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The analysis of doxorubicin-loaded poly(butyl cyanoacrylate) nanoparticles in *in vitro* glioma models

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

vorgelegt beim Fachbereich 14
Biochemie, Chemie und Pharmazie

der Johann-Wolfgang-Goethe University
in Frankfurt am Main

von

Berta Sanchez de Juan

aus
Madrid, Spanien

Frankfurt am Main
November 2005

Dedicado a Joaquin y Merche

Acknowledgements

The following thesis benefited from the insights and direction of several people. First, I would like to acknowledge Professor Dr. Jörg Kreuter for allowing me to work on his group as PhD-student and particularly for encouraging me to develop independent and self-sufficient approach to my work. I also wish to thank Hagen von Briesen for his scientific support and I specially would like to thank Dr. Svetlana Gelperina for her very refreshing scientific support, and for her limitless passion for science.

I am very grateful to the Deutsche Forschungsgemeinschaft (DFG), Graduiertenkolleg Arzneimittel: Entwicklung und Analytik and INTAS grant 00-838, who supported this work.
I am also very grateful to the Chemotherapeutic Institute Georg-Speyer-Haus, where the most important work was carried out.

I would like to express my sincere thanks to all the member of the institute of pharmaceutical technology at the University of Frankfurt, and in particular to my colleagues Alessandra Ambruosi, Alexander Bootz, Telli Hekmatara for a great work atmosphere and very unforgettable moments.

I am very especially grateful to Christian Kunz for his wonderful help to accomplish this work.
Finally I would like to thank my family and all my friends, who at different moments and in different ways made everything easier.

1 Introduction

1.1 The glioblastoma

Brain tumours, especially malignant gliomas belong to the most aggressive human cancers with a short survival time. Despite the numerous advances in neurosurgical operative techniques, adjuvant chemotherapy, and radiotherapy (1-3), the therapeutic progress is still limited. Even the chemotherapeutic drugs most effective in glioblastoma, nitrosurea, platinum compounds, or temozolomide, increase the survival time of patients only slightly (4). Reasons responsible for the aggressive character of glioma include rapid proliferation, diffuse growth, and invasion into distant brain areas in addition to extensive cerebral edema and high level of angiogenesis.

Gliomas are a class of tumours that develops from glial (neuroepithelial or support) cells. Astrocytes, ependymal, and oligodendroglial cells are all examples of glial cells that compose the supportive tissue of the brain. Gliomas comprise nearly one-half of primary brain tumors and one-fifth of all primary spinal cord tumours. Contemporary classification of gliomas is based on the World Health Organization (WHO) system, which classifies the tumors according to the cell of origin and histologic features identified by the pathologist or neuropathologist. Low-grade gliomas are slowly growing, and are assigned either a I or II grade. From a practical standpoint, grade I tumours (such as the pilocytic astrocytoma) are usually excluded from conversation dealing with gliomas, as they constitute a distinctive pathologic and clinical entity. High grade (malignant) gliomas grow much more quickly, and are assigned either a III (anaplastic) or IV (glioblastoma multiforme) grade. Combined, grade III and IV gliomas represent about 40% of all primary brain tumours in patients aged 40-49 years, and 60% in patients older than 60 years. In most clinical series, grade III tumours comprise approximately 10% and grade IV 90% of the total number of high grade, malignant primary brain tumours.

In order to treat gliomas, models systems are necessary to evaluate the therapeutic value of the different potiential drugs. In this perspective, rat brain tumour models have been widely used in experimental neuro-oncology for almost three decades. The rat models that are available have provided a wealth of information on *in vitro* and *in vivo* biochemical and biological properties of brain tumours and their in vivo responses to various therapeutic modalities. Ideally, valid brain tumour models should be derived from glia cells, be weakly or non-immunogenic, and their response to therapy, or lack thereof, should be similar to human brain tumours (81).

Before the effects of the potential therapeutic compounds are analyzed in rat tumour models, their efficiency has to be characterized in cell culture systems. The glioma most commonly used tumour cell lines are: GS-9L, RG-2 and F-98. GS-9L gliosarcoma, an immunogenic tumour, was chemically induced in an inbred Fischer rat, has been one of the most widely used of all rat brain tumour models. The F-98 and RG-2 gliomas were both chemically induced tumours that appear to be either weakly or non-immunogenic (81).

It is important to evaluate the efficiency of the therapeutic drug to kill the tumour cells, as well its delivery to the target tissue. But successful entry of therapeutic drugs into the brain is very rare because the blood-brain-barrier (BBB) makes the brain practically inaccessible for lipidinsoluble compounds (hydrophilic) such as polar molecules and small ions. The delivery of the cancer drug through the BBB is therefore one of the major obstacles in glioma treatment.

1.2 Mechanisms conferring drug resistance in glioma treatment

1.2.1 The Blood-Brain Barrier

One of the most important fields studied in drug targeting is the targeting to the brain due to its complexity, and only very few approaches are successful.

The brain is a delicate organ with very efficient mechanisms to protect it. Unfortunately, the same mechanisms that maintain its homeostasis and thus protect it against intrusive chemicals can also inhibit the access of therapeutic agents.

Many existing pharmaceuticals, which are potentially effective, are ineffective in the treatment of brain diseases due to the inability to effectively deliver and sustain them within the brain. This failure of systematically delivered drugs to effectively treat many CNS diseases, such as brain tumours, can be explained by a number of biological barriers that inhibit or hinder the drug delivery to the brain. These barriers are: the blood-brain-barrier, the blood-cerebrospinal fluid barrier, and the blood-tumour-barrier.

The Blood-brain barrier (BBB), which is formed by the tight junctions within the capillary endothelium of the brain, forms a formidable barrier to the CNS inhibiting the delivery of therapeutic agents (mostly with high molecular weight and/or hydrophilic drug). Although selective transport mechanisms are present in the BBB such as diffusion, carrier-mediated transport, receptor-mediated, adsorptive and fluid-phase endocytosis, the transport of therapeutic agents via systemic mechanisms is limited. Principal mechanisms involved in the restriction of brain drug uptake by the BBB include: (1) the absence of paracellular openings, (2)

the lack of pinocytosis, and (3) the presence of significant protein efflux pumps. Therefore, important research is dedicated to develop methods and technologies to circumvent the BBB for brain drug delivery.

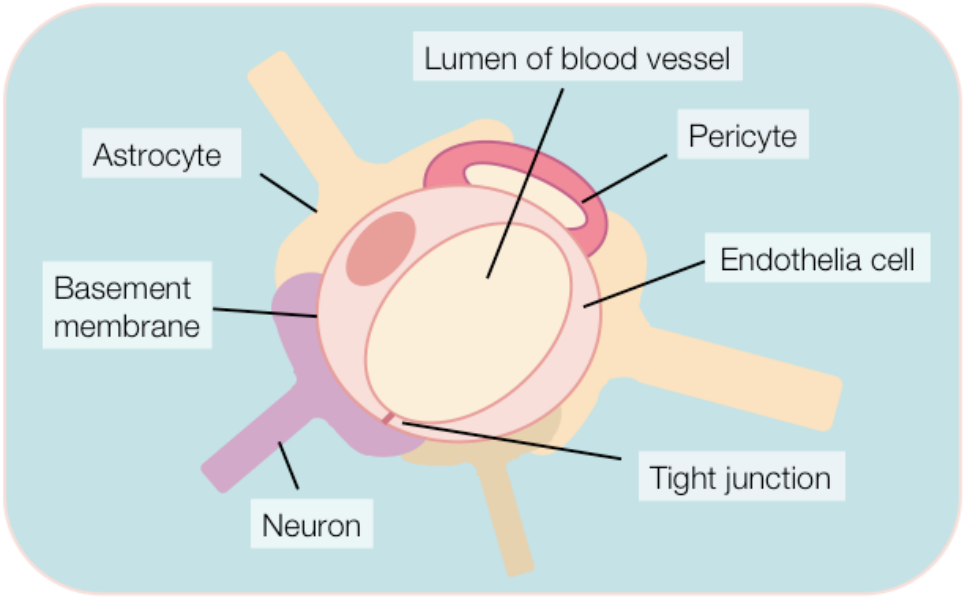


Figure 1. A schematic picture of the blood-brain-barrier with astrocytes associated

In order to overcome the limited access of drugs through the BBB to the brain, different delivery methods have been developed. Many of these methods are characterized by the manipulation of the BBB by temporary disruption of tight junctions to allow paracellular movement by way of osmotic opening (13, 14) or by the use of biologically active agents (e.g. histamine, serotonin, free oxygen radicals, calcium entry blockers, etc.), (15, 16). The problem with this method is that it is very invasive because it also allows the free passage of non-desired drug, resulting in a high toxicity of the brain.

Other important factors in limiting the entry of drugs into the brain are their physico-chemical properties (e.g. hydrophilicity, lipophilicity, hydrogen bonding potential). These characteristics largely determine the passive transport of drugs across the BBB. The lack of efficacy of some drugs such as cisplatine and doxorubicin against gliomas has been attributed to their lack of lipid solubility. This is responsible for the increased focus on lipid-soluble drugs in glioma treatment. However, this property does not necessarily ensure a passage through the BBB,

because the BBB is further reinforced by the presence of significant protein efflux pumps, represented by a high concentration of P-glycoprotein (Pgp). Pgp is an active drug efflux transporter protein, which is in the luminal membranes of the cerebral capillary endothelium. This efflux transporter actively removes a broad range of drug molecules from the endothelium cell cytoplasm before they cross into the brain.

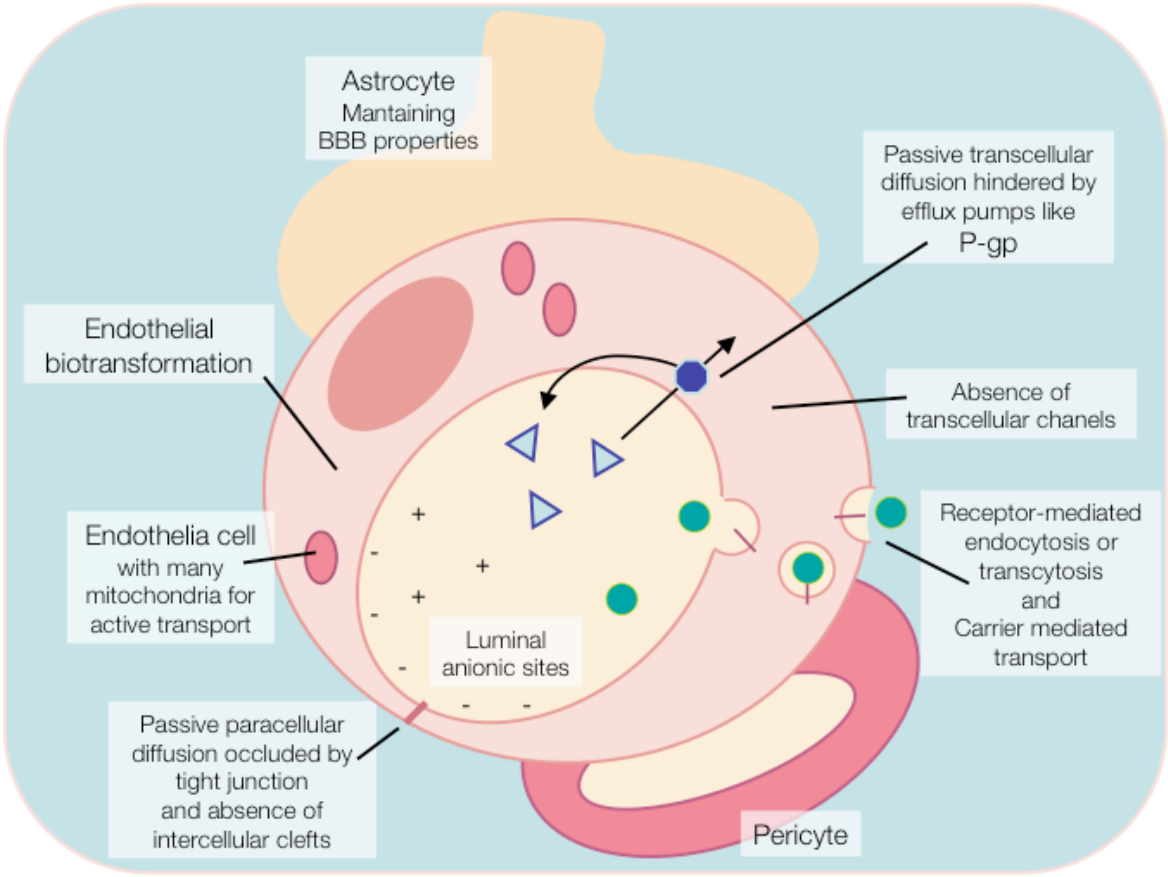


Figure 2. Properties of the transport in the blood-brain-barrier

The presence of P-gp in tumours causes multidrug resistance (MDR), and P-gp in the BBB is also responsible for multidrug resistance (MDR) in the case of brain tumours. Using P-gp inhibitors in cancer therapy can therefore be beneficial in two ways. The pharmacokinetics of the therapeutic drugs can change and particularly, CNS concentrations can increase. The intracellular drug concentration in brain tumours can be increased (provided that the inhibitor is distributed to the brain tumour). The P-gp protein will be introduces in detail in chapter 1.3.

1.2.2 The Tumour resistance

As mentioned before, the insufficient response to anti-cancer drugs is caused in part by the inaccessibility of the brain for most chemotherapeutical compounds due to limited transport through the blood-brain barrier. But the BBB is only one obstacle, which has to be overcome in order to successfully treat brain tumours.

Therapeutic anticancer drugs must reach tumours by overcoming problems such as drug resistance at the tumour level due to physiological barriers (non-cellular mechanism) and drug resistance at the cellular level (cellular mechanism). In addition, they must successfully have the following attributes: distribution, biotransformation and clearance of anticancer drugs in the body. There are different mechanisms by which a tumour can be resistant to any therapeutic drug. This resistance is the cause of frequent failure in chemotherapy treatment with any chemotherapeutic drug.

The mechanisms of tumour resistance can be classified as
• Non-cellular drug resistance mechanisms could be due to poorly vascularized tumour regions, which can effectively reduce drug access to the tumour and thus protect it from the cytotoxicity of the drug. The acidic environment in tumours can also confer a resistance mechanism against basic drugs. These compounds would be ionized, preventing their internalization across the membrane cellular. • Cellular drug resistance mechanisms compromise altered activity of specific enzyme systems, altered apoptosis regulation, or transport based mechanisms, like P-glycoprotein efflux system, responsible for the multidrug resistance (MDR), or the multidrug resistance associated protein (MRP).

Another problem faced by the use of anticancer drugs is their toxicity to both, tumour and normal cells, resulting in a limited efficacy of chemotherapy due to significant side effects. Furthermore, until recently, frequently repeated doses of pharmaceutically active agents were required for patient treatment to maintain desired drug levels and thereby also prolonged the significant side effects of these drugs.

All of the above mentioned facts favoured the introduction of controlled drug release delivery formulations, such as nanoparticles, liposomes, microspheres, etc. Controlled-release technology has attracted much attention since it is possible to overcome such non-cellular and cellular based mechanisms of resistance. Moreover, it is possible to increase the selectivity of drugs to cancers cells reducing their toxicity towards normal tissues by means of these

formulations. Therefore, the development of drug carriers for controlled release of drugs and targeting has been extensively studied and it was shown that drugs bound to the surface-modified nanoparticles are able to cross impermeable barriers such as the BBB. The use of drug carrier systems will be introduced in more detail in chapter 1.4.

1.3 The P-glycoprotein (P-gp).

The resistance of tumour cells to multiple drugs, referred to as multidrug resistance (MDR), is a major obstacle in the successful treatment of various human cancers. In some types of tumours, MDR is inherent, while in others it is usually acquired. The two best-described transporters mediating MDR are P-glycoprotein (P-gp) and the multidrug-resistance protein (MRP) (37-39). Although both are members of the large ATP binding cassette (ABC) superfamily of transport ATPases, one of the largest protein families found in all phyla. Both proteins are located in the plasma membrane they share less than 15 % amino acids identity (39).

The transport protein P-gp is located on the luminal side of the brain capillary endothelium and acts as a transmembrane pump, which removes drugs from the cell membrane and cytoplasm. ATP hydrolysis provides the energy for its active drug transport, which can occur against a steep concentration gradient. P-gp is the product of the MDR1 gene (mdr1 and mdr1b in rodents, and MDR1 in humans), which can be phosphorylated and localized in the plasma membrane as glycosylated transmembrane protein (57).

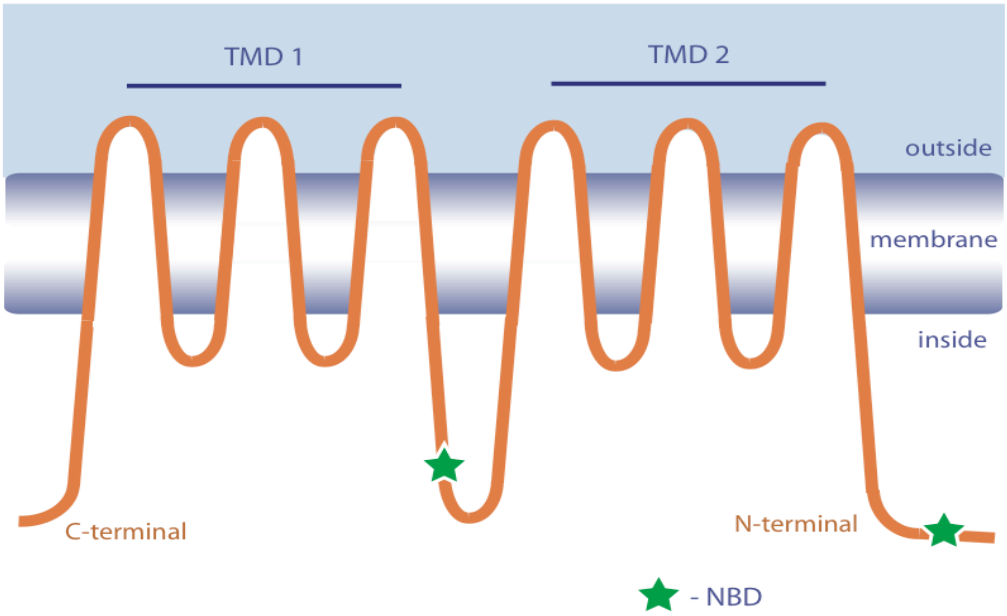


Figure. 3 P-gp Structure

P-gp has been shown to channel the ATP-mediated extrusion of MDR drugs, an activity that can be inhibited by P-gp modulators in intact cells. However, P-gp modulators do not inhibit multidrug-resistant associated protein MRP-dependent drug efflux (40). The discovery of P-gp modulators capable of reversing MDR in cells overexpressing P-gp raised hope that the administration of these modulators along with chemotherapeutics agents could overcome the MDR phenomenon. But the treatment with P-gp modulators was unsuccessful due to the fact, that MDR is not solely mediated by P-gp and additional mechanisms exist which cannot be reversed by P-gp modulators (41-44).

A typical ABC transporter generally consists of at least one evolutionarily conserved ABC, also known as NBD (Nucleotide Binding Domain) with about 215 amino acids, as well as several predicated α-helical TMD (Transmembrane Domains). The NBD and TMD are normally arranged in a duplicated forward (TMD6-NBD) 2 (see Fig.3) or reverse (NBD-TMD) 2 configurations, although some other arrangements exist.

Molecules interacting with P-gp may be classified as substrates or antagonists (inhibitors). Cancer drugs frequently are P-gp substrates, but do not block the transport of other substrates while compounds in the antagonist or inhibitor groups, which fail to be transported, prevent the transport of other compounds.

