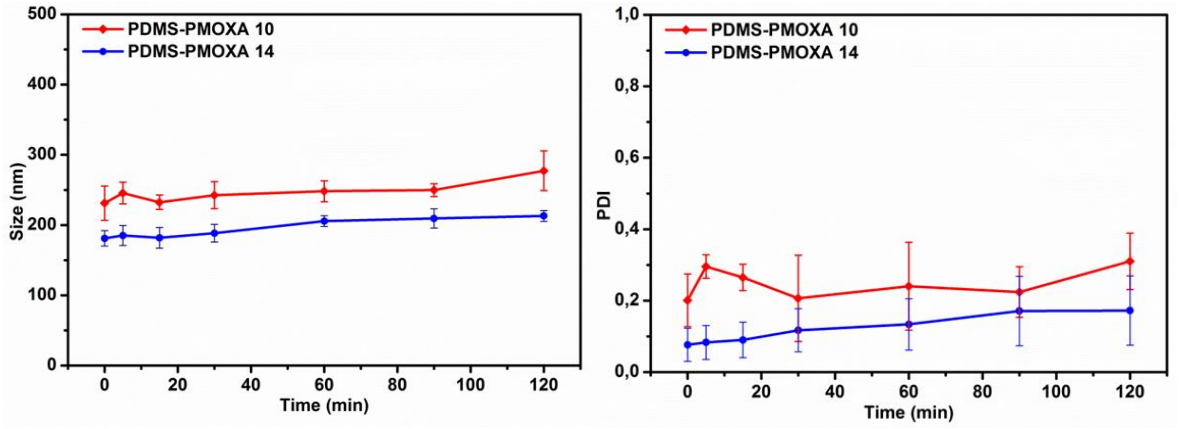


Figure 25. *Influence of the 100 mM PBS+ 100 mMNaCl(pH 7.4) on the Ps size (left) and PDI (right) of the PDMS-PMOXA Ps. Stability studies were performed using the DLS technique (Zetasizer Nano) for 2h at the room temperature. Values denote the mean ±s.d. (n ≥ 3).*

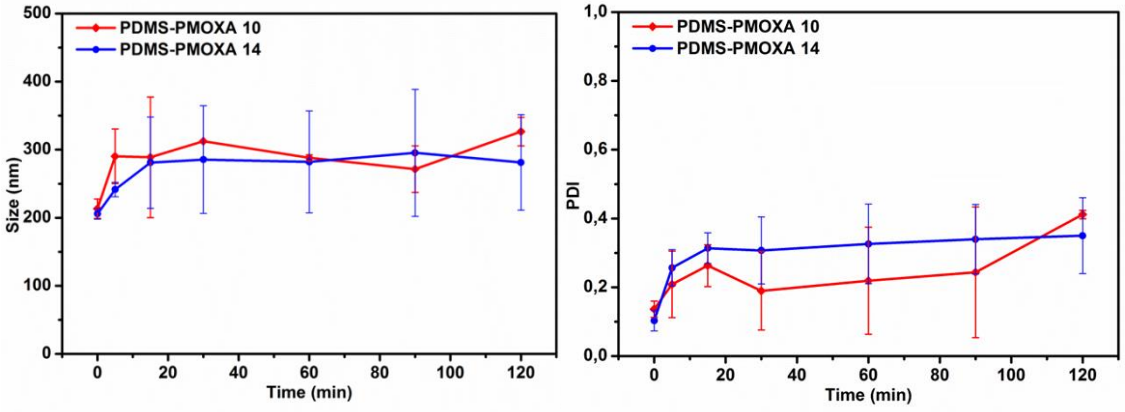


Figure 26. *Influence of the HBSS–HEPES (pH 7.4) on the stability of the tested nanosystems over time. Influence on the size distribution (left) and PDI (right). Stability studies were performed using the DLS technique (Zetasizer Nano) for 2h at the room temperature. Values denote the mean ±s.d. (n ≥ 3).*

