UNIVERSIDADE DA BEIRA INTERIOR

Faculdade de Ciências da Saúde

Development of intelligent vehicles for delivery of anti-tumoral drugs

Helena Carina Canhoto de Andrade Pissarra

Dissertação para obtenção do Grau de Mestre em

Ciências Biomédicas

(2º ciclo de estudos)

Orientador: Prof. Doutor Ilídio Joaquim Sobreira Correia

Covilhã, outubro de 2014

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“Nobody said it was easy
It's such a shame for us to part
Nobody said it was easy
No one ever said it would be this hard
Oh, take me back to the start”

Coldplay – Scientist

Acknowledgments

At the end of this master thesis, there are a lot of people that I would like thank, by the support and courage. One way or another, they all contributed for the realization of this work.

First, I would like to thank to my supervisor Professor Ilídio Correia for the unconditional support, guidance, patience, and help during my master's degree. His wise advices help me to grow as an investigator and person. I also feel the need to acknowledge him for having believed in my capabilities and for making me believe in myself. It has been a privilege to work with him.

I also would like to thank to Sónia Miguel, MSc for the help in acquiring scanning electron microscopy and Confocal Laser Scanning Microscopy images of the produced nanoparticles.

I would like to thank to all my group colleagues for their support, knowledge sharing, support and courage that they gave me in the bad moments, but also for all the moments of fun and happiness that we had together, without a doubt, I have been blessed with a friendly and cheerful group.

To my closest friends that have always accompanied me. They always believed in me and they helped me to always have a smile in my face. Thank you all from the bottom of my heart.

Lastly, and most importantly, I would also like to express my sincere thanks to my family, particularly to parents and my brothers. Thanks for the unconditional support and for always believing in me when I doubted about myself. To them I dedicate this thesis.

To all of you… a thank you…

Abstract

The female reproductive system is essential for the maintenance of the human species. However, it can be affected by several diseases such as ovarian cysts or cancer, pelvic infection, cervical cancer, cervical cysts and polyps, endometrial cancer, endometriosis, endometrial polyps, hyperplasia and fibroids, tipped uterus, prolapsed uterus and breast cancer, which can lead to infertility problems or patient death. Cervical cancer remains as one of the leading causes of women's death worldwide, and it does not present any signs or symptoms. It has been intimately related whit Human Papilloma Virus duo to the genetic mutations that this virus can cause, when it enters in the cervical cells. For the treatment of cervical cancer surgery, radiotherapy or chemotherapy, alone or combined can be used. Chemotherapy use different drugs as therapeutic agents and the most used in cervical cancer are cisplatin, ifosfamide, paclitaxel, irinotecan and gemcitabine. These drugs can be administered either orally or intravenously. The lack of specificity of these chemotherapeutics agents avoids their accumulation at the target tissue and allows its spreading along the body causing side effects in the healthy tissues. In an attempt to minimize side effects and improve the efficacy of the currently available treatments, new therapeutic approaches are currently being developed. New drug delivery systems are currently being developed to allow the transport of a therapeutic substance through the body and a sustained drug release at the target tissue. Among the different drug delivery systems, nanoparticles are the most used due to their higher capacity to be internalizing by cells and release the drug that they transport inside them. There are several types of nanoparticles that belong to two main groups: organic and inorganic. The magnetic nanoparticles constitute the larger group of inorganic nanoparticles, whereas liposomes, dendrimers and polymeric nanoparticles belong to the organic group.

The objective of the present work was to produce polymeric poly-ε-caprolactone/poly-methyl methacrylate nanoparticles with a sustained drug release to be used for cancer treatment. The produced nanoparticles were characterized by Scanning Electron Microscopy, Transmission Electron Microscopy, Dynamic Light Scattering and Fourier Transform Infrared spectroscopy. Moreover, Cisplatin was used as model drug for the characterization of the loading and release profiles of these nanocarriers, as well as to evaluate the therapeutic potential of the produced system. In addition the *in vitro* studies were performed to evaluate the cellular internalization and biocompatibility of the produced nanocarriers, and also characterize the release profile and therapeutic efficacy of the loaded drug to cancer cells. The results obtained, suggest that the produce nanosystem has suitable properties to be used as a drug delivery system for cancer therapy.

Keywords:

Cervical cancer, Drug delivery systems, Nanoparticles, Poly-ε-caprolactone, Poly-methyl methacrylate.

Resumo

O sistema reprodutor feminino é essencial para garantir a continuidade da espécie humana. No entanto este está sujeito a várias doenças tais como quistos ou cancro nos ovários, infeções pélvicas, cancro, quistos e pólipos no colo do útero, cancro do endométrio, endometriose, pólipos no endométrio, hiperplasia, miomas e cancro da mama, que podem levar a problemas de infertilidade ou mesmo à morte das doentes. O cancro do colo do útero continua a ser uma das principais causas de morte entre mulheres em todo o mundo, e não apresenta sinais ou sintomas. Esta doença está intimamente relacionada com o Papiloma Vírus Humano, devido ao poder mutagénico que este vírus tem após infetar as células do colo do útero. No tratamento do cancro do colo do útero pode ser usada cirurgia, radioterapia e quimioterapia, sozinhas ou combinadas. A quimioterapia usa fármacos como agentes terapêuticos, sendo os mais usados a cisplatina, ifosfamida, paclitaxel, irinotecano e gencitabina, que podem ser administrados quer por via oral ou intravenosa. A falta de especificidade dos fármacos usados faz com que eles não se acumulem apenas no tecido alvo mas também se espalhem pelo corpo afetando os em tecidos saudáveis e destruindo órgãos vitais. Na tentativa de minimizar os efeitos colaterais e melhorar os resultados obtidos com as terapias disponíveis, tem sido desenvolvidas novas abordagens que assentam no desenvolvimento de sistemas de entrega de fármacos. Estas formulações/dispositivos podem transportar uma substância terapêutica através do corpo e promover a sua libertação durante o período de tempo necessário e no órgão alvo. As nanopartículas são os sistemas mais utilizados devido à grande capacidade para entrar nas células e libertar o fármaco no seu interior. Na atualidade existem vários tipos de nanopartículas, sendo divididas em dois grandes grupos: orgânicas e inorgânicas. Dentro das nanopartículas inorgânicas podemos encontrar as nanopartículas magnéticas. No que se refere as nanopartículas orgânicas as mais estudadas são os lipossomas, dendrímeros, e as nanopartículas poliméricas.

O objetivo do presente trabalho foi produzir nanopartículas poliméricas de poli-ε-caprolactona/poli-metil metacrilato que permitissem o transporte de fármacos e a sua libertação em células cancerígenas para serem utilizadas no tratamento do cancro. As nanopartículas produzidas foram caracterizadas por microscopia eletrónica de varrimento, microscopia eletrónica de transmissão, dispersão dinâmica de luz e espectroscopia de infravermelho. Estudos *in vitro* foram realizados para avaliar a biocompatibilidade e a capacidade das nanopartículas serem internalizadas pelas células cancerígenas, assim como avaliar a eficiência de encapsulação do fármaco no interior da nanopartículas e a sua libertação, usando Cisplatina como fármaco modelo, de forma a permitir caracterizar a eficiência terapêutica do sistema apresentado. Os resultados obtidos sugerem que as nanopartículas produzidas constituem bons candidatos para futuras aplicações de entrega de fármacos à nano-escala para terapia do cancro.

Palavras-chave

Cancro do colo do útero, Nanopartículas, Poli-ε-caprolactona, Poli-metil metacrilato, Sistema de entrega de fármacos.

Resumo Alargado

O sistema reprodutor feminino é essencial para garantir a continuidade da espécie humana. Ele é responsável pela receção do óvulo para possível fertilização, suportar o desenvolvimento do feto e permitir o seu nascimento. Este sistema tem ainda a capacidade de produzir certas hormonas, como o estrogénio e progesterona, que influenciam não só o sistema reprodutor feminino mas também outros órgãos. Da sua composição fazem parte os ovários e ovidutos, o útero, a vagina, a genitália externa e as glândulas mamárias. Tal como todos os sistemas do corpo, este está sujeito a diferentes desequilíbrios dos quais podem resultar doenças como quistos ou cancro nos ovários, infeções pélvicas, cancro, quistos e pólipos no colo do útero, cancro do endométrio, endometriose, pólipos no endométrio, hiperplasia, miomas e cancro da mama. O cancro do colo do útero continua a ser uma das principais causas de morte de mulheres em todo o mundo. Este tipo de cancro não apresenta sinais ou sintomas e tem sido intimamente relacionada com o Papiloma Vírus Humano (HPV). No entanto, existem outros fatores de risco como sejam o tabagismo, outras doenças sexualmente transmissíveis, o uso prolongado de contracetivos orais e alterações genéticas. A prevenção desta doença pode ser feita através de vacinação contra o vírus HPV e pela redução da exposição aos outros fatores de risco. A quimioterapia é uma das terapias mais usadas para tratar esta doença. A cisplatina, ifosfamida, paclitaxel, irinotecano e gencitabina são exemplos dos fármacos mais usados no tratamento deste tipo de cancro individualmente ou de uma forma combinada. No entanto, sendo uma terapia sistémica envolve todo o corpo e tem uma elevada toxicidade associada. Esta toxicidade traduz-se em náuseas, vómitos, diminuição da produção de glóbulos brancos e vermelhos, diminuição da resposta a infeções, danos nos rins, entre outros. Na tentativa de minimizar estes efeitos colaterais e melhorar a eficácia terapêutica das terapias disponíveis, têm sido desenvolvidas novas abordagens terapêuticas que incluem o desenvolvimento de sistemas direcionados de entrega de fármacos. Estas formulações/dispositivos podem transportar substâncias terapêuticas através do corpo promovendo uma libertação prolongada do fármaco, durante o período necessário e no órgão alvo. Na atualidade existem vários tipos de sistemas entrega de fármacos, como sejam microesferas, nanopartículas, ou implantes biodegradáveis para libertação controlada e sustentada de fármacos (géis, filmes, andaimes, etc). No entanto, as nanopartículas são as mais utilizadas devido à possibilidade de lhes ser conferida seletividade através da funcionalização da sua superfície com ligandos específicos, e ainda devido à maior capacidade entrar destes entrarem nas células e libertar o fármaco dentro destas. Os fármacos podem encontrar-se ligados à superfície do nanotransportador, encapsulados no seu interior ou dissolvidos na sua matriz. As nanopartículas têm também a vantagem de conferir proteção aos fármacos, aumentar do tempo de meia-vida do fármaco na circulação sanguínea, e ainda permitir a entrega de mais do que um composto ao mesmo tempo. Atualmente existem vários tipos de nanopartículas, sendo divididas em dois grandes grupos: orgânicas e inorgânicas. Dentro das nanopartículas inorgânicas podemos encontrar as nanopartículas magnéticos que

podem ser usadas não só na entrega de fármacos, mas também em técnicas de diagnóstico e noutros tipos de terapias. As nanopartículas poliméricas mais estudadas são os lipossomas, dendrímeros e nanopartículas poliméricas. As nanopartículas poliméricas podem ser produzidas com polímeros naturais ou sintéticos que podem ou não ser biodegradáveis. Existem vários métodos para a produção de nanopartículas poliméricas que podem determinar o tamanho e a carga a superfície que as partículas possuem.

O objetivo do presente trabalho foi produzir nanopartículas de poli-ε-caprolactona/ poli-metil metacrilato que permitissem efetuar uma libertação controlada do fármaco ao longo do tempo, a fim de serem utilizadas no tratamento do cancro. As partículas foram produzidas através de uma adaptação do método de nanoprecipitação anteriormente descrito na literatura. A Microscopia Eletrónica de Varrimento e a Microscopia Eletrónica de Transmissão foram usados para analisar a morfologia das partículas. A dispersão dinâmica de luz foi usada para avaliar o tamanho e o zeta-potencial da nanopartículas produzidas. A Espectroscopia Infravermelha permitiu também a análise da composição química das nanopartículas produzidas. A quantidade de fármaco incorporado nas nanopartículas e o seu perfil de libertação foi também caracterizado para avaliar a aplicabilidade do sistema na entrega de fármacos, usando a Cisplatina como fármaco modelo. Este fármaco permitiu também verificar a eficácia terapêutica do sistema apresentado. Para avaliar a internalização das nanopartículas pelas células cancerígenas procedeu-se à obtenção de imagens de Microscopia Confocal. A citotoxicidade dos nanoveículos produzidos foi avaliada através de estudos *in vitro* usando uma linha de células cancerígena (Hela) e uma linha celular normal (fibroblastos humanos). As nanopartículas com o fármaco incorporado provocaram uma diminuição significativa da proliferação das células cancerígenas para 30% ao fim de 7 dias. Os resultados obtidos revelam que as nanopartículas produzidas possuem as propriedades necessárias para serem usados na entrega de fármacos na terapia do cancro.

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List of Abbreviations

BSA	Bovine serum albumin
CLSM	Confocal Laser Scanning Microscopy
CEA	Carcinoembryonic antigen
Cis-PCL/PMMA NP	Cisplatin loaded Poly-ε-caprolactone/Poly-methyl metacrylate nanoparticles
CTR1	Copper transporter 1
DDS	Drug delivery system
DMEM-F12	Dulbecco's Modified Eagle's Medium - F12
DMEM-HG	Dulbecco's Modified Eagle's Medium – High Glucose
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EE	Encapsulation Efficiency
EtOH	Ethanol
FBS	Fetal Bovine Serum
FTIR	Fourier Transform Infrared Spectroscopy
GM-CSF	Granulocyte–macrophage colony-stimulating factor
Hela	Human cervical cancer cells
HFib	Human Fibroblast cells
HPV	Human Papilloma Virus
k^-	Negative control
K^+	Positive control
LTTs	Ligand-targeted therapeutics
MPS	Mononuclear phagocytic system
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H tetrazolium reagent
NGR	Asn–Gly–Arg tripeptide
PAA	Poly-acrylic acid
PCL	Poly-ε-caprolactone
PAMAM	Polyamidoamine
PCL/PMMA NP	Poly-ε-caprolactone/Poly-methyl metacrylate nanoparticles
PCR	Polymerase chain reaction
PEG	Poly(ethylene-glycol)
PFA	Paraformaldehyde
PLA	Poly-lactic acid
PLGA	Poly-lactic-co-glycolic acid
PMMA	Poly-methyl methacrylate
pRB	Retinoblastoma protein

Pt	Platinum
PVA	Poly-vinyl alcohol
PVP	Poly-vinyl pyrrolidone
RGD	Arg–Gly–Asp tripeptide
ROS	Reactive oxygen species
SEM	Scanning electron microscopy
SiO2	Silicon dioxide
$SnCl_2$	Tin(II) chloride
TAG72	Oncofetal antigen tumour-associated glycoprotein-72
TCA	Tricarboxylic acid
TiO_2	Titanium dioxide
VEGFR	Vascular endothelial growth-factor receptor

Chapter I:

Introdution

1. Introduction

The female reproductive system is essential for the maintenance of the human species. It is responsible for receiving the oocytes for fertilization, supports the development of the fetus and allows child birth (Kobayashi and Behringer, 2003). Furthermore, it is also involved in different functions such as the production of oocytes and sexual hormones, such as estrogen and progesterone, which have influence in the reproductive system and other organs such as brain, skin, bone and vascular system (Junqueira and Carneiro, 2004).

According with GLOBOCAN 2012, an estimate of the incidence of mortality and prevalence from the world health organization, there were 14.1 million new cancer cases and 8.2 million cancer deaths, in 2012 worldwide (WHO, 2014). Among all types of cancer, the cervical cancer is the fourth most abundant, accounting for 8% of all cancers in women, and it is responsible for 7% of deaths caused by cancer in 2012. (WHO, 2014). Despite the great efforts done so far, the survival rate of patients suffering this disease is still not satisfactory. Due to that, it is essential to develop new therapies that reduce side effects for patients, mortality rate, and also decrease costs associated with treatment in clinical practice (Jin et al., 2014, Lohcharoenkal et al., 2014).

1.1. Female Reproductive System

Female reproductive system is comprised by a pair of ovaries and oviducts, uterus, vagina, external genitalia (see Figure 1) and mammary glands (Spencer, 2009).

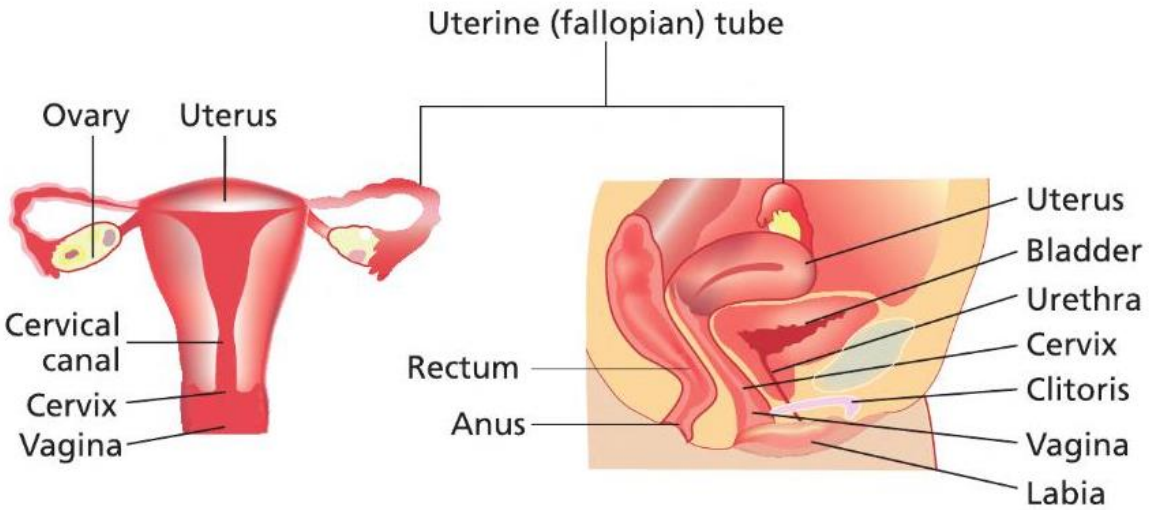

Figure 1: Illustration of the human female reproductive system (adapted from (Spencer, 2009)).

As we can see in figure 1, the ovaries are two small oval glands located in the pelvic portion of the abdomen (Markovic and Markovic, 2008). They are responsible for the production of the female gametes (oocytes) and for the secretion of hormones namely estrogen and progesterone. The external surface of each ovary is covered by a epithelium (Jones and Lopez, 2006), also known as germinal epithelium (Van De Graaff, 2002). This layer is composed by cuboidal epithelial cells. The tunica albuginea, a collagenous connective tissue, is located immediately bellow (Van De Graaff, 2002). The interior of the ovary has two regions: the cortex and medulla (Saladin and Miller, 1998). The outer cortex is responsible for the development of the germ cells while the central medulla contain arteries, veins, lymphatic vessels and nerves (Saladin and Miller, 1998, Jones and Lopez, 2006).

The oviducts (or fallopian tubes) are funnel-shaped long muscular tubes that connect the ovary to the upper part of the uterus (Markovic and Markovic, 2008, Van De Graaff, 2002, Saladin and Miller, 1998). It can be divided in infundibulum (at ovarian end), the ampulla (the middle part of the tube) and the isthmus (at uterus end) (Saladin and Miller, 1998). This is a very important site because it is where the ovum meets sperms and the fertilization takes place (Markovic and Markovic, 2008). They are composed by three layers: the internal mucosa composed by ciliated columnar epithelium, the muscularis (the middle layer) composed by a circular and a longitudinal layer of smooth muscle, and the serous layer (the external layer) that is part of the visceral peritoneum (Van De Graaff, 2002, Junqueira and Carneiro, 2004). The mucosa epithelium has secretory cells that secret mucus which covers the internal surface of the fallopian tubes. This mucus is responsible for nutrition and protection of the oocyte. This mucus is also important for the sperm activation (Junqueira and Carneiro, 2004). The muscular layer is responsible for the movement of the oocyte through the lumen of the uterine tube (Van De Graaff, 2002). The main function of serous layer is the structural support (Tate et al., 2003).