Table 1. P-gp substrates and inhibitors

A. P-gp substrates (Chemicals transported by P-gp)	
Cancer drugs	HIV protease inhibitors
Doxorubicin	Amprenavir
Daunorubicin	Indinavir
Vinblastine	Nelfinavir
Vincristine	Ritonavir
Actinomycin D	Saquinavir
Paclitaxel	Cardiac drugs
Teniposide	Digoxin
Etoposide	Quinidine
Immunosuppressive drugs	Antiemetic
Cyclosporin A	Ondansetron
Lipid-lowering agent	Anti-diarrheal agent
Lovastatin	Loperamide
Antihistamine	Anti-gout agent
Terfendine	Colchicine
Steroids	Antibiotic
Aldosterone	Erythromycin
Hydrocortisone	Anti-helminthic agent
Cortisol	Ivermectin
Corticosterone	Anti-tuberculuos agent
Dexamethasone	Rifampin
Dopamine antagonist	Florescent dye
Domperidone	Rhodamine-123

B. P-gp Inhibitors (Chemicals that block P-gp-mediated transport)	
Cyclopropyldibenzosuberane	Anti-estrogen cancer agent
Immunosuppressant	Tamoxifen
Cyclosporin A	Antiarrhythmic
Valspodar (PSC833)	Quinidine
HIV protease inhibitors	Antifungal agent
Ritonavir	Keteconazole
Saquinavir	Sedative
Nelfinavir	Midazolam
Cacium Channel Blocker	
Verapamil	

P-gp activity is dependent on the signal transduction pathway and is connected to protein kinase C (PKC). PKC phosphorylates P-gp, and overstimulation of PKC results in an increase of Pgp-mediated substrate movement. PKC inhibitors produce a built-up of toxic chemicals in MDR cells expressing P-gp, however, PKC inhibitors are also known to interact directly with the Pgp protein. P-gp activity can be detected by different methods. One of them is the measurement of ATP activity

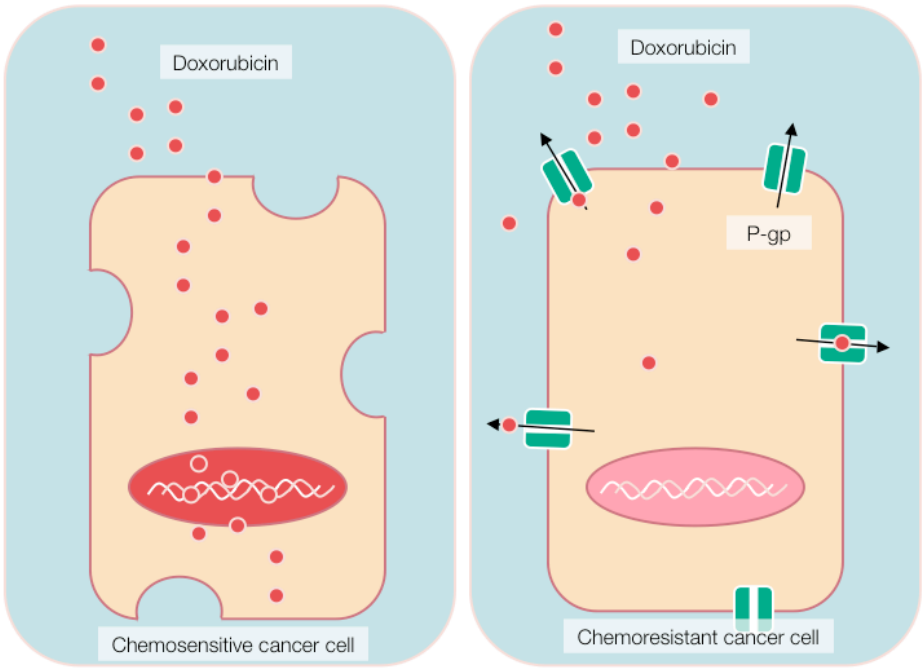


Figure 5. Doxorubicin transport mechanism in chemosensitive cancer cells and chemoresistant cancer cells.

The cancer drug doxorubicin is one of the therapeutic candidates for brain tumours, which has proven to be effective in glioblastome cell lines (17). But doxorubicin lacks the ability to cross the BBB because it is a P-gp substrate. For this reason, a strategy, which emerged to enhance the drug delivery of doxorubicin in the brain, was the administration in a formulation that may overcome P-glycoprotein-mediated efflux. One option would be the use of a drug delivery system to deliver doxorubicin to glioma cells lines.

1.4 Nanoparticles as a drug delivery system

1.4.1 Features of a drug delivery system

A considerable interest was established in recent years in the development of biodegradable carriers for effective delivery of therapeutic molecules at a controlled rate to the required site in the body. Such a delivery is assumed to avoid the unwanted effects of drug molecules because of controlled biodistribution. Polymeric nanoparticles are one of the promising drug delivery devices, which could meet these requirements. The requirements for an ideal delivery system are listed below.

Properties of an ideal delivery system
• Selectivity: delivery of the drug preferentially to tumour cells or an enhanced effect on the desired site. • Controlled/sustained rate of release • Low immunogenicity (not causing immune responses and hypersensitivity). • Scope of disease • Pharmaceutically acceptable characteristics (stability, administration, biodegradability, and ease of sterilization).

Nanoparticles as a delivery system are able to efficiently transport therapeutic drugs, which are unable to across cellular membranes and other types of biological barriers due to their physicochemical properties, such as lipophilicity and molecular weight. Drugs or compounds that are not ionized at physiological pH, lipophilic, and of low molecular weight are able to across the BBB by diffusion mechanisms. But others essential compounds, such as amino acids, neuropeptides, and hexoses, normally need specific carries to permeate into the brain, and for many others therapeutic drugs it is difficult to cross these biological barriers and to

internalize into cells. Therefore the use of this delivery system, such as nanoparticles has been used in a number of studies to obtain a high efficiency in certain pharmacological treatment and to increase the drug concentration in the target tissues.

1.4.2 Poly(butyl cyanoacrlate) nanoparticles.

Nanoparticles are nanosized polymeric colloidal particles with a therapeutic agent encapsulated within the polymeric matrix, or adsorbed, or conjugated onto the surface of the nanoparticle. They are made of natural or synthetic polymer ranging in size between 10-1000 nm (34). Nanoparticles can be prepared from synthetic biodegradable polymers by polymerization techniques of suspension, emulsion, or micelle polymerization. Various polymers have been tested in drug delivery research. Poly(butyl cyanoacrylate) nanoparticles meet ideal requirements for targeting, it is for instance biodegradable, has the ability to alter the biodistribution of drugs and is easy to synthesize and purify. Furthermore, the use of biodegradable polymeric nanoparticles for controlled release of anticancer drugs has the advantages of enhancing the drug therapeutic efficacy and reducing drug systemic side effects.

The nanoparticles used in this work are made from butyl cyanoacrylate monomer by emulsion polymerization. This reaction involves the dispersion of the monomer liquid, butyl cyanoacrylate, in an aqueous phase to form particles.

The employment of poly(butyl cyanoacrylate) nanoparticles have previously been shown to facilitate the transport of doxorubicin into the brain (76). Moreover, this particles very significantly increased the survival time of rats with intracranial transplanted glioblastoma 101/8 and even led to long-term remission in 25% of the animals (72). Consequently, poly(butyl cyanoacrylate) nanoparticles were successful in enabling the treatment of glioma tumours, as also shown *in vivo* experiments by Kreuter et al. 2003 (33). Unfortunately, the mechanism by which the drug encapsulated in the nanoparticles reached the brain is presently not fully elucidated.

1.4.3 Surfactants as surface modifications of nanoparticles

As already indicated, the association of cytostatic drugs to nanoparticles leads to modifications of the drug biodistribution profile, which can improve the efficacy. However, one of the major problems in targeted drug delivery is the rapid opsonization and uptake of the nanoparticles by the mononuclear phagocytes system (MPS) (58). Once in the bloodstream, nanoparticles are opsonized and cleared by macrophages, resulting in a decreased efficacy on the target cells or tissues. The tendency of MPS organs to capture nanoparticles especially occurs in those nanoparticles, which have not undergone any modification of their surface. They are called non-modified nanoparticles. The predisposition of MPS macrophages for endocytosis/phagocytosis provides an opportunity to efficiently deliver therapeutic agents to these cells and to enhance this specific anticancer drug efficacy, using these non-modified nanoparticles.

The usefulness of non-modified nanoparticles is limited by their massive capture by the macrophages of the MPS, therefore modification of nanoparticles is necessary to target to other types of tumours. The ability of cells of the MPS to capture the nanoparticles depends on the characteristics of the nanoparticle surface, such as charge and hydrophobicity surfaces. It is possible to modify the surface of the nanoparticles to avoid capture by the MPS, protecting the nanoparticles from phagocytosis (36). In modified nanoparticles, the surface characteristics and the size of the nanoparticles are the key for their biological fate. Larger particles are filtered out by the first capillary bed. A small size seems to improve the reduction in opsonization reactions and subsequent removal by macrophages. Particles smaller than 100 nm, with hydrophilic surfaces appear undergo relatively less opsonization and clearance by MPS uptake (102).

The coating of the surface with surfactants has been studied with the objective of the development of the modified nanoparticles. The use of hydrophilic surfactants to coat the surface creates a hydrophilic surface around the nanoparticles and avoids the capture of nanoparticles by macrophages, giving long-circulating properties (36). The long-circulating carriers thereby also increase the possibility of the nanoparticles to reach the brain (83; 20). Thus, the coating of the nanoparticles surface with non-ionic surfactants promotes an enhancement of the internalization of the nanoparticles into cells (12, 36, 56).

A hydrophilic surface can be obtained by adsorbing hydrophilic surfactants, such as polysorbate 80 on the nanoparticles surface or using block/branched copolymers, such as poloxamines and poloxamers. Polyethylene oxide (PEO) or poly ethylene glycol (PEG) are the

most successful non-ionic hydrophilic polymer moiety employed for this purpose (36). Poloxamers consist of two ethylene oxide (EO) polymers attached to the ends of one propylene oxide (PO) polymer. Poloxamines consist of four EO polymers attached to four PO polymer and all four are coupled to an ethylene diamine core.

All these surfactants, because of their structure and properties, are used to modify the nanoparticle surface. However, the chemical nature of the coating surfactant is of importance, because poloxamine 908-coated nanoparticles showed long-circulating characteristics but failed to increase nanoparticle brain concentration (98), whereas doxorubicin adsorbed onto polysorbate 80-coated nanoparticles was successfully delivered into the brain (76, 33, 72). Polysorbate 80 so far is the most successful surfactant for brain delivery (33, 34). Overcoating the surface with polysorbates has resulted in an increased transport of nanoparticles-associated drugs across the BBB, allowing targeting to the brain after intravenous injection (35). The mechanism responsible for this enhancement in the transport of the nanoparticles is not yet completely elucited. It is possible that polysorbate present on the nanoparticles surface acts as an anchor for apolipoprotein E (present in plasma), resulting in its adsorption onto the nanoparticles. The particles thus would mimic LDL particles and interact with LDL receptors on endothelial cells in the brain capillaries leading to their cellular uptake (35). Others authors (79, 80, 101) indicated polysorbate 80 as a surfactant able to inhibit the efflux pump protein, P-glycoprotein (P-gp), mainly localized in the endothelial cells, and consequently it would allow the internalization of the nanoparticles and/or the drug by blocking its P-gp-mediated transport.
Usually, the mode of attachment of these substances onto the nanoparticle surface often involves physical adsorption. For instance, the polymeric surfactants of the poloxamers and poloxamines series can attach themselves onto the surfaces of particles via their central hydrophobic region (polyoxypropylene or polyoxypropylene ethylene diamine oxide respectively), while leaving the hydrophilic portion (ethylene oxide) protruding out into the surrounding medium.
Polysorbate 80, poloxamer 188, and poloxamine 908, three non-ionic surfactants that provide hydrophilic properties to the nanoparticle system, provoked different effects on the properties of the nanoparticles. As above already mentioned, polysorbate 80-nanoparticles were able to overcome the blood-brain-barrier (72), whereas poloxamer 188 and poloxamine 908 only provided long-circulating properties to the nanoparticles system (97, 98). For this reason, in the present study these three surfactants were used to coat poly(butyl cyanoacrylate) nanoparticles (PBCA-NP) to analyse their effect on the cytotoxicity of the Dox-PBCA-NP analysed.

In summary, nanoparticles may provide different opportunities for targeting by surface modifications, which would allow specific biochemical interactions with the proteins/receptors expressed on the targeted cells. Thus, nanoparticles represent an emerging tool for intracellular drug targeting of insoluble and sensitive drugs (such as proteins, oligonucleotides, or DNA). Nanoparticles not only act as an effective carrier for such drugs, protecting them from the undesirable physiological conditions, but also may allow a selective and controlled release of the drugs at the target sites.

This is of particular importance in cancer chemotherapy, where cellular uptake of drugs is limited due to the removal of the drugs by efflux protein pumps, such as P-glycoprotein. Anticancer drugs encapsulated in nanoparticles cannot be recognized by this efflux mechanism and therefore could circumvent the multidrug resistance.

1.5 The chemotherapeutic agent doxorubicin

Doxorubicin, an anthracycline antibiotic, is one of the most important anticancer drugs. Anthracycline antibiotics are very broad-spectrum antitumor drugs and very effective neoplastic agents, which are widely used clinically. However, these drugs induce many dose-limiting adverse effects such as cardiac myopathy, alopecia, etc. In order to minimize these adverse affects in clinical cancer chemotherapy, anthracyclines must be selectively transported into tumour cells. To reach these selective target cells or tissues, carrier systems for these drugs have been developed (nanoparticles, nanocapsules, and liposomes). Moreover, hydrophilic molecules such as anthracycline antibiotics show a restricted transport through the cellular membrane, thus nanoparticles also could be used to overcome the restriction of the transport through biological membranes. For instance, doxorubicin, which is mainly hydrophilic, leads to minimal drug internalization through the cellular membrane and is unable to overcome biological barriers such as the blood-brain-barrier. Therefore, it does not reach the brain after systemic administration. Due to this fact, free doxorubicin cannot successfully be used for the treatment of brain tumours by this route.

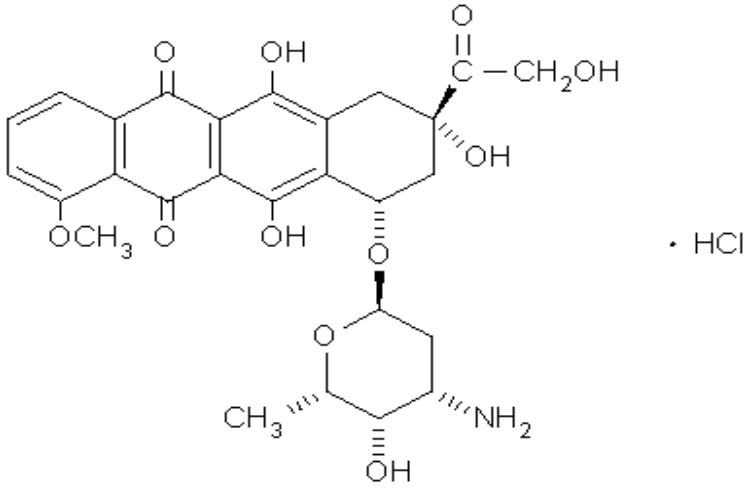


Figure 4. Doxorubicin structure.

Doxorubicin has two major functional groups: a primary amine group in a sugar moiety and a primary hydroxyl group of -C=OCH_2OH group in the aliphatic chain ring.

The drug may be bound in the nanoparticle in form of a solid solution or dispersion, or may be adsorbed to the surface of the nanoparticles, depending on the physical characteristics of both, polymer and drug. If a physical binding to the nanoparticles is not possible incorporation of the drug into the nanoparticles can be achieved by two methods: by incorporation of the drug during nanoparticle production or by absorption after nanoparticle formation by incubation in the drug solution. In our study, doxorubicin-poly(butyl cyanoacrylate) nanoparticles were produced by incorporating the doxorubicin at the time of production, this resulted in a polymer matrix with doxorubicin partly incorporated into the matrix but also adsorbed onto the surface.

1.6 The use of nanoparticles for drug delivery in glioma tumour cells

1.6.1 Overcome the multidrug resistance in tumour cells

A decrease of drug accumulation in cells may be the result of both, a decrease in drug influx and an increase in drug efflux. Since most of the chemotherapeutic drugs enter cells by way of passive diffusion through the cell membrane, changes in drug influx can be linked to

changes in cell membrane structure. This could cause either changes in drug trafficking through the cell membrane or influence on signalling pathways controlling apoptosis.
Drug efflux from resistant cell is considered to be the main cellular method of decreasing drug accumulation in cells. Drug efflux is mediated by the activity of P-gp, transporters of the MRP family, and some others proteins.
MDR cells overexpress P-glycoprotein, resulting in a higher degree of resistance to anticancer agents. For instance, cancer cells frequently acquire MDR against doxorubicin. Treatment of these P-gp expression tumours with anticancer drugs in combination with a chemosensitizing agent (modulator agent) that can modulate the P-gp activity has been shown to improve the efficacy of the anticancer drug and reverse the P-gp mediated MDR phenotype. The modulators function by blocking the transporter-mediated drug efflux and thereby cause in combination with an anticancer drug the drug accumulation within the cells, leading to the death of the tumour cell.

As many tumour cells are resistant to doxorubicin, the incorporation of this drug into biodegradable poly(alkyl cyanoacrylate) nanoparticles has been investigated in resistant cells (59, 60). Interestingly, it has been also shown that the association of doxorubicin with poly(alkyl cyanoacrylate) nanoparticles also reversed the resistance to doxorubicin in a large number of multidrug-resistant cell lines (11). The nanoparticle-associated doxorubicin accumulated within the cells and appeared to avoid the P-gp-dependent-efflux. This reversal was only seen with poly(alkyl cyanoacrylate) nanoparticles and was not observed to be due to nanoparticle endocytosis. Rather, the formation of a complex between positively charged doxorubicin and negatively charged polymer degradation products seemed to favour the diffusion across the membrane (10). However, the exact mechanism, by which doxorubicin-nanoparticles bypasses the multidrug-resistance mediated by P-gp still remains unclear.

The broad-spectrum of P-gp substrates, containing different structural and chemical characteristics, makes it very difficult to elucidate which mechanism is used to pump doxorubicin out of the cell. Furthermore, it is also unclear at which level P-gp is acting. The drug-substrate can penetrate into the cell through the membrane and then once it reaches the cytoplasm it will be pumped out by P-gp-mediated transport. But it could also be at membrane level where the P-gp acts, extruding the drug situated in the transmembrane.
Due to this, the identification of methods for circumventing these drug efflux transport systems has attracted significant attention as potential ways to improve the internalization and accumulation of therapeutic agents in the organism.

In accordance with all of above mentioned findings, we were interested in optimizing the internalization of doxorubicin into the tumour cells and employed poly(butyl cyanoacrylate) nanoparticles to enhance the doxorubicin transport across the membrane as well as the used of a P-gp inhibitor that could inhibit the pump efflux of doxorubicin out of the cells.

Recent studies suggested that Pluronic® block copolymers (poly(ethylene oxide)-block-poly(propylene oxide)-block-poly(ethylene oxide) are potent non-ionic surfactant inhibitors of P-gp in cells (9-13). In MDR cancer cells, Pluronic® block copolymers enhanced the accumulation of drugs and increased their cytotoxicity effects (9, 10).