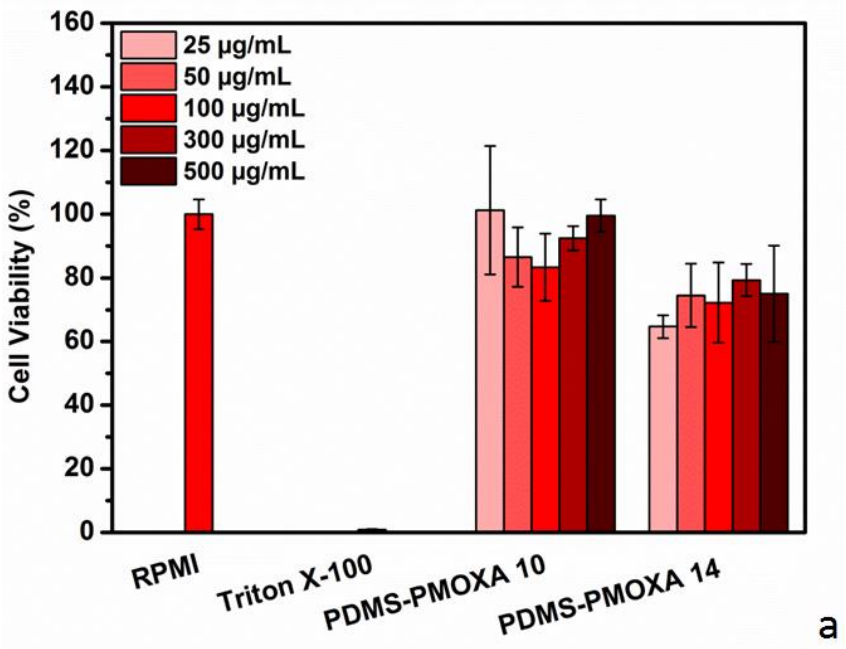
The results conducted using the DLS method showed that the size of the $PDMS_{65}$-b-$PMOXA_{10}$ Ps was ca.231 nm after the extrusion and at the beginning of the experiment, while at the end (after 2 h) the size was ca.277 nm (**Fig. 25a**). Furthermore, the PDI changes were not significant during the measurement period (from 0.2 at the beginning to 0.3 after 2h of measurement). As for the $PDMS_{65}$-b-$PMOXA_{14}$ Ps, the starting size measured was ca.180 nm up to 213 nm by the end of the experiment, while the PDI changed for 0.1, as in the case of the first Ps nanosystem.

In the case of the HBSS-HEPES (pH 7.4) stability, the size of both the $PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$ Ps, there was an increase in the size at the beginning of the

experiment, while later through the experiment the size of the nanosystems did not change drastically. As for the PDI, it also changed at the beginning of the experiment, and stayed mostly the same throughout the rest of the experiment with the exception of $PDMS_{65}$-b-$PMOXA_{14}$ Ps nanosystems between the last two time-points, where the increase in the PDI was again high.

5.3. Cell viability assay

Considering the fact that the purpose of the tested nanosystems is to apply them in cancer therapy treatment, we carried out some studies to evaluate the cytotoxicity of the Ps itself on the chosen cancer cell lines (MCF-7 and MDA-MB-231 breast cancer cells). The procedure and the method itself was described in **4.3.5.**, where in our studies we used CellTiter-Glo® Luminescent Cell Viability Assay (Promega Corporation, USA) based on the quantification of the ATP (Adenosine triphosphate) in the cell culture, produced by the metabolically active breast cancer cells.