Connected with the oviducts we can find the uterus. Uterus is a pear-shaped muscular organ located in the pelvic cavity behind the bladder and in front of the bowel. It is responsible for receiving the blastocyst and providing a site for his implantation, for the gestation takes place, and also contracts during the bird of the baby (Van De Graaff, 2002). It can be divided in corpus uteri (the wider upper portion of the uterus) and the cervix uteri (the lower, narrower part of the uterus) (Markovic and Markovic, 2008). The wall of the corpus uteri is composed by three layers: the perimetrium, myometrium and endometrium (Jones and Lopez, 2006, Van De Graaff, 2002, Junqueira and Carneiro, 2004, Saladin and Miller, 1998, Tate et al., 2003). The perimetrium is the most external layer of the uterus and is composed by a thin visceral peritoneum (Van De Graaff, 2002, Tate et al., 2003). The myometrium, the higher layer of the uterus, is composed of smooth muscle running in all directions (Saladin and Miller, 1998, Van De Graaff, 2002). The endometrium is the most internal layer and is composed by a stratum functionalis and stratum basalis (Jones and Lopez, 2006). The stratum functionalis consists in a lining epithelium, uterine glands and is shed during every

menstruation (Jones and Lopez, 2006, Saladin and Miller, 1998). The stratum basalis is a highly vascularized structure and serves to regenerate the stratum functionalis after every menstruation (Van De Graaff, 2002).

Underneath the uterus is located the vagina that connects the uterine cavity with external genitals (Markovic and Markovic, 2008). It is a muscular tube that receives the sperm during the coitus, acts as a birth canal during parturition and provides an exist for the menstruation to get out of the body (Van De Graaff, 2002, Saladin and Miller, 1998). The vagina wall is composed by an external fibrous layer, a middle muscle layer and an internal mucosal layer (Van De Graaff, 2002). The fibrous layer is an elastic connective tissue that supports nerve bundles that control blood flow and the contraction of smooth muscle of the vaginal tissue. Moreover, it has some free sensory nerve endings, mainly near the vaginal opening (Jones and Lopez, 2006, Van De Graaff, 2002). The muscle layer is composed by smooth muscle that allows the vagina dilatation (Tate et al., 2003). The mucosal layer consists of nonkeratinized stratified squamous epithelium that forms a series of transverse folds called vagina rugae (Van De Graaff, 2002). Vagina has no glands, but it is lubricated by the transudation of serous fluid through its walls and by mucus from the cervical glands above it (Saladin and Miller, 1998). The epithelial cells are rich in glycogen. This glycogen is metabolised to produce lactic acid, which is responsible for decrease of pH value, for about 3.5-4, and consequently protect vagina against some microorganisms (Junqueira and Carneiro, 2004, Saladin and Miller, 1998).

The vulva (pudendum) is the external part of the female reproductive system and consists of several female organs such as labia major, labia minor, clitoris, opening of the vagina and the urethra (Markovic and Markovic, 2008). It is a highly vascularized region with a sympathetic and parasympathetic innervation as well as extensive somatic neurons that respond to sensory stimulation (Van De Graaff, 2002).

1.2. Female Reproductive System Disorders

The female reproductive system can be affected by various diseases/disorders such as ovarian cysts or cancer, pelvic infection, cervical cancer, cervical cysts and polyps, endometrial cancer, endometriosis, endometrial polyps, hyperplasia and fibroids, tipped uterus, prolapsed uterus and breast cancer. These diseases/disorders can be induced by infections caused by virus and bacteria or genetic mutations, which lead to uncontrolled growth of cells. Also other diseases may potentiate the appearance of these diseases, such as obesity or diabetes (Jones and Lopez, 2006).

1.2.1. Cervical cancer

As already mentioned, cervical cancer remains as one of the leading causes of death in women across the world (Markovic and Markovic, 2008). This type of cancer doesn't present

any signs or symptoms. It has been intimately related whit Human Papilloma Virus (HPV) (Mota, 2012). HPV was identified as viral particles in 1949 and nowadays are known almost 100 different types (from these 100 types about 18 have been identified as being implicated in the majority of cervical cancers) (Jones and Lopez, 2006). The prevalence of HPV DNA in women with cervical cancer has been shown to range from 63% to 98% (Chin'ombe et al., 2014). The HPV can integrate the deoxyribonucleic acid (DNA) of the host cell and promote the synthesis of some viral proteins such as E6 and E7. These proteins are oncogenic proteins that bind to p53 and retinoblastoma protein (pRB), which are two tumor suppressors' proteins and cause their inactivation. Thereby, with the inactivation of these two proteins, cell cycle arrest is prevented without causing significant transformation (Mota, 2012, Tornesello et al., 2013). However, there are other factors that promote this type of cancer such as smoking, sexually transmitted diseases, prolonged use of oral contraceptives and genetic alterations (Mota, 2012). Women with a greater number of sexual partners have an increased risk of be infected with the virus (HPV) or other pathogenic agents (Jones and Lopez, 2006).

1.2.1.1. Diagnosis of cervical cancer

The diagnosis of this disease is extremely important so that the treatment can be administered as soon as possible. There are some methods to investigate the presence of cervical cancer, such as, cervical cytology (pap smear), DNA identification of oncogenic HPV and colposcopy with directed biopsy (Mota, 2012).

Pap smear is one of the most commonly used tests for screening and diagnosis cervical cancer. It consists of 3 basic steps: sample collection with a medical device to scrap cervical epithelium, sample processing using Papanicolaou stains and interpretation of the results using cytopathology (Markovic and Markovic, 2008).

Other possible method is the identification of oncogenic HPV DNA in the host (namely the high-risk HPV types include types 16, 18, 31, 33, 35, 39, 45, 51, 52, 55, 56, 58, 59, 66, 68, 73, 82 and 83) (Al Moustafa et al., 2014) that can be performed by polymerase chain reaction (PCR) . This technique consists in a chemical reaction that results in the synthesis of a large number of target DNA strands, in this case HPV DNA (Olesen et al., 2014).

At last, colposcopy arises as a diagnostic medical procedure where a speculum is used to see female organs such as vulva, vagina and cervix. If a sick-looking tissue is observed, a biopsy can be collected and then histologic analysis be performed (Markovic and Markovic, 2008).

1.2.1.2. Prevention and treatment of cervical cancer

The prevention of cervical cancer is a very important step and may depend not only on the reduction of risk factors (mentioned above), but also by vaccination against HPV (Markovic and Markovic, 2008). Moreover, a deep knowledge of the disease will contribute to decrease the number of infected women in the near future. However these measures will not allow

eradicates the disease, so it is important understand and improve the currently available therapies.

There are three conventional therapies that can be used for cervical cancer treatment (depending on the stage): surgery, radiotherapy and chemotherapy. These therapies can be used alone or combined.

The surgery can be done in 3 ways: the removal of the tumor mass without invasion of the surrounding tissues; to remove the tumor mass and part of surrounding tissue; remove involves the removal of part of the tumor mass for the restitution of function of other organs affected by the tumor. The application of this therapeutic approach is mainly dependent on the stage of development of the cancer (Markovic and Markovic, 2008).

Radiotherapy uses ionizing radiation (beta particles, alpha particles or X-rays) for cancer treatment, causing DNA damage of cells (Markovic and Markovic, 2008). The main disadvantage of this technique is the lack of selectivity; therefore it damages not only the cancer cells but also the healthy ones. However, healthy cells have DNA repair systems available and allow their survival (Khan and Khan, 2003). Radiotherapy is classified according to the type of energy used. There are two radiation therapeutic techniques used in the treatment of cervical cancer: teletherapy and brachytherapy (Khan and Khan, 2003, Rosenberg, 2008). Teletherapy uses X-ray applied from the outside of the body, while brachytherapy involves the intake of a radioactive agent that will release alpha or beta particles, depending on the radioactive agent (Khan and Khan, 2003).

An alternative to surgery and radiology is chemotherapy, which uses drugs as therapeutic agents. Inversely to the surgery and radiotherapy, chemotherapy is a systemic therapy that involves all body and causes toxicity (Markovic and Markovic, 2008). The drugs are frequently administered orally or intravenously. The observed lack of selectivity of drugs leads to unspecific uptake of drugs by all type of cells, leading to various side effects (Jain, 2008). This toxicity and side effects are caused by the accumulation of drugs within non-target tissues. This can damage vital organs such as liver and heart and trigger future complications. The drugs that are commonly used for cervix cancer treatment are cisplatin, ifosfamide, paclitaxel, irinotecan, and gemcitabine in combination with cisplatin (Markovic and Markovic, 2008).

Cisplatin is part of a unique and important class of platinum (Pt) antitumor agents (Cepeda et al., 2007). The mechanism of action of cisplatin involve the binding of the drug to DNA, causing the subsequent cell death by apoptosis, necrosis or both (Fuertes et al., 2003a, Galluzzi et al., 2013). Cisplatin has been used in the treatment of other types of cancer such as testicular, ovarian, bladder, head and neck, oesophageal and small cell lung cancer (Giaccone, 2000). It has been reported that this drug is able to enter into the cell either by passive diffusion or through a gated channel (Ciarimboli, 2012). The input of cisplatin through

the cell via passive diffusion is unspecific and only depends of the concentration of cisplatin in the medium (Ciarimboli, 2012). On the other hand cisplatin can enter into the cell through some specific channels. A channel that has been most closely associated with the input of cisplatin to the cell is a high-affinity copper transporter 1 (CTR1) (Florea and Büsselberg, 2011). Some studies suggest that CTR1 is predominantly in perinuclear vesicles of some cell lines, whereas in others it is at the plasma membrane (Liang et al., 2014, Safaei and Howell, 2005, Zisowsky et al., 2007). The mechanism used for the entrance of cisplatin into the cell trough CTR1 is not yet fully known, however there are some hypotheses to explain this transport: platinum (Pt) drugs may be transported across the plasma membrane through a pore formed by the three transmembrane domains of CTR1 (Klomp et al., 2003, Safaei and Howell, 2005, Petris et al., 2003).

However, there are other factors that influence the uptake of the Pt drugs by CTR1 channel, such as temperature, pH, K^+ ions and reducing agents (Safaei and Howell, 2005). The uptake of cisplatin leads to an accumulation of the drug inside the cell, where the Cl^- concentration lower than that found in blood. In such conditions, cisplatin Cl^- group is released, conferring a positive charge to cisplatin that is now able to interact with nucleophilic sites on intracellular macromolecules like proteins, RNAs, and DNA adducts. Adduct formation results in the inhibition of DNA replication, RNA transcription, arrest at the G2 phase of the cell cycle, and/or programmed cell death (Kartalou and Essigmann, 2001, Galluzzi et al., 2013, Ciarimboli, 2012)(see figure 2).

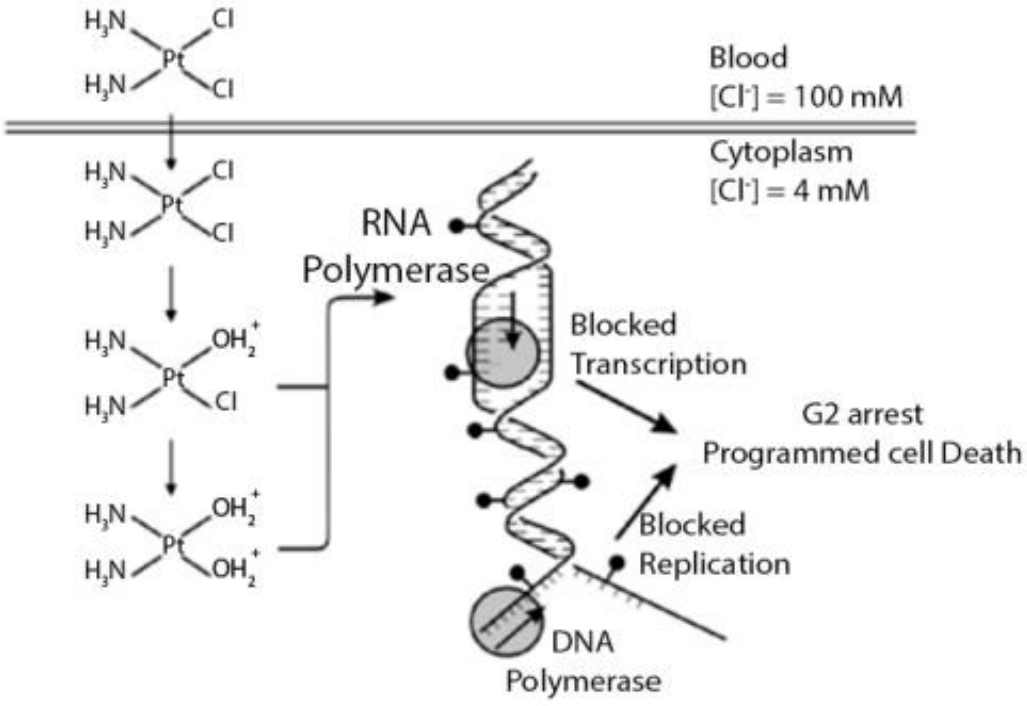

Figure 2: Mechanism of action of cisplatin (adapted from (Kartalou and Essigmann, 2001)).

Due to its high affinity for nucleophilic groups, cisplatin reacts with different cellular components such as membrane phospholipids, cytoskeletal microfilaments, and thiol-containing molecules which leads to only approximately 1% of the intracellular cisplatin will be able to interact with nuclear DNA (Galluzzi et al., 2013). These nuclear and protein damages leads to the activation of apoptotic pathways, through the activation of caspase-3, caspase-6, and caspase-7 with the subsequent cleavage of key substrates (Fuertes et al., 2003b).

However, in cisplatin resistant cell lines a decreased uptake or increased efflux of the drug has been observed. Moreover, the increased inactivation of cisplatin by intracellular proteins such as glutathione, the increased repair of cisplatin-DNA adducts and defects in the apoptotic response pathway also promote the resistance of cancer cells (Galluzzi et al., 2013, Galluzzi et al., 2011). The decrease in the uptake can be explained by the loss of efficiency of CTR1 (due to the factors affecting the uptake), low concentration of cisplatin outside the cell and decreased expression of CTR1 (Ciarimboli, 2012). On the other hand, it has been previously reported in literature that the two copper efflux transporters ATP7A and ATP7B regulate the efflux of cisplatin. These two copper efflux transporters are located in trans-Golgi network and export cisplatin by exocytosis (Prohaska and Gybina, 2004, Ciarimboli, 2012, Kuo et al., 2007). There are other studies that described the multidrug resistance is associated with protein 2 (ABC-transporter 2) (Liedert et al., 2003). ABC-transported 2 works as an ATP-dependent conjugate export pump that require the formation of Pt-glutathione conjugates for the cisplatin efflux from the cell (Kuo et al., 2007).

Therapies based on this PT drug family have many side effects such as nausea and vomiting, decreased production of blood cells and platelets in bone marrow, decreased response to infection, induce damage in the kidneys, neurons and hearing loss (Florea and Büsselberg, 2011).

In an attempt to minimize the side effects and also improve the efficacy of the currently available therapeutics, new approaches are currently being explored. New drug delivery systems (DDS) are being designed and developed to encapsulate and deliver therapeutic agents (Jain, 2008, Haley and Frenkel, 2008).

1.3. Drug Delivery Systems

DDS are formulations/devices that can be loaded with a therapeutic substance through the body. Furthermore, these carriers can promote a sustained release of the therapeutic substance along time, at a specific target organ (Jain, 2008).

Conventional therapies to cancer, like chemotherapy, involve the administration of free therapeutic agents to the patient. However, they have a limited effectiveness, lack of

selectivity and poor biodistribution (Wilczewska et al., 2012). Such therapies can be improved by encapsulating the drug in a DDS. These systems upgrade the efficacy and safety of the treatments (Jain, 2008), since the drug is carried till the place of action, minimizing the interaction with others tissues, and protecting the drug from rapid degradation and clearance from the body. Furthermore, a lower concentration of drug is required to achieve the aimed therapeutic effect in the organism (Wilczewska et al., 2012) .

Several types of DDS such as microspheres, nanoparticles (NPs), gels, films and scaffolds have been developed so far (Jain, 2008). Between them NPs are the most used ones, since they have several advantages including small size, which allows their transport along the entire body. Furthermore, the surface of these carriers can also be functionalized with specific ligands that drive the NPs to the target cell and have a high loading capacity (Jain, 2008, Davis, 2008).

1.3.1. Nanotechnology

Nanotechnology is a field of research that involves the development of new materials at the nanoscale level (Bhushan, 2010), ranging from 10nm to 1000nm (Mohanraj and Chen, 2007). Such materials can be used in different fields such as energy, environment and medicine (Gu et al., 2013). In energy field it allows the production of photovoltaic solar cells with higher efficiency, while reducing their manufacturing and electricity production costs at an unprecedented rate (Serrano et al., 2009). In the environment field, sensor system based in nanocrystalline materials with high spatial resolution and sensitivity, can complement and improve the effectiveness of conventional analytical instruments for environmental monitoring (Rickerby and Morrison, 2007). In medicine these materials (NPs) can be used in imaging and diagnostic proposes, and also as vehicles for therapeutic agent/drug delivery (Gu et al., 2013).

Nanomedicine focused on cancer therapeutics is a particularly interdisciplinary field, which gathers knowledge from Biology, Chemistry, Engineering, Physics and Medicine. The developed of DDS, that can increased drug bioavailability in diseased tissues and also minimize side effects in healthy cells is of crucial importance for improve patients' quality of life and also increase life expectancy.

1.3.2. Types of nanoparticles

The different types of NPs produced are classified according to their composition into two groups: inorganic and organic NPs (Yezhelyev et al., 2006).

Inorganic NPs are characterized by their stability, good loading capacity and controlled release of drug. However, inorganic NPs often present cytotoxic effects to the human body.

Magnetic NPs constitute the larger group of this type of NPs (Xu et al., 2006, Sahoo and Labhasetwar, 2003).

Organic DDS have suitable properties for their application as anti-cancer therapeutics. They have high biocompatibility, biodegradability, high loading capacity and versatile chemical composition that allows their modification with bioactive macromolecules. Liposomes, dendrimers and polymeric NPs are members of this group (López-Dávila et al., 2012, Zhang et al., 2008a).

1.3.2.1. Inorganic Nanoparticles

1.3.2.1.1. Magnetic nanoparticles

Magnetic NPs are generally composed by a magnetic core and can be coated with several materials (silica, gold, polymers, etc.). Their applications involve the delivery of a drug to a specific tissue, diagnostic (Magnetic Resonance imaging) or therapy (hyperthermia/thermal ablation) (Arruebo et al., 2007) (see Figure 3). A very interesting property of magnetic NPs is their ability to obey to the Coulomb's law. Such feature enables an external magnetic gradient to guide them to a specific site, maintaining them at the site of action until needed and then allow their removal (Pankhurst et al., 2003).

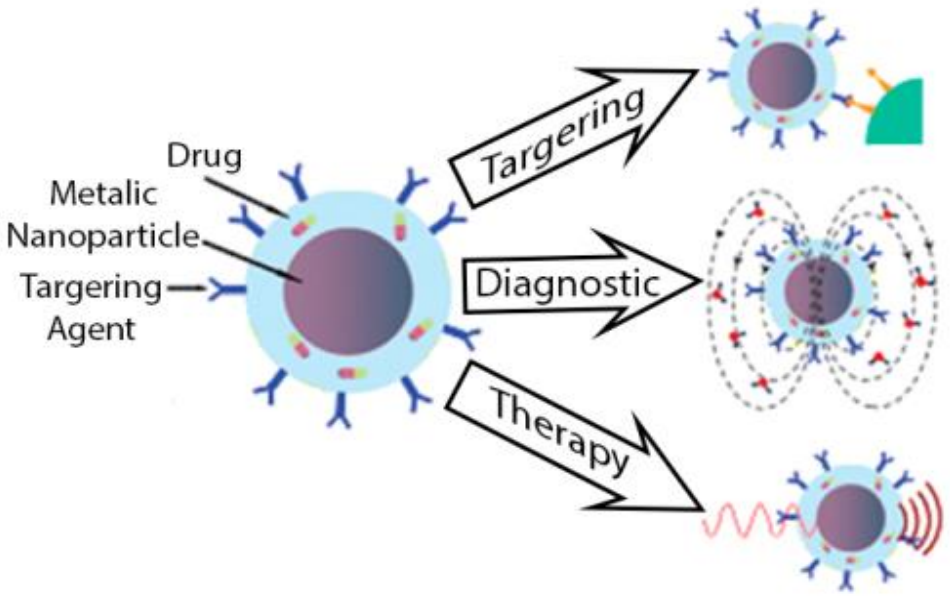

Figure 3: Biomedical applications of magnetic nanoparticles (adapted from (Umut, 2013)).