1.6.2 Poloxamer 185 (Pluronic® P85), a P-gp Inhibitor

Initial work using Pluronic® block copolymers was focused on their ability to incorporate drug molecules and transport the drugs within the body. The discovery of the effect of these compounds on multiple drug resistance (MDR) of tumours was later recognized and had important implications for drug delivery.

One important factor for the activity in MDR cancer cells is the effect of the concentration and composition of Pluronic® block copolymers (24). Pluronic® block copolymers have been shown to increase doxorubicin absorption in P-gp expressing cancer tumour cell lines (10). It has been demonstrated that Pluronic® block polymers hypersensitize MDR cells resulting in an increase of the cytotoxicity activity of anticancer drugs with respect to these cells by 2 to 3 orders of magnitude (9). Due to this fact, the drug accumulation within the cells is increased, which enhances the drug activity.

Pluronic® block copolymers or amphiphilic block copolymers (ABCs) have been used in pharmaceutical applications ranging from sustained release to gene delivery applications. The use of ABCs for delivery of therapeutic agents was developed from their exclusive chemical structure consisting of a hydrophilic block, which is chemically bound to a hydrophobic block. The ethylene oxide (EO), hydrophilic block, and the propylene oxide (PO), hydrophobic block, arrange in a basic A-B-A structure: EO_x -PO_y -EO_x. Due to their amphiphilic nature, Pluronic® block copolymers are able to self-assemble into micelles in aqueous solutions at or above CMC (critical micelle concentration). The pluronic molecules assemble into dense micelles with PO blocks (lipophilic) remaining inside the micelles and EO blocks (hydrophilic) on the outside of

micelle structure. Upon micellezation, the hydrophobic core regions (PO blocks) serve as reservoirs for hydrophobic drugs, which may be loaded by chemical, physical or electrostatics means, depending on the specific functionalities of the core-forming block and the solubilization properties. The presence of a hydrophilic polymeric on the surface of polymeric micelles induces steric repulsive forces and stabilizes the micellar surface. This prevents the adsorption of proteins to the delivery system. As a result, polymeric micelles may efficiently escape the uptake of RES. The extent of steric stabilization is dependent on the hydrophilic block (EO) and its density on the colloidal particles. In fact, copolymers were originally used as stabilizers for colloidal suspension as emulsions, liposomes, and nanoparticles. The adsorption of block copolymers on the surface of those carriers was found to affect the pharmacokinetics and biological fate of the delivery system, leading to a long circulation and accumulation in sites with leaky capillaries. (61). Variations in the number of hydrophilic EO units and lipophilic PO units result in copolymers with different molecular mass and distinct hydrophilic-lipophilic balance (HLB). Copolymers with short hydrophilic poly-EO block and/or long lipophilic poly-PO block are highly lipophilic and are characterized by a relatively low CMC and low HLB. In contrast, copolymers with a long hydrophilic poly-EO block and/or a short lipophilic poly-PO block are hydrophilic and are characterized by a relatively high CMC and high HLB. Pluronic® compounds such as poloxamer 185 are intermediate in their lipophilicity and have CMC and HLB values that fall between the two extremes identified above.

The effect of Pluronic® on the P-gp efflux system is mediated by unimers. Batrakova et al. (18) showed that an increase in the accumulation of Rhodamine-123 (Rho-123), a P-gp substrate, and hypersensitization effects induced by Pluronic® block copolymers in MDR cancer cells occur at Pluronic® concentrations below the CMC. This means that both effects are caused by the block copolymer single chains, termed unimers. As a result, Rho123 accumulation and doxorubicin toxicity increase with increasing concentrations of Pluronic® until the CMC is reached and the unimer concentrations level off. Previous work demonstrated that the exposure of cells to Pluronic® decreases intracellular levels of the P-gp substrates (19). This can be one reason for the effect of Pluronic® copolymers on the function of energy dependent transporters such as P-gp. Batrakova et al. (25) demonstrated that the effect of poloxamer 185 (P85) on the decrease of ATP available in BBMEC monolayer was not due to leakage of intracellular ATP out of the cells but rather a result of inhibition of cellular metabolism. The mitochondria are responsible for carrying out much of the metabolic activities in the cell and might be a potential site of action for this compound. A confocal microscopy study showed that P85 is transported inside the cells and spreads throughout the cell, where it may interact with intracellular organelles including the mitochondria (25). A study from Rapoport (26) showed that two

Pluronic® copolymers, P85 and P105, reduce the activity of the electron transport chain in the mitochondria. This study indicates that these molecules are transported inside the cells and reach the mitochondria (27, 28). In addition, Batrakova et al. suggested that P85 significantly enhanced the brain penetration of a P-gp substrate, digoxin, in wild-type mice expressing functional P-gp, resulting in brain/plasma levels of digoxin similar to those observed in P-gp-deficient knock-out mice (29).

Pluronic® block copolymers have a tri-block structure containing a central hydrophobic poly(propylene oxide) segment flanked by two hydrophilic poly(ethylene oxide) segments. Hydrophobic poly(propylene oxide) can incorporate itself into the lipid membrane and induce changes in membrane structure (30). It is also hypothesized that Pluronic® copolymers can increase the microviscosity of the membrane, resulting in a decrease of the fluidity.

Accumulation studies using radioactively labelled poloxamer 185 indicate that the single molecule chains of the block copolymer are more efficiently taken up in the cells than the P85 chains incorporated within the micelles (25). This is probably due to the smaller size of the unimers compared with the micelles as well as the ability of unimers to bind with the cell membranes. Cellular accumulation of poloxamer 185 at micellar concentrations of block copolymers is likely a result of the combination of both, unimer and micellar transport processes. Poloxamer 185 induces drastic changes in the microviscosity of the cell membranes in BBMCE. Similar changes were observed in cancer cells treated by other Pluronic® block copolymers (36). These changes can be attributed to the alterations in the structure of the lipid bilayer because of the adsorption of the block copolymer molecules on the membrane.

At concentrations below CMC, poloxamer 185 apparently inhibits P-gp function, thereby reducing drug efflux out of the brain microvessel endothelial cells. At concentrations above the CMC, this surfactant appears to increase vesicular transport of the drug into the brain microvessels (32). Above the CMC, this block copolymer is able to form micelles. Under these conditions the Rho-123 and doxorubicin levels reveal a tendency for first levelling off and then decreasing. At high concentrations of Pluronic®, Rho-123 is incorporated into micelles and is transported through a vesicular route, resulting in different accumulation kinetics (21). Furthermore, exposure of the cells to the micelles induces removal of the drug from the cells through an unclear mechanism.

2 Perspective of the work

The objective of this study is to expand the capacity of doxorubicin of treating several types of tumours for the use in brain tumour therapy. As already known, tumour cells display resistance to doxorubicin because of drug resistance phenomena. Doxorubicin is a P-gp substrate and therefore presently is not used for the treatment of brain tumours.

The employment of poly(butyl cyanoacrylate) nanoparticles have previously been shown to facilitate the distribution of doxorubicin into the brain (76). Moreover, these particles very significantly increased the survival time of rats with intracranial transplanted glioblastoma 101/8 (72). Poly(butyl cyanoacrylate) nanoparticles were produced in order to carry doxorubicin and to enhance its internalization through the cellular membrane of the tumor cells thus overcoming the resistance presented in these cells.

Based on previous results, the modification of the surface characteristics of the nanoparticles has been demonstrated to be of great importance for reaching the target tissue and/or cell. The different chemical nature of the coating surfactants enables different surfactant properties, and hence it was decided to compare the effect of several surfactants, polysorbate 80, poloxamer 188, and poloxamine 908 on the drug uptake.

In the present study, the capacity of these three surfactants on the transport of doxorubicin through the cellular membranes of the glioma tumour cells as well their influence on the cytotoxicity on the glioma cell lines after incubation with free doxorubicin and after adsorption to nanoparticles was studied. The sensitivity of either doxorubicin alone or doxorubicin formulated in poly(buty cyanoacrylate) nanoparticles towards glioma cells was analysed using three cytotoxicity detection assays, the MTT, the LDH, and the ATP tests.

An *in vitro* evaluation was carried out using different glioma cell lines, the GS9L, the RG-2, and the F-98 cell lines, to investigate cell-line-specific differences in the degrees of resistance to chemotherapy. In addition, the P-gp expression was analysed in the three cell lines.

Since, the drug resistance mechanism of the three cell lines, GS9L, F-98, and RG-2 cell lines also involves the P-gp expression, poloxamer 185, a known P-gp inhibitor, was employed as an additional new coating surfactant.

3 Materials and Methods

3.1 Preparation of poly(butyl cyanoacrylate) nanoparticles

3.1.1 Reagents and Laboratory equipment

Reagents

n-Butyl-2-cyanoacrylate	Sicomet® 6000)
Poloxamer 188 (Pluronic F-68)	Sigma Steinheim, Germany
poloxamin 908 (Tetronic® 908)	Sigma Steinheim, Germany
Polysorbate 80 (Tween® 80)	ICI Chemicals Essen, Germany
Poloxamer 185 (Pluronic® P85)	BASF, Ludwighafen, Germany
Dextran 70000	Sigma Steinheim, Germany).
Doxorubicin	Sicor, Rho, Italy
Mannitol	Merck, Darmstadt, Germany
0.01N HCl	Merck, Darmstadt, Germany
0.1N NaOH	Merck, Darmstadt, Germany
1N NaOH	Merck, Darmstadt, Germany
2N NaOH	Merck, Darmstadt, Germany
0.9 % (w/v) NaCl	Merck, Darmstadt, Germany
Dichloromethane	Merck, Darmstadt, Germany
n-Butanol, p.A.	Merck, Darmstadt, Germany
n-Pentanol, p.A.	Fluka, Seelze, Germany
Sodiumsulfate	Merck, Darmstadt, Germany

Laboratory equipment

GC-System:

GC: HP5890 Series II — Hewlett-Packard, Bad Homburg, Germany
Integrator: HP3396 A
Column:
Fused Silica Capillary Column, Permabon-FFAP-DF-0,1, 25 m x 0,25 mm ID
Machery-Nagel, Düren, Germany
Centrifuge-Filter (Ultrafree MC, 100,000 NMWL) Millipore, Eschborn, Germany

HPLC-System:

Mobile phase :

70% Water	Milli-Q- Millipore, Eschborn, Germany
30% Acetonitril	Merck, Damstadt, Germany
+ 0,1 % Trifluoracetic acid	Fluka, Seelze, Germany

Interface: D-7000 — Merck-Hitachi, Darmstadt, Germany
Pump: D-7120 — Merck-Hitachi, Darmstadt, Germany
Column: LiChroCart 250.-4 with LiChrospher 100 RP-18 (5µm)
Autosampler: D-7200
Detector: D-7420, UV/VIS; D-7480, Fluoreszenz

Lyovac GT2 freeze dryer	Leybold AG, Köln, Germany
G2 glass filter	Schott, Mainz, Germany
Magnetic stirrer RCT basic	KA Labortechnik, Staufen, Germany
Ultrasonicbath Sonorex RK31	Bandelin electronic GmbH & Co. KG., Berlin
Ependorf Centrifuge 5415 D	Eppendorf AG, Hamburg, Germany
Analyse Balance Sartorius CP 224S	Sartorius, Göttinegen, Germany
Malvern Zetasizer 3000 Has	Malvern, Wores, UK
Laminar-Flow sterilbench	Heraeus, Hanau, Germany

3.1.2 Nanoparticles production procedure

3.1.2.1 Poly(butyl cyanoacrylate) nanoparticles (PBCA-NP)

Unloaded polybutylcyanoacrylate nanoparticles (unloaded-PBCA nanoparticles) nanoparticles were obtained by anionic polymerization at pH below 3. 1% (w/v) butylcyanoacrylate monomer was added to a 1% solution of dextran 70.000 in 0.01N HCl (pH 2) under magnetic stirring for 4h. After 4 h the nanoparticle suspension is neutralized with NaOH (0.1 or 1N) and filtered through a sintered G2 glass filter (pore size of 90-150µm) to remove larger. The nanoparticles were lyophilized adding 3% mannitol as cryoprotector and using a Lyovac GT2 free dryer. The nanoparticles were stored in 2 ml vials at 4°C.

The unloaded-PBCA and Dox-PBCA-NP preparation were carried out under aseptic conditions. To achieve this, all material and components used for nanoparticle preparation were autoclaved and the solutions were filtered through 0.22 µM filter membranes. The polymerization was carried out under a laminar flow sterile bench.

The nanoparticles were resuspended in 0.9 % (w/v) NaCl solution prior use.

3.1.2.2 Doxorubicin-loaded-poly(butyl cyanoacrylate) nanoparticles (Dox-PBCA-NP)

Doxorubicin-polybutylcyanoacrylate nanoparticles (Dox-PBCA nanoparticles) were prepared using the same procedure of polymerization but with only 2.5 h polymerization time instead of 4 h. 30 min after the start of polymerization a doxorubicin solution (25mg/ml in 0.01N HCl) was added to the polymerization medium. The final concentration of doxorubicin was 0.25 % (w/v). After 2.5 h the nanoparticle suspension is neutralized with NaOH (0.1 or 1N) and filtered through a sintered G2 glass filter The nanoparticles were lyophilized in vials of 1-2 ml

using a Lyovac GT2 freeze dryer and a solution of 3% Mannitol was used as cryoprotector. The nanoparticles were resuspended in 0.9 % (w/v) NaCl solution prior to use.

3.1.2.3 Coating Procedure

The poly(butyl cyanoacrylate) nanoparticles were coated with polysorbate 80 (Tween® 80), poloxamer 188 (Pluronic® F-68), poloxamin 908 (Tetronic® 908), or poloxamer 185 (Pluronic® P85). These surfactants are always used in solution at a 1 % concentration. An amount of 100 µl of 10 % surfactant solution was added to 900 µl of the nanoparticle suspension or in the case of the doxorubicin control to 900 µl doxorubicin solution. After this these nanoparticles were stirred for 30 min and then used immediately for cell incubation.

3.1.3 Characterisation of Dox-loaded- and unloaded-PBCA-NP

3.1.3.1 Size determination

The size of the nanoparticles was determined by photon-correlation spectroscopy (PCS), i. e. the hydrodynamic diameter.

The size of the nanoparticles was found to be 190 ± 30 nm

3.1.3.2 Yield polymerization

The polymerisation yield was measured by gas chromatography (GC) as described (103). Briefly, nanoparticle suspension was hydrolysed with 2 N NaOH shaking overnight at room temperature. 50 µl of this solution were mixed with 50 µl of internal standard (pentanol approx. 0.5 %) and diluted with 900 µl of Milli-Q water. 500 µl of the diluited solution were extracted with 1000 µl of methylene chloride. The methylene chloride was dried with water-free sodium sulphate and 1µl of the sample was injected into the GC system. The following instrument parameters and conditions were used:

Instrument Parameter

Injector: split / splitless HP 19251 – 60540, 250°C
Detector: FID, 250°C
Oven-Temperature: Gradient, 45°C (3 min) → 10°C 1/min → 130°C (4 min)

Gas

Aux gas: Helium (Qualität 5.0)
Column flow: 1,0 ml / min
Split flow: 10,0 ml /min
Septum flow: 1,1 ml / min
Column + Aux: 35 ml / min
Column + Aux + Air: 430 ml / min
Column + Hydrogen + Aux: 65 ml / min
Column head pressure: 94 kPa

3.1.3.3 Drug loading

To determinate the doxorubicin entrapment efficiency, lyophilized Dox-poly(butyl cyanoacrylate) nanoparticles were resuspended in water and the particles were separated from the solvent by centrifugation. 100 µl of nanoparticle suspension were transferred to microcentrifuge filters and centrifuged at 16000 g for 20 min. Concentration of free doxorubicin dissolved in supernatant was determined by HPLC. The filtered solution containing the free doxorubicin was injected after dilution by 1:100 to the HPLC system and the amount of doxorubicin was calculated.

The chromatographic separation was performed on a 250x4 mm column packed with LiChrospher® 100 RP-18(5µm). The mobile phase was phosphate buffer (pH 4.2; 0,01 M) acetonitrile (60:40). The detector was set to 250 nm. The following analysis parameters were used

HPLC-System:

Liquid chromatgraph: D-7000	Merck-Hitachi
Packing: LiChrospher® 100 RP-18 (5µm)	Merck, Germany
Column: LiChroCART® 250-4 HPLC-Cartridge	Merck, Germany

Guard column: LiChroCART® 4-4
Injection volume: 20 µl
Flow rate: 0,8 ml / min.
Detection:
- Absorption = 250 nm
- Fluorescence = λ_{ex} 480 nm, λ_{emm} 560 nm

Temperature: ambient
Mobile phase: mixture phosphate buffer (pH 1,4; 0,01M) - acetonitrile (60:40)

3.2 Cell Culture

3.2.1 Cell line and Growth Medium

GS-9L

GS-9L-Medium

500 ml DMEM	Gibco, Pasley, UK
50 ml Fetal calf serum, inactive	Gibco, Pasley, UK
10 ml L-Glutamin [200 nM]	Gibco, Pasley, UK
5 ml Penicillin/Streptomycin [10.000 µg/ml]	Gibco, Pasley, UK
5 ml MEM non-essential amino acids (NNAA)	Invitrogen, Karlsruhe

This is a cell line of non-human/non-primate origin. Its morphology corresponds to glia cells and its specie is rat. The GS-9L rat glioma cell line was derived from N-nitrosomethylurea-induced tumour. The rat glioma GS9L cell line was cultured as a monolayer in DMEM medium supplemented with 2% L-glutamine, 1% penicillin/streptomycin, 1% non-essential amino acids and 10% foetal bovine serum under cell culture conditions (37°C, 5% CO2). GS-9L is an adherent cell lineGS-9L cell line was supplied by ECCC

RG-2

RG-2 Medium

500 ml DMEM	Gibco, Pasley, UK
50 ml Fetal calf serum, inactive	Gibco, Pasley, UK
10 ml L-Glutamin [200 nM]	Gibco, Pasley, UK
5 ml Penicillin/Streptomycin [10.000 µg/ml]	Gibco, Pasley, UK

The rat glioma cell line RG-2 was produced by inoculation with a single dose of N-ethyl-N-nitrosourea in a pregnant CD Fischer rat. The biological characteristics of this tumour closely resemble those of human glioblastome. The cells come from brain tissue and its morphology is glia. The cells were cultured as monolayer, growing as adhederent monolayer in DMEM medium supplemented with 2% L-glutamine, 1% penicillin/streptomycin, and 10% foetal calf serum. RG-2 cell line was supplied by ATCC.

F-98

F-98-Medium

500 ml DMEM	Gibco, Pasley, UK
50 ml Fetal calf serum, inactive	Gibco, Pasley, UK
10 ml L-Glutamin [200 nM]	Gibco, Pasley, UK
5 ml Penicillin/Streptomycin [10.000 µg/ml]	Gibco, Pasley, UK

The tumour was produced by inoculation with a single dose of N-ethyl-N-nitrosourea. The biological characteristics of this tumour closely resemble those of human glioblastome. The cells come from brain tissue and its morphology is glia. The rat glioma cell line F-98 was cultured as monolayer, growing as adherent monolayer in DMEM medium supplemented with 2% L-glutamine, 1% penicillin/streptomycin, and 10% foetal calf serum. F-98 cell line was supplied by the Institute of Virology (Frankfurt am Main, Germany).