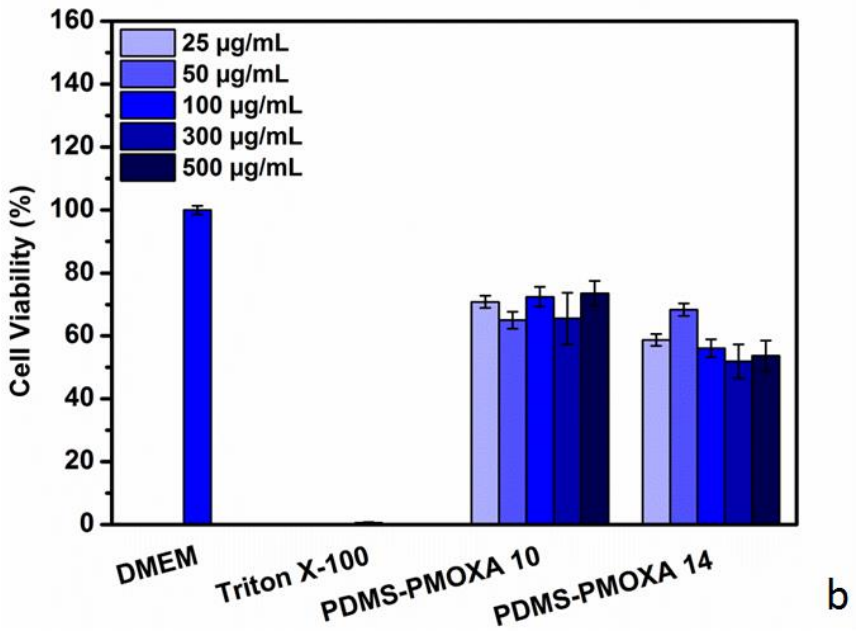


Figure 27. *Cell viability values (%) of MDA-MB-231 (a) and MCF-7 (b) obtained using the luminescent assay after 6 h incubation time with empty PDMS-PMOXA Ps (both types used in the study) at fivedifferent concentrations. 10% HBSS and Triton® X-100 (1%) were used as negative and positive controls, respectively, in this study. Error bars represent mean ±s.d. (n>3).*

After incubation of 6 h at 37°C, we measured the luminescence of the two breast cancer cell lines and determined the cell viability, as shown in the **Fig.27**. In both cases, we tested five different concentrations of empty Ps (25, 50, 100, 300 and 500 µg/mL), as well as the both types of nanosystems ($PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$). The results showed that the cell viability was mainly above 60% for both cell lines and with both type of Ps. In the case of the MDA-MB-231 cell line, it can be seen that the cell viability was 10–20% better when tested with $PDMS_{65}$-b-$PMOXA_{10}$ than in the case of the other Ps. However, the viability percentage was always above 60% (even with the higher concentration of the polymer tested), thus we can conclude that the Ps tested are non-toxic at the concentrations and for both the cell lines tested in 6 h time period.

Furthermore, in case of the MCF-7 breast cancer cell, $PDMS_{65}$-b-$PMOXA_{10}$ can be described as non-toxic (viability percentage above 60%, even with the highest polymer concentration tested), while the $PDMS_{65}$-b-$PMOXA_{14}$ showed cell viability percentages below the value of 60% for the higher concentrations tested (100, 300, and 500 µg/mL), which means that after 6 h incubation these Ps nanosystems have a toxic influence on the MCF-7 breast cancer cells.

In this study (*in vitro* part of the study), we also tested the MCF-7 cell viability after 24 h incubation period and the values obtained are shown in **Fig.28**. $PDMS_{65}$-b-$PMOXA_{10}$ Ps at the lower concentrations (25, 50, and 100 mg/mL) of $PDMS_{65}$-b-$PMOXA_{14}$ can be described as non-toxic for the cell line tested, because the cell viability after 24h was always ca. 90–100%, while for the concentrations 300 and 500 mg/mL in case of $PDMS_{65}$-b-$PMOXA_{14}$, the cell viability was ca. 60%, which can still be described as non-toxic.