The most investigated magnetic NPs are those produced with iron, their alloys and oxides (Wilczewska et al., 2012). These NPs are the only magnetic NPs approved for clinical use by Food and Drug Administration (Wilczewska et al., 2012). When they were used as a DDS the drug can be linked to the NP by a covalent bound, electrostatic interaction, absorbed or

adsorbed (Figuerola et al., 2010, Yallapu et al., 2011, Wu et al., 2010). Magnetic NPs can cause oxidative stress by promoting the production of Reactive oxygen species (ROS) and the activation of phagocytosis and cytokine-release function of macrophages. However, these NPs have several advantages such as simple synthesis, chemical stability in physiological conditions and the possibility of chemical modification through their coating (Figuerola et al., 2010, Asmatulu et al., 2005, Karimi et al., 2013). The coating of these NPs with polymers such as gelatine, chitosan, poly(ethylene-co-vinyl acetate), poly(vinyl pyrrolidone) (PVP), poly(lactic-co-glycolic acid) (PLGA), poly(ethylene glycol) (PEG), and poly(vinyl alcohol) (PVA), oleic acid and dextran or other materials like proteins (like albumin) can improve their biocompatibility (Wilczewska et al., 2012, Jain et al., 2005, Berry et al., 2003, Peng et al., 2008, Karimi et al., 2013).

1.3.2.2. Organic Nanoparticles

1.3.2.2.1. Liposomes

Liposomes are spherical, self-closed structures, that have an aqueous core, composed of phospholipids and steroids (Wilczewska et al., 2012, Torchilin, 2005). Liposomes usually present a concentric lipid bilayer but in some cases can have several bilayers. Their size can vary between 100 and 5000 nm, depending on the number of bilayers that they have on their composition. The number of bilayers and size allow the classification of the liposomes into four categories: small unilamellar, oligolamellar, large unilamellar and multilamellar vesicles (Mallick and Choi, 2014). They are able to load hydrophobic and hydrophilic drugs as presented in Figure 4. The water-soluble drugs are carried inside the liposomes, in the aqueous phase, whereas the water-insoluble drugs can be incorporated into the liposomal membrane.

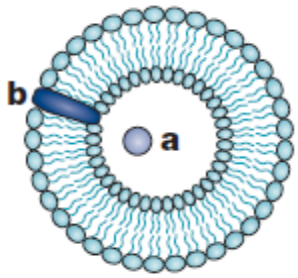

Figure 4: Representation of the traditional phospholipids liposomes. a- water-soluble drug, b- water-insoluble drug (adapted from (Torchilin, 2005)).

These carrying systems are biocompatible, biodegradable and can carry a variety of different molecules like neurotransmitters, antibiotics, anti-inflammatories, genes and drugs.

Liposomes are formed through the hydrophilic/hydrophobic interactions between lipid/lipid and lipid/water. In the production process the surface of the liposomes can be modified with specific proteins, antigens or other biological substances, which will improve their selectivity to the target tissue. Liposomes present some limitations such as low encapsulation efficiency, fast burst release of drugs and poor storage stability (Bamrungsap et al., 2012). In the market there are some liposome based formulations for cancer therapy, being Doxil® the most used in the clinical (Barenholz, 2012).

1.3.2.2.2. Dendrimers nanocarriers

Dendrimers have well-defined size and structure and they have been produced with different molecules such as glycogen, amylopectin and proteoglycans (Wilczewska et al., 2012).

These structures are composed by a core, dendrons and surface active groups (Figure. 5). Dendrimers can be produced by two methods: the divergent approach and the convergent approach (Svenson and Tomalia, 2012). The main difference between these two methods is the direction of dendrimer growth. In divergent method, dendrimer growth starts from a polyfunctional core and proceeds radially (Hierold et al., 2010). In the convergent method the dendrimer growth begins from the exterior of the molecule and continues inward by coupling end groups to each branch of the monomer (Ledin et al., 2011). The modification of the surface amine groups of dendrimers with neutral or anionic groups can be easily produced in order to reduce its toxicity. This functionalization consists in a reaction between the amine groups in the dendrimer surface and the functionalization agent. The drug can be covalent or noncovalent bonded to the dendrimer. In the noncovalent approach the drug, generally hydrophobic, is incorporated into dendrimer core and it is difficult to control their release from the dendrimer core, which limits their use. when the drug is covalently bond to the dendrimer periphery, its release can be controlled by incorporation of degradable linkages between the drug and the dendrimer (Gillies and Frechet, 2005).

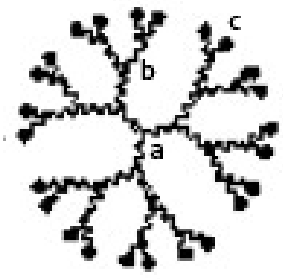

Figure 5: Representation of a dendrimers. a- core, b- dendrons, c- active groups (adapted from (Svenson and Tomalia, 2012)).

Polyamidoamine (PAMAM) is one of dendrimers already in commercialization and one of the most used for biomedical applications (Gillies and Frechet, 2005). Polypropyleneimine

dendrimers have been commercialized and investigated for their biological application, but the presence of multiple cationic amine groups leads to a significant toxicity (Malik et al., 2000). Furthermore, polyaryl ether dendrimers, have been tested for drug delivery applications, but their poor water solubility demands the extensive use of solubilizing groups at their periphery (Liu et al., 2000).

1.3.2.2.3. Polymeric nanoparticles

Polymeric NPs are core-shell spherical structures that can be produced with natural (Serum albumin, Gelatin, Chitosan, etc.) and/or synthetic (Poly-ε-caprolactone (PCL), Poly lactic acid (PLA), Poly methyl methacrylate (PMMA), Poly glycolic acid, etc.) polymers (Pinto Reis et al., 2006a). They can be designated as nanocapsules, if the drug is confined in the core of nanoparticle, or nanospheres, if the drug is physically and uniformly dispersed in the polymeric matrix (see Figure 6).

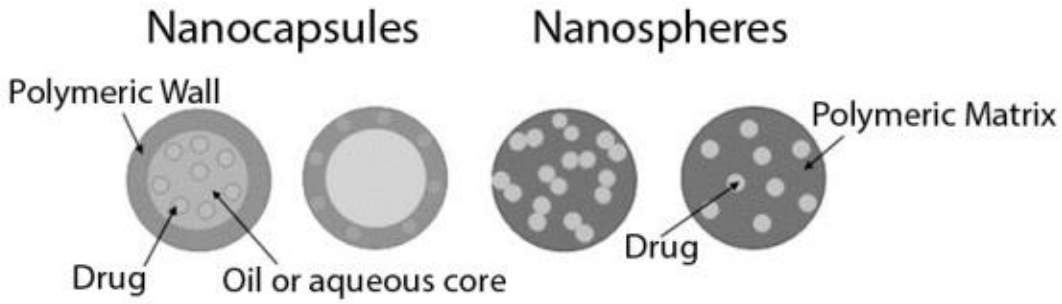

Figure 6: Schematic representation of polymeric nanoparticles (adapted from (Pinto Reis et al., 2006b))

Polymeric nanocarriers provide several advantages, such as better overall drug/carrier stability (Sutton et al., 2007) and sustained drug controlled release (Soppimath et al., 2001). The synthetic polymers have some advantages over the natural, since they can provide a temporally controlled drug delivery of bioactive pharmaceutics for longer periods than those presented by some natural polymers, such as chitosan or alginate, which suffer from extensive swelling and also some biodegradation (Panyam and Labhasetwar, 2012).

Different methods, like nanoprecipitation, emulsification/solvent evaporation, emulsification/solvent diffusion, salting out, dialysis, superficial fluid technology, spray-drying and crystallization have been used for polymeric NPs synthesis (Mohanraj and Chen, 2007, Pinto Reis et al., 2006a, Vauthier and Bouchemal, 2009, Rao and Geckeler, 2011, Miladi et al., 2013, Mora-Huertas et al., 2010).

The emulsification/solvent evaporation (see Figure 7) was the first method developed to prepare polymeric NPs from a preformed polymer (Rao and Geckeler, 2011, Vanderhoff et al., 1979). This technique consists on the formation of a simple or double emulsion (using high-

speed homogenization or ultrasonication) and the evaporation of the organic solvent, by continuous magnetic stirring or under reduced pressure, which leads to the precipitation of the polymer and the subsequent obtention of the particles (Miladi et al., 2013). The size of the obtained particles can be controlled by changing the stirring rate, type and amount of dispersing agent, viscosity of organic and aqueous phase and temperature (Pinto Reis et al., 2006a). Generally, this method of simple emulsion is used for the encapsulation of hydrophobic drugs. On the other hand, when hydrophilic drugs are aimed to be encapsulated, the double emulsion is more appropriate. This double emulsion consists in the dispersion of the primary emulsion in a second aqueous phase, before organic solvent evaporation (Miladi et al., 2013).

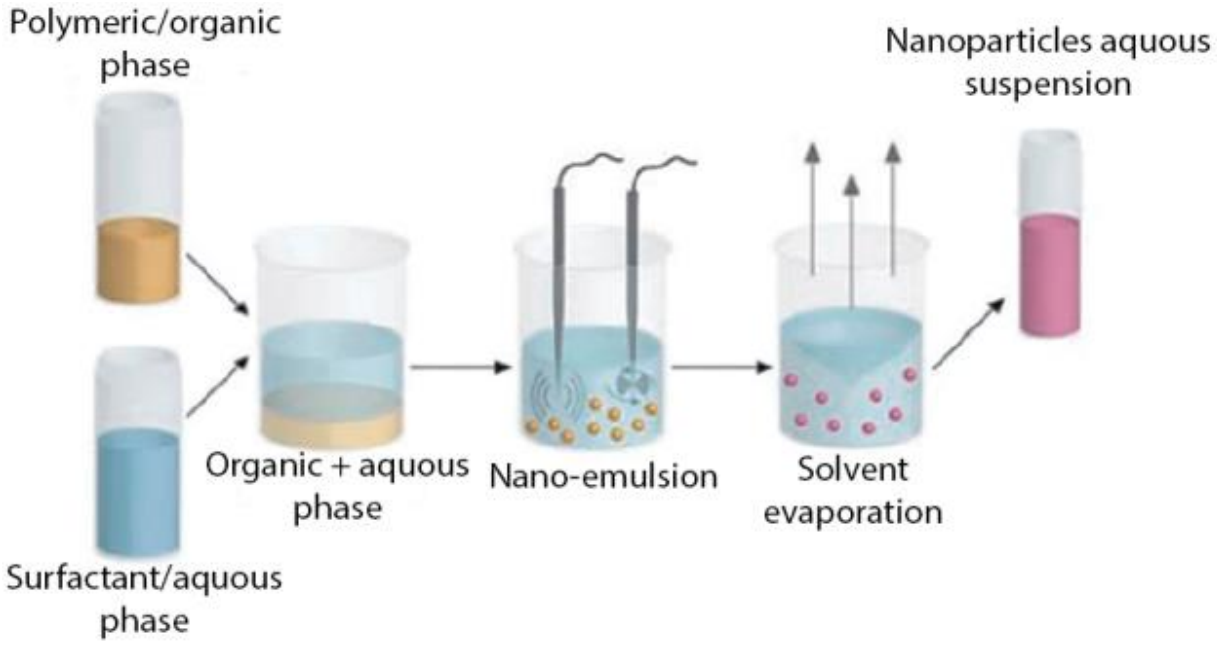

Figure 7: Schematic representation of the emulsification/solvent evaporation technique (adapted from (Raemdonck et al., 2014)).

Another method described in the literature is nanoprecipitation or solvent displacement method (see Figure 8). This method was developed by Fessi *et al.* (Fessi et al., 1989) and consist in a spontaneous emulsification of the organic internal phase containing the dissolved polymer into the aqueous external phase (Pinto Reis et al., 2006a). The organic phase must be miscible in the aqueous phase and easy to remove by evaporation. It can be an organic solvent or a mixture of organic solvents. The solvents frequently used are acetone, dimethyl sulfoxide, isopropyl alcohol, ethanol or ethyl lactate, ethyl acetate, acetonitrile, etc. (Anton et al., 2008).

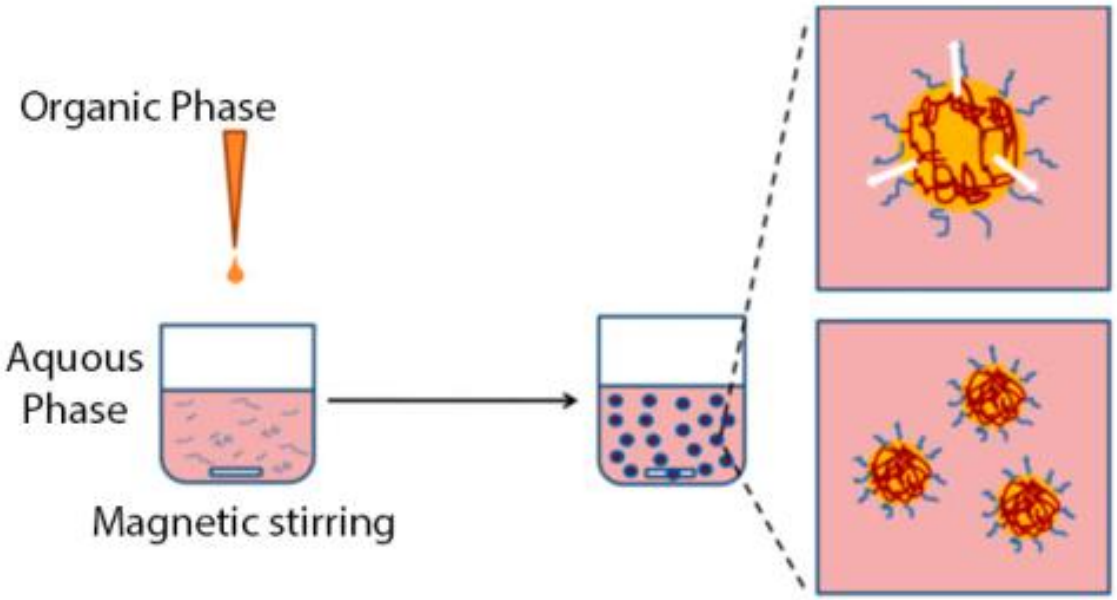

Figure 8: Representation of the nanoprecipitation method (adapted from (Khan and Schneider, 2013)).

The decrease of interfacial tension between the two phases results in the rapid diffusion of the organic phase (organic solvent and polymer) into the aqueous phase (non-organic solvent supplemented with one or more naturally or synthetic surfactants). This increase the surface area and leads to the formation of small droplets of organic solvent (Rao and Geckeler, 2011). The main variables that induce the formation of the spontaneous emulsification are the conditions used for adding the organic phase into the aqueous phase such as injection and agitation rate, the method of addition and the proportions between the two phases. One of the limitations of this technique is that only water-miscible solvents can be used, in which the diffusion rate is enough to produce spontaneous emulsification (Pinto Reis et al., 2006a). This technique is more adequate to encapsulate lipophilic drugs than hydrophilic drugs. Such can be explained by the week interaction between the drug and the polymer. In the hydrophilic drugs, the drug will tend to diffuse from the organic phase to the external aqueous medium during the spontaneous emulsification process of the polymer, reducing the amount of encapsulated drug (Khan and Schneider, 2013).

The emulsification/solvent diffusion (see Figure 9) is other technique used to produce polymeric NPs and was developed by Leroux and collaborators (Leroux et al., 1995). In this method it is required an organic phase, that contains the partial water-soluble organic solvent, the polymer, the hydrophobic drug, and two aqueous phases. The first aqueous phase contains the stabilizer agent solution and the second that is called the dilution phase, is mainly composed for a large volume of water (Miladi et al., 2013). In the encapsulation of a hydrophilic drug it is needed the use of an aqueous inner phase to dissolve the drug. To

obtain the emulsification both the organic phase and the aqueous phase must be saturated to ensure the initial thermodynamic equilibrium of both liquids (Pinto Reis et al., 2006a, Miladi et al., 2013).

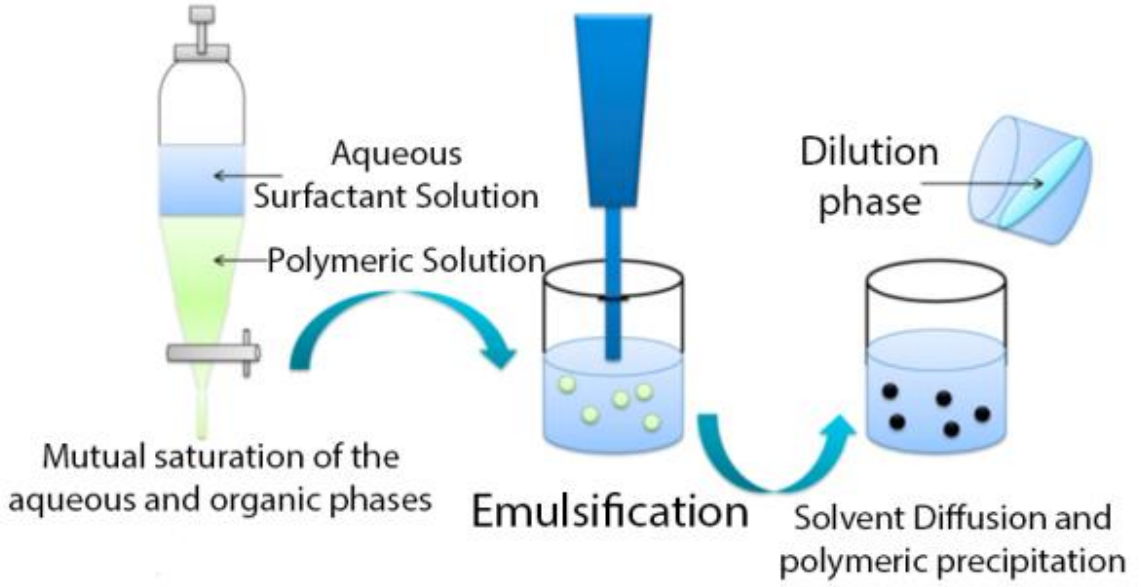

Figure 9: Schematic illustration of the emulsification/solvent diffusion technique (adapted from (Pinto Reis et al., 2006a)).

The emulsification can be obtained under vigorous agitation. The next step is the dilution of the previous emulsion with a large amount of dilution phase (usually pure water). This dilution results in the diffusion of the organic solvent contained in the dispersed droplets leading to the precipitation of the polymer (Vauthier and Bouchemal, 2009), which results in the formation of the NPs. The organic solvent can be then eliminated by distillation or cross-flow filtration (Mora-Huertas et al., 2010). The main conditions that affect the size of the particles are the organic/aqueous phase ratio, emulsification stirring rate, volume of water for the dilution and the temperature (Mora-Huertas et al., 2011).

The salting out method (see Figure 10) is currently used for the preparation of polymeric NPs from the preformed polymer, and it was first developed by Bindschaedler *et al.* (Bindschaedler et al., 1990). It is very similar to the emulsion/solvent diffusion method (Pinto Reis et al., 2006a). This method requires a water-miscible solvent and involves a salting-out process, that, by turn, requires the dissolution of a high concentration of salt in the aqueous phase which will result in the loss of miscibility between the two phases. The polymer and the drug (lipophilic) are dissolved in a water-miscible solvent and then the mixture is emulsified into an aqueous phase containing the salting-out agent and the stabilizer.