Caco-2

Caco-2-Medium

500 ml DMEM	Gibco, Pasley, UK
50 ml Fetal calf serum, inactive	Gibco, Pasley, UK
10 ml L-Glutamin [200 nM]	Gibco, Pasley, UK
5 ml Penicillin/Streptomycin [10.000 µg/ml]	Gibco, Pasley, UK
5 ml MEM non-essential amino acids (NNAA)	Invitrogen, Karlsruhe

The Caco-2 intestine cell line consists of human colonocyte derived adenocarcinoma cells. To apical to basolateral uptake transport, highly differentiated Caco-2 cells express variable levels of several efflux pump proteins depending on cell source and culture conditions.
The cells were grown in DMEM (Dubelcco´s Modified Eagle´s Medium) supplemented with 10% fetal bovine serum, 0.1mM nonessential amino acids, 100 U/ml penicillin, and 0.1mg/ml streptomycin. Caco-2 cell line was supplied by ATCC.

3.2.2 Cell Culture Reagents and Materials

Trypsin/EDTA	Gibco, Pasley, Scottland
Cytoperm Incubator	Heraeus, Hanau, Germany
Cell culture flasks (50ml, 75ml, 250ml)	Greiner, Frickenhausen, Germany
Glasstic® Slide 10 with Grids	Hycor, Penicuik, UK.
Microtiter 12-, 24-, 96-well plate	Greiner, Frickenhausen, Germany
Multichannel pipette	Costar, Wiesbaden, Germany
Sterile Pipettes (5 ml, 10 ml, 25ml)	Sarstedt, Nürnbrecht, Germany
Plastic tubes Falcon® (15ml, 50ml)	Greiner, Frickenhausen, Germany
Laminar Flow sterile bench	Heraeus, Hanau. Germany
Cytoperm Incubator	Heraeus, Hanau. Germany

3.3 Cytotoxic detection assays

3.3.1 Materials

Trypsin/EDTA	Gibco, Pasley, Scottland
Cytoperm Incubator	Heraeus, Hanau, Germany
Glasstic® Slide 10 with Grids	Hycor, Penicuik, UK
Microtiter 12-, 24-, 96-well plate	Greiner, Frickenhausen, Germany
Multichannel pipette	Costar, Wiesbaden, Germany
Sterile Pipettes (5 ml, 10 ml, 25ml)	Sarstedt, Nürnbrecht, Germany
Plastic tubes Falcon® (15ml, 50ml)	Greiner, Frickenhausen, Germany
3-(4,5-dimethylthiazol-2-yl)-2,5-diphynyl-tetrazoliumbromid (MTT)	Sigma, Steinheim
LDH test (Cyto kit)	Roche, Basel, Switzerlanf
HS Vialight, BioWhitaker	Cambrex, UK
NRR (Nucleotid Releasing Agent)	
AMR (ATP Monitoring Agent)	
Photometer Spectra Max 340	MWG Biotech, Ebersberg, Germany
Luminometer MicroLumat LB 96P	Berthold, Bad Wildbad, Germany
Triton 100-X	Merck, Darmstadt, Germany

To measure the ability of the cell to survive, and to continue proliferate, different proliferation or cytotoxicity assays were carried out.

For testing, the cells are trypsined and seeded at $1*10^5$ cells/ml in 96-well plate and were incubated overnight to allow them to adhere to the bottom. The cells are counted using trypan blue by hemocytometer technique. The cells were incubated with the different formulations for 24h. The medium is removed, at the end of the drug exposure and the cells were washed to

eliminate any rest of samples and further incubated with 200 µl of fresh medium for 24 h in the absence of the drug in order to determine an irreversible effect of doxorubicin on cell survival and not only the direct effect of the drug on a metabolic pathway or process. Thus, we are certain to measure the mitochondrial activity or enzyme activity of the cells, which are able to proliferate after long exposure to doxorubicin. For the three tests the positive control are untreated cells and negative control are cells treated with 1% NaN_3.

3.3.2 MTT-Test

This assay is based on the measurement of the mitochondrial activity of viable cells by the reduction of the tetrazolium salt MTT (3-(4,5-dimethyathiazol-2-yl)-2,5-diphenyl tetrazolium bromide) to a blue product formazan. Formazan is insoluble in water and must be solubilized in isopropanol/HCl. A stock solution of 5 mg/ml was prepared in PBS and diluted 1:10 before use (1 mg/ml). The MTT solution was filtered through a 0.22 µM filter membrane. The cells were plated into 96-well microtiter plates and 50 µl of sterile filtered of 1 mg/ml MTT solution was added to each well. After 2 – 3 h the medium is removed and the plate was protected from the light and incubated for 10 min to allow a better solubilization of formazan crystal. The absorbance of formazan dye was recorded at 540 nm with an ELISA plate reader. Since the absorbance directly correlated with the number of viable cells, the viability was calculated from the absorption. The wells, which contained medium, MTT but no cells, were used as blank in the plates reader.

3.3.3 LDH-Test

The cells were plated into 96-well microtiter plates for this assay. The LDH assay (Roche) analyses the activity of the cytoplasma enzyme lactate deshydrogenase, LDH, released from the cytosol of damaged cellular membrane into the supernatant. The LDH activity was determined using a commercial kit, containing a salt (tetrazolium salt INT) and an enzyme (catalyst). The reduction of nicotinamide (NAD+) to NADH/H+ in presence of lactate and LDH, and posterior reduction of tetrazolium salt INT in presence of NADH/H+ and catalyst (diaphorase) results in a red product, formazan, which could be measured at 590 nm using a spectrophotometer. The absorbance then was correlated with the number of viable cells.
The cells, which were able to survive after drug exposure, were lysed with 0.1% TritonX-100, and the released LDH started the reaction that was measured.

3.3.4 ATP-Test (HS Vialight, BioWhitaker, Cambrex)

The cells were platted on a 96 well microtiter plate.

The ATP assay (Cambrex) is based on the measurement of ATP present in all metabolically active cells by using the enzyme luciferase that catalyses the formation of light from ATP and luciferin. The light produced by the previous reaction is recorded in relative light units (RLU) by a luminometer, and corresponds with the number of viable cells.

3.4 Detection of P-glycoprotein (Western Blot)

3.4.1 Material and Reagents

Trypsin/EDTA	Gibco, Pasley, Scottland
Cytoperm Incubator	Heraeus, Hanau, Germany
Sterile Pipettes (5 ml, 10 ml, 25ml)	Sarstedt, Nürnbrecht, Germany
Plastic tubes Falcon® (15ml, 50ml)	Greiner, Frickenhausen, Germany
Cell culture flasks (50ml, 75ml, 250ml)	Greiner, Frickenhausen, Germany
0,2 µm Nitrocellulose membrane	Schleicher x Schnell
HP chemiluminescence film Hyperfilm™	Amershams BS, Uppsala, Sweden.
Bio-Rad Protein assay (Bio-Rad)	München, Germany.
C219 (mdr1 polyclonal antibody)	Calbiochem, San Diego, USA.
Mouse monoclonal anti-rabbit Ig peroxidase	Dianova, Hamburg, Germany
ECL Plus western Blotting Detection Reagents	Amersham BS, Uppsala, Sweden
Lysis Mix	
Lysis Buffer: 10 mM KCl; 1,5 mM $MgCl_2$; !0 MM Tris-HCl (pH 7,4)	
PMSFS	
Protese inhibitors	Roche, Basel, Switzerland
Branson Ultraschall-Desintegrator Sonifier 250	G. Heinemann, Schwäbisch Gmünd, Germany

3.4.2 Procedure

Membrane samples were prepared from Caco-2, GS-9L, F-98, and Rg-2 cells. The cells were washed with PBS and detached by trysinized and collected by centrifugation at 3000 g for

5 min. The pellets were incubated with lysis buffer and put them on ice for 10 min. Cells were shortly 3 times sonificated, avoiding the formation of foam and keeping the samples on ice.
After that the lysates were centrifuged 500 g for 10 min to remove nuclei and debris. Protein concentration was determined using the Bradford assay. Equal amounts of protein were loaded for each sample (40µg). Samples were run on a 7,5% SDS-polyacrylamide gel at 100 volt for 90 min, and the proteins were transferred to a nitrocellulose membrane at 12 volt for 30 min. The membrane was blocked with 5% non-fat dry milk for 1 h then incubated with 1:1000 dilution of anti-P-glycoprotein C219 (mdr1 polyclonal antibody) overnight, followed by three times washings, and incubation with a 1:10000 dilution of a secondary antibody, mouse monoclonal anti-rabbit Ig peroxidase conjugated for 60 min, followed by further three times washings. Inmunoreactive proteins were subsequently detected using the ECL Plus western Blotting Detection Reagents and exposing the membrane to high performance cheminoluminescence film Hyperfilm™.

3.4.3 Protein determination by Bradford

The concentration of protein in the cellular lyses was detected by a commercial detection assays, Bio-Rad-Assay, Bio-Rad. The detection consists in a unspecific interaction of Coomassie-Blue with the aromatic amino acids (Bradford, 1976). 2 µl of the cellular lyses added in 800 µl of the reagent and incubated for 5-10 min on ice. The measurement was done in a BioRad Spectrophotometer at 595 nm.

3.5 Cellular accumulation of doxorubicin

3.5.1 Flow Cytometer (FACS Analysis)

Flow cytometry is the measurement of characteristics of single cells (cyto) suspended in a flowing saline stream. A focussed beam of laser light hits the moving cell and light is scattered in all directions. Detectors placed forward of the intersection point or side-on (with respect to the laser beam) receive the pulses of scattered light and they are converted into a form suitable for computer analysis and interpretation. The total amount of forward scattered light detected is closely correlated with cell size, whereas the amount of side scattered light can indicate nuclear

shape or cellular granularity. The results were analysed by the CellQuest Pro programm.

3.5.1.1 Material

FACSCalibur	Becton-Dickenson, Heidelberg, Germany
CellQuestPro	Becton-Dickenson, Heidelberg, Germany
Trypsin/EDTA	Gibco, Pasley, Scottland
Cytoperm Incubator	Heraeus, Hanau, Germany
24-well plates	Greiner, Frickenhausen, Germany
FACS tubes	Gibco, Pasley, Scottland
Steril Pipetten (5 ml, 10 ml, 25ml)	Sarstedt, Nürnbrecht, Germany
Plastic tubes Falcon® (15ml, 50ml)	Greiner, Frickenhausen, Germany
PBS-Buffer pH 7,4	Biochrom, Berlin, Germany

3.5.1.2 Procedure

For this study we used FACS (Fluorescence Activated Cell Sorter) analysis. The cells were split at 10^5 cell/ml in 24 well plate overnight to allow them to adhere on the bottom of the plate. Next day the incubation with the different samples is started. The cells are incubated for 4 h with the different formulations. After the incubation time the cells are washed, trypsinized, and collected in 1 ml of medium in FACS tubes. The cell suspension is centrifugated at 14000 g for 10 min. Then the medium is discarded and the pellets resuspended in 500 µl FACS-PBS (PBS with 3 % FCS) by vortex and further centrifuged. The cells were washed three times before measurement by FACS.

3.5.2 Confocal Laser Scanning Microscopy (CLSM)

3.5.2.1 Material and Reagents

Concavalin A Alexa Fluor 594 conjugate	Molecular Probes, Eugene, Oregon,

USA	
Leica DM IRBE	Leica, Heidelberg, Germany
Leica Confocal Software	Leica, Heidelberg, Germany
8-chambre slides	Nunc, Wiesbaden, Germany

3.5.2.2 Procedure

In order to study the uptake of doxorubicin by the different cell lines, confocal laser scanning microscopy (CLSM) was used. The intracellular localization of doxorubicin in the different cell lines is analysed by CLSM since fluorescence images can be made using the autofluorescence of doxorubicin. A krypton-argon laser line (488nm) was used for excitation of doxorubicin, and a long pass filter (550nm) was used for detection of the emitted light.

For this assay the GS9L, F-98, RG-2 and Caco-2 cell lines were seeded in 8-chambre slides, and incubated overnight to allow them to adhere on the bottom. At the next day, the incubation with the doxorubicin formulations was made over 6 h. This is the time that it was established to see a maximal accumulation of doxorubicin within the cells, assuring still a viable state of the cells. The coating of the nanoparticles was just done before the start of the incubation period. The control was always untreated cells. Then, the cells were carefully washed three times with PBS, to avoid detaching the cells. The cellular membrane was stained with the dye Cy5 ConA, adding 100µl of Cy5 from a stock solution and waiting 2 min for each well. The dye then is discarded and three times with PBS washed, leaving a small amount of PBS within the well.

3.5.3 Cellular membrane study

3.5.3.1 Material and Reagents

Concavalin A Alexa Fluor 594 conjugate	Molecular Probes, Eugene, USA
Leica DM IRBE	Leica, Heidelberg, Germany
Leica Confocal Software	Leica, Heidelberg, Germany
8-chambre slides	Nunc, Wiesbaden, Germany

3.5.3.2 Procedure

The GS9L cells were seeded in 8-chambre slides, and incubated overnight. The surfactants solutions prepared and 50 µl of each solution added to each well containing 450 µl medium. The concentrations were prepared 10x concentrated to obtain the desired final concentration.

The cells were incubated for 24 h with the surfactant solutions. At the end of the treatment, the cells are washed with PBS three times and the membrane stained with 100 µl Cy5 solution for 2 min. The cells washed three times with PBS, leaving a small volume of PBS within the wells.

4 Results

4.1 Preparation and characterization of poly(butyl cyanoacrylate) nanoparticles (PBCA-NP) and Dox-poly(butyl cyanoacrylate) nanoparticles (Dox-PBCA-NP)

4.1.1 Preparation procedure

The nanoparticles used in this study were prepared by an emulsion polymerization process. The polymerization mechanism was an anionic process, initiated by bases such as OH^- resulting from the dissociation of water.

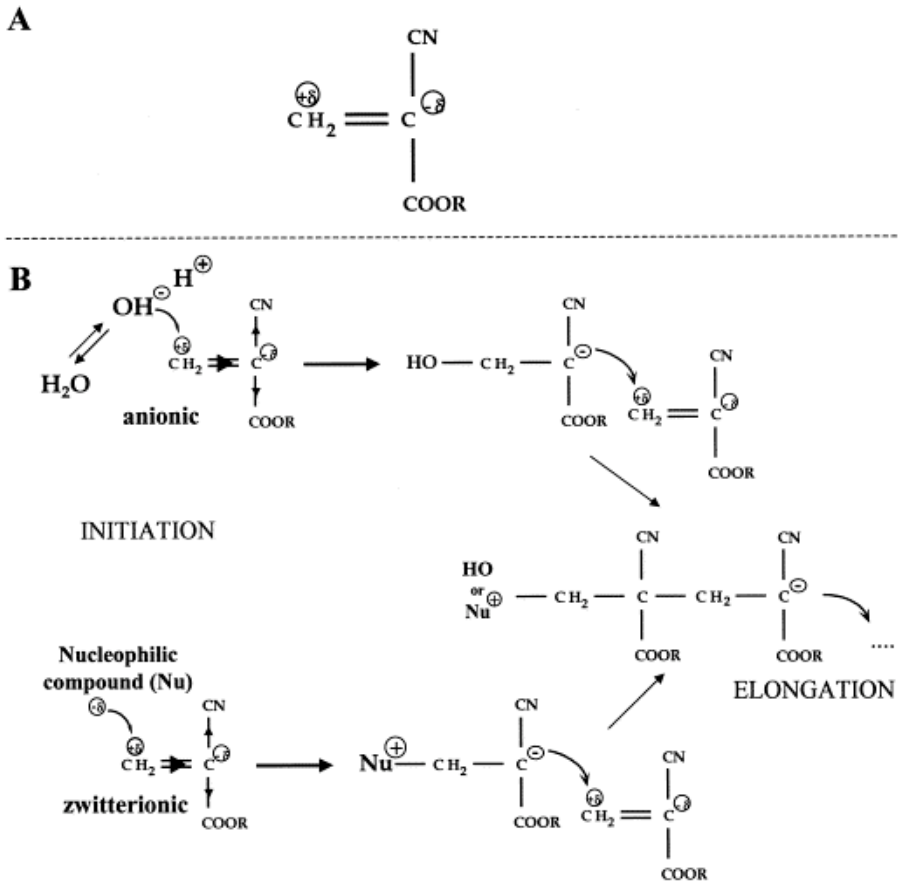


Figure 6 A. Alkyl cyanoacrylate molecule. B. Anionic emulsion polymerization process of alkyl Cyanoacrylates

The polymerization procedure is carried out by the addition of the monomer, butyl cyanoacrylate, into the polymerization medium, which consists of an aqueous acidic solution of 0.01 N HCl (pH 2), and the stabilizer dextran 70000 under constant stirring.
The molecular weight of the polymer depends on the pH. In the presence of high concentration of H^+ the polymerization termination is rapid, leading to small molecular weights. At lower concentrations of H^+ the termination rate is reduced and, consequently, the resulting molecular weights increase. At low molecular weights, the growing particles can be very soft and prone to agglomeration. For this reason, stabilizers have a significant influence on the particle size and the molecular weight (52, 53). The influence of the stabilizer was found to also be very important for product yield (77,78). The yield was assessed by gas chromatography (GC), described thoroughly in the Material and Methods section and was found to be 60 %.

Other factors contributing to the particle size of the nanoparticles include monomer concentration and stirring speed. Because the solubility of the cyanoacrylate is exceeded at concentrations above 0.05%, monomer droplets are formed and the system has to be stirred constantly. The particle size increases slightly with increasing stirring speed (52). This increase is caused by the higher kinetic energy of the system at higher agitation forces. The increased kinetic energy level of the system enables some oligomers, small semisolid particles, and even larger particles to overcome the interfacial energy barrier surrounding the particles, leading the coalescence with other particles (53, 54). The preparation of nanoparticles in the present study took place at 1% butyl cyanoacrylate and 500 rpm speed.

4.1.2 Size of the nanoparticles

The particle size determines the surface area available for contact with the cell membrane and has an influence on the mechanism by which the nanoparticles will be internalized into these cells.
There are several factors, which can determine the size. Different pH values of the polymerization medium influence on the particle size differently: a particle size minimum exits at around pH 2, whereas the polydispersity falls with increasing pH.
The stabilizers also have a significant influence on the uniformity of particles size (77,78). The particles size was measured by Photon Correlation Spectroscopy (PCS). The particle size of our

nanoparticles ranged from 190 nm to 200 nm and the distribution was monomodal.

4.1.3 Zeta potential

PBCA NP and coated PBCA NP showed negative zeta-potentials in distilled water, a general phenomenon of theoretically uncharged polymeric particle. Adsorption of doxorubicin on the surface modifies the charge of nanoparticles towards a positive value, caused by the positive charge of this drug.

4.1.4 Loading of Dox-poly(butyl cyanoacrylate) nanoparticles

The load of the Dox-PBCA-NP was assessed in the supernadant of the nanoparticles suspension after centrigugation. Relative drug loading (% of total amount) was calculated by subtraction of free drug in the supernatant from the total amount in the vials and was found to be 70 % of loading.

4.2 The Growth Inhibition of Glioma Cells after Doxorubicin and Dox-PBCA-NP incubation. Cytotoxicity Assays

Drug targeting systems, such as PBCA-NP, have to face three challenges, one is the avoidance of the macrophages of the mononuclear macrophages system (MPS), the second is to direct the carriers to the desired site of action, and the third is to yield a high delivery of the drug to the targeted cells. Previous works already demonstrated quite successful avoidance of the MPS by the poly(butyl cyanoacrylate) nanoparticles (83) and, therefore, in many cases an enhanced transport of the drug to the target tissue (76, 82). Moreover, it was found that binding of doxorubicin to nanoparticles increased the efficacy against glioma tumours (72). Responsible for this achievement, was a selective alteration of the surface characteristics of these nanoparticles that represent the key factors to determine the organ distribution of these carriers. Therefore, one of the objectives of this work was the analysis of the influence of such

modification on the action and transport of the doxorubicin into the targeted tumour cells, the glioma cells. For this purpose, the doxorubicin-poly(butyl cyanoacrylate) nanoparticles were coated with three non-ionic surfactants.