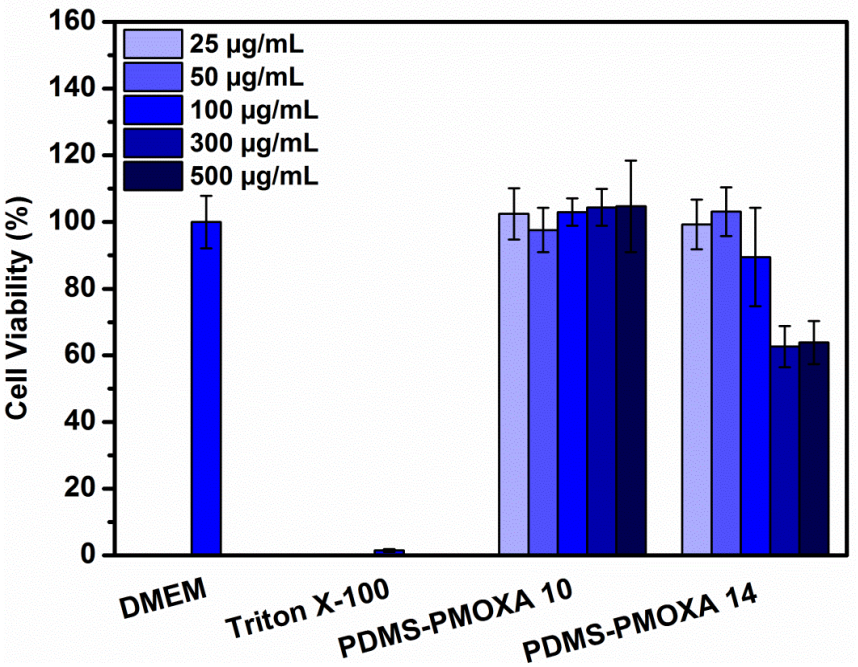


Figure 28. *MCF-7 cell viability values (%) obtained using the luminescent assay after 24 h incubation time with empty PDMS-PMOXA Ps ($PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$) at five different concentrations. 10% HBSS and Triton® X-100 (1%) were used as negative and positive controls, respectively, in this study. Error bars represent mean ±s.d. (n>3).*

6. Conclusions

The results in this study, and presented through the thesis, led to the following main conclusions:

- DLS measurements of the size and PDI for both Ps nanosystems ($PDMS_{65}$-b-$PMOXA_{10}$ and $PDMS_{65}$-b-$PMOXA_{14}$) showed that the particles were prepared successfully using the film rehydration technique, resulting in spherical nanosized particles with a good size homogeneity. The results obtained meet the expectations of the study and find support in the previously conducted research.

- TEM measurements confirmed the spherical shape of the particles and emphasised the slight difference in the appearance of the membrane of those two nanosystems.

- The results of the stability studies showed that the PDMS-b-PMOXA Ps were quite stable in the tested buffers within the timeframe tested, and the pH tested in the study. However, with the HBSS–HEPES buffer (pH 7.4) there were some instabilities within the first few time-points, after which the size and the PDI of the Ps was again stable.

- Considering the cell viability studies on both cancer cell lines tested for both Ps nanosystems, we can say that the particles were non-toxic for all concentrations tested for the MDA-MB-231 cells after 6h, while they showed somewhat higher cytotoxicity for the MCF-7 breast cancer cells after the period of 6h, especially for the higher concentrations. In the case of the 24 h study on the MCF-7 cells, Ps appeared to be non-toxic for the cells, with exception of two highest concentrations of the $PDMS_{65}$-b-$PMOXA_{14}$ Ps.

Overall, the results stated above prove that PDMS-b-PMOXA Ps have a great potential to serve as carriers for cancer nanotherapy, but the system needs further improvements and more tests concerning the influence of the Ps on the cells and their stability in the physiological media.

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8. Summary

Breast cancer, as well as cancer in general, is one of the most therapeutically challenging fields in a matter of drug treatment. Since the conventional anticancer drugs encounter many obstacles such as poor specificity, high toxicity and susceptibility to induce drug resistance there is a need for new solutions in anticancer treatment. Nanotechnology offers a wide range of possibilities among which are polymersomes as potential drug delivery systems.

The aim of this study was to prepare and characterize polymersomes (Ps) made out of Poly(dimethylsiloxane)-block-poly(2-methyloxazoline) (PDMS-b-PMOXA) diblock copolymer, to test their stability and finally their behaviour towards two different breast cancer cell lines. We also conducted research using two different nanosystems, differing in the number of hydrophilic PMOXA units.

The results of the study included size and polydispersity index (PDI) of the nanosystems determined by using dynamic light scattering (DLS). Furthermore, imaging of the particles was done using transmission electron microscopy (TEM) while the stability was tested in Hank's balanced salt solution– 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer HBSS–HEPES at pH 7.4 and in Phosphate buffer saline (PBS) + sodium chloride (NaCl). *In vitro* experiments, in particular cell viability assay, was done on MDA-MB-231 and MCF-7 breast cancer cell lines.