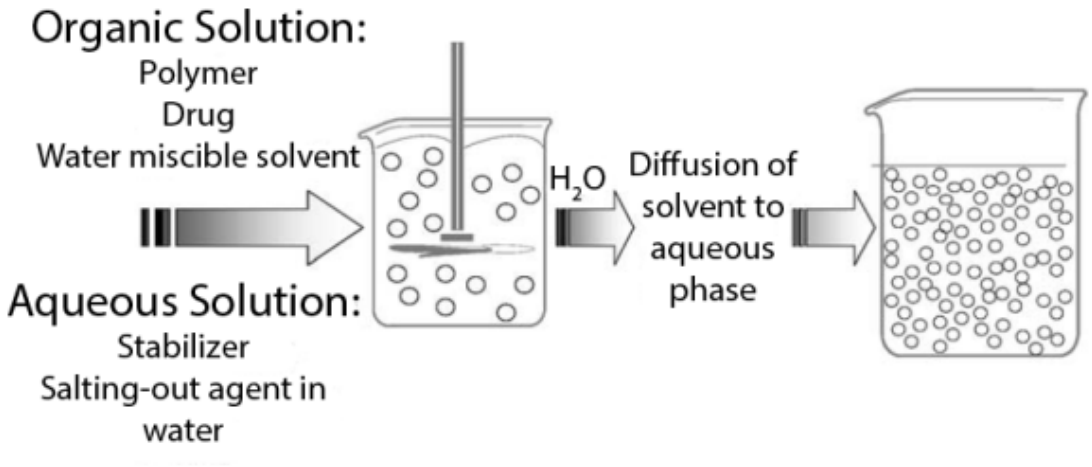

Figure 10: Schematic illustration of the salting-out technique (adapted from (Pinto Reis et al., 2006a))

After the emulsion, like in the emulsion/solvent diffusion, a large amount of water is added to this solution, in a way that the concentration of salting out agent will decrease and the miscibility of the two phases will increase (Rao and Geckeler, 2011). Then the water-miscible solvent diffuse in the aqueous phase, the polymer precipitation is induced and forms the NPs. So, the polymer solvent and the salting-out agent are eliminated (Vauthier and Bouchemal, 2009). In this technique the increase of temperature is not necessary, allowing the encapsulation of heat-sensitive substances. However, this technique present the disadvantage of requiring extensive nanoparticle wash steps (Pinto Reis et al., 2006a).

1.3.3. Poly-ε-caprolactone nanoparticles

PCL is a synthetic, hydrophobic, semi-crystalline and biodegradable polymer obtained by polymerization of ε-caprolactone (see Figure 11). The monomer (ε-caprolactone) can be obtained from the oxidation cyclohexanone by peracitic acid (Rocca et al., 2003). One of the most used methods for PCL preparation is the ring opening polymerization. There are four main mechanisms of ring opening polymerization: anionic, cationic, monomer-activation and coordination insertion. All of them result in a polymeric chain composed of several replications of the extended monomer (Labet and Thielemans, 2009).

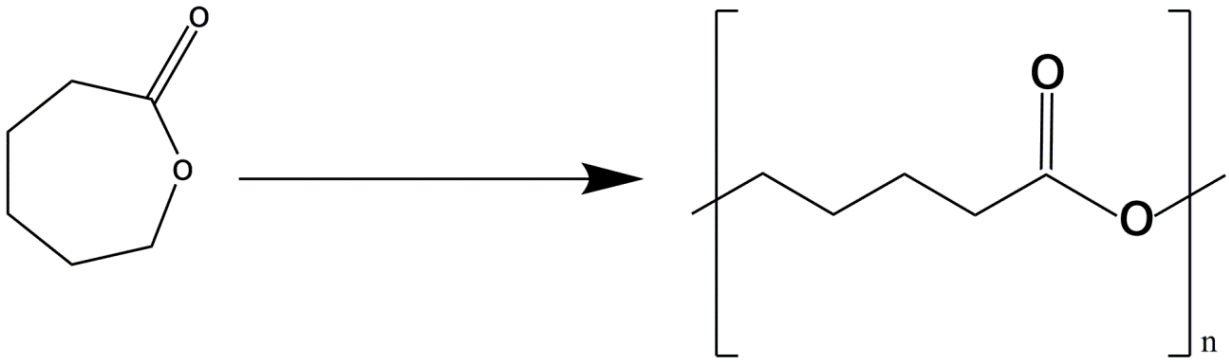

Figure 11: Representation of ε-caprolactone (left) and poly-ε-caprolactone (right) structure (adapted from (Labet and Thielemans, 2009)).

PCL is insoluble in water and alcohols, has a low solubility in polar solvents (acetone, acetonitrile, dimethylformamide, etc) and is soluble in aromatic and chlorine solvents (dichloromethane, chloroform, carbon tetrachloride, benzene, etc) (Sinha et al., 2004, Pohlmann et al., 2013). PCL suffers slow degradation by enzymatic or non-enzymatic processes. In the non-enzymatic cleavage of PCL (hydrolytic degradation) the water permeability is the limiting step because it starts in the amorphous regions. This region is auto-catalyzed by carbonyl end groups of fragmented polymeric chain (Jenkins and Harrison, 2006, Sinha et al., 2004). This process of PCL degradation is too slow and can take several months or even years (Woodruff and Hutmacher, 2010). Alternatively, the enzymatic fragmentation is faster and involves the activity of lipase. This enzyme is responsible for the cleavage of esters groups' with the release of carboxyl groups. This group can undergo phagocytosis and used in tricarboxylic acid (TCA) cycle (Woodruff and Hutmacher, 2010).

PCL can be combined with different polymers to modify its mechanical, physical and ionic properties and the biodegradability profile. This polymer, alone or conjugated, have been used for the production of implants, nanofibres, scaffolds, composites, films, hydrogels, micelles, microspheres and NPs (Dash and Konkimalla, 2012).

In the literature there are several examples of NPs produced with PCL are described (Table 1).

Table 1: Examples of PCL nanoparticles reported in literature. BSA - Bovine serum albumin; MPEG - Methoxy poly(ethylene glycol); PEG - Poly(ethylene glycol); EE – Encapsulation Efficiency.

Polymer	Drug	Method of Production	Mean size	EE%	Reference
PCL	BSA	Double emulsion/solvent evaporation	276 -308 nm	55-80%	(Lamprecht et al., 1999)
PCL	Exemestane	Interfacial deposition method	115 – 350 nm	23 – 84%	(Kumar and Sawant, 2013)
PCL	Uncaria tomentosa	Emulsion solvent evaporation	223 – 408 nm	33 – 88%	(Ribero et al., 2013)
PCL/ Dextran	Doxorubicin	Nanoprecipitation method	95 – 123 nm	42-52%	(Li et al., 2013)
PCL	tamoxifen	Solvent displacement	100 – 300 nm	64 %	(Chawla and Amiji, 2002)
PCL - MPEG	Ibuprofen	Emulsion/solvent diffusion	85 – 97 nm	48 – 64 %	(Baimark, 2009)
PCL - PEG	Honokiol	Solvent diffusion method	~132 nm	20%	(Gou et al., 2009)
PCL – l-Lactide	nimodipine	Precipitation method	81 – 132 nm	19 – 91 %	(Ge et al., 2000)
PCL/ magnetic	Cisplatin	Single emulsion/solvent evaporation	~160 nm	7%	(Yang et al., 2006)
	Gemcitabine			25%	

As we can see in table 1, several PCL based NPs have been produced and used over the years (Lamprecht et al., 1999, Kumar and Sawant, 2013, Ribero et al., 2013, Li et al., 2013, Chawla and Amiji, 2002, Baimark, 2009, Gou et al., 2009, Ge et al., 2000, Yang et al., 2006). They can be produced with several sizes, depending mainly on the molecular weight of the polymer used. This system based on PCL are characterised by an initial burst followed by a sustained release during several hours or days. PCL NPs can be loaded with several drugs either hydrophilic or hydrophobic. Their good biocompatibility makes them good candidates to be

used in the treatment of several diseases such as neuroblastoma, breast and ovarian cancer (Li et al., 2013, Chawla and Amiji, 2002, Gou et al., 2009).

Shenoy and collaborators loaded PCL NPs with tamoxifen and evaluated their biodistribution *in vivo* using female athymic mice (4/6 weeks old) (Shenoy and Amiji, 2005). Their results suggested that the produced NPs were transferred from the circulatory compartment to the tumor site. They concluded that after 1 hour of injection, 90% of free drug administered reached the liver, while only 70% attained this organ, when loaded NPs were used. 6 hours post injection the concentration in the liver of both formulations (free drug and inside NPs) was 2 and 7%, indicating possible degradation/metabolism of the drug. These results suggest that PCL NPs delivery the drug at the tumour tissue, minimizing the amount of drug that reaches the liver and subsequently degraded.

1.3.4. Poly (methyl methacrylate) nanoparticles

PMMA is a synthetic, biocompatible and non-degradable polymer obtained through the polymerization of the methyl methacrylate monomer (see Figure 12) (Bettencourt and Almeida, 2012). PMMA is soluble in several organic solvents like trichloromethane and trichloroethylene, dimethylformamide and chloroform (Evchuk et al., 2005). Despite its low solubility in water, this polymer has been reported as being soluble in a mixture of water and alcohol (Hoogenboom et al., 2010).

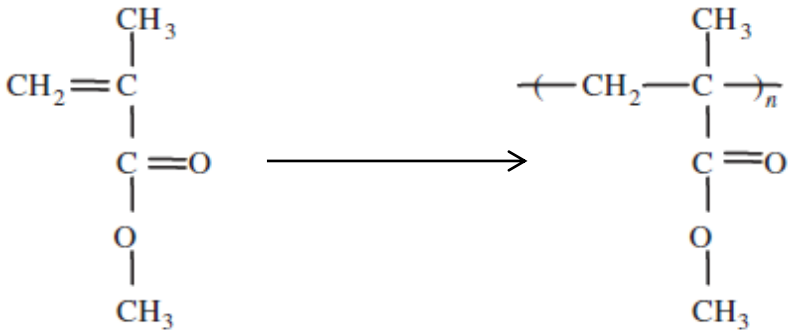

Figure 12: Representation of methyl methacrylate (left) and poly (methyl methacrylate) (right) structure (adapted from (Bettencourt and Almeida, 2012)).

PMMA has been reported to be used in several biomedical applications such as a bone cement (in total hip replacement), vertebral stabilization agent in patients with osteoporosis, a prosthetic material in dental and mandibular corrections and as a permanent implant in the form of intraocular lens used in cataract surgery (Carvalho Costa et al., 2009, Schade and Roukis, 2010). Furthermore, PMMA particles have been the first ones to be developed for vaccination purposes (Kreuter and Speiser, 1976, Kreuter et al., 1976). Since then several

micro and NPs have been produced using this polymer alone or combined with others materials (Table 2).

Table 2: Examples of PMMA nanoparticles produced so far. PAA – Poly acrylic acid; TiO_2 - Titanium dioxide; SiO_2 - Silicon dioxide; EE - Encapsulation Efficiency.

Polymer	Drug	Method of Production	Mean size	EE%	Reference
PMMA	Coenzyme Q10	Solvent evaporation method	50-250 nm	96 %	(Hsu et al., 2003)
PMMA/ PAA	Cisplatin	Free radical emulsion polymerization	150 nm	5-10 %	(Lee et al., 2013)
PMMA/Silver	-	Silver reduction	15-25 nm	-	(Singh and Khanna, 2007)
PMMA/ Latex	-	Soap-free emulsion polymerization	162–626 nm	-	(Anancharungsuk et al., 2010)
PMMA/ cationic groups	Antisense oligoribonucleotide	Emulsion polymerization technique	417 nm	20-100 %	(Rimessi et al., 2009)
PMMA/ TiO_2	-	Dispersion polymerization method	264-306 nm	-	(Park et al., 2006)
PMMA	Repaglinide	Solvent Evaporation Method	108 nm	65-90 %	(Poovi et al., 2010)
PMMA/ Chitosan	Paclitaxel	Radical polymerization	140-250 nm	95-98 %	(Akhlaghi et al., 2010)
PMMA/ SiO_2	-	Suspension dispersion polymerization	20-30 nm	-	(Zhu et al., 2008)

Nowadays, there are PMMA based NPs that are used as DDS for the treatment of colorectal adenocarcinoma, hypersensitivity, muscular dystrophy, diabetes and breast cancer (Lee et al., 2013, Anancharungsuk et al., 2010, Rimessi et al., 2009, Poovi et al., 2010, Akhlaghi et al., 2010). However, PMMA needs to be further characterized and investigated in order to expand its application in the biomedical field.

The combination of biostable PMMA with biodegradable PCL polymers provide materials with a good mechanical integrity, that may be used as an alternative to other copolymers, that are currently be tested to be used to allow a sustained release of bioactive compound (Elvira et al., 2004, Méndez et al., 2002).

1.3.5. Characterization of nanoparticles properties

NPs can be characterized based on their size, shape, surface properties, drug loading and release capacity. Depending on the purpose for which the particles are designed, these properties may be further optimized.

1.3.5.1. Particle size and shape

Particle size is one of the most important characteristics of NPs. This property influence the *in vivo* distribution and toxicity of NPs, and also the others characteristics such as drug loading capacity, drug release, targeting ability and stability (Mohanraj and Chen, 2007).

The influence that size has on drug release can be explained by the relation between the surface area and the size of the core. Small NPs have larger surface area, therefore, most of the loaded drug is at or near the particle surface, leading to fast drug release. On the contrary, larger particles have larger cores, allowing more drug to be encapsulated and a slower release of drug is expected (Redhead et al., 2001).

Smaller particles also present a higher risk of aggregation when they are stored for long periods, although they present a lower rate of polymer degradation. It was thought that in smaller particles, the degradation products formed can diffuse out of the particles easily while in large particles, the degradation products remain within the polymeric matrix for longer periods, causing autocatalytic degradation of the polymeric material, leading to two phases in the polymeric degradation process (Dunne et al., 2000, Panyam et al., 2003). The polymer degradation products can be used in cells metabolism (Woodruff and Hutmacher, 2010, Danhier et al., 2012), or accumulated in the liver, spleen and kidney, or excreted by kidney depending of the size of the product (Park et al., 2009)

Particles size is also responsible for the clearance and biocompatibility presented by NPs (Figure 13). Particles with sizes smaller than 5 nm will suffer clearance in kidney, while particles with sizes higher than 200 nm will accumulate in liver and spleen. NPs with sizes from 5 to 200 nm are able to remain in the systemic circulation for longer periods than those above 200 nm or behind 5 nm, increasing the probability of these system reach the target tissue (Ernsting et al., 2013).

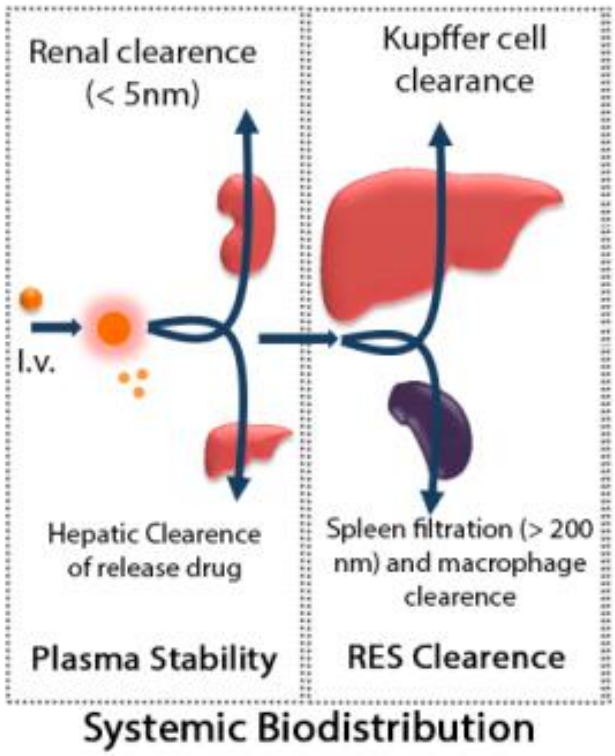

Figure 13: Biodistribution and clearance of nanoparticles (adapted from (Ernsting et al., 2013)).

Besides size, the shape of NPs has been also described as being important for the interaction between the NPs and the cell (Truong et al., 2014). Spherical particle have a higher cellular uptake, which is explained by greater membrane wrapping time required for elongated particles (Verma and Stellacci, 2010, Chithrani and Chan, 2007, Nel et al., 2009). Moreover, recently it has been demonstrated that the uptake of rod-shaped NPs by macrophages was more efficient than for spherical NPs. In addition spherical NPs were uptaken by cervical cancer and human lung epithelial cells more efficiently than those with rod-shaped NPs (Bartneck et al., 2010).

1.3.5.2. Nanoparticles Surface properties

The NPs surface properties also influence the effectiveness of these system (Verma and Stellacci, 2010). The zeta (ξ) potential or surface charge of the particle has been shown to be important in the cellular uptake, because opposite charges at the surfaces cytoplasm

membrane of cell and the NPs will promote their interaction (Zhang et al., 2008b, Patil et al., 2007, He et al., 2010). The rate of clearance from the blood was significantly higher for the negatively charged particles (ξ-potential ~ -40 mV) than for neutral ones (ξ-potential ±10 mV). The negatively charged particles also showed an increased rate of Mononuclear Phagocytic System (MPS) uptake in the liver compared to the neutral NPs, indicating that phagocytic cells favoured the uptake of negatively charged particles, and thus increase the rate of clearance of particles from the blood (Li and Huang, 2008).

The presence of ligands, such as antibodies or other molecules, such as amino acids, glycoproteins or proteins (see Table 3) also influence NPs therapeutic applicability. The anti-bodies ligands have a higher degree of specificity for the target tissue then the others However, the antibody stability, immunogenicity and half-life can limit their use (Scott et al., 2012). They are also expensive and time-consuming to produce, and problems related with stability and storage might exist (Allen, 2002). The non-anti-bodies ligands are simpler accessible, inexpensive to manufacture and easy to handle, but are not so specific (Allen, 2002).

Table 3: Ligands that have been used for functionalized the NPs surface. CEA - carcinoembyonic antigen; GM-CSF - granulocyte–macrophage colony-stimulating factor; LTTs - ligand-targeted therapeutics; NGR - Asn–Gly–Arg tripeptide; RGD - Arg–Gly–Asp tripeptide; TAG72 - oncofetal antigen tumour-associated glycoprotein-72; VEGFR - vascular endothelial growth-factor receptor (adapted from (Allen, 2002)).

Targeting ligands	Target	Tumour target
Non antibodies		
RGD	Cellular adhesion molecules	Vasculature endothelial cells in solid tumour
NGR	Aminopeptidase	
Folate	Folate receptor	Cancer cells that overexpress the folate receptor
Transferrin	Transferrin receptor	Cancer cells that overexpress the transferrin receptor
GM-CSF	GM-CSF receptor	Leukaemic blasts
Galactosamine	Galactosamine receptor	Hepatoma
Anti-bodies		
Anti-VEGFR	Vasculature endothelial growth-factor receptor	Vasculature endothelial cells in solid tumour
Anti-ERBB$_2$	ERBB$_2$ receptor	Breast, ovarian cancers and others
Anti-CD20	CD20, a B-cell surface antigen	Non-Hodgkin's lymphoma and other B-cell lymphoproliferative diseases
Anti-CD22	CD12, a B-cell surface antigen	
Anti-CD19	CD19, a pan-B-cell surface epitope	
Anti-CD33	CD33, a T-cell epitope	Acute myeloid leukaemia
Anti-CD25	CD25, α-subunit of the interleukin-2 receptor on activated T cells	Hairy-cell leukaemia, Hodgkin's and other CD25+ lymphoma haematological malignancies
	Interleukin-2 receptor	Cutaneous T-cell lymphoma
Anti-HLA-DR10ß	HLA-DR10ß subunit	Non-Hodgkin's lymphoma and other B-cell lymphoproliferative diseases
Anti-tenascin	Extracellular-matrix protein overexpressed in many tumours	Glial tumours, breast cancer
Anti-CEA	CEA	Colorectal, small-cell lung and ovarian cancers
Anti-MUC1	MUC1, an glycosylated epithelial mucin	Breast and bladder cancer
Anti-TAG72	TAG72, oncofetal antigen tumour-associated glycoprotein-72	Colorectal, ovarian and breast cancer

1.3.5.3. Drug loading and release profile

The loading/release drug capacity of NPs is another important property. For therapeutic purposes it is fundamental that the DDS are able to encapsulate drugs without suffering any degree of degradation. Drug loading in NPs systems can be done by two methods: during the preparation of particles (incorporation) or after the formation of particles (incubation). The drug becomes physically embedded into the matrix or is adsorbed onto particles surface (Agnihotri et al., 2004).