The present *in vitro* study involves the evaluation of the chemosensitivity of three rat glioma cell lines towards: (1) doxorubicin in solution, (2) doxorubicin-poly(butyl cyanoacrylate) nanoparticles, and (3) coated-doxorubicin-poly(butyl cyanoacrylate) nanoparticles (coated-Dox-PBCA NP).

In order to determine the effect of the nanoparticles formulations on the cells as well as the sensitivity of the glioma cells to doxorubicin, the cytotoxic effect of doxorubicin was evaluated by its inhibitory effect on the cell proliferation. Widely employed, cell viability tests or proliferation tests were used to determine the potential carcinogenicity or cytotoxicity of the anticancer drug. The existing well described proliferate assays to evaluate the cellular sensitivity to chemotherapeutics drugs are employed according to the authors preferences because of their ease of use, optimal condition, measurement requirement, etc. In the present *in vitro* study the cytotoxicity of different formulations was evaluated using three assays measuring different parameters. The assays were the MTT, the LDH, and the ATP assays.

In the MTT assay, a tetrazolium (MTT) salt is added to the media and upon internalizing into the cells, is reduced to a blue product by the mitochondria dehydrogenase into the mitochondria. The absorbance is recorded and the extend of the viability was defined as the relative reduction of the absorbance, which is directly correlated with the amount of viable cells in relation to the cell control (= 100 %).

The LDH is a cytosplamic enzyme, which after cellular membrane damage is released from the cytosol into the extracellular media. This enzyme catalyzes the reduction of lactate to a formazan (a red product), which absorbance is recorded. For this purpose in our experiments, the cells were subjected to doxorubicin either in free form or in the nanoparticles formulations for 24 h. The surviving still viable cells were then cultivated with doxorubicin-free medium for another 24 h. After this time these remaining cells were lysed with a 1 % Triton X 100 solution, and released LDH by this lysis was determined, indicating the percentage of cells that survived the doxorubicin treatment. The decrease in the formazan absorbance in relation to the untreated cell control (= 100 %) corresponded to a decrease in the LDH activity, and therefore was a decrease of the expression in the cellular viability caused by the doxorubicin action.

In the ATP assays, the amount of ATP contained in the cells is used to give the conversion from luciferin to oxiluciferin with the corresponding emittance of light, which is recorded. Thus, the decrease of the amount of ATP, due to a decrease in the cell number of living cells indicates a viability decrease.

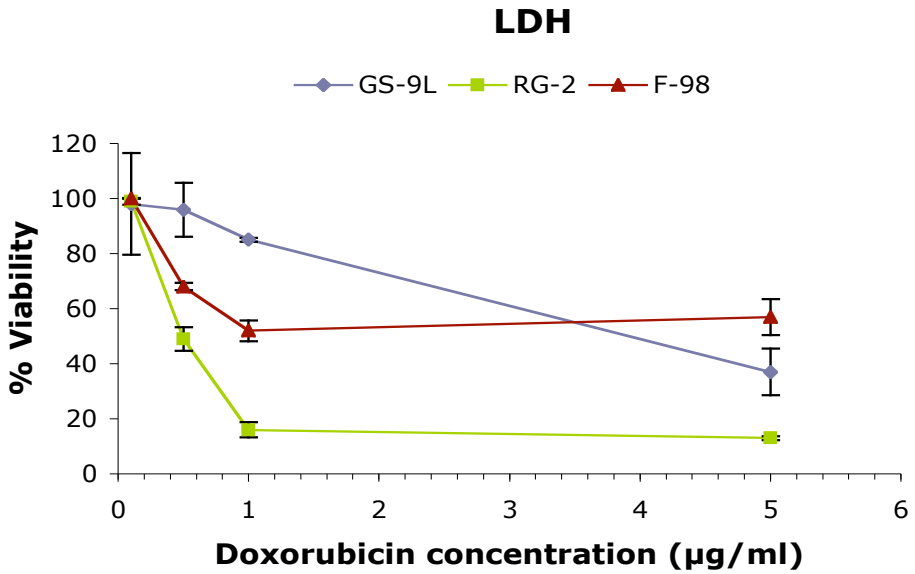
4.2.1 Effect of doxorubicin solution on the GS-9L, RG-2, and F-98 glioma cell lines

At first, the sensitivity of the glioma cell lines, GS9L, RG-2, and F-98 towards free doxorubicin was analysed in order to identify cell-line-specific differences in the degrees of resistances to doxorubicin.

The cytotoxic effect of doxorubicin in solution was tested at concentrations ranging from 0.1 µg/ml to 5 µg/ml doxorubicin. The cells were incubated for 24 h in the presence of different concentrations of doxorubicin and keeping them a further 24 h in the absence of the drug. The survival rate of the cells was calculated and results are expressed as:

Percent of survival = 100 x (number of treated cells surviving) / (number of untreated cells)

The mean percentages of cell survival (viability) and the corresponding standard deviations were calculated. The curves of viability percentages represented in Figure 7.



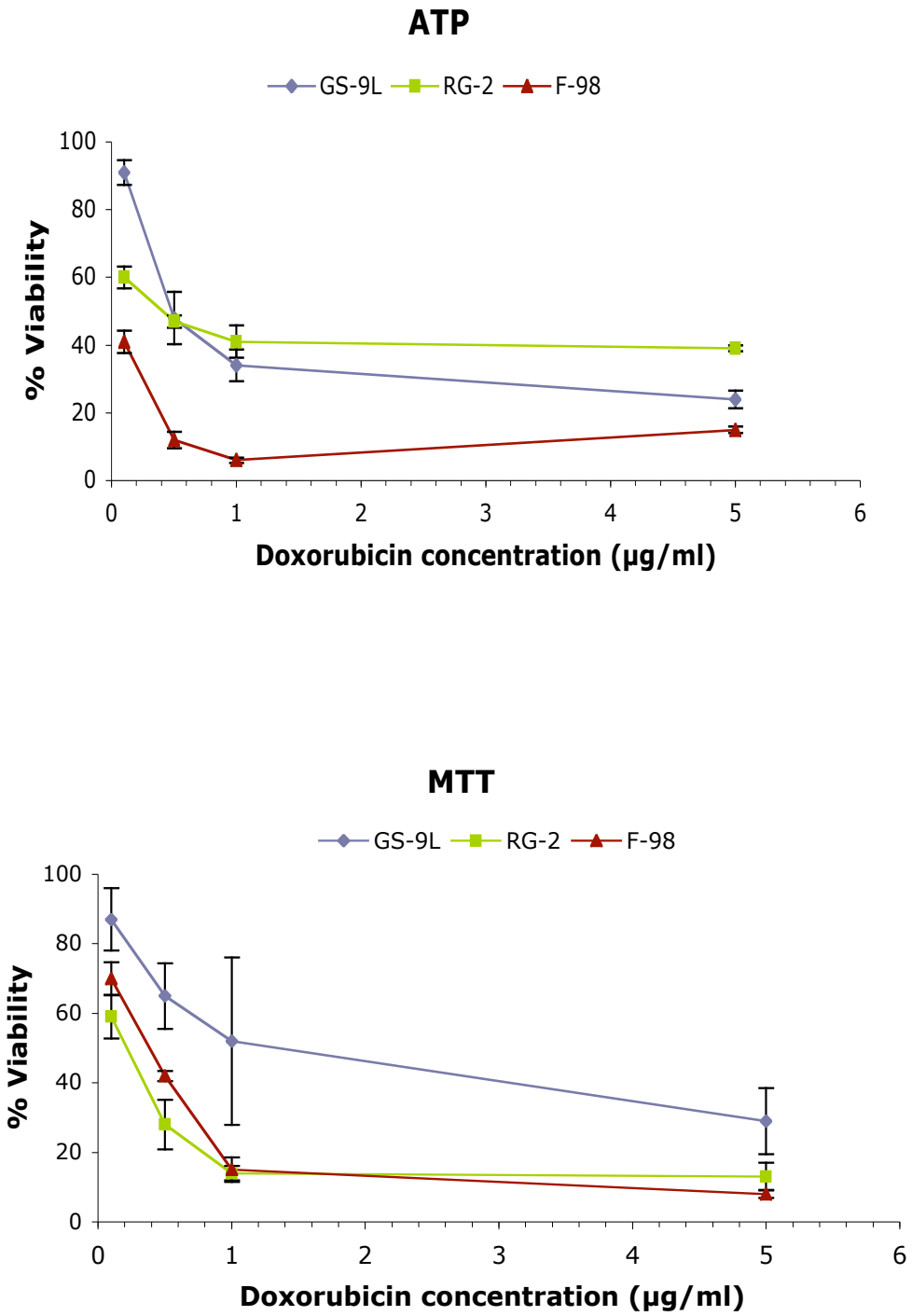


Figure 7. Viability of the GS-9L, RG-2, and F-98 glioma cell lines after exposure of doxorubicin concentrations, according the three assays.

As shown in figure 7, the cytotoxic effect of doxorubicin measured by LDH, or ATP MTT test was concentration-dependent in all cell lines. The decrease of viability was most pronounced at low doxorubicin concentrations below 1 µg/ml. At higher concentrations, the viability of RG-2 and F98 cells did not change significantly up to the highest concentration of 5 µg/ml. Only in

the GS-9L cell line, a further marked decrease in cell viability depending on the assay was observable, which was most considerable in the LDH test and statistically insignificant in the ATP test. Moreover, the GS-9L cell line appeared to be the least sensitive to the drug: The viability of this cell line at doxorubicin concentrations between 1 µg/ml and 5 µg/ml was ~ 50 - 30%, whereas the viability of the F-98 and the RG-2 cells was below 20 % in most cases. At the same time, it has to be mentioned that the cytotoxicity induced by doxorubicin measured by the three tests was different between the cell lines, this difference being most pronounced for the GS-9L cells. The concentration capable of reducing the cell proliferation by 50 %, IC_{50}, was calculated. As expected, different values of IC_{50} observed between the three cell lines is caused by a different response of the cell lines to doxorubicin, resulting in a varied degree of resistance presented by the cells, cell lines-specific resistance differences. However, when we compare the values obtained for one cell line using the three assays, a discrepancy is observed, which is clearer once the IC_{50}, is calculated for each cell line, depicted in Table 1. The difference in the calculated IC_{50} values was most pronounced for the F-98 cell line in the ATP test

The cytotoxicity of free doxorubicin was not increased by the presence of either surfactant (data not shown). Only the effect of polysorbate 80 occasionally, at low concentrations of doxorubicin, showed a slightly increase in the cytotoxicity of free doxorubicin. The effects of poloxamer 188 and poloxamine 908 were negligible.

Table 1. IC_{50} values for the GS9L, RG-2, and F-98 cell lines, according to the MTT, LDH, and ATP assays

Assay	Cell line (IC_{50} values µg/ml)		
	GS9L	RG-2	F-98
MTT	1.4	0.2	0.4
LDH	3.9	0.6	1
ATP	0.6	0.6	0.01

The different values of IC_{50} observed between the three assays show variability in the results and it is therefore not easy to establish to what degree the different rat glioma cell lines are chemosensitive towards the doxorubicin solution. Nonetheless, the IC_{50} for GS-9L is always the highest independent of the assay used, meaning that the GS-9L cells present the lowest growth inhibition, and therefore the highest resistance towards doxorubicin. RG-2 has the lowest resistance in LDH and MTT test whereas F-98 shows less resistance than RG-2 in ATP test. In

the MTT test, no significant difference between RG-2 and F-98 appeared. The growth-inhibiting effect of doxorubicin is variable, depending on the cell line. Therefore, the different responses to doxorubicin can be accounted for by a relationship of doxorubicin with a variety of the resistances presented by the cells.

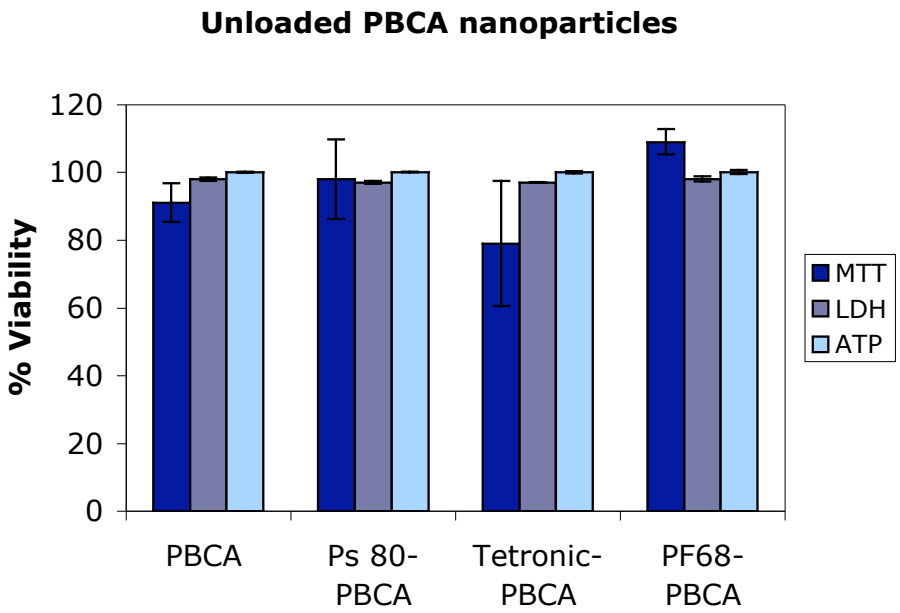
4.2.2 Effect of unloaded-PBCA-NP on the viability of the GS-9L, RG-2, and F-98 glioma cell lines

The absence of cytotoxicity of the unloaded nanoparticles is an important requirement for their usefulness as a carrier system for doxorubicin.

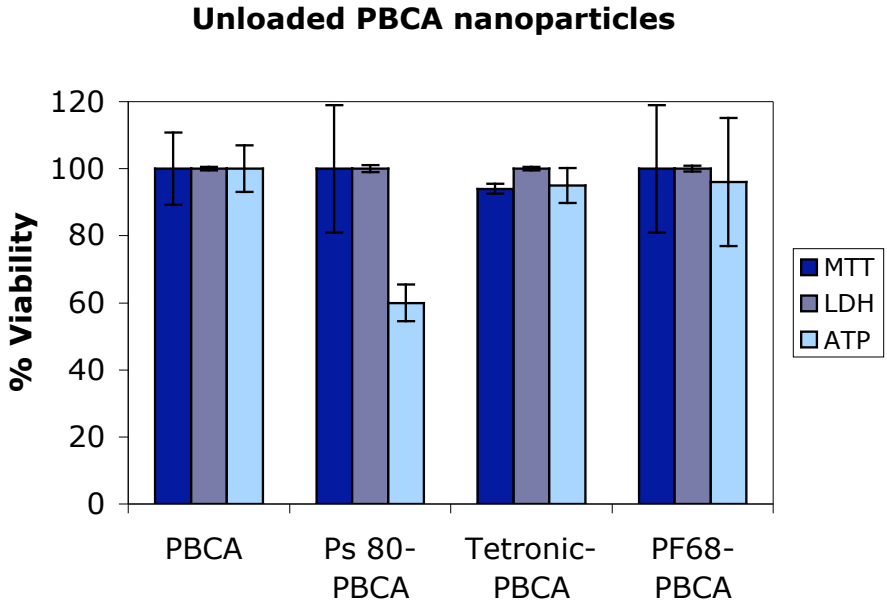
To prove the absence of cytotoxicity of unloaded-PBCA-NP, the cell lines were incubated with the unloaded-PBCA-NP in a range of concentrations between 0 µg/ml and 25 µg/ml of polymer with the objective to establish a maximum-non-toxic concentration of unloaded-PBCA-NP. From the results obtained in this experiment, it was established that the maximum concentration, which did not produce any toxic effect in any of the all cell lines was 15 µg/ml of polymer. Consequently, this concentration was used as a reference for all following experiments.

The effect of the coating of the unloaded nanoparticles on the cell proliferation was also assessed. The surfactants used were polysorbate 80 (Ps 80), poloxamer 188 (PF68), and poloxamine 908 (Tetronic®). The coating of the nanoparticles was done just before incubation with 1 % solution of each surfactant. Then, the cells were incubated with a concentration of 15 µg/ml of uncoated- and coated-, unloaded-PBCA-NP for 24 h. Figure 8 depicts the viability values for the three cell lines according to the MTT test, the LDH test, and the ATP test.

A.


Unloaded PBCA nanoparticles
% Viability
120
100
80
60
40
20
0
PBCA
Ps 80-PBCA
Tetronic-PBCA
PF68-PBCA
MTT
LDH
ATP

B.


Unloaded PBCA nanoparticles
% Viability
120
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C.

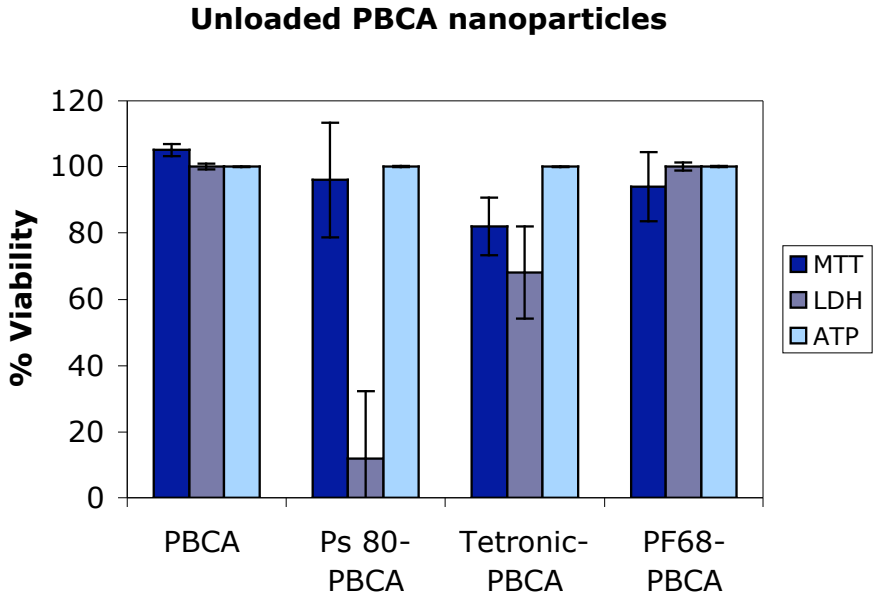

Figure 8. Cellular survival after 24 h incubation with unloaded-PBCA-NP, according to MTT, LDH, andATP assays on (A.) GS-9L, (B.) F-98, (C.) RG-2 cell lines.

As shown in figure 8, no significant decrease of the cell number was observed and the nanoparticles coated with the different surfactants did not exhibit a significant cytotoxic effect with the exception of polysorbate 80 (Ps80-PBCA-NP) in the LDH test after incubation of the RG-2 cells, where the viability decreased considerably.

4.2.3 Effect of Dox-PBCA-NP on the viability of the GS-9L, RG-2, and F-98 glioma cell lines compared to doxorubicin solution

Once proved that the unloaded-PBCA-NPs were non-toxic for the cells, the question arose if the cytotoxic activity of doxorubicin against the glioma cells could be enhanced by means of nanoparticles and if the use of surfactants as surface coatings improves the drug delivery into the glioma cells. For this reason, the nanoparticles were loaded with doxorubicin and coated as described in Material and Methods section. The effect produced by the Dox-PBCA-NP (uncoated and coated) was compared with the effect produced by doxorubicin in solution at equivalent doxorubicin concentrations.