9. Sažetak

UVOD

Područje medicine koje danas nailazi na najveći broj prepreka kada se radi o pozitivnim ishodima liječenja je svakako antitumorska terapija. Konvencionalni lijekovi na tržištu pokazuju mnoge nedostatke kao što su, primjerice, neselektivnost i prebrza eliminacija putem retikuloendotelnog sustava. Upravo iz tog razloga nanotehnologija nastoji ponuditi nova rješenja tih prepreka u vidu nanosustava za dostavu lijekova među kojima se nalaze i polimersomi, a koji bi trebali poboljšati broj pozitivnih terapijskih ishoda u antitumorskoj terapiji zahvaljujući vlastitim svojstvima.

OBRAZLOŽENJE TEME

Svrha ovog rada bila je pripremiti i provesti karakterizaciju polimersoma, potencijalnih nanosustava za dostavu lijekova, građenih od poli(dimetil)siloksan-blok-poli(2-metiloksazolin) (PDMS-b-PMOXA) diblok kopolimera, s tim da su pripremljena dva sustava, jedan od 10 a drugi od 14 hidrofilnih poli(2-metiloksazolin) jedinica. Nadalje, ispitali smo i stabilnost sustava u određenom vremenskom periodu u dva različita pufera te smo se dotakli i *in vitro* pokusa na dvije različite kulture stanica raka dojke gdje smo ispitivali toksičnost ovih nanosustava od interesa.

MATERIJALI I METODE

Za pripremu polimersoma iz diblok kopolimera PDMS-b-PMOXA koristili smo metodu rehidracije polimernog filma pri čemu smo pripremali dvije vrste polimersoma (sa 10 i 14 hidrofilnih PMOXA jedinica). Za modificiranje veličine čestica koristili smo metodu ekstruzije kroz 200 nanometarske polikarbonatne filtere (20 puta) a utvrđivanje dobivene veličine proveli smo koristeći Da bismo dobili bolju predodžbu o stvarnoj slici nanosustava primjenili smo transmisijsku elektronsku mikroskopiju kojoj je prethodilo tretiranje uzorka uranil acetatom. Stabilnost smo ispitivali u vremenskom periodu od 2 sata, koristeći metodu dinamičkog zakretanja svjetlosti (DLS) za mjerenje veličine čestica u određenim vremenskim točkama. Ispitivanje stabilsnosti smo radili, uz konstantno mješanje, u dva različita pufera, fosfatnom puferu uz dodatak natrijevog klorida (PBS+NaCl) i puferu balansiranom Hankovim solima (engl. Hank's balanced Salt Solution, HBSS pufer) uz dodatak hidroksietil-piperazineetan-sulfonske kiseline (engl. hydroxyethyl-piperazineethane-sulfonic acid, Hepes). *In vitro* dio ove studije podrazumijeva ispitivanja toksičnosti praznih polimersoma na dvije

različite kulture stanica raka dojke, MDA-MB-231 i MCF-7 u 5 različitih koncentracija (25, 50, 100, 300 i 500 μg/ml) a rezultati su dobiveni kalkulacijama nakon mjerenja luminescencije uzoraka.