The release of the drug encapsulated in the NPs along the time, is usually characterized *in vitro* by using phosphate buffer solution (Chawla and Amiji, 2002), simulated body fluid (Rai et al., 2005), Tris buffer (Chen and Du, 2013), or with the addition of some specific enzymes that degrade the polymeric matrix in order to mimic the physiological conditions (Chawla and Amiji, 2002). It is important that the period of release of the drug is enough to allow drug to arrive to the target site with a concentration within the therapeutic window.

1.3.6. Mechanisms of cellular uptake

NPs can accumulate at the tumour microenvironment, through active (see section 1.3.3.2) and passive targeting (Danhier et al., 2010).

Passive targeting takes advantage of the biological characteristics of the tumor microenvironment itself, namely by exploiting the existence of a highly vascularized network of leaky blood vessels that surround the tumor tissues (see Figure 14) (Federman and Denny, 2010).

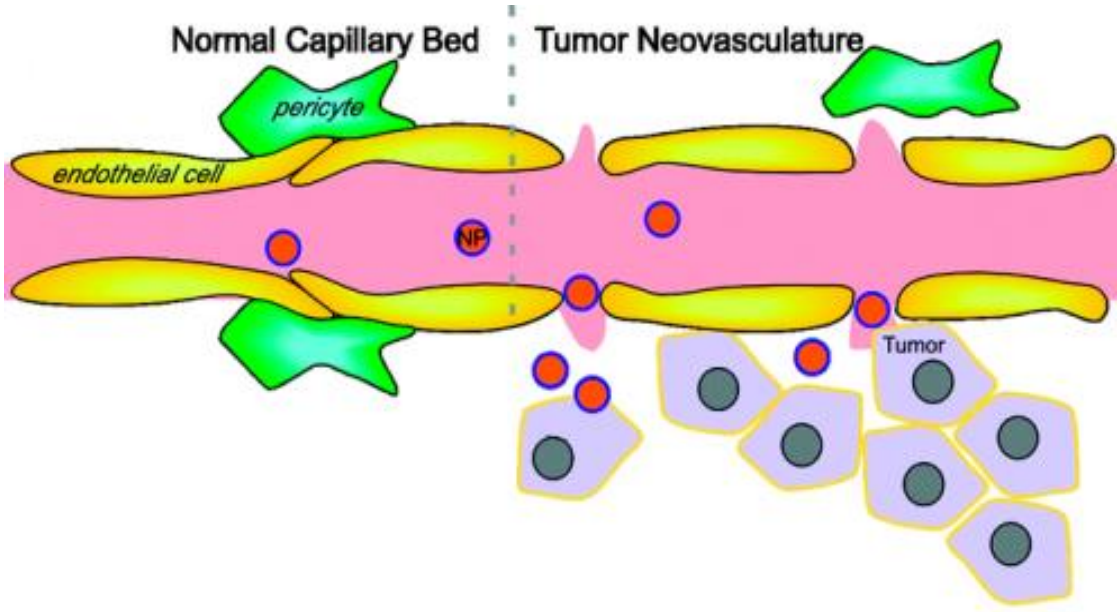

Figure 14: Representation of targeted nanoparticle delivery to cancer cells. The nanoparticles migrate from the intact circulatory system into the disordered tumour vasculature. Nanoparticles are able to accumulate and extravasate through fenestrations (Federman and Denny, 2010).

Once the nanocarriers interact with biological fluids their physicochemical properties are changed in such an extent that can influence the nanocarrier-cell interactions, and consequently the target cells (M Rabanel et al., 2012). Opsonisation is one of the major barriers that DDS need to overcome (Albanese et al., 2012). Opsonisation followed by phagocytosis is mediated by the MPS. This system is comprised by immune system cells, like phagocytic cells, which are especially present in the spleen and liver. Depending on nanoparticle surface potential and size, opsonins, proteins that are present in the blood, rapidly bind to NPs enabling macrophages to remove them from blood circulation (Albanese et al., 2012, Owens III and Peppas, 2006). Once nanocarriers successfully evade MPS, they can accomplish their accumulation at the tumor tissues. The accumulation of DDS in diseased tissues after intravenous administration is critical, since it will determine the therapeutic efficacy of the DDS into target cells.

Contrarily to normal tissues vasculature, in tumour ambient, the capillary bed is constructed by an assortment of malformed branching structures, juxtaposed vessels of random calibre and dimension. The vessels themselves are immature, consisting of loosely fitted endothelial cells lacking pericyte support. Tumour vessels have large endothelial fenestrations ranging from 200 to 800 nm in contrast to normal endothelium with pores with 5–10 nm (Torchilin, 2011, Aslan et al., 2013). As a result, there is an increased capillary permeability leading to extravasation of plasma proteins and others molecules resulting in an increased extravascular pressure within many solid tumours (Federman and Denny, 2010). Taking advantage of the unique pathophysiological characteristic of tumour vessels, the nanocarriers accumulate in this zone by the enhanced permeability and retention (EPR) effect. This effect is taken into account for the formulation of nanodevices suitable for anti-cancer therapy (Stylianopoulos, 2013).

When the NPs can't diffuse through the cell membrane, there are others mechanisms (endocytosis) that allow their entrance into cell (Kettler et al., 2014). Generally, endocytosis can be divided into two broad categories — phagocytosis and pinocytosis (Sahay et al., 2010). Pinocytosis can also be divided in caveolin-mediated endocytosis, clathrin-mediated endocytosis, caveolin-clathrin independent endocytosis and macropinocytosis (Thurn et al., 2007, Treuel et al., 2013) (see Figure 15).

Phagocytosis is responsible for the uptake of large particles, pathogens (bacteria or yeast), remnants of dead cells and arterial deposits of fat. This mechanism is mainly performed by specialized cells such as macrophages, monocytes and neutrophils (Conner and Schmid, 2003, Kettler et al., 2014). It is a highly regulated process that involves specific cell-surface receptors and signalling cascades mediated by Rho-family GTPases (Conner and Schmid, 2003, Hall and Nobes, 2000).

Pinocytosis is a type of endocytosis in which small particles, fluids and solutes are transported into the cell. It is a non-specific and non-absorptive process and as above described can be divided into 4 types (Petros and DeSimone, 2010).

Macropinocytosis is a regulated form of endocytosis that mediates the non-selective uptake of solute molecules, nutrients and antigens, forming vesicle with sizes between 100nm to 5 μm (Kettler et al., 2014). It is an actin-dependent process that originates the formation of large endocytic vacuoles (macropinosomes) (Lim and Gleeson, 2011).

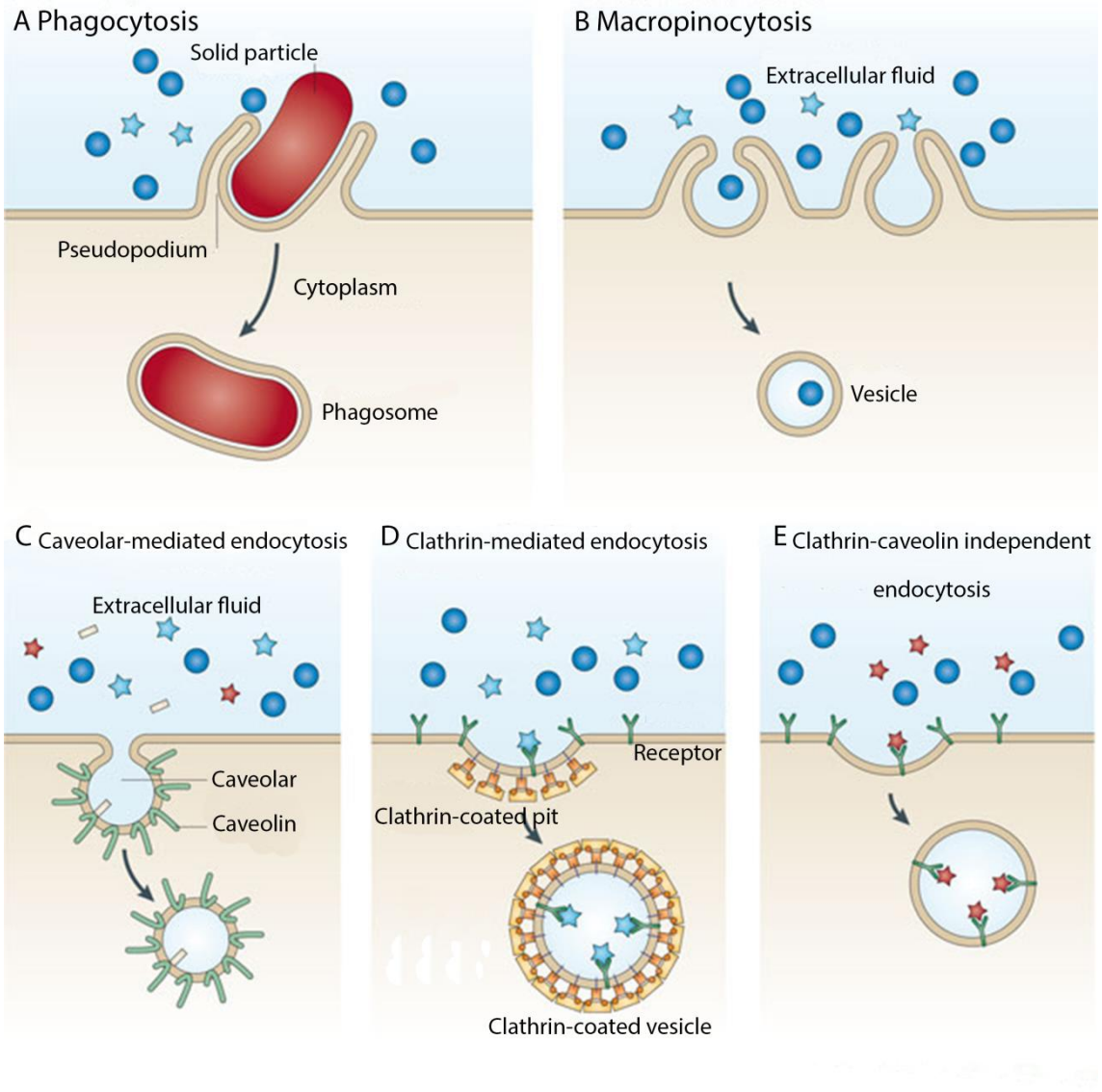

Figure 15: Mechanisms of nanoparticles internalization in cells. Internalization of large particles is facilitated by phagocytosis (A). Nonspecific internalization of smaller particles can occur through macropinocytosis (B). Smaller nanoparticles can be internalized through several pathways, including caveolar-mediated endocytosis (C), clathrin-mediated endocytosis (D) and clathrin-independent and caveolin-independent endocytosis (E). Nanoparticles are represented by blue circles (< 1 μm), blue stars (about 120 nm), red stars (about 90 nm) and yellow rods (about 60 nm) (adapted from (Petros and DeSimone, 2010)).

Clathrin mediated endocytosis is the classical route for the uptake of essential nutrients, and is also involved in the uptake of the majority of the NPs in non-macrophage cells. Several accessory proteins (such as clathrin, Adaptor protein -180, EPS 15, EPS 15-interacting protein, Dynamin 1 and Endophilin) are implicated in the formation of clathrin-coated vesicles that result from the accumulation of extracellular macro/micro/nano molecules into clathrin coated vesicles that fuse to early endosomal vesicles eventually becoming degradative lysosomes (Mousavi et al., 2004). Endosome escape has been reported for NPs with a positively charged surface (Thurn et al., 2007).

Caveolar mediated endocytosis occurs through the invagination of the plasma membrane (Conner and Schmid, 2003). The shape and structural organization of caveolae are conferred by caveolin, a dimeric protein that binds to cholesterol, inserts a loop into the inner leaflet of the plasma membrane, and assembles to form a striated caveolin coat on the surface of the membrane invaginations (Kiss and Botos, 2009). This protein is abundant in muscle, endothelial cells, fibroblasts and adipocytes and absent in neurons and leukocytes (Doherty and McMahon, 2009). The internalization of caveolae vesicles can be triggered through a sinalling cascade that results in tyrosine-phosphorylation of caveolae constituents (Pelkmans and Helenius, 2002).

Caveolae and clathrin independent endocytosis mediates the internalization of extracellular fluid, such as glycosylphosphatidylinositol-linked proteins, interlukin-2, growth hormones, etc. (Sahay et al., 2010). This requires specific lipid compositions, such as actin, GRAF, PAK flotillin, and are dependent on cholesterol (Doherty and McMahon, 2009).

1.4. Objectives

In the present study polymeric NPs based in PCL and PMMA were produced to be applied in cancer treatment. The present work plan had the following aims:

- Design, production and characterization of polymeric nanocarriers composed of PMMA and PCL;
- Characterization of the morphology and structural properties of NPs by Scanning Electron Microscopy (SEM) and Fourier Transform Infrared (FTIR);
- Characterization of the drug loading and release profiles of the nanovehicles;
- Evaluation of the cytotoxic profile of the NPs;
- Characterization of the cellular uptake of the NPs produced.

Chapter II:

Materials and Methods

2. Materials and Methods

2.1. Materials

Human cervical cancer cells (HeLa) were obtained from ATCC (Middlesex, UK). The cell culture plates and T-flasks were obtained from Orange Scientific (Braine-l'Alleud, Belgium). Ibidi cell imaging chambers plates were acquired from Ibidi GmbH (Munich, Germany). Cisplatin, Collagen type I, Dimethyl sulfoxide (DMSO), Dulbecco's Modified Eagle's Medium High Glucose medium (DMEM-HG), Ethanol (EtOH), Paraformaldehyde (PFA), Penicillin G, Phosphate-buffered saline solution (PBS), PCL (Mw 10000 kDa), PMMA (Mw 15000 kDa), Mowiol 4-88, Rhodamine B Isothiocianate, Streptomycin and Trypsin were purchased from Sigma-Aldrich (Sintra, Portugal). Anti-GFP and Hoescht 33342® were purchased from Life technologies (Porto, Portugal). 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) was purchased from Promega (Canada). Sodium hydrogen carbonate was bought from Panreac (Barcelona, Spain). Fetal bovine serum (FBS) was acquired to Biochrom AG (Berlin, Germany).

2.2. Methods

2.2.1. Synthesis of PCL/PMMA nanoparticles

PCL/PMMA NPs (PCL/PMMA NP) were produced through an adaptation of the nanoprecipitation method previously described by Fessi *et al.* (Fessi et al., 1989). Briefly, PCL and PMMA were dissolved in acetonitrile by mild heating and sonication. The polymeric solutions were gently poured drop wise into deionized distilled water, containing PVA under strong magnetic stirring. The resulting suspension of NPs was centrifuged at 8 000 g, for 20 min. The supernatant was discarded. The pellet was washed three times with distilled water and stored at 4ºC. To produce Cisplatin loaded PCL/PMMA NP (cis-PCL/PMMA NP) an amount of cisplatin (0.05mg of cisplatin per 1 mg of polymer) was dissolve in DMSO and then added to the acetonitrile polymer solution before be added drop wise to the aqueous phase.

2.2.2. Scanning electron microscopy analysis of nanoparticles

The NPs morphology was characterized by scanning electron microscopy (SEM) analysis.

For the preparation of the samples, one drop of the NPs suspension was added to a 15 mm cover glass. After being dried overnight, the sample was placed on an aluminium board using a double-side adhesive tape and covered with gold using a Quorom Q 150 R ES (United Kingdon) sputter coater. Samples analysis was performed by using a Hitachi S-3400N (Tokyo,

Japan) scanning electron microscope operated at an accelerating voltage of 20 kV, at diferent amplifications (Ribeiro et al., 2009).

2.2.3. Transmission electron microscopy analysis of nanoparticles

The morphological of PCL/PMMA NPs and cis-PCL/PMMA NPs was conducted by transmission electron microscopy (TEM) using an Hitachi HT7700 (Tokyo, Japan). The samples were suspended in mili-Q water and placed on copper grids with Formvar® films for TEM analysis.

2.2.4. Characterization of Size and Zeta Potential of nanoparticles

The size and zeta potential (surface charge) of produced PCL/PMMA NPs and cis-PCL/PMMA NPs was determined by dynamic light scattering (DLS). Prior to analysis, NPs were resuspended in 1000 µL of mili-Q water, sonicated and then analyzed immediately. Sample analysis was performed at 25 °C by using a disposable folded capillary cell. All sample measurements were performed in a Zetasizer Nano Zs instrument (Malvern Instruments, Worcestershire, UK).

2.2.5. Fourier Transform Infrared spectroscopy analyses of nanoparticles

The produced PCL/PMMA NP, cis-PCL/PMMA NP, commercial cisplatin, PCL, PMMA and PVA spectra were acquired in the range of 4000–400 cm^{-1}, using a Nicoletis 20 FTIR spectrophotometer, operating in ATR mode (MKII GoldenGate™ Single Reflexion ATR System). Data collection was performed with a 4 cm^{-1} spectral resolution and with 128 scans (Coimbra et al., 2011).

2.2.6. Characterization of the loading profile of the nanoparticles

The amount of cisplatin encapsulated in NPs was determined by the tin(II) chloride ($SnCl_{2)}$ method (Nishiyama et al., 1999). 100 µL of the supernatant obtained in the first step of centrifugation of cis-PCL/PMMA NPs was mixed with 100 µL of 2N HCl-0.2M SnCl2 solution. The Pt content in the solution was then determined spectrophotometrically, by reading the absorbance of the Pt complex (complex formed between the pt of cisplatin and Sn at 403 nm. The non-encapsulated drug was subtracted to the initial drug concentration and the percentage of encapsulation efficiency determined (Yu et al., 2008). A UV-1700 PharmaSpec

spectrophotometer from Shimadzu (Kyoto, Japan) was used to acquire the data and UV Probe Shimadzu 2.0 software was used for data analysis.

2.2.7. Characterization of the release profile of the nanoparticles

The release profile of the carriers was characterized by re-suspending the cis-PCL/PMMA NPs in PBS 1% at a concentration of 1mg/mL. At different time points (1h, 6h, 12h, 24h, 72h and 168h) 100 µL of PBS solution was collected and the amount of cisplatin released was determined through the procedure previously described in 2.2.4.

2.2.8. Characterization of the cell proliferation in the presence of PCL/PMMA nanoparticles

Hela cells were cultured in DMEM-HG medium supplemented with heat-inactivated FBS (10% v/v) and antibiotic/antimitotic (1%v/v) in an incubator at 37ºC, with a 5% CO_2 humidified atmosphere. Human fibroblast (HFib) cells were cultured in DMEM-F12 medium supplemented with heat-inactivated FBS (10% v/v) and antibiotic/antimitotic (1%v/v) in an incubator at 37ºC, with a 5% CO_2 humidified atmosphere. To evaluate cell proliferation in the presence of NPs, cells were seeded into a 96-well cell culture plates, at a density of 10×10^3 cells/well in contact with different concentrations of PCL/PMMA NP (between 65.5 and 1000 µg/mL) (Ribeiro et al., 2009, Gaspar et al., 2011). The cell growth was monitored at 24, 48 and 72 hours using an Olympus CX14 inverted light microscope (Tokyo, Japan) equipped with an Olympus SP-500 UZ digital camera. The positive controls (k+) were treated with EtOH 96%, while in the negative control (k^-) cells were cultured in the absence of NPs.

2.2.9. Characterization of the biocompatibility of PCL/PMMA nanoparticles

PCL/PMMA NPs biocompatibility was evaluated through an MTS assay. HFib or Hela were seeded in a 96-well cell culture plates, at a density of 10×10^3 cells/well, with DMEM-HG or DMEM-F12 medium, respectively, supplemented with FBS (10% v/v) and incubated at 37ºC, with a 5% CO_2 humidified atmosphere. In the following day, the medium was replaced and cells were incubated with crescent concentrations of PCL/PMMA NPs, ranging from 65.5 to 1000 µg/mL. After an incubation of 24, 48 and 72h, the medium of each well was removed and replaced by a mixture of 100µL of fresh culture medium and 20µL of MTS reagent solution. The cells were incubated for 4h, at 37°C, under a 5% CO_2 humidified atmosphere. Cell viability was assessed through the reduction of the MTS into a water-soluble brown formazan product. Subsequently, the absorbance of the samples was measured at 492nm

using a microplate reader (Sanofi, Diagnostics Pauster). The values of absorbance obtained for the production formazan are directly proportional to the metabolic activity of cells, which is directly dependent on the number of viable cells. The k^+ was treated with EtOH 96%, and in the k^- cells were kept in culture only with culture medium (Ribeiro et al., 2009).