The loaded doxorubicin-nanoparticles were coated at a 1% concentration with the previous surfactants as used to coat the empty NPs in the earlier tests (polysorbate 80, poloxamer 188, and poloxamine 908). The effect of doxorubicin in solution and doxorubicin bound to nanoparticles was assessed using the MTT test and LDH test only, because the ATP was not available any more. The evaluation of the effect of doxorubicin solution and Dox-PBCA-NP (uncoated- and coated-) was carried out at different concentrations of doxorubicin ranging from 0.1 µM to 5 µM. The maximum concentration of polymer used was 15 µg/ml for all experiments. The cells were incubated for 24 h with the samples, at which time the medium was changed and the cells were incubated for a further 24 h in the absence of the drug. The cells capable of proliferating after the drug exposure were quantified by measuring their mitochondrial activity using the MTT assay, and the LDH activity by the LDH assay.

4.2.3.1 MTT results for doxorubicin solution and Dox-PBCA-NP

In the Figure 9, the MTT data again shows a concentration-dependent decrease in all three cell lines. This decrease, as seen in doxorubicin solutions (Figure 7), was most pronounced at low doxorubicin concentrations up to 1 µM and in most cases did not change up to a doxorubicin concentration of 5 µM. However, a different response of the cell lines towards doxorubicin formulations was observed. In general, doxorubicin in solution displayed a lower cytotoxicty towards the cells as compared to Dox-PBCA-NP, throughout at low concentrations. The F-98 cell line especially demonstrates this effect and shows a very low viability at high concentrations. For the RG-2 cells a drastic drop in viability to very low levels was observed at 1µM doxorubicin.

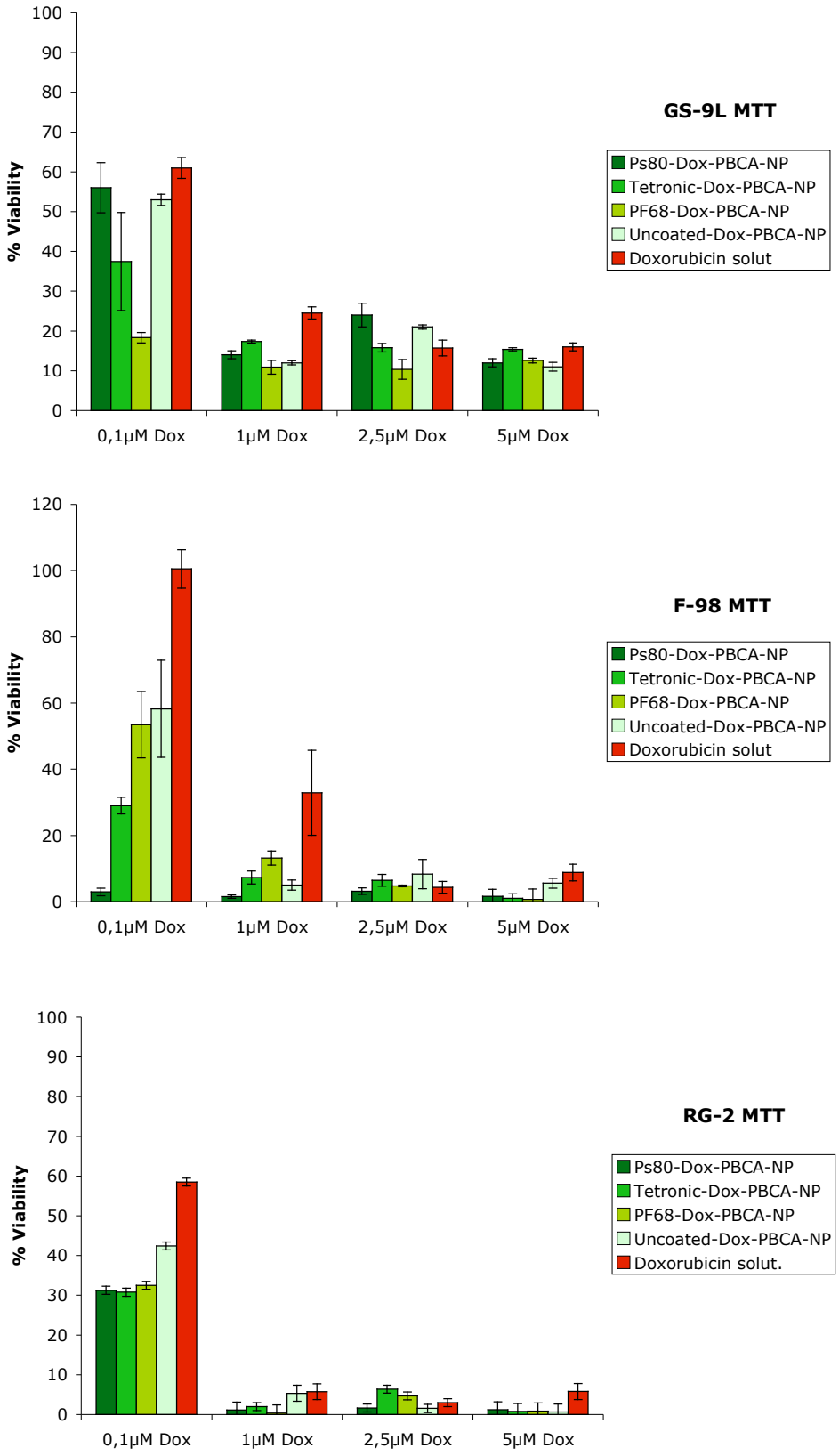

Figure 9. Viability of GS-9L, F-98, and RG-2 according to MTT assay after 24 hours of incubation with different concentrations of doxorubicin formulations.

As already shown in Figure 7, again the GS-9L cell line appeared to be the least sensitive to doxorubicin, since the cell viability of GS-9L cells was much higher than in the F-98 and RG-2 cell lines at doxorubicin concentrations between 1 µM and 5 µM. In the latter cell lines (F-98 and RG-2) the viability was considerably below 20 % in most cases.

However, in general the effect of coating of the nanoparticles with the surfactants on the increase of efficacy of doxorubicin although statistically significant in some cases was practically not important. Only in the case of GS-9L cells, poloxamine 908 and poloxamer 188 coating showed a visible decrease in the viability, which was more pronounced at low concentrations. Additionally the viability of the RG-2 and F-98 cell lines decreased significantly after Ps 80-Dox-PBCA-NP exposure.
According to the MTT assay, it is obvious that the GS-9L cell line shows a higher resistance to every formulation of doxorubicin than the RG-2 and F-98 cell lines, and the RG-2 cell line seems to be more sensitive than the F-98 cell line for every formulation.

4.2.3.2 LDH assay results for doxorubicin solution and Dox-PBCA-NP

The LDH assay data show a slight concentration-dependent cytotoxic effect, for doxorubicin in solution as well as for the nanoparticulate formulations (Figure 10). Again the GS-9L cell line was, even at high concentrations, the most resistant towards the action of doxorubicin. The RG-2 cells were the most sensitive towards doxorubicin, as well as to the other formulations.
In the GS-9L cells, only Ps 80-Dox-PBCA-NP caused a higher decrease in viability than doxorubicin in solution with a dose dependent effect leading to zero viability at 5 µM doxorubicin. The rest of coatings did not improve the doxorubicin activity in relation to uncoated nanoparticles and doxorubicin alone.
In the F-98 cell line the three coatings had a significant effect on the viability of the cells even more at lower concentrations, with a marked dose dependent effect leading to zero viability for Ps80-Dox-PBCA-NP.

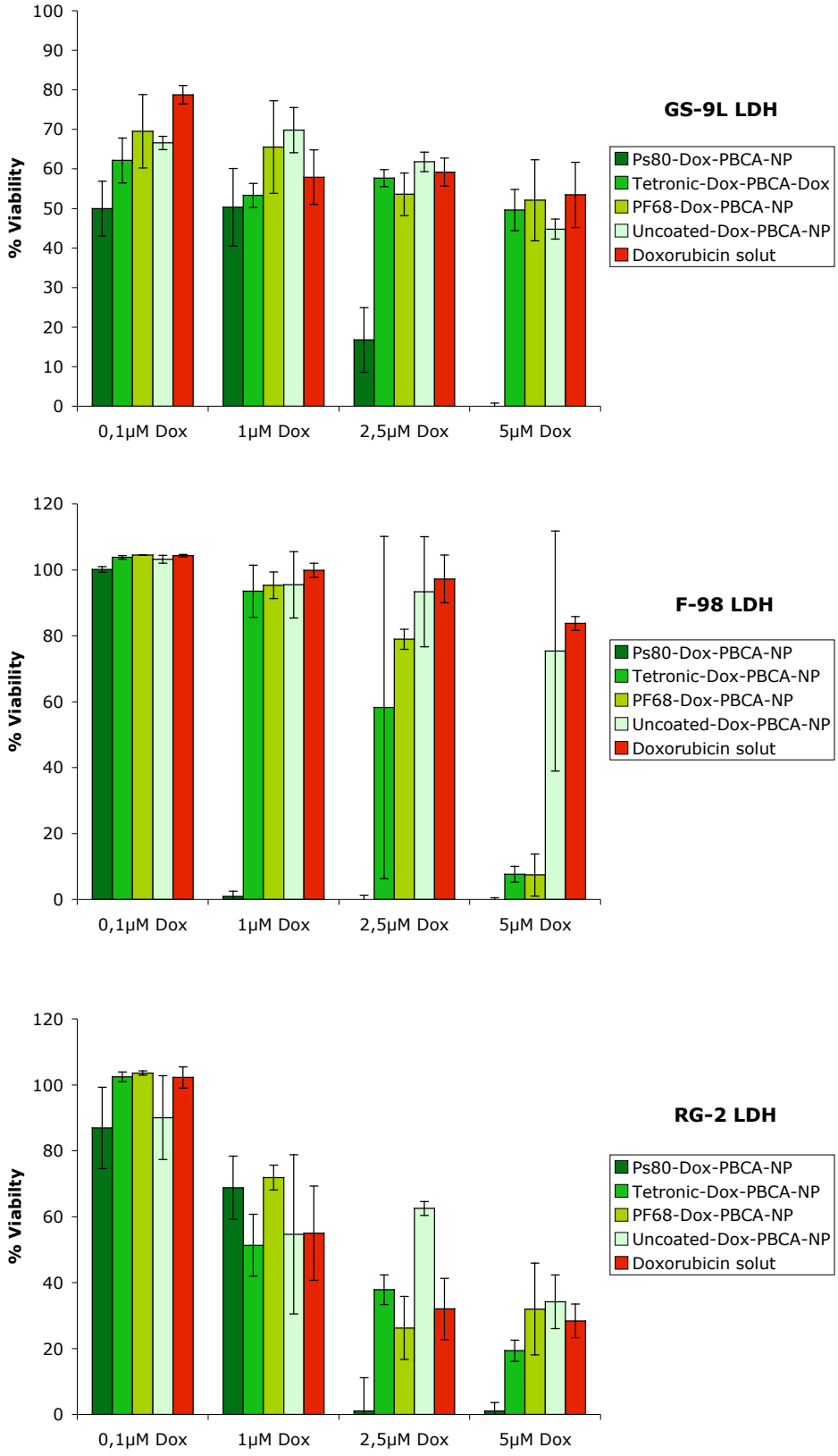

Figure 10. Viability of GS-9L, F-98, and RG-2 according to the LDH assay after 24 hours incubation with different concentrations of several doxorubicin formulations.

However, the uncoated nanoparticles and doxorubicin solution do not display such a decrease, resulting in a similar effect for both formulations.

On the other hand, RG-2 presented a marked viability decrease with an increase in the concentration of doxorubicin for all formulations tested, that was even more pronounced at low concentrations of doxorubicin.

At higher concentrations, polysorbate 80 (Ps 80) also was the most effective surfactant in the LDH assay in all cell lines.

The difference between Ps80-Dox-PBCA-NP and doxorubicin in solution was clear in all three cell lines showing a higher viability decrease after treatment with Ps80-Dox-PBCA-NP than with free doxorubicin. A marked influence of poloxamine 908 and poloxamer 188 was observed in F-98 cell line and a somewhat lesser influence was visible in RG-2 cell lines compared to uncoated Dox-PBCA-NP (Fig. 10).

Throughout the viability values obtained by the LDH assay were lower than those obtained using the MTT assay under the same conditions. There is a concordance in the two assays showing the GS-9L as the most resistant cell line.

4.3 Intracellular Accumulation of Doxorubicin and P-gp expression in the Glioma Cell Lines

As stated above, doxorubicin showed a different growth inhibition effect on the three cell lines. To explain this different sensitivity to doxorubicin observed in the three cell lines, which was largely independent of the formulation used, the uptake of doxorubicin into the cells was analysed. This intracellular accumulation of doxorubicin was measured by flow cytometer (FACS), exploiting the fluorescence capacity of doxorubicin. The cells were incubated with 1 µM doxorubicin solution for 4 h and the mean fluorescence of the cells was recorded, corresponding with the uptake of doxorubicin into the cells. As seen in Figure 11, the amount of doxorubicin accumulated after 4 h differed in the three cell lines.

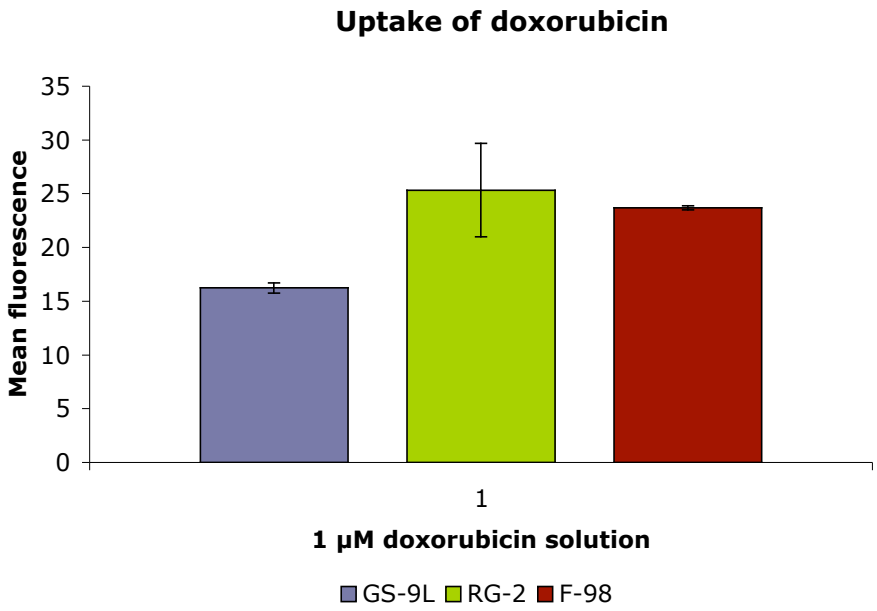

Figure 11. Intracellular accumulation of doxorubicin in GS-9L, RG-2, and F-98 cell lines after 4 h incubation, measured by flow cytometer analysis.

As expected, the uptake of doxorubicin by the GS-9L cells was significantly lower, as compared to the uptake by the RG-2 and F-98 cells. The difference in the uptake between the RG-2 and F-98 was not significant. Therefore, the low sensitivity of the GS-9L cells seen in the cytotoxic assays corresponds to a lower accumulation of doxorubicin into the cells.

One of the possible reasons of low accumulation in tumour cells, such as glioma cells, may be multidrug resistance (93, 94, 95). The accumulation of doxorubicin into the cells depends on its transport across the cellular membrane. The transport of doxorubicin into resistant cells is governed by the multidrug resistance (MDR). Given that one of the prime transporters mediating multidrug resistance is the P-glycoprotein (P-gp) and that doxorubicin is a P-gp substrate (91, 92), we analysed the expression of this efflux pump protein in the different cell lines. The expression of this transmembrane transport protein was assessed by Western blot technique (Figure 12). The Caco-2 cell line was used as a positive P-gp expressing cell line.

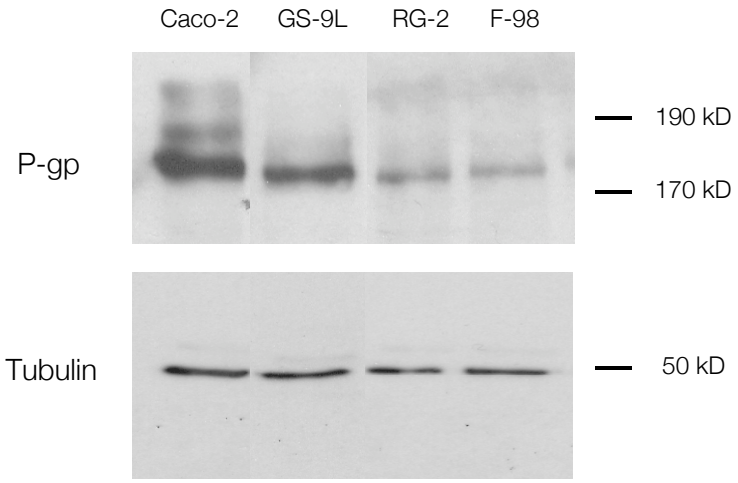

Figure 12. Western blot analysis of P-gp in GS-9L, RG-2, and F-98 rat glioma cell lines. Caco-2 cell line was used as positive P-gp expressing cell line. P-gp was detected using the monoclonal P-gp antibody C219 (1:1000 dilution).

The Western blot shows that the different cell lines express the P-gp protein to different degrees. The first band corresponds to the positive P-gp expressing control cell line, Caco-2. The second band, GS-9L cell line shows a stronger expression of this efflux pump, showing a more pronounced protein expression than the RG-2 and F-98 cell lines.

The expression of P-gp normally corresponds with drug resistance. Therefore, the different degrees of expression of P-gp in the cell lines are probably responsible for the variation of the responses to doxorubicin in the three cell lines. This means that this transmembrane protein is responsible for the efflux transport of doxorubicin, decreasing the doxorubicin accumulation and therefore increasing the resistance to the cytostatic drug. The low level of accumulation of doxorubicin in the GS-9L cell line showed by FACS analysis corresponded to a high expression level of P-gp, which pumps doxorubicin out of the cells, resulting in a decrease of its activity on the cells, corroborating the results observed by the cytotoxic assays in the GS-9L cell line.
In contrast, the increased sensitivity of RG-2 and F-98 towards doxorubicin is caused by an increased intracellular accumulation of doxorubicin due to the fact that the lower P-gp expression level in these cells results in less extend of the efflux transport of doxorubicin. This further backs the fact that the expression of P-gp on the RG-2 and F-98 cell surfaces is lower than on GS-9L.

4.4 Uptake and Localization of Doxorubicin into Glioma Cells

The conclusions deducted from the previous cytotoxic assay results led us to presume that Dox-PBCA-NP, even at low concentrations of doxorubicin, enhances the uptake of doxorubicin into the tumour cells. Consequently their cytotoxic activity against tumour cells should be higher than doxorubicin in solution. The different responses shown for each cell line using the same range of concentrations of doxorubicin and the same assay conditions would be justified by specific resistances at the cellular level presented by the tumour cells. As said previously, the P-gp expression in the cellular membrane of the glioma cells seems to be the responsible for the different sensitivity towards doxorubicin.