REZULTATI I RASPRAVA

Korištenjem prethodno spomenute metode rehidracije polimernog filma i nakon modulacije veličine čestica ekstruzijom kroz filter od 200 nm dobili smo veličinu čestica oko 200 nm, točnije oko 205 nm u slučaju $PDMS_{65}$-b-$PMOXA_{14}$ te oko 203.5 nm u slučaju $PDMS_{65}$-b-$PMOXA_{10}$. Takav rezultat, tj. veličina nanosustava odgovara rezultatima već provedenih studija koje su koristile istu metodu priprave. Indeks polidisperznosti (PDI), dobiven također metodom DLS-a, bio je poprilično nizak i također sličan kao u postojećim studijama na koje smo se referirali. Da čestice zaista imaju vezikularni izgled i uniformiranu veličinu potvrdili smo koristeći TEM. Uzorak $PDMS_{65}$-b-$PMOXA_{10}$ pokazao je istu veličinu kao što je izmjerena DLS-om te uniformiranost u veličini u čitavom uzorku promatranom pod mikroskopom. U slučaju $PDMS_{65}$-b-$PMOXA_{14}$ pokazalo se svojevrsno "urušavanje" membrane, moguće zbog povećanja duljine hidrofilnog dijela. Također, kod većeg povećanja, u slučaju drugog nanosustava, vidi se nepodudarnost u veličini izmjerenoj DLS-om i onoj koja se može isčitati prema skali na slici. Razlog može biti što konkretan uzorak vizualiziran TEMom nije prošao potpunu ekstruziju pa je veličina čestica ostala oko 400 nm. Unatoč tome oblik čestica potvrđuje literarne navode i očekivanja i može se dobro vidjeti membrana građena od diblok kopolimera. Kod ispitivanja stabilnosti sustava u slučaju HBSS-Hepes pufera veličina čestica je porasla nakon prvog mjerenja nakon čega je ostala konstantna, uz manje varijacije, tijekom 2 sata dok je u slučaju fosfatnog pufera veličina čestica bila gotovo nepromijenjena kroz čitavo mjerenje. Indeks polidisperznosti je također pokazao varijacije na samom početku nakon čega se pokazao relativno stabilnim. *In vitro* testovi toksičnosti na staničnim linijama pokazuju različite rezultate za dvije stanične kulture te su rađeni nakon perioda od 6h te 24 h za MCF-7 staničnu liniju. U slučaju MDA-MB-231 stanične linije toksičnost $PDMS_{65}$-b-$PMOXA_{10}$ se pokazala 10-20% manjom nego u slučaju $PDMS_{65}$-b-$PMOXA_{14}$ iako je u oba slučaja i kod svih koncentracija postotak živih stanica bio iznad 70%. Kada se radi o MCF-7 staničnoj liniji pokazalo se kako su oba nanosustava otprilike jednako (ne)toksična, s time da je postotak živih stanica bio nešto manji nego u slučaju MDA-MB-231 iako je kod svake koncentracije bio iznad 60%. Prethodno spomenuti rezultati odnose se na vremenski period od 6h dok smo za vrijeme od 24 sata istraživanje proveli na MCF-7

staničnoj liniji. Nakon 24 sata inkubacije stanica sa suspenzijom praznih polimersoma ($PDMS_{65}$-b-$PMOXA_{10}$ i $PDMS_{65}$-b-$PMOXA_{14}$) u 5 različitih koncentracija postotak živih stanica bio je oko 100% uz iznimku dviju najvećih koncentracija za sustav sa većim brojem hidrofilnih jedinica gdje je postotak živih stanica nakon 24 sata bio oko 60%.

ZAKLJUČCI

Ovom smo studijom pokazali uz koje parametre je moguća uspješna priprema polimersoma građenih od PDMS-b-PMOXA diblok kopolimera te da je nakon ekstruzije veličina čestica oko 200 nm, kako je i očekivano. Unatoč razlici u duljini hidrofilnog dijela kopolimera nema znatne razlike u veličini nanočestica izmjerenoj DLS metodom. TEM metoda je, međutim, pokazala vizualnu razliku čestica u smislu izgleda njihove membrane koja je u slučaju $PDMS_{65}$-b-$PMOXA_{14}$ "kolabirala" dok se u slučaju $PDMS_{65}$-b-$PMOXA_{10}$ vide pravilne sferične strukture sa tankim membranama građenim od dvosloja diblok kopolimera. Stabilnost nanosustava u oba pufera kroz period od 2 sata se pokazala relativno dobrom uz početno povećanje, nakon čega je krivulja koja pokazuje veličinu u odnosu na vrijeme relativno nepromijenjena. Indeks polidisperznosti (PDI) je pokazao isti trend promjene (nakon početnog povećanja krivulja ovisnosti PDI o vremenu je relativno nepromijenjena). Treba naglasiti i kako je veća stabilnost zabilježena u slučaju fosfatnog pufera gdje su odstupanja tijekom pokusa zaista minimalna. *In vitro* ispitivanja toksičnosti pokazala su da nakon 6 h inkubacije obe stanične linije pokazuju smanjenje broja živih stanica. Smanjenje je veće u slučaju $PDMS_{65}$-b-$PMOXA_{14}$ te su se suspenzije polimersoma pokazale toksičnijima za MCF-7 staničnu liniju. Nakon 24 h inkubacije izmjerena je luminescencija uzoraka te je utvrđeno kako je postotak živih stanica ostao nepromijenjen osim u slučaju dvije najveće koncentracije $PDMS_{65}$-b-$PMOXA_{14}$. Ovi rezultati *in vitro* pokusa toksičnosti svakako zahtjevaju daljnja ispitivanja s obzirom da bi trend porasta toksičnosti u odnosu na duljinu vremena inkubacije trebao biti suprotan u slučaju MCF-7 stanične linije. Nadalje, za MDA-MB-231 staničnu liniju nismo vremenski bili u mogućnosti provesti ispitivanja toksičnosti za vremenski period od 24 sata stoga i to ostaje potrebno detaljnije istražiti.