2.2.10. Characterization of the cellular uptake of PCL/PMMA nanoparticles

Hela cells were seeded in a µ-slide 8 glass bottom dishes at a density of 20×10^3 cells/well with 200 µL of DMEM-HG medium supplemented with FBS (10% v/v) without antibiotic, in order to allow cell transfection (Cunningham et al., 2007). The wells were previously coated with collagen to promote cell adhesion to the surface of the plate. 24 hours later cells were transfected with the produced NPs. After 4 hours the transfection process was stopped and cells were fixed with paraformaldehyde. The NPs used for transfection were previously loaded with fluorescein Rhodamine B, by mixing it with the polymer and then the protocol described in 2.2.1 was followed.

Subsequently, confocal Laser Scanning Microscopy (CLSM) was used to evaluate the capacity of the NPs to enter into cells, and also to evaluate their intracellular distribution. Previously to cell transfection, cell nucleus were stained with a Hoechst 33342® molecular probe and incubated for 20-30 min. Moreover, the actin of adhered cells was stained with anti-GFP. The glass bottom dishes with transfected cells were visualized using a Zeiss LSM 710 laser scanning confocal microscope (Carl Zeiss SMT Inc., New York, USA) equipped with a plane-apochromatic 63x/DIC objective. Data analysis of CLSM images was performed with Zeiss software (Axio Vs40 V4.5).

2.2.11. IC50 determination of cisplatin

To evaluate the concentration of cisplatin that is needed to eliminate an half of the population (IC50) of Hela cells, an Alamar Blue was used. Hela cells were seeded into a 96-well cell culture plates, at a density of 10×10^3 cells/well, with DMEM-HG medium supplemented with FBS (10% v/v) and incubated at 37ºC, with a 5% CO_2 humidified atmosphere. In the following day, the culture medium was replaced and cells were incubated with crescent concentrations of Cisplatin ranging from 2-100 µM. After an incubation of 24h, the medium of each well was removed and replaced with a mixture of 100µL of fresh culture medium and 10µL of resazurin reagent solution. The cells were incubated for 24h, at 37°C, in a 5% CO_2 humidified atmosphere. Cell viability was assessed through the reduction of the resazurin into resorufin product. Fluorescence measurements were then performed in a plate reader spectrofluorometer (Spectramax Gemini XS, Molec-ular Devices LLC, USA) at an

excitation/emission wavelength of 560/590 nm, respectively. The values of absorbance obtained are directly proportional to the metabolic activity of cells, which is also directly proportional to the number of viable cells. The positive controls (k^+) were treated with EtOH 96%, and in the negative control (k^-) cells were cultured in the in culture with medium.

2.2.12. Evaluation of the cytotoxic profile of the produced loaded nanoparticles

The anti-tumoral activity of the produced cis-PCL/PMMA NP was determined through an MTS assay. First, Hela cells were seeded into a 96-well cell culture plates, at a density of 10×10^3 cells/well, with DMEM-HG medium supplemented with FBS (10% v/v) and incubated at 37ºC, with a 5% CO_2 humidified atmosphere. In the following day, the medium was replaced and cells were incubated with the produced loaded NPs containing a final concentration of 75µM and 80µM of encapsulated drug, and 100µM of free cisplatin. These values were chosen because they are around the value of IC50. After an incubation period of 24h, 72h and 7d the medium of each well was then removed and replaced with a mixture of 100µL of fresh culture medium and 20µL of MTS reagent solution. The cells were incubated for 4h, at 37°C, under a 5% CO_2 humidified atmosphere. Cell viability was assessed through the reduction of the MTS into a water-soluble brown formazan product. The absorbance was measured at 492nm using a microplate reader (Sanofi, Diagnostics Pauster). The values of absorbance obtained for formazan are directly proportional to the metabolic activity of cells, and subsequently equivalent to the number of viable cells. In K^- cells were kept in culture with medium.

2.2.13. Statistical analysis

One-way analysis of variance (ANOVA) with the post-hoc Newman-Keuls test was used to compare the results obtained for the different groups. A $p< 0.05$ was considered statistically significant. Additional p values ($p<0.01$ and $p<0.001$) were used to ascertain higher degrees of significance. The analysis of all data was performed in the GraphPad Prism v.5.0 software (Trial version, GraphPad Software, CA, USA).

Chapter III:

Results and Discussion

3. Results and Discussion

3.1. Particles morphology, size and zeta potential characterization

Surface morphology and shape of the produced NPs were analyzed through SEM and TEM. As can be observed in the Figure 16, the produced unloaded NPs present a spherical-like morphology which is crucial to maximize cellular uptake and contribute for a better hydrodynamic behavior in the blood stream (Albanese et al., 2012). The surface of the NPs is smooth.

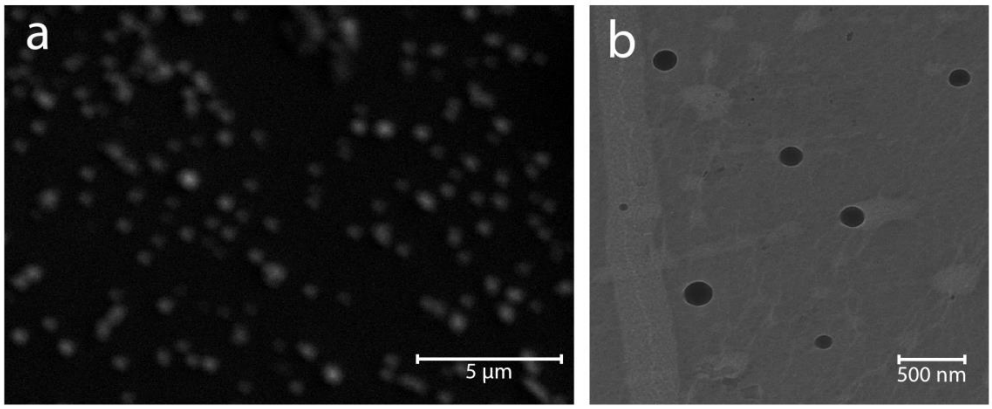

Figure 16: SEM and TEM images of PCL/PMMA NPs.

Cis-PCL/PMMA NPs also present a spherical-like morphology and a smooth surface (see Figure 17).

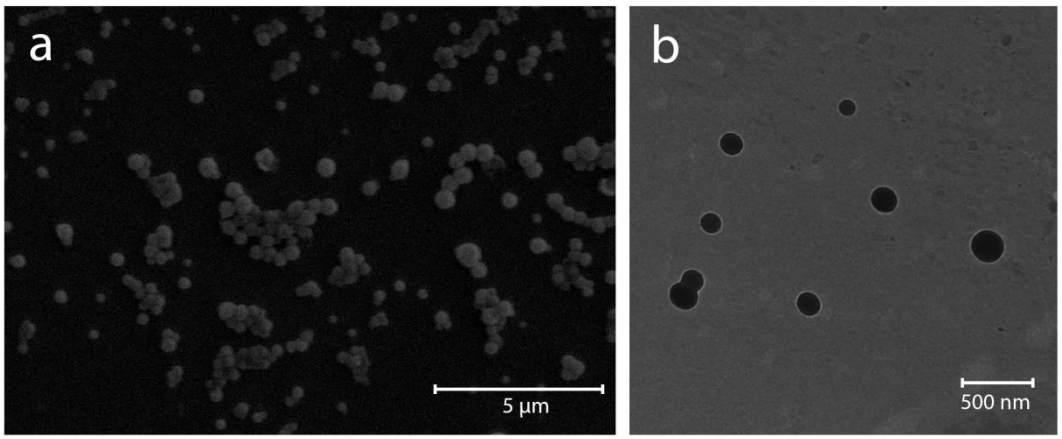

Figure 17: SEM and TEM images of cis-PCL/PMMA NPs.

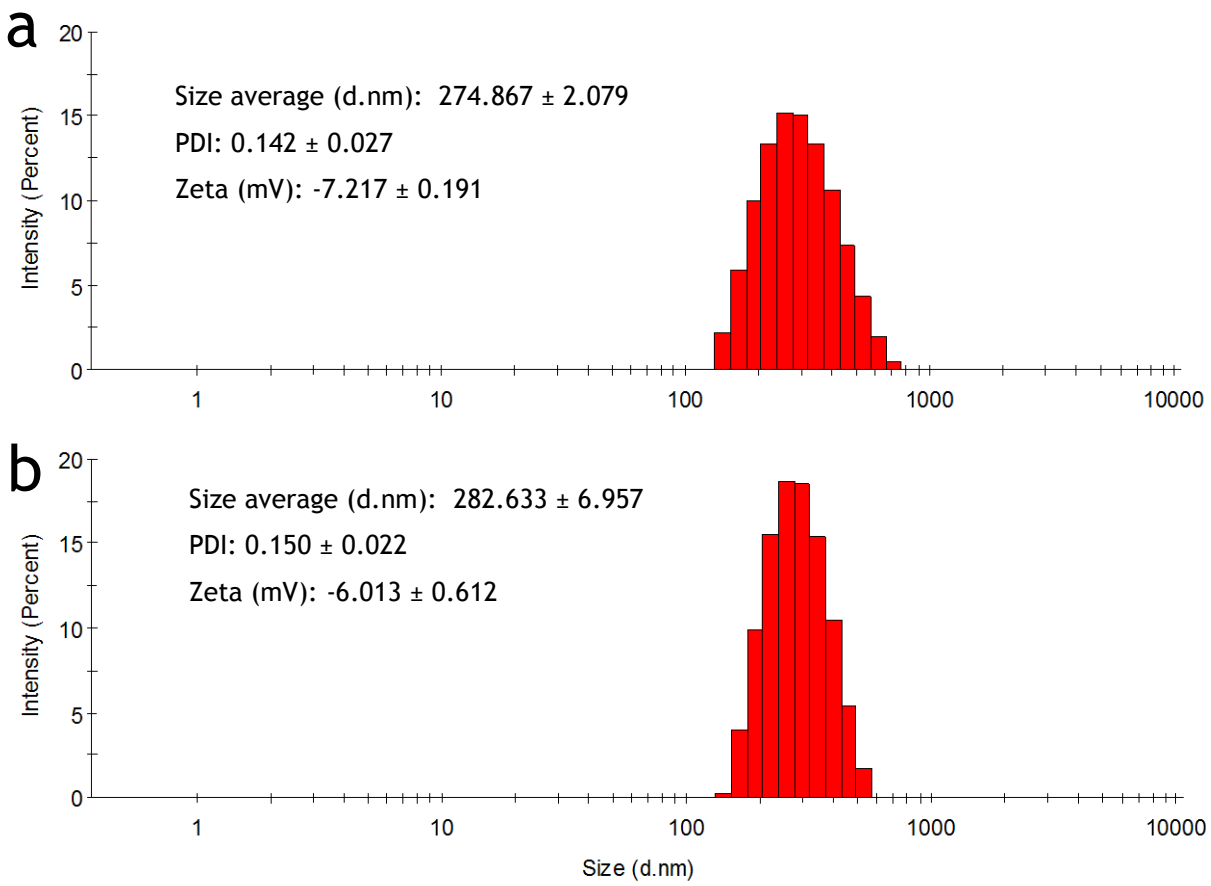

Figure 18: Size, PDI and zeta potential of PCL/PMMA NPs and cis-PCL/PMMA NPs (a and b), respectively. n=3. Data is presented as mean ± s.d.

NPs size is a critical characteristic since it ultimately determines nanocarrier accumulation in tumor tissues through the EPR effect (Ernsting et al., 2013). The size characterization was evaluated through DLS analysis. The results show that both the PCL/PMMA and cis-PCL/PMMA NPs are able to form nano-sized particles (figure 18). PCL/PMMA NPs produced here present a mean size of 274 nm, while cis-PCL/PMMA NPs have a mean size of 282 nm. This difference in NP size can be explained by the fact that cis-PCL/PMMA have drug encapsulated, with more core space being occupied.

In the literature there are several examples of PCL or PMMA NPs. For PCL it is easy to found nanoparticles with a size range between 85 and 408 nm (Lamprecht et al., 1999, Kumar and Sawant, 2013, Ribero et al., 2013, Li et al., 2013, Chawla and Amiji, 2002, Baimark, 2009, Gou et al., 2009, Ge et al., 2000, Yang et al., 2006). PMMA nanoparticles have been produced with sizes between 20 and 626 nm (Hsu et al., 2003, Lee et al., 2013, Singh and Khanna, 2007, Anancharungsuk et al., 2010, Rimessi et al., 2009, Park et al., 2006, Poovi et al., 2010, Akhlaghi et al., 2010, Zhu et al., 2008). The results obtained in this work are within the range founded in the literature.

It is not expected that the produced nanoparticles are eliminated through urine. However it may be expected that they will suffer some retention in the liver and spleen once particles with sizes higher than 200 nm are retained (Ernsting et al., 2013). So, in the ideal case, the produced nanoparticles should have a size ranging between 5 and 200 nm.

The production NPs with suitable size ranges is very important. Polydispersity index (PDI) values lower than 0.3 indicated uniform size distribution of NPs in all batches (Aminu et al., 2013). Herein, the NPs produced had an uniform size distribution, since their values are lower than 0.15.

The value of zeta potential obtained was similar for both NPs formulations (-7 mV for PCL/PMMA NPs and -6 mV for cis-PCL/PMMA NPs) In the literature there are some reports that show that NPs based on PCL or PMMA that present a zeta potential around the same value of those obtained for the produced NPs (Lee et al., 2013, Ribeiro et al., 2013). Another important aspect involved in cell-nanoparticle interaction is the surface charge of nanoparticles. Positively charged nanoparticles are quickly involved by serum proteins, which impairs their function, since they are eliminated by mononuclear phagocytic system in spleen and liver (Albanese et al., 2012). So, particles that have a zeta potential between -10 and +10 mV improve the time of blood circulation and the escape from retention in liver and spleen. This is a critical finding for the envisioned application of this DDS, since its administration route will be primarily by systemic injection.

3.2. Fourier Transform Infrared Spectroscopy Analysis of the produced nanoparticles

ATR-FTIR technique was substantial advantages in relation to others spectroscopic techniques in signal/noise ratio, resolution, speed, detection limits and the ability to identify almost all compounds present in the samples (Bacsik et al., 2004). ATR is based in the phenomenon of total internal reflection. When the beam of radiation found a material that selective absorbs radiation, the beam loses energy at a specific wavelength and the resultant attenuated radiation is measured and plotted as a function of wavelength by the spectrometer and gives rise to the absorption spectral characteristics of the sample (Griffiths and De Haseth, 2007, Stuart, 2004, Schrader, 2008).

In figure 19 are presented the spectra acquired for PVA (purple), PMMA (blue), PCL (green) and PCL/PMMA NP (orange).

The spectrum of commercial PVA FTIR show five characteristic peaks are found: one nearly 3259 cm^{-1} that belongs to O-H group; another band is visible nearly 2900 cm^{-1} resulting from C-H group; in the 1750 cm^{-1} region it is possible identify the peak resulting from C=O group;

between 1461 and 1417 cm^{-1} a peak of CH_2 group; nearly 1150 cm^{-1} there are a peak due to C-O-C and closely to 1140 cm^{-1} a peak of C-O group can be observed (Mansur et al., 2008).

FTIR spectrum of PMMA present four characteristics peaks: nearly 3000 cm^{-1} a peak that belongs to C-H groups, close to 1700 cm^{-1} from C=O groups, in the zone of 1400 cm^{-1} one from CH_3 groups and nearly 1200 cm^{-1} another from OCH_3 groups (Ramesh et al., 2007).

The FTIR spectrum of the commercial PCL used in this work present one characteristic peak at 1750 cm^{-1} from C=O groups (Correia et al., 2013).

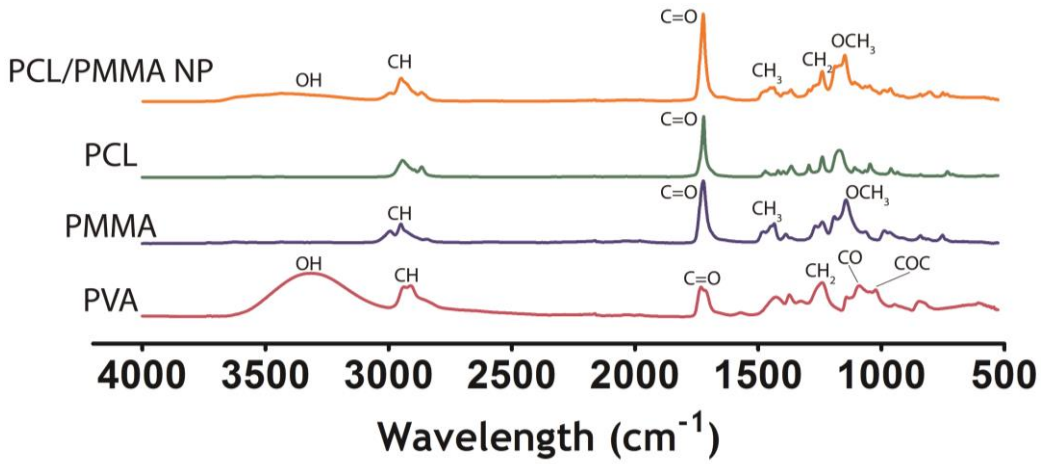

Figure 19: FTIR spectra of the produced nanoparticles and of the used compounds used for their production: purple – PVA; blue – PMMA; green – PCL; orange – PCL/PMMA NP.

In PCL/PMMA NP FTIR spectrum it is possible to see an intense peak at 1700 cm^{-1}. This peak correspond to C=O and is characteristic both for PCL and PMMA. In the zone of 3250 cm^{-1} it is possible to see a week peak corresponding to O-H group of PVA. Between 1461 and 1417 cm^{-1} exist a peak of CH_2 group, also characteristic of PVA. This shows that the formed NPs have a low percentage of PVA. Nearly 3000 cm^{-1}, a peak resulting from CH groups is observed, characteristic both from PVA and PMMA. In the zone of 1400 cm^{-1} and nearly 1200 cm^{-1} more peaks are visible and belong to CH_3 and OCH_3, respectively, that is also characteristic of PMMA. The spectra do not show any new bands for the blends that can be associated with some specific interactions between PCL, PMMA and PVA, thus suggesting the absence of specific interactions in these blends. This fact was already described in some previous works found in the literature, where these polymers have been described as do not be able to establish bounds between them (Kim and Lee, 2011, Liu and Kiran, 2008).

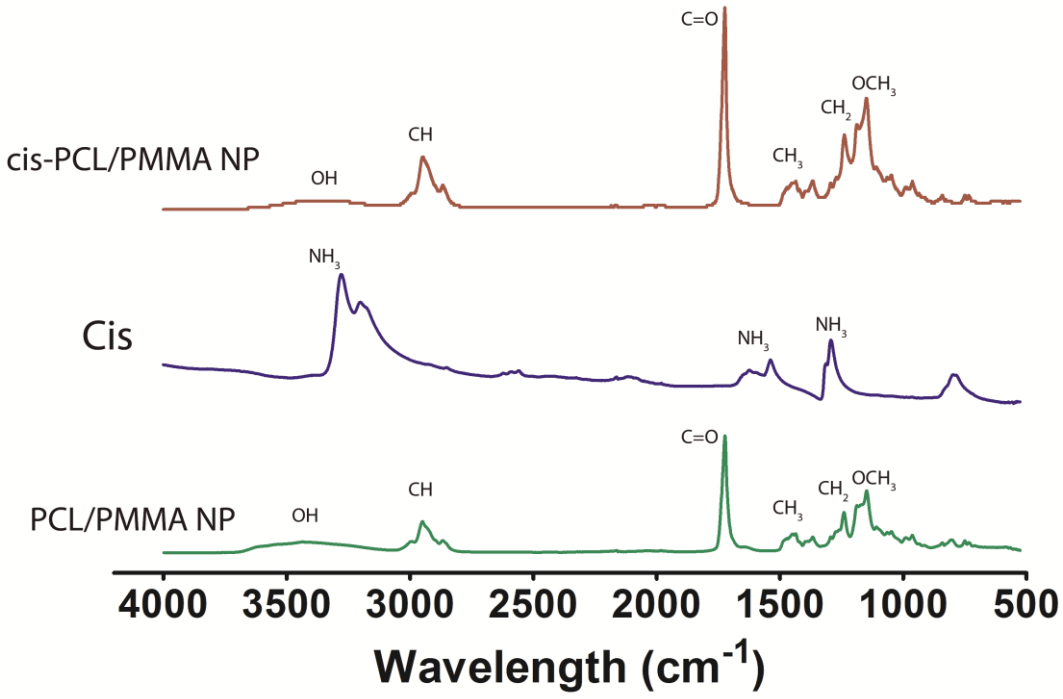

Figure 20: FTIR spectra of the produced nanoparticles and cisplatin: green - PCL/PMMA NP; blue – cisplatin; red - cis-PCL/PMMA NP.