Drugs such as doxorubicin appear to enter the cell by passive diffusion through the lipid bilayer (96), resulting in sensitive cells towards doxorubicin. However with resistant cells, upon entering doxorubicin into the cells, the drug binds to the P-gp protein and is pumped out of the cell (85, 86). To circumvent this multidrug resistance at the cellular level many authors have proposed the use of competitive inhibitors such as the calcium channel blocker, verapamil (86). One problem faced by the clinical use of verapamil is the serious adverse effects of this compound. Another strategy suggested for delivery of anticancer drugs, with the aim of overcoming the resistance phenomena, is the association of the drug with nanoparticles (86, 87). Couvreur et al. carried out studies where doxorubicin was encapsulated in various types of nanoparticles (6, 59) and the sensitivity of resistant cells to the doxorubicin-loaded-nanoparticles was then evaluated by measuring the cytotoxic effect produced by increasing the concentration of the doxorubicin-loaded-nanoparticles. Resistant cells treated with doxorubicin-loaded poly(alkyl cyanoacrylate) (PACA) nanoparticles showed a much higher sensitivity than to the free drug (6, 59, 84, 87, 88).

As expected, by our previous cytotoxicity results, the doxorubicin either in solution or absorbed on the PBCA nanoparticles showed different growth-inhibiting effects on the three glioma cell lines at equivalent concentrations of doxorubicin. This fact was due to the resistance phenomena, above described, presented by the glioma cells towards cytotoxic drugs, such as doxorubicin. The results also clearly revealed that such resistance was presented in a different degree by each cell line due to the specific resistance at the cellular level, represented by the expression of the P-glycoprotein (see figure 12). Thus, all said above led us to evaluate the capacity of doxorubicin to overcome the P-gp mediated efflux once doxorubicin was formulated as nanoparticles and/or with a P-gp inhibitor.

Given that the spectrum of P-gp substrates and P-gp inhibitors is very wide, the P-gp

inhibitor for our analysis was chosen taking as reference the Batrakova´s studies where it was demonstrated that poly(ethylene oxide)-poly(propylene oxide) block copolymers (Poloxamers) are potent inhibitors of the P-gp drug efflux system (7, 8, 9, 65). Formulation of P-gp-dependent drugs with poloxamer 185 (Pluronic® P85) resulted in significant increases in their transport to P-gp expressing cells. Experimental studies have been demonstrated to show that Pluronic® block-copolymers sensitize MDR cells, resulting in an increase in the cytotoxic activity of the anthracyclines and others drugs.

In this study, poloxamer 185 was formulated with doxorubicin solution and Dox-PBCA nanoparticles. In the case of Dox-PBCA nanoparticles it was used to coat the nanoparticle surface and in the case of doxorubicin solution it was just mixed with a poloxamer 185 solution. The aim of this analysis was to use the P-gp inhibition capacity of poloxamer 185 to improve the transport of doxorubicin into the cells, avoiding its efflux out of the cells by the P-gp and increasing its intracellular accumulation.
It was also observed in the cytotoxic assay data that polysorbate 80 used as a coating was responsible for a marked effect on the viability of all the cell lines. Moreover some studies proposed that polysorbate 80 also possesses a P-gp inhibition activity (79, 80). For this reason it was decided to analyse the two surfactant, poloxamer 185 and polysorbate 80, comparing their effect on the P-gp activity and, therefore, their effect on the doxorubicin uptake.

4.4.1 Uptake of doxorubicin into GS9L cell line

For these experiments a concentration of 1 µM of doxorubicin was used, either in Dox-PBCA nanoparticles or in doxorubicin solution. FACS analysis and CLSM analysis were carried out to evaluate this P-gp inhibition on the GS-9L glioma cell line.

4.4.1.1 FACS Analysis

Using FACS analysis, it was possible to determine to which grade doxorubicin entered into the different cell lines. Doxorubicin in solution and Dox-PBCA nanoparticles were formulated with poloxamer 185 and polysorbate 80. Doxorubicin solution was mixed with 1 % solution of poloxamer 185 and 1 % polysorbate 80, and the Dox-PBCA nanoparticles were

coated with 1 % poloxamer 185 and 1 % polysorbate 80 solutions. The fluorescence coming from doxorubicin quantified to the accumulation of doxorubicin into the cells. Figure 13 shows the percentage of fluorescence measured, which is directly proportional to the amount of cells that took up doxorubicin.

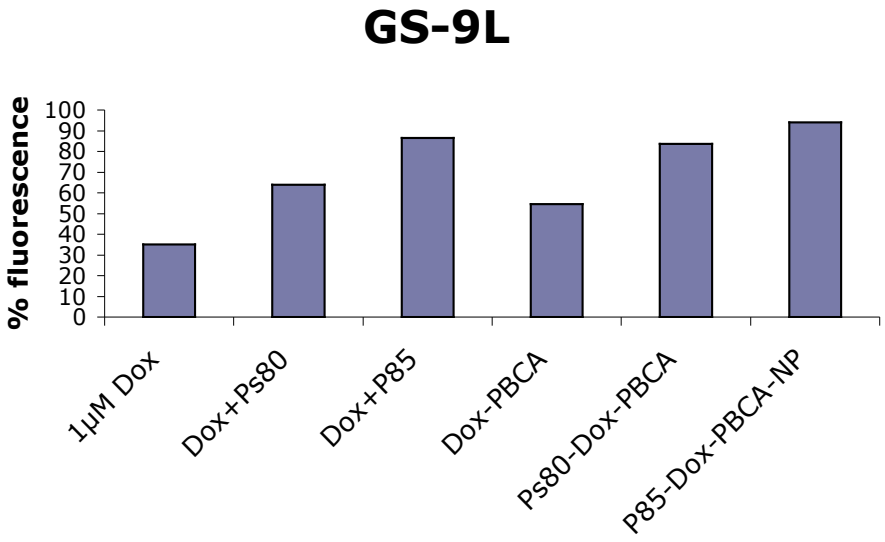

Figure 13. Fluorescence signal of doxorubicin at 590 nm (λ_{exp} = 480 nm) after incubation with the cells for 4 h.

As can be seen, the maximal fluorescence signal came from the formulations containing the poloxamer 185, especially in the case of P185-Dox-PBCA-NP where the signal is the highest. The formulations containing polysorbate 80 as well showed a significant fluorescence signal in comparison to doxorubicin in solution and uncoated Dox-PBCA-NP. Accordingly, an improvement of the uptake of doxorubicin by the cells was observed after incubation with both polysorbate 80 and poloxamer 185, poloxamer 185 showing a greater improvement. It has to be mentioned that the doxorubicin bound to uncoated nanoparticles showed a better internalization that free doxorubicin.

Therefore, the three nanoparticle formulations yielded a better cell uptake than the simple solutions of doxorubicin, either alone or in solution with a surfactant in the GS-9L cell line.

Thus, the different drug resistances showed by the three cell lines were responsible for a variable entry of doxorubicin into the cells. It was demonstrated that this resistance was higher in cells with higher P-gp expressions (see above results). According to this, the inhibitory effect of poloxamer 185 on the P-gp activity is visible on the GS9L P-gp expressing cell line, enhancing the accumulation of doxorubicin into the cell.

4.4.1.2 CLSM Analysis

Subsequently, the accumulation of doxorubicin into the cells was visualized by CLSM assay after 6 h incubation. The same doxorubicin formulations as in the FACS experiment were used: doxorubicin solution, doxorubicin+1% P185 solution, doxorubicin+1%Ps80 solution, Dox-PBCA nanoparticles, P185-Dox-PBCA nanoparticles, and Ps80-Dox-PBCA nanoparticles.

In the Figure 14 the confocal microscope show the effect of poloxamer 185 and polysorbate 80 on the uptake and localization of doxorubicin for the GS-9L cell line.

Differences in accumulation of doxorubicin into the cells were clearly observed with respect the different formulation employed. The microphotographs of the GS-9L glioma cell line show that doxorubicin in solution scarcely internalizes into the cells, showing the lowest uptake of the drug after 6 h incubation. However, after incubation with Dox-PBCA nanoparticles, the cells did take up doxorubicin better than doxorubicin in solution. The presence of polysorbate 80 in the formulations also showed an improved internalization of doxorubicin but to fewer degrees than in the presence of poloxamer 185. Therefore, in the presence of poloxamer 185, either as a solution or as a coating of the nanoparticle surface the uptake of doxorubicin was clearly increased in comparison to the rest of all of the formulations.

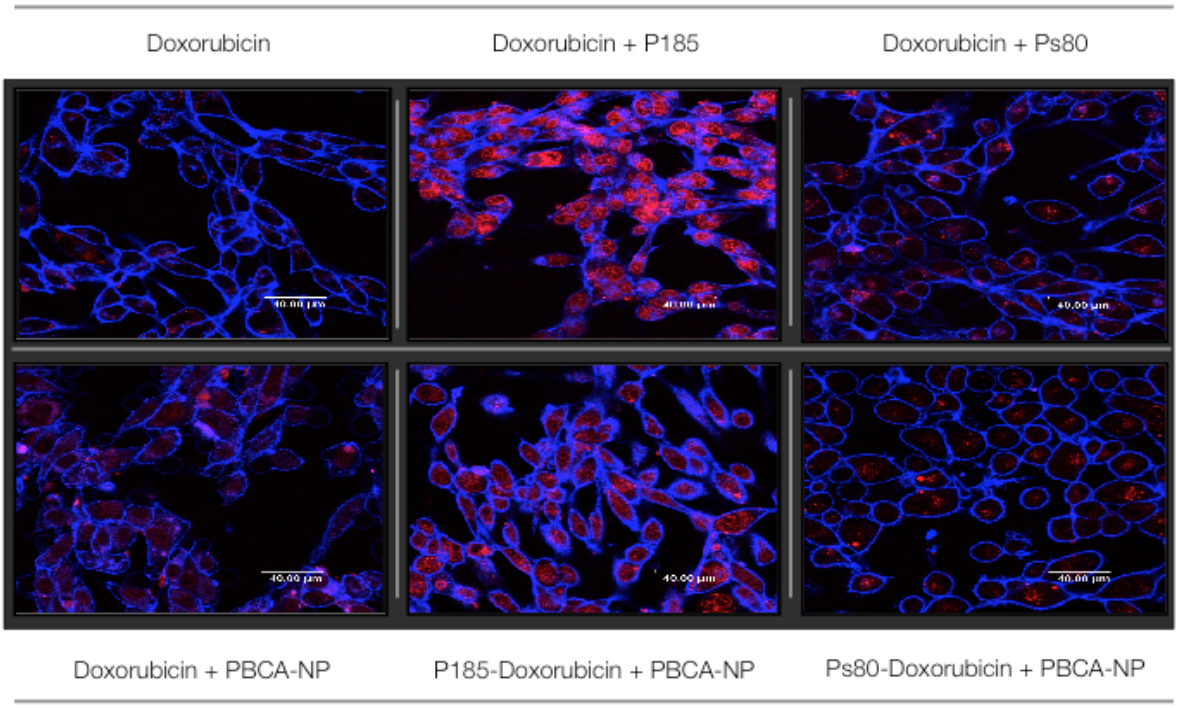

Figure 14. Intracellular localization of doxorubicin in GS-9L cell line by confocal microscopy. Cells were exposed to various Dox-PBCA-NP and doxorubicin formulations for 6 h and examined by fluorescent confocal microscopy.

The intracellular localization on doxorubicin was also markedly different with the employed formulations. Poloxamer 185 allowed the accumulation of doxorubicin in the nucleus with both, doxorubicin solution or bound to nanoparticles (Fig. 14). In the case of Ps80-Dox-PBCA-NP the doxorubicin was localized in certain areas of the cytoplasm, whereas after incubation with uncoated Dox-PBCA nanoparticles there is a diffused localization of doxorubicin.

4.4.2 Uptake of doxorubicin into RG-2 cell line

4.4.2.1 FACS Analysis

The same FACS experiments were carried out with the RG-2 cell line. As Western blot analysis demonstrated, we need to take into consideration that the P-gp expression in this cell line was very low. In the next figure, the intracellular accumulation of doxorubicin after 4 h for RG-2 cell line is shown.

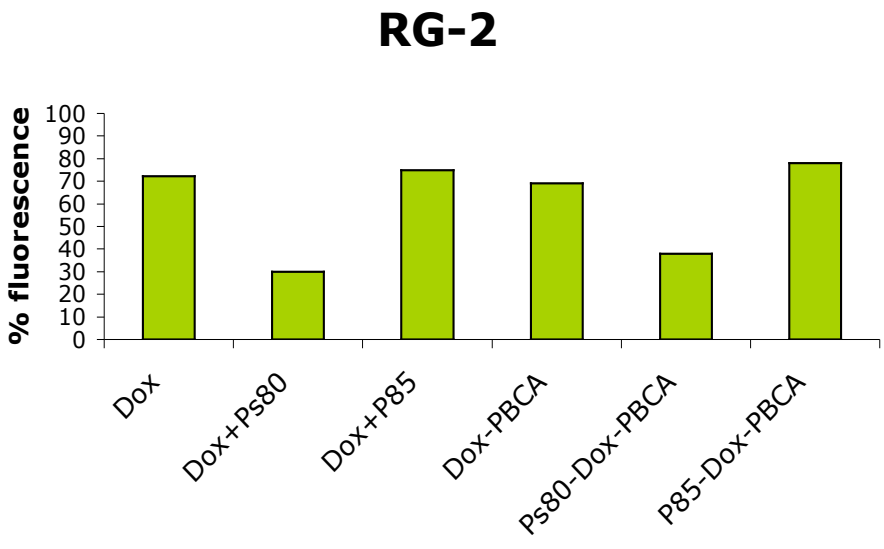

Figure 15. Fluorescence signal of anthracyclines at 590 nm (λ_{exp} = 480 nm) after incubation with the cells for 4 h.

Contrary to that what was seen in the GS-9L cell line, the RG-2 cell line showed a high

fluorescence signal after incubation with the doxorubicin in solution, which was almost comparable to the signal produced by Dox-PBCA-NP and the formulations containing poloxamer 185. FACS analysis also showed that there was no significant difference between the uncoated Dox-PBCA-NP and the P185-Dox-PBCA-NP. Dox+Ps80 and Ps80-Dox-PBCA-NP showed the weakest signals with a slight increase of the fluorescence for the Ps80-Dox-PBCA-NP.

4.4.2.2 Confocal microscopy analysis

In the figure 16, the intracellular accumulation of doxorubicin after 6 h for RG-2 cell line is shown. As seen in the confocal microscopy images, an effect of poloxamer 185 on the RG-2 cell line was only slightly detectable because a high internalization was also detectable with doxorubicin alone. The improvement of the uptake of doxorubicin into the cells using uncoated Dox-PBCA-NP and P185-Dox-PBCA-NP was very similar, as also shown by FACS analysis. Doxorubicin in solution, as well as doxorubicin containing poloxamer 185, crosses the membrane successfully. On the other hand, the nanoparticles coated with polysorbate 80 and doxorubicin+Ps80 do not present a large improvement. Nevertheless, the Ps80-Dox-PBCA-NP facilitates the uptake of doxorubicin compared to the uptake of doxorubicin after incubation with doxorubicin+Ps80. In addition, the Dox-PBCA-NP formulation seemed to yield a higher uptake of doxorubicin than doxorubicin alone or doxorubicin+Ps80. The intracellular localization of doxorubicin lead to accumulation in the nucleus poloxamer 185 treatment, whereas Ps80-Dox-PBCA-NP showed marked perinuclear localization of doxorubicin.

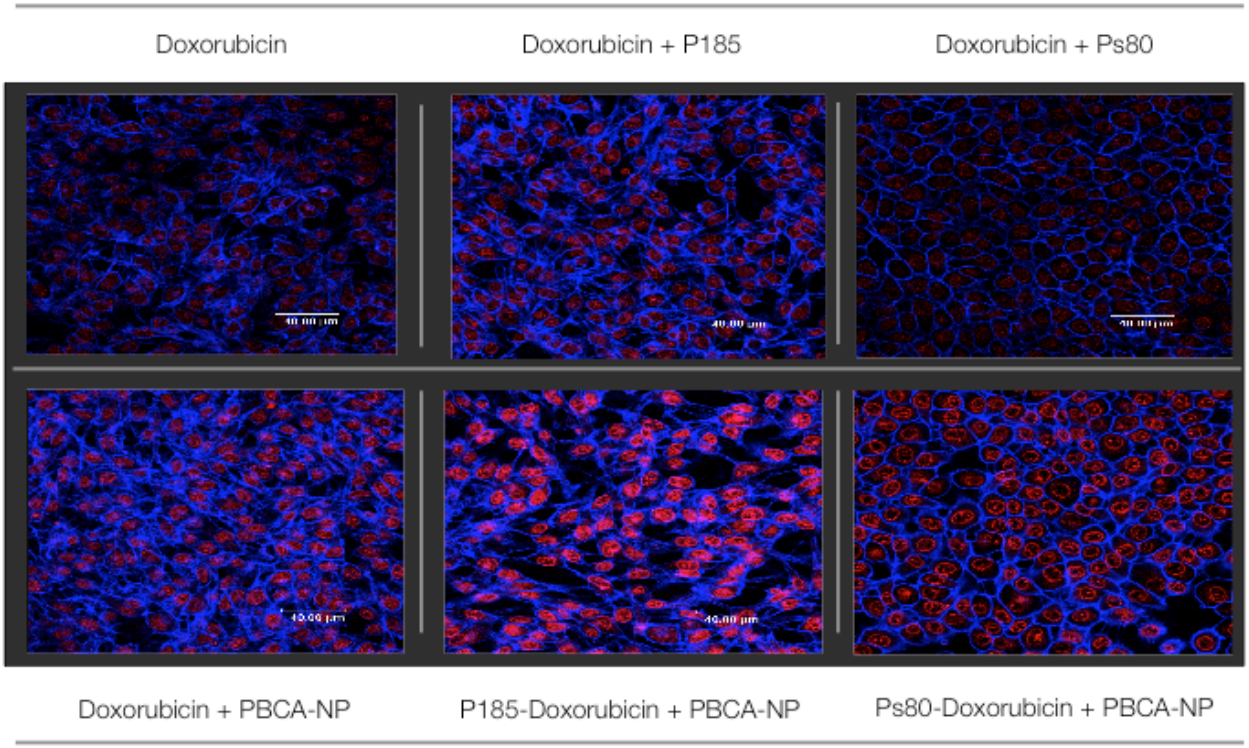

Figure 16. Intracellular localization of doxorubicin in RG-2 cell line by confocal microscopy. Cells were exposed to various Dox-PBCA-NP and Doxorubicin formulations for 6 h and examined by fluorescent confocal microscopy.

With this cell line, an enhancement in the uptake of doxorubicin by the use of PBCA-NP was not observed. Moreover, the poloxamer 185 effect in the RG-2 cell line was not as pronounced as in the GS-9L cell line, where the amount of doxorubicin taken up was significantly lower than that of the poloxamer 185 formulation, almost by 50 %. Polysobarte 80 had a stronger effect on the RG-2 cell line than on the GS-9L cel line.

4.4.3 Uptake of doxorubicin into the F-98 cell line

4.4.3.1 FACS Analysis

The FACS data for F-98 cell line are depicted in Figure 17. There were not many differences in the accumulation of doxorubicin between the several formulations and poloxamer 185. No significant increase of the intracellular doxorubicin was observed in any formulations.