U svakom slučaju, ovi polimerni nanosustavi pokazali su veliki potencijal za primjenu u antikancerogenoj terapiji, ali sigurno je da zahtjevaju još veliki broj ispitivanja njihovih svojstava i modulacije samog sustava.

Basic documentation card

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Diploma thesis

PREPARATION AND CHARACTERIZATION OF POLYMERSOMES FOR APPLICATION IN CANCER THERAPY

Nikolina Dakić

SUMMARY

Since the conventional anticancer drugs encounter many obstacles, nanotechnology offers a wide range of new possibilities among which are polymersomes (Ps) as potential drug delivery systems. The aim of this study was to prepare and characterize Ps made out of poly(dimethylsiloxane)-block-poly(2-methyloxazoline) (PDMS-b-PMOXA) diblock copolymer, to test their stability and finally their behaviour towards two different breast cancer cell lines.

The results of the study included size and polydispersity index (PDI) of the nanosystems determined by using dynamic light scattering (DLS). Furthermore, imaging of the particles was done using transmission electron microscopy (TEM) while the stability was tested in two different buffers relevant for the study. *In vitro* experiments, in particular cell viability assay, were done on MDA-MB-231 and MCF-7 breast cancer cell lines.

The thesis is deposited in the Central Library of the University of Zagreb Faculty of Pharmacy and Biochemistry.

Thesis includes: Cancer, nanosystems, polymersomes, PDMS-b-PMOXA, stability, biocompatibility

Keywords: 65 pages, 27 figures, 1 table and 136 references. Original is in English language.

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The thesis was accepted: June 2016.

Temeljna dokumentacijska kartica

Sveučilište u Zagrebu
Farmaceutsko-biokemijski fakultet
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PRIPREMA I KARAKTERIZACIJA POLIMERSOMA ZA ANTITUMORSKU TERAPIJU

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SAŽETAK

Nanotehnologija može značajno pridonijeti razvoju antitumorskih lijekova. Uklapanjem antitumorskih lijekova u odgovarajuće terapijske nanosustave kao što su polimerosomi može se povećati njihova djelotvornost i/ili sigurnost. Cilj je ovog diplomskog rada bio priprema i karakterizacija polimerosoma građenih od poli(dimetilsiloksan)-blok-poli(2-metiloksazolin) (PDMS-b-PMOXA) diblok kopolimera, ispitivanje njihove stabilnosti i biokompatibilnosti.

Veličina čestica te indeks polidisperznosti (PDI) određeni su metodom raspršenja laserske svjetlosti, a oblik i morfologija polimerosoma metodom transmisijske elektronske mikroskopije. Stabilnost polimerosoma ispitana jr koristeći dva različita pufera. Od *in vitro* pokusa provedeo je ispitivanje biokompatibilnosti koristeći MDA-MB231 i MCF-7 stanične linije.

Rad je pohranjen u Središnjoj knjižnici Sveučilišta u Zagrebu Farmaceutsko-biokemijskog fakulteta.

Rad sadrži: 65 stranica, 27 grafičkih prikaza, 1 tablica i 136 literaturnih navoda. Izvornik je na engleskom jeziku.

Ključne riječi: Rak, terapijski nanosustavi, polimersomi, PDMS-b-PMOXA, stabilnost, biokompatibilnost

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Rad prihvaćen: lipanj 2016.