In figure 20 are presented the spectra acquired for PCL/PMMA NP (green), cisplatin (blue) and cis-PCL/PMMA NP (red).

The characteristics peaks of cisplatin in the FTIR spectrum are found between 3400-3200 cm^{-1}, corresponding to the amine stretching peak, 1600-1500 cm^{-1} belonging to asymmetric amine bending and 1300-1200 cm^{-1} corresponding to symmetric amine bending (Yan and Gemeinhart, 2005).

The FTIR spectrum of Cis-PCL/PMMA NP is very similar to that of PCL/PMMA NP. As in the case of PCL/PMMA NP, this spectrum does not show any new bands for the blend,, thus suggesting the absence of specific interactions between PCL, PMMA, PVA and cisplatin (Kim and Lee, 2011, Liu and Kiran, 2008). The method used to produce the nanoparticles (nanoprecipitation) is reported to produce nanocapsules, where the drug is confined to the core of the nanoparticles (Mora-Huertas et al., 2010). Due to this fact in the FTIR spectrum does not show peaks belonging to cisplatin. Also, the fact of their reduced amount compared to the amount of polymer can explain the fact that no characteristic peak of cisplatin was clearly identified in the spectrum.

3.3. Characterization of the loading profile of the produced nanoparticles

The loading capacity of the PCL/PMMA produced nanoparticles was characterized using an indirect method. The amount of cisplatin in the supernatant was determined and subtracted to the initial amount of drug used to load NPs. The drug was chosen based on the different reports that describe its use in the treatment of cervix cancer (Waggoner, 2003, Cepeda et al., 2007, Fuertes et al., 2003b, Kamel et al., 2012, Markovic and Markovic, 2008).
A calibration curve of cisplatin was performed using water as solvent in order to determinate the loading profile of the nanoparticles (Figure 21). The absorbance of several cisplatin concentrations was determined at 403 nm, as described in section 2.2.4., to obtain the calibration curve.

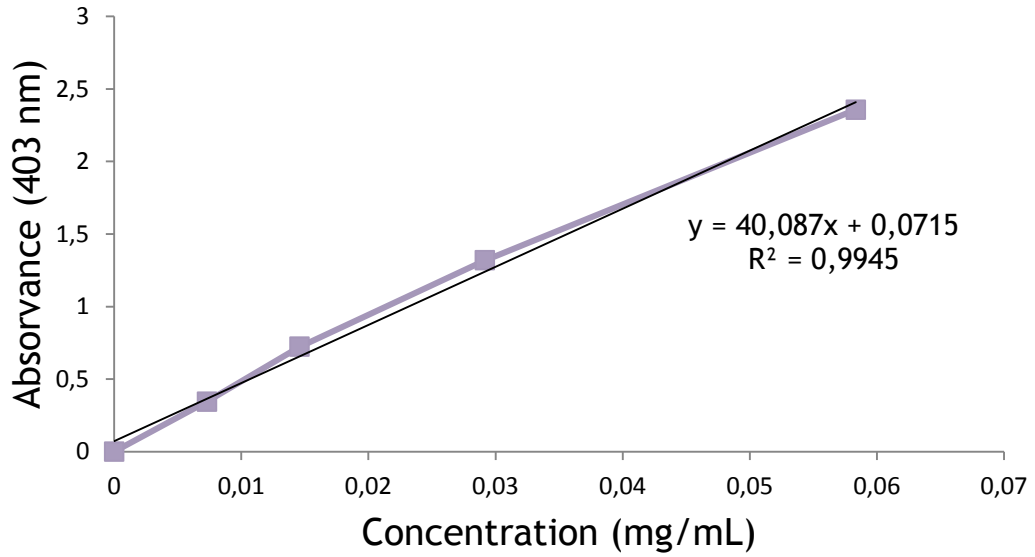

Figure 21: Calibration curve and its respective equation used to calculate the encapsulation efficiency of cisplatin in PCL/PMMA NP.

Two different formulations were produced by changing the stirring time during the production process (table 4). It was noticed that depending on the period of incubation the loading efficiency of the nanoparticles changed. So, a stirring time of 30 min was chosen to obtain drug encapsulation efficiency of 66.78% and the morphology and size shown in the section 3.1. The following tests were done using this formulation.

Table 4: Stirring time and encapsulation efficiency of different formulations.

Stirring time	EE%
30 min	66.78%
60 min	41.76%

3.4. Characterization of the release profile of the vehicles

It is important that nanoparticles can retain the drug in its inner core during relatively long periods of time, in order to provide its release at the target site with an appropriate concentration. In addition, increasing the period among which drug is released is important to reduce de number of administrations, whilst always assuring that the drug concentrations are within the therapeutic window (Taurin et al., 2013). Moreover, the fact that the particle does not release a significant amount of drug before reaching the target site is also an advantage, since it contributes for the reduction of the side effects that drug release could cause (Alexis et al., 2008).

The produced PCL/PMMA nanoparticles were maintained in a PBS solution at 37º C, pH=7.4, to study the drug release profile. PBS solution samples were collected after 1h, 6h, 12h, 24h, 72h and 168h. Samples absorbance was measured at 403 nm to evaluate the release of cisplatin from the vehicles. A calibration curve (figure 21) was performed to characterize the drug release profile from nanovehicles.

Figure 22 shows the release profile obtained for the produced cis-PCL/PMMA NP. The graph shows that the release of the drug occurs for at least 7 days (168 hours). In the first 24 hours about 21% of the drug is released, what corresponds to an initial burst release profile. After the first 24 hours the drug continues to be released, but at a slower rate reaching a 30% release after 168 hours. This fact can be very interesting in the medical approach since almost all the 30% of the loaded drug released from the nanoparticles is released after 6 hours. This increases the probability of the drug to be released at the target site.

The drug release from particles based on PCL is usually faster (Chawla and Amiji, 2002) than those based on PMMA (Poovi et al., 2010). This fact can be explained by the higher rate degradation presented by PCL in comparison to that of PMMA. The mixture of these materials is expected to promote an extended release along time. However, the release profile depends not only on the materials used to make the nanovehicle, but also on the type of drug encapsulate within NPs since the release depends of the drug solubility. Hydrophilic drugs will interact more easily with the water then hydrophobic ones, causing a easer release for the hydrophilic ones (Mohanraj and Chen, 2007).

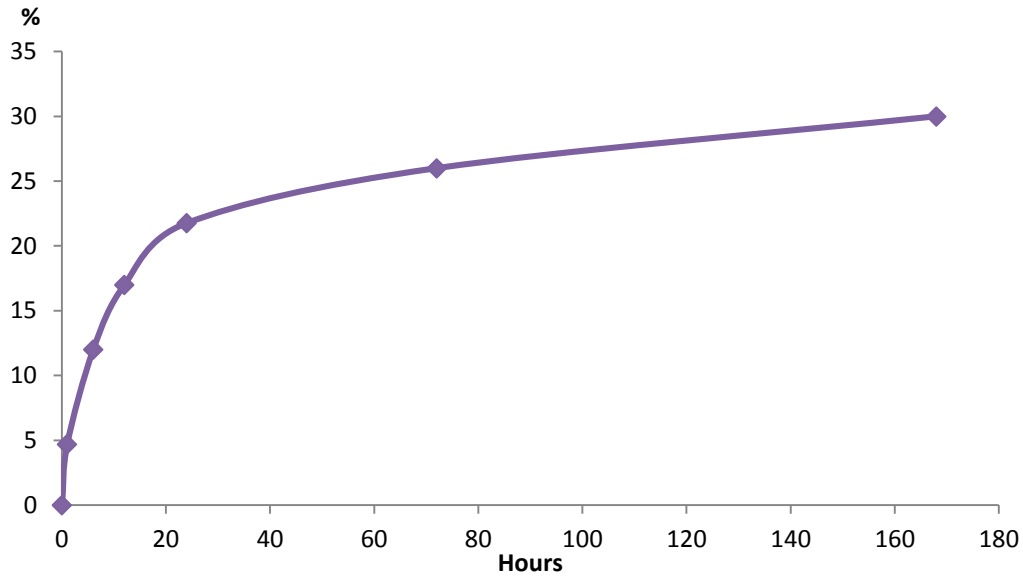

Figure 22: Release profile of cisplatin from cis-PCL/PMMA NP formulations at pH = 7.4.

3.5. Evaluation of PCL/PMMA nanoparticles biocompatibility

The cytotoxic profile of the produced nanoparticles was characterized through *in vitro* assays. This study was performed to address if the synthesized nanoparticles formulations are toxic for cells, using Hela and HFib as models.

After 24, 48 and 72 hours, cell adhesion and proliferation was noticed in wells, where cells were in contact with different concentrations of the produced nanoparticles, between 65.5 and 1000 µg/mL, and in the K^-, after 24 and 72 hours (Figure 25 and 26). In K^+, no cell adhesion or proliferation was observed; dead cells with their typical spherical shape are seen in Figure 23 and 24.

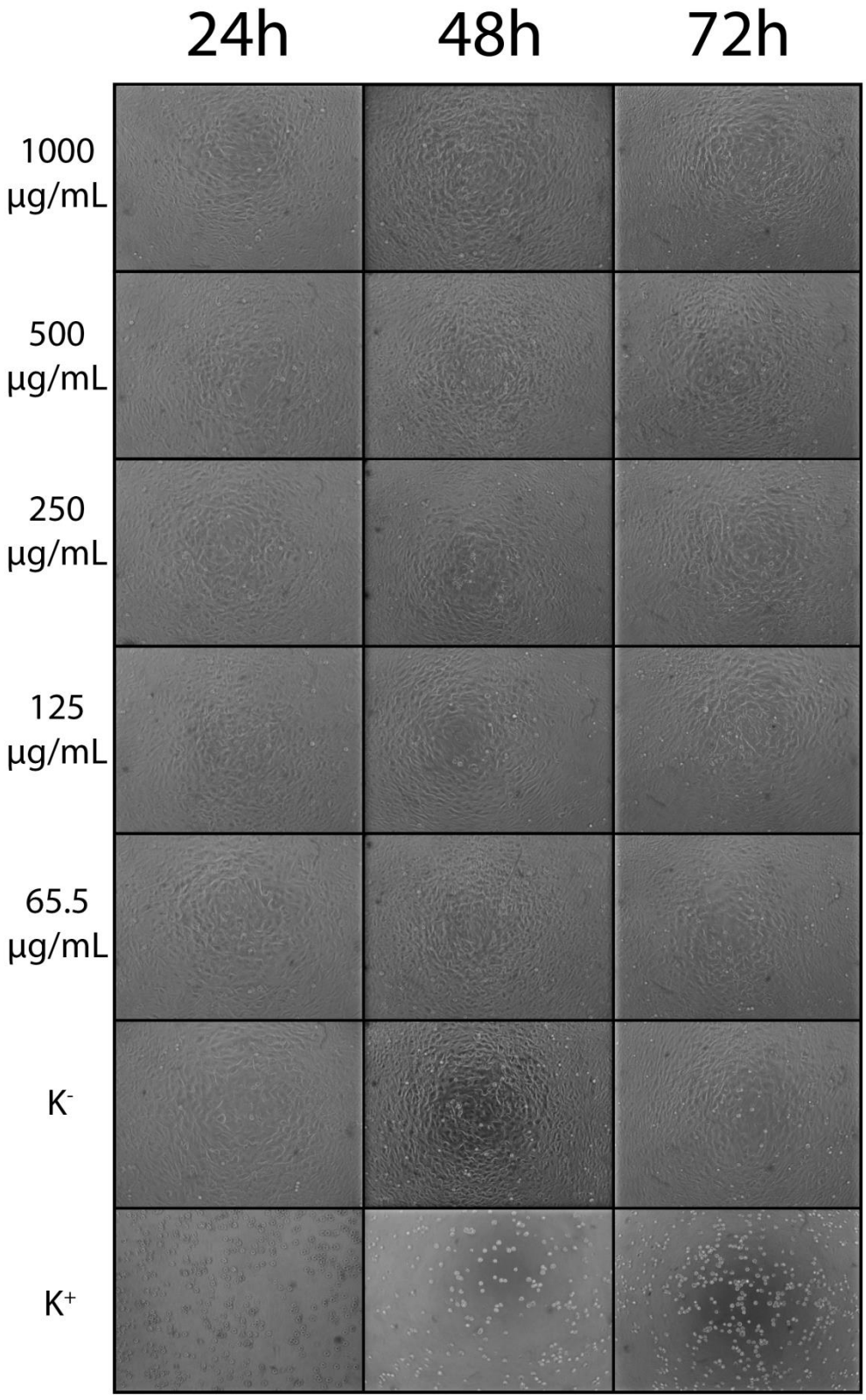

Figure 23: Inverted Microscope Images of HFib cells 24, 48 and 72h after being seeded with PCL/PMMA nanoparticles. K^- (live cells), K^+ (dead cells). Original magnification x100.

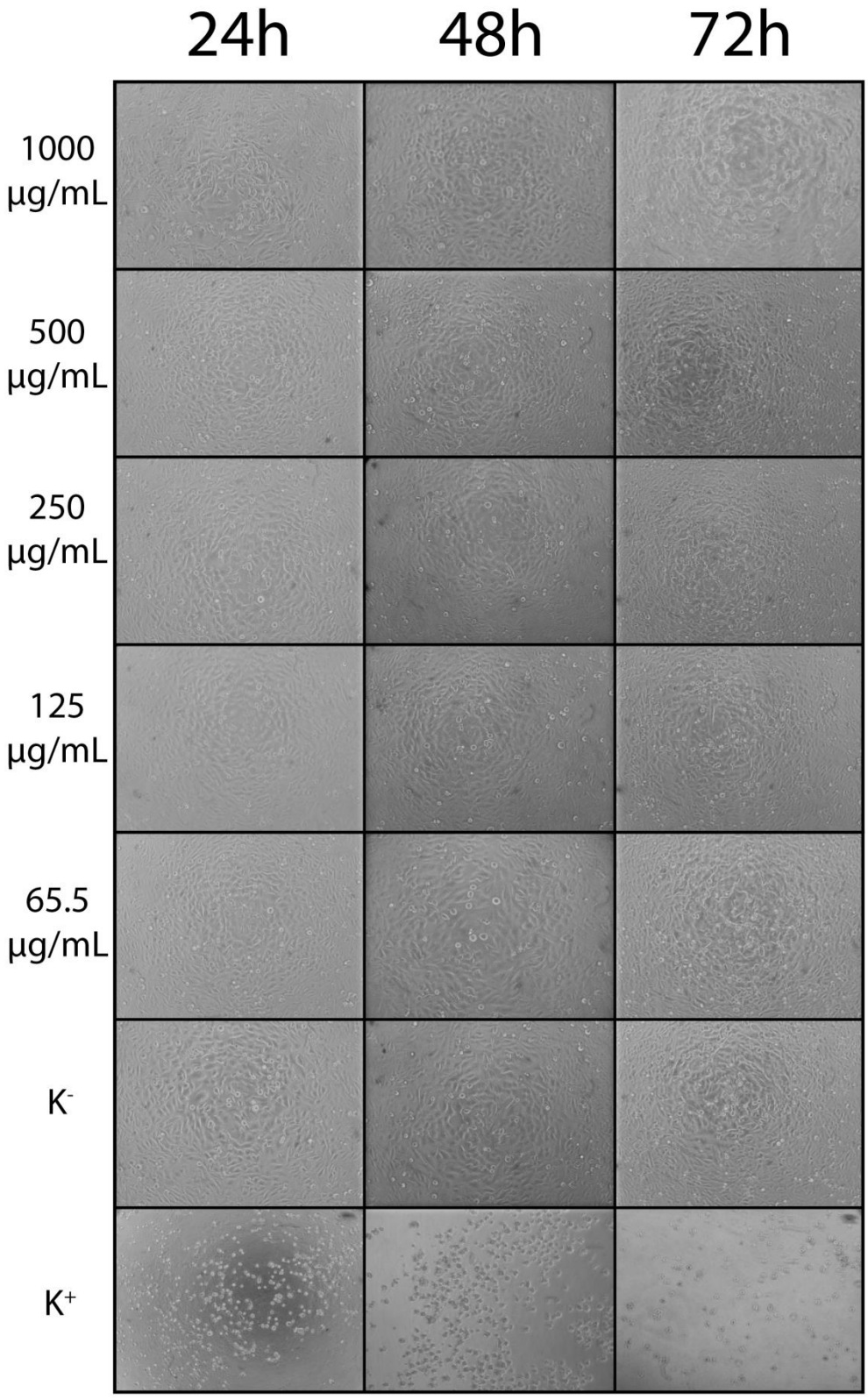

Figure 24: Inverted Microscope Images of Hela cells 24, 48 and 72h after being seeded with PCL/PMMA nanoparticles. K^- (live cells), K^+ (dead cells). Original magnification x100.

These results are in concordance with that founded in the literature both for PCL and PMMA, that are reported to be biocompatible when they were in contact with different types of cells (Bettencourt and Almeida, 2012, Woodruff and Hutmacher, 2010). PCL was been largely used either alone or conjugated with inorganic materials to improve their biocompatibility between 0.125 and 2 mg/mL (Filipović et al., 2014, Lale et al., 2014, Zhang et al., 2011). Several studies have shown that nanoparticles based on PMMA did not induce any cytotoxicity in a wide range of concentration (between 0.1 and 100 µg/mL) (Efthimiadou et al., 2014, Varchi et al., 2013). The concentration of polymers used in this work is within the range founded in the literature for PCL, and is higher than that of PMMA, and also present high cell viability in contact with them.

The biocompatibility of PCL/PMMA nanoparticles was characterized through an MTS assay. As already mentioned, Hela and Fib-H were seeded in contact with different concentrations of the nanoparticles, between 65.5 and 1000 µg/mL. The cellular viability was assessed over time (24, 48 and 72h). Due to the slow degradation of both the polymers used, it is not expected that the polymers suffer degradation during the period along which this experience is held.

The MTS assays results (Figure 25) showed that cells in contact with the nanoparticles had higher viability than the positive control, around 100%, during the period of incubation. This value is similar to that of K-. It also shows a statistically significant difference between the positive control and the negative control and cells in contact with nanoparticles.