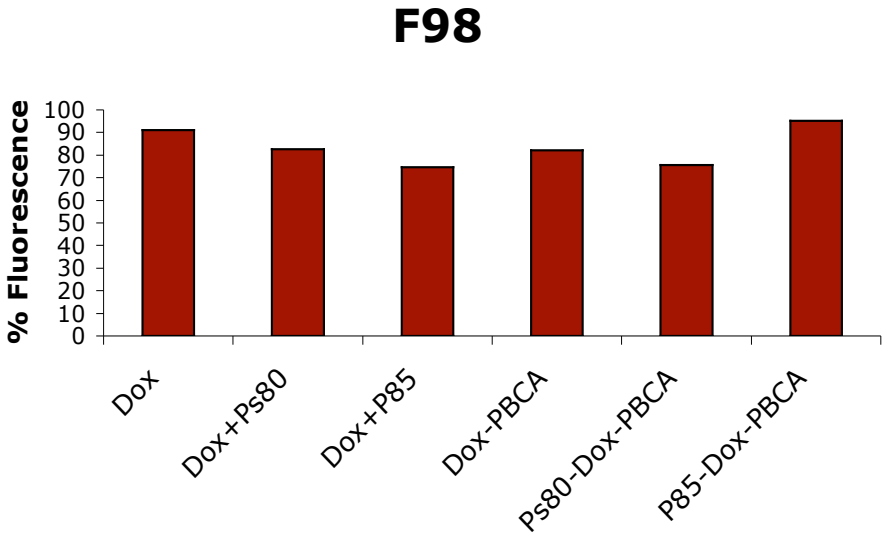

Figure 17. Fluorescence signal of doxorubicin at 590 nm (λ_{exp} = 480 nm) after incubation with the cells for 4 h.

Therefore, it can be concluded that, as with RG-2 cell line, in the F-98 cell line doxorubicin alone could cross the membrane very efficiently as FACS analysis shows. Moreover, PBCA-NP did not show a significant enhancement of the doxorubicin transport and poloxamer 185 showed a poor effect on the improvement of the accumulation of doxorubicin into the cells. No significant difference was seen in the presence or in the absence of poloxamer 185. One reason for this is that the P-gp expression in the F-98 cell line was very low. The effect of this was that the doxorubicin was not subjected to P-gp-mediated transport due to the lack of P-gp on the surface of the cells. Only in the case of nanoparticles coated with poloxamer 185 showed the highest signal.

4.4.3.2 Confocal microscopy analysis

In the case of F-98 cell line, after 6 h incubation with the different formulations, the internalization of doxorubicin in the cells increased for all formulations, as shown in the Figure 18. The accumulation of doxorubicin into the cells appeared to be much lower, almost insignificant with the Ps80-Dox-PBCA-NP and with doxorubicin+Ps80 as compared to the rest of the formulations.

The rest of the formulations showed an important accumulation of the drug independently of the presence of either poloxamer 185 or PBCA-NP. The F-98 cells took up doxorubicin to a high extent even after incubation with the doxorubicin in solution alone. These results were very similar to those with the RG-2 cell line.

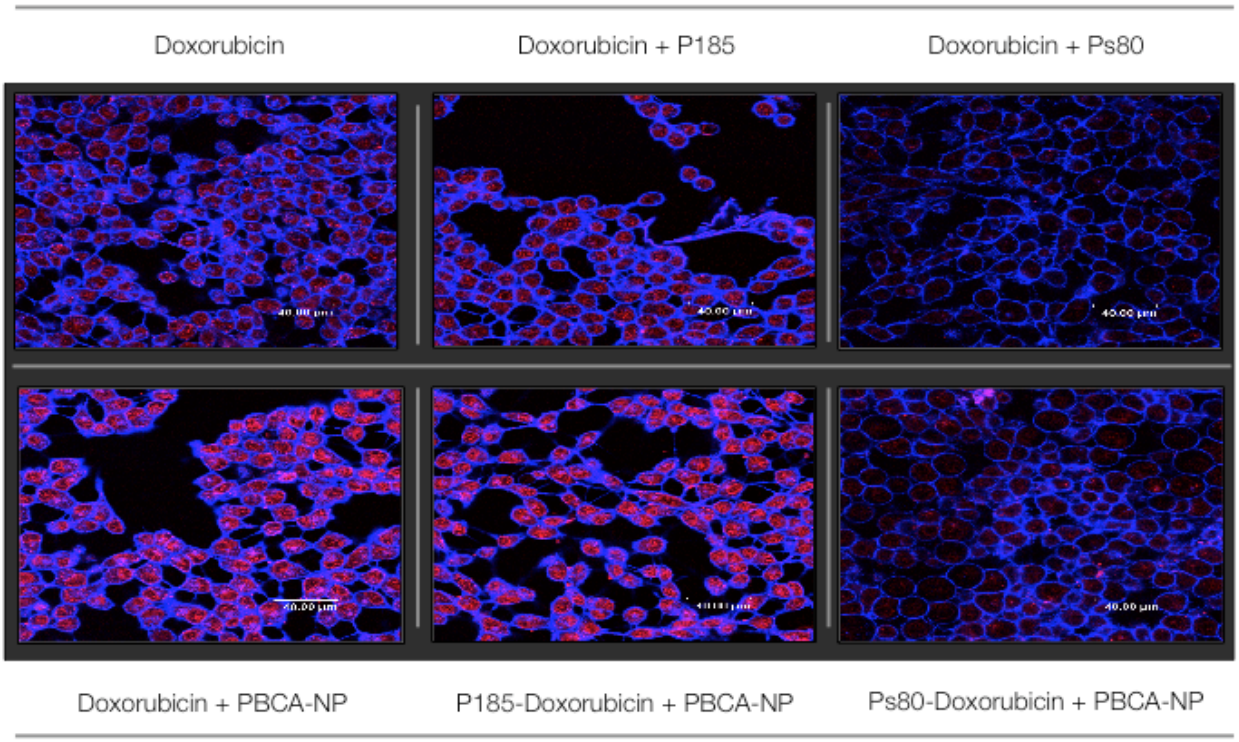

Figure 18. Intracellular localization of doxorubicin in F-98 cell line by confocal microscopy. Cells were exposed to various Dox-PBCA-NP and doxorubicin formulations for 6 h and examined by fluorescent confocal microscopy.

The confocal microscopy analysis shows similar results to those acquired by FACS analysis. Only one discrepancy to the FACS results was found. The decreased uptake of doxorubicin after incubation with polysorbate 80 formulations shown by CLSM was not apparent by the FACS analysis.

4.4.4 Uptake of doxorubicin into Caco-2 cell line

The Caco-2 cell line is frequently used as a model of the human intestinal mucosa representing a biological barrier model and it is a useful *in vitro* system for studying the P-gp function (87). Thus, this cell line also was employed in the present study because of its high expression of the P-gp protein.

4.4.4.1 FACS Analysis

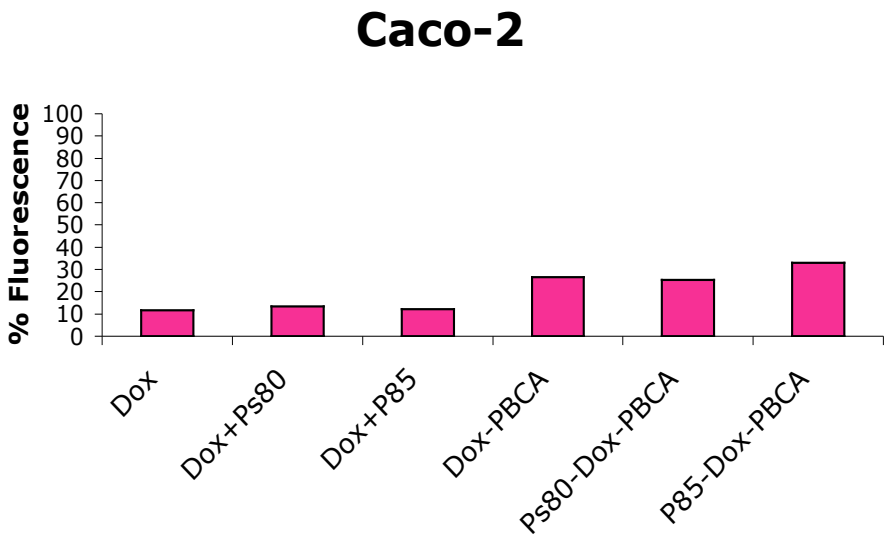

Figure 19. Fluorescence signal of doxorubicin at 590 nm (λ_{exp} = 480 nm) after incubation with the cells for 4 h.

The figure 19 shows the cellular fluorescence after treatment with the different doxorubicin formulations. Unexpectedly, the effect of poloxamer 185 to improve the intracellular accumulation of doxorubicin was negligible. Doxorubicin, with either P185 or Ps80 did not enhance its cell accumulation. A significant increase was observed after binding to nanoparticles and the P185-Dox-PBCA-NP yielded the highest uptake.

4.4.4.2 Confocal microscopy analysis

The Caco-2 cells were split in an 8-chamber slide and incubated for 2-3 days allowing them to grow to a confluent monolayer. After a minimum confluence of 80 % was reached the experiment was started.

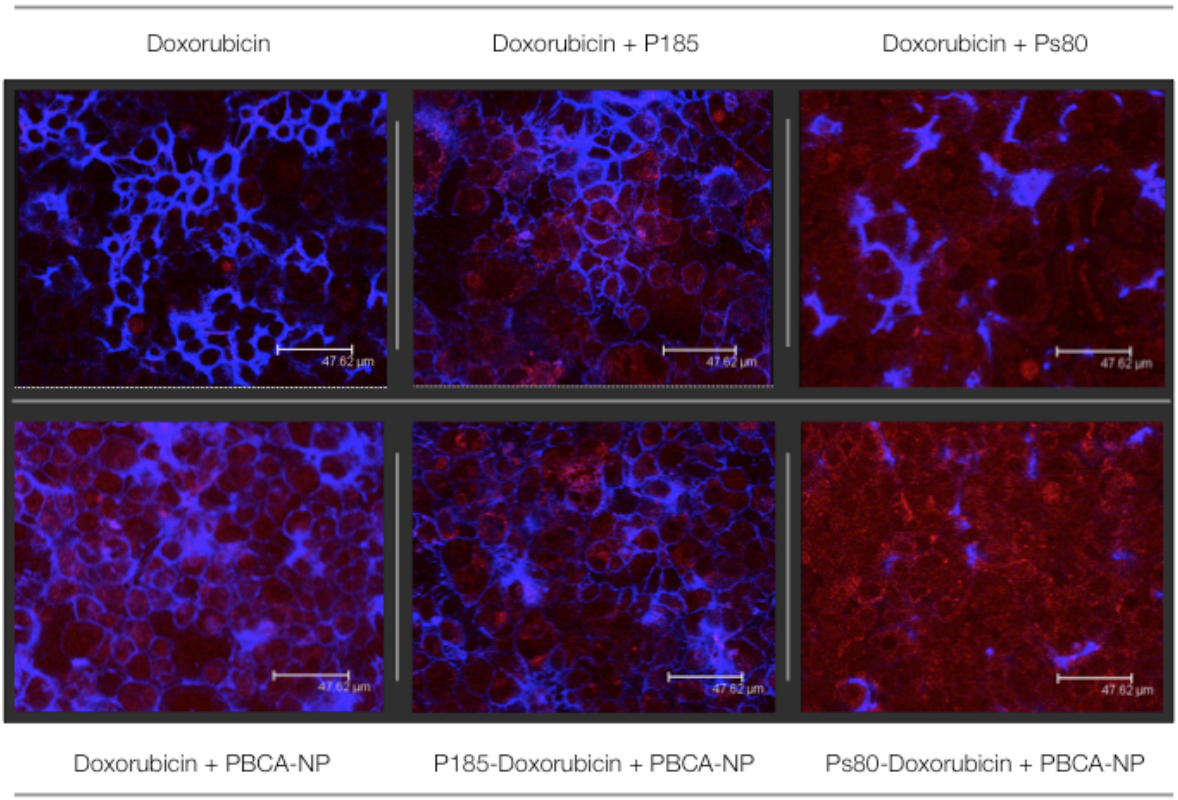

Figure 20. Intracellular localization of doxorubicin in Caco-2 cell line analysed by confocal microscopy. The cells were exposed to various Dox-PBCA-NP and doxorubicin formulations for 6 h and examined by fluorescence confocal microscopy.

The intracellular accumulation of doxorubicin in the Caco-2 was increased in the presence of PBCA nanoparticles formulations, which was even more pronounced in the presence of poloxamer 185 and polysorbate 80. However, it has to be mentioned that in the case of doxorubicin+Ps80 and Ps80-PBCA-NP the dye of the cellular membrane was not clearly visible, therefore the exact localization of doxorubicin cannot be assured. As expected, doxorubicin in solution led to the lowest accumulation in the Caco-2 cells. Only poloxamer 185 and polysorbate 80 yielded a slight accumulation of doxorubicin in solution.

In the following figure the different uptake of doxorubicin in the four cell lines is represented.

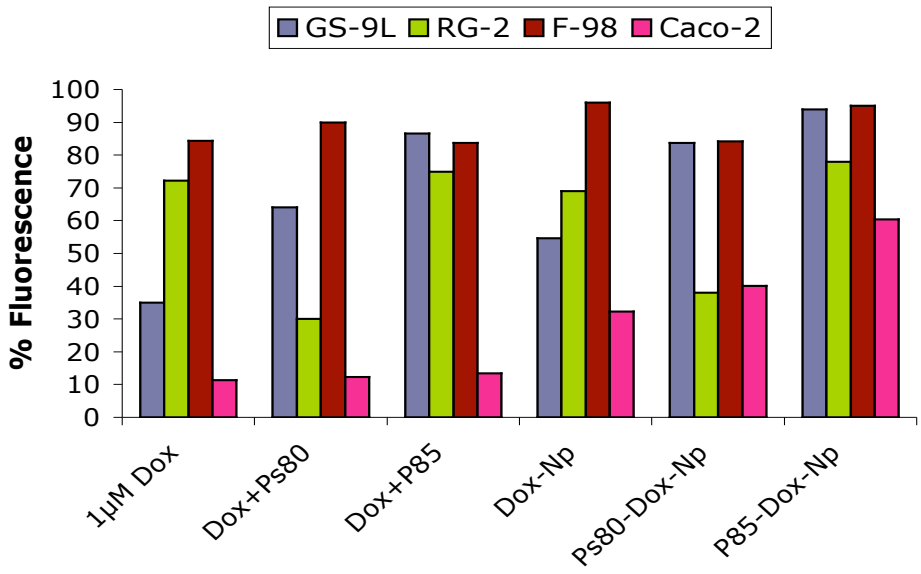

Figure 21. Cellular fluorescence after the treatment of doxorubicin formulations in the GS-9L, Caco-2, F-98 and RG-2 cell lines

The Caco-2 and the resistant GS-9L cells showed the lowest uptake of doxorubicin, which was increased only when polysorbate 80 or poloxamer 185 were present, being more efficient poloxamer 185 than polysorbate 80. In both cell lines, the nanoparticles enhanced the doxorubicin internalization. In the case of the doxorubicin sensitive cell lines, F-98 and RG-2, the F-98 cells showed higher accumulation of doxorubicin than RG-2 cells, which was largely independent on the formulation used. However, the presence of polysorbate 80 in the RG-2 cells led to a reduced intracellular accumulation of doxorubicin, whereas poloxamer 185 did not alter or improve the doxorubicin uptake by the cells in comparison to doxorubicin alone.

In many cases the presence of poloxamer 185 also clearly provoke a nuclear accumulation of doxorubicin, whereas after treatment with polysorbate 80 formulations, the doxorubicin is accumulated in certain locations in the cytoplasms.

4.5 Study of the effect of poloxamer 185 and polysorbate 80 on the integrity of the cellular membrane

In the literature poloxamer 185 and polysorbate 80 surfactants are both well-described as P-gp inhibitors (8, 9, 65, 79, 80). However, in our previous experiments, it was observed that the uptake of doxorubicin was increased in the presence of poloxamer 185 in cells with higher P-gp expression level whereas polysorbate 80 even led to a reduction in the intracellular accumulation of doxorubicin in doxorubicin sensitive-cell lines RG-2 and F-98.

The non-ionic surfactant polysorbate 80 has been shown to increase the permeability of certain transport markers in Caco-2 cells as well as improving the permeation of proteins susceptible to P-gp-mediated efflux (66, 67). Yamazaki et al. (68) demonstrated that polysorbate 80 enhanced the cellular accumulation of an epipodophyllotoxin derivate susceptible to P-gp-mediated efflux, and this was attributed to an increased influx of the drug rather than an inhibition of P-gp-mediated efflux. Poloxamer (Pluronics®) copolymers have also been shown to enhance the diffusion of compounds such as doxorubicin across model lipid bilayers (69). These investigations suggest that surfactant interactions with membranes leading to increased membrane permeability may play an important role in enhancing the transmembrane diffusion of a P-gp substrate and its intracellular accumulation, independently of the inhibitory effects of these surfactants on P-gp. However in the previous FACS data and confocal microscopy images shown, the accumulation of doxorubicin was enhanced in almost all cases due to the presence of poloxamer 185 and not to the same extend by polysorbate 80. Given that both surfactants are P-gp inhibitors, this means that the P-gp inhibition activity of both surfactants differs with the different properties of poloxamer 185 and polysorbate 80 for the uptake of doxorubicin in the P-gp expressing cell lines. Therefore, the different mechanism by which both surfactants inhibit P-gp activity could be explained by different effects of poloxamer 185 and polysorbate 80 on the uptake of doxorubicin by the cells.

Consequently, we focused on the effects of both surfactants on the permeability of the cellular membrane of the GS9L cell lines possessing the highest P-gp expression. The effects of both surfactants on cell permeability were assessed after incubation with poloxamer 185 and polysorbate 80. For this experiment, the cells was exposed to different concentrations of both

poloxamer 185 and polysorbate for 24h, then the membrane was stained and analysed by confocal microscopy (see Figure 22).

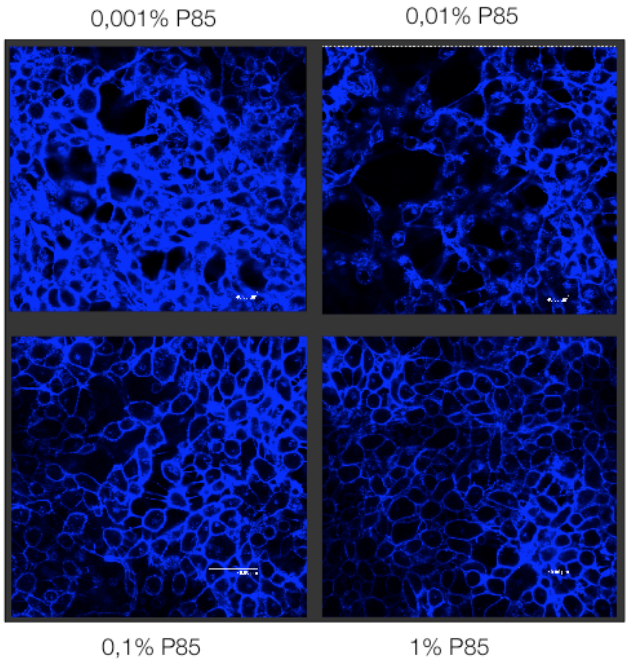

Figure 22. Integrity of membrane cellular of GS-9L cell line after incubation for 24 h with different concentrations of poloxamer 185.

The GS-9L cells were incubated with several concentrations of poloxamer 185 ranging from 0.001 % to 1 %. After 24 h of incubation, the surfactant solutions were removed. The cells were washed, the cellular membrane stained, and the cells were observed under the confocal microscopy.

The same procedure was carried out for polysorbate 80. The GS9L cells were incubated with several concentrations of polysorbate 80, ranging from 0.001 % to 1 % surfactant solution. After 24 h of incubation, the surfactant solutions were removed. The cells were washed and the cellular membrane stained, which was observed under the confocal microscopy.
In Figure 22 and 23 the Cy5-dyed cellular membranes of the GS-