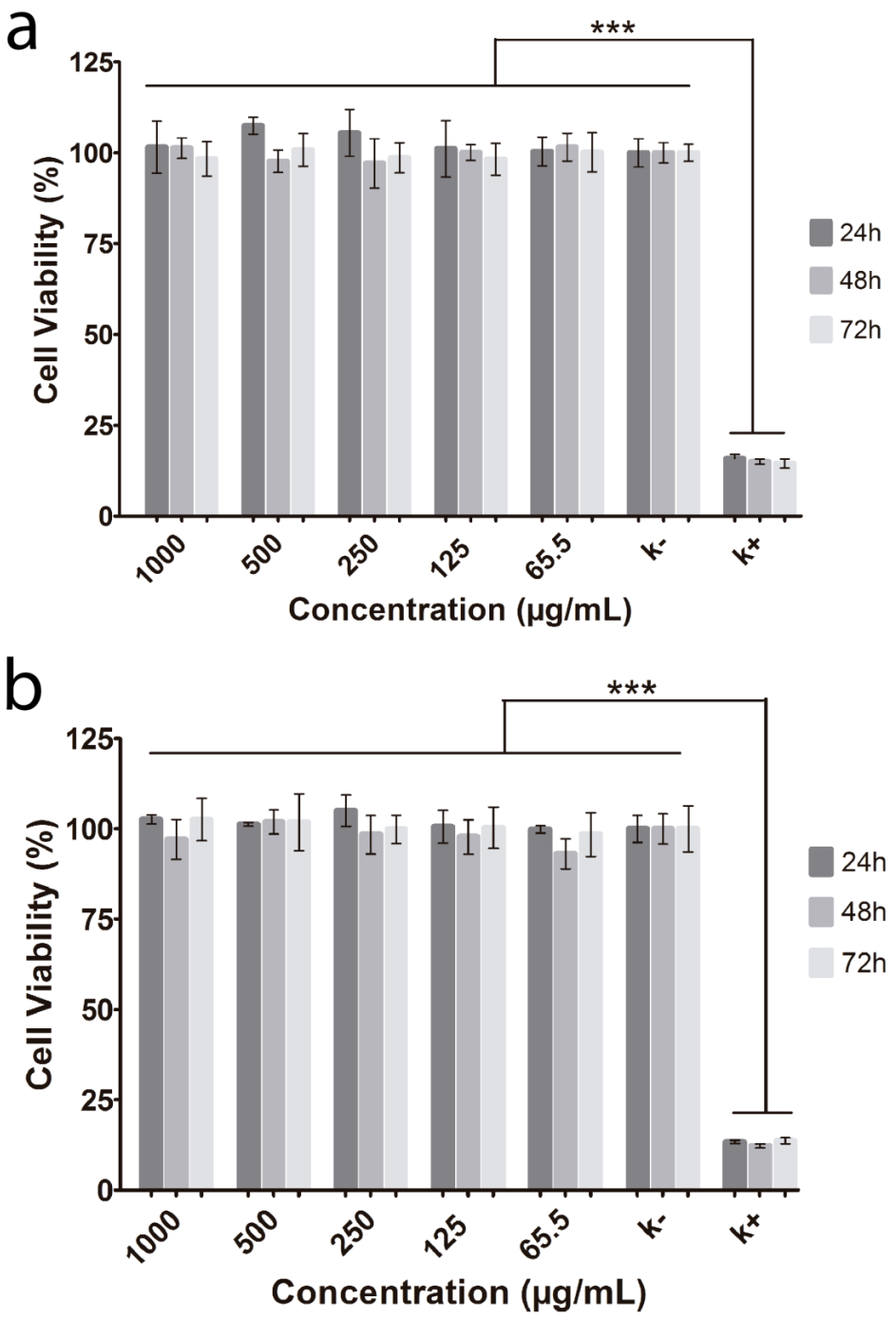

Figure 25: Evaluation of the cellular viability after cells being exposed to the produced nanoparticles with concentrations ranging from 65.5 to 1000 µg/mL in HFib (a) and Hela (b). Dark grey bars represent cell viability at 24h. Medium grey bars represent cell viability at 48h. Light grey bars represent cell viability at 72h. Cells culture in absence of nanoparticles was used as K^-. Cells treated with EtOH (96%) were used as K^+. Each result is mean ± standard error of the mean of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Dunet's post hoc test (***$p<0,001$).

3.6. Qualitative evaluation of the in vitro transfection

The CLSM microscopy was used to evaluate the internalization of the produced PCL/PMMA nanoparticles in Hela cells. CLSM is an optical microscopy technique that has found tremendous utility in biology, biophysics, chemistry and materials science (Van Gough, 2008). This technique offers many advantages over the conventional optical microscopy, including enhanced contrast and 3D analysis. As depicted in Figure 26, PCL/PMMA nanoparticles were internalized by cells, reaching their cytoplasm and some of them were also founded in the cell nucleus (red dots in figure). These results showed that the produced nanoparticles have the ability to cross the cytoplasmic membrane of cells. However the nuclear pores of the cells are too small (~10 nm) to allow nanoparticles to enter to the intact nucleus (Sadhukha and Prabha, 2014), during cell division, the nuclear membrane dissolves, and due to that nanoparticles are able to enter into the nuclear space (Di Tomaso et al., 2013). For Hela cells clathrin-mediated endocytosis and caveolin-mediated endocytosis have been proposed has the main mechanism used for nanoparticles uptake (Sahay et al., 2010, Oba et al., 2008, Gratton et al., 2008).

The ability of the produced nanoparticles to reach the cytoplasm and the nucleus of cells allows the delivery of the entrapped drug inside the cell. This capacity gives drugs the possibility to be applied directly within cells cytoplasm, increasing their efficiency and reducing the drug required dose. However, it is necessary to give specificity to the nanoparticles to allow them to target diseased cells in order to reduce side effects in healthy cells.

Once cisplatin is liberated from de NPs in the cytoplasm, due its size, it is able to reach the nucleus and exert its therapeutic potential (Ciarimboli, 2012).

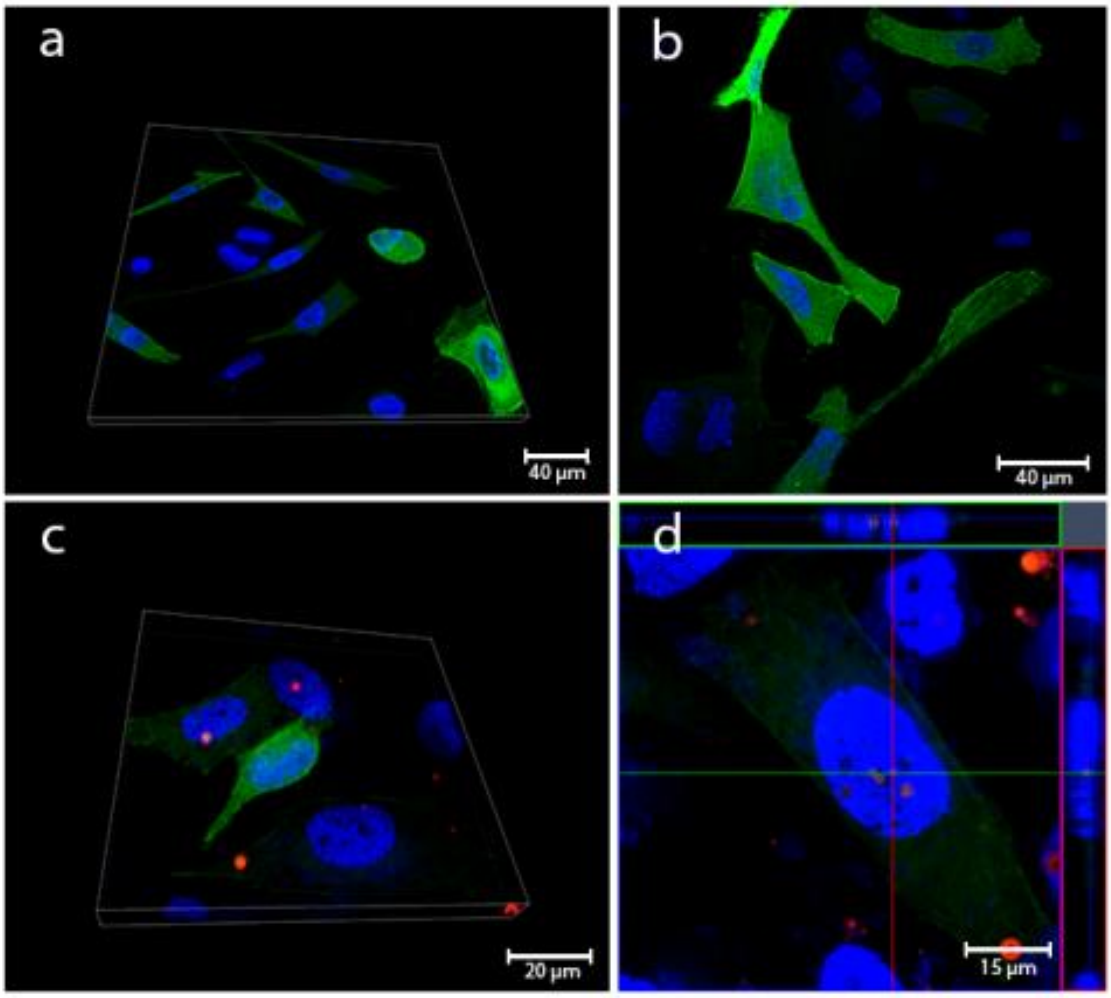

Figure 26: CLSM images of Hela cells. Nucleus stained with Hoechst® 33342 (blue) and cytoplasm with GFP (green) (a) 3D reconstruction of control; (B) control; (C) 3D reconstruction of transfected cells with rhodamine-PCL/PMMA nanoparticles; (D) orthogonal projection of transfected cells with rhodamine-PCL/PMMA nanoparticles.

3.7. Determination of the inhibitory concentration of free Cisplatin in Hela-Cells

After the characterization of the biocompatibility it is important to address the actual anti-tumoral activity of Cisplatin before further studies be performed. Therefore the IC 50 of Cisplatin was determined (Figure 27). The IC50 of a drug is the minimum concentration that is able to kill half of the tumor cell population. Due to possible variations in the *in vitro* cell line models during cell passages, it is critical to assess the experimental IC50. Experimental IC50 was calculated through the graphic obtained with the experimental data. The value of the cisplatin concentration that is able to kill 50% of <hella cells is about 16 µM. This value is almost 1.7 to 12 fold higher than that reported in the literature 1.2 to 9.3 µM (Kamel et al., 2012, Takara et al., 2006). This fact can be explained by the possible acquisition of a resistant phenotype from Hela cells due to their culture for long periods.

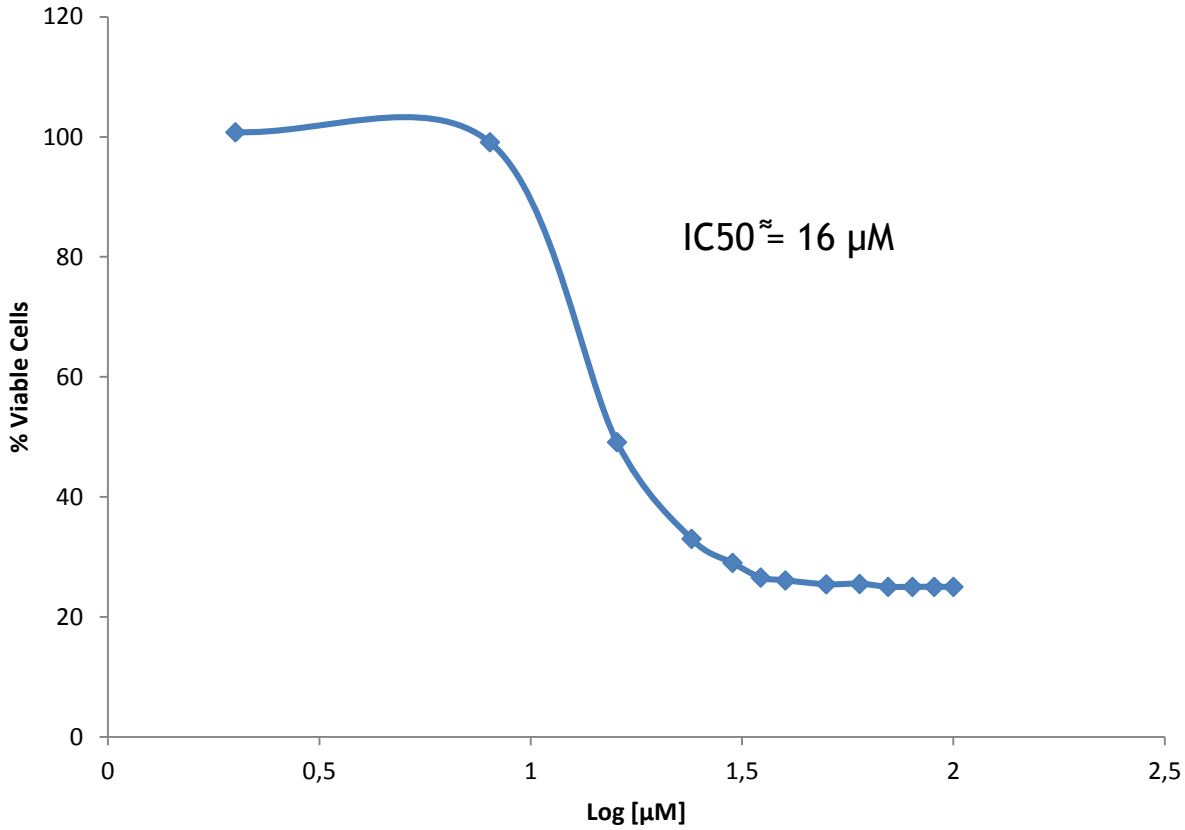

Figure 27: IC 50 determination of Cisplatin anti-tumoral activity in Hela cancer cells. Blue curve represents the mathematical fitting performed for IC50 calculation with n=13.

3.8. Characterization of the cytotoxic profile of the produced cisplatin load nanoparticles

After establishing the effect of cisplatin in the Hela cells and determined of IC50, cells were put in contact with the produced cis-load PCL/PMMA NP. These experiments were performed using 80µM of free cisplatin as positive control and two concentrations of drug, 100µM (1.25 the concentration of cisplatin used as control) and 75µM (0.93 the concentration of cisplatin used as control). When drugs were delivered by the nanoparticles to Hela cells, a significant decrease in cell proliferation was observed (Figure 28). After 24 hours of incubation there were no differences in cell viability between all the groups. At 72 hours the cell viability starts to decreases. It drops to near 25% in the positive control and 75% for both group tests. After 7 days the viability in the positive control remained at in 25% while the group of 75 µM drops to near 60% and the group of 100 µM almost reaches the value of the positive control, i.e. 30% cell viability.

Although, some properties can be changed in order to improve the presented system, as for example, the weight of the used polymers (Woodruff and Hutmacher, 2010). Since polymers with low molecular weight suffer an easiest and faster degradation, the degradation of the polymeric matrix of the nanoparticle leads to an higher release profile of the drug along time (Mohanraj and Chen, 2007).

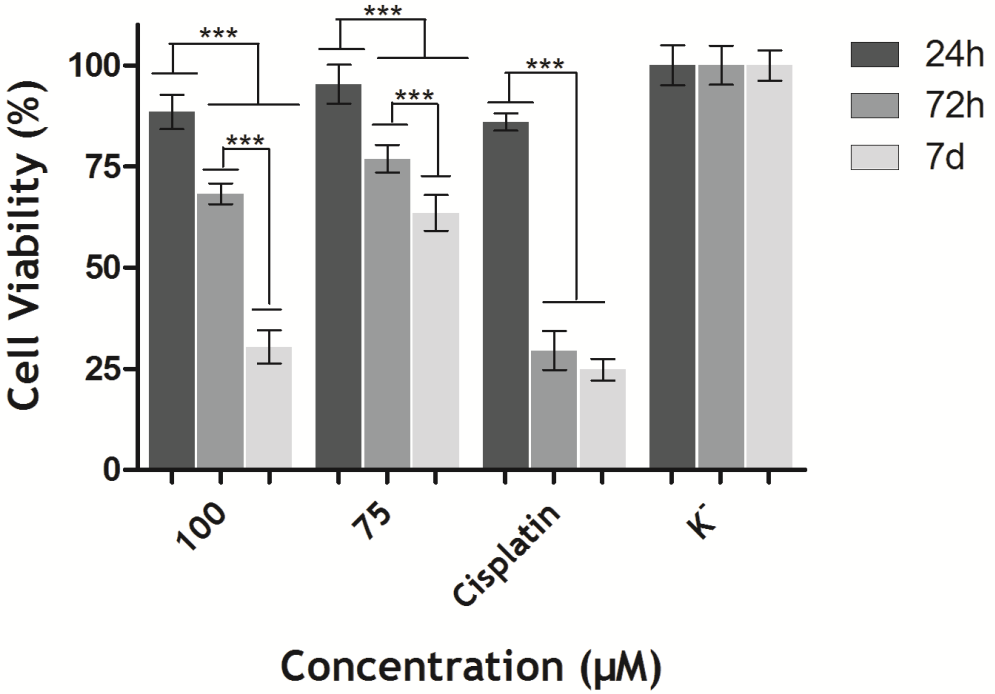

Figure 28: Evalution of the cytotoxic effect of Cisplatin when delivered by PCL/PMMA NP at 24, 72h and 7 days. Cisplatin has used with a concentration of 80 µM; K^- is the negative control cells. n=5; ***p < 0.001. Data is presented as mean ±s.d.

The produced nanoparticles don't have any specificity for any type of cells. However, in *vivo*, due to the EPR effect and the tumor microenvironment it is expected that the nanoparticles accumulate in higher percentage in cancer tissues than health tissues. Furthermore, the higher metabolic rate presented by cancer cells it is expected that they uptake more NPs than normal cells.

Chapter IV:

Conclusions and Future Perspectives

4. Conclusions and Future Perspectives

Cervical cancer is one of the leading causes of death in women worldwide. Like in other types of cancer, chemotherapy is widely used for the treatment of this disease. However this type of therapy was several side effects associated with lack of specificity. In an attempt to minimize side effects and to improve the efficacy of the current available therapies, new therapeutic approaches are currently being explored. Among them DDS are the most used. Nanoparticles due to their size are transported along smallest blood vessels of the body and they are able to migrate from the tumor neovasculature to the cancer cells. Others important NPs characteristics are shape, surface properties, and drug loading capacity and release profile.

Different polymers have been used for the production of polymeric nanoparticles. In the present study, PCL and PMMA were used in the production of polymeric nanoparticles through an adaptation of the nanoprecipitation method. Cisplatin is one of the most used drugs in the treatment of cervical cancer, and here it was used as drug model and loaded into PCL/PMMA NP. Then the suitability of these nanoparticles for cancer therapy was performed.

Different techniques were used in order to evaluate the NPs characteristics. The morphology of the nanoparticles was characterized by SEM and TEM analysis. The produced NP present spherical-like shapes either loaded with cisplatin or unload. Trough DLS analysis it was possible determinate that the unload PCL/PMMA NP had a mean size of 275 nm while the cis-PCL/PMMA NP had a mean size of 282 nm. The zeta potential is -7 mV for PCL/PMMA NPs and -6 mV for cis-PCL/PMMA NPs. FTIR analysis was also performed and the presence of PCL, PMMA and PVA in the nanoparticles. Although, the presence of cisplatin has not detected due to the low amount used and by its internal location in the particles. Moreover, the loading and release profiles of cis-PCL/PMMA NP were characterized. For the produced nanosystem an efficiency of drug encapsulation between 41 and 70% was achieved. The sustained release profile over 7 days showed that cisplatin was successfully loaded in NPs, and being the release profile within the appropriate therapeutic window. The produced nanosystem were biocompatible since the two types of cells tested adhered and proliferated in the presence of the NPs. Cis-load PCL/PMMA significantly reduced the cell proliferation to 30% after 7 days. This reduction in cell viability also demonstrates the loading of the cisplatin inside the NP was successfully achieved.

In the future, several aspects can be changed to improve the therapeutic efficacy of this nanosystem. The application of monomers instead the preformed polymer allows a better control of the size of the nanoparticles. The reducing of size of produced nanoparticles for value near 100 nm would benifit their blood circulation and reduces their possible retention in the liver and spleen.

Furthermore, due to the great influence that the type of encapsulated drug has in the loading and release profile, it would be interesting to load different drugs, such as paclitaxel, ifosfamide, irinotecan or gemcitabine (also used for cervical cancer treatment) or a combination of drugs within the nanoparticles to access if the loading or the release profiles of PCL/PMMA nanoparticles are kept. A cross-linker can be used in order to improve drug loading capacity on NPs. Another newsworthy option will be the loading of genes or growth factors for the treatment of other diseases besides the cancer. Furthermore, NPs surface may be functionalized with anti-bodies, proteins or others, in order to grant specificity to NPs for cancer cells.

Chapter V:

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5. Bibliography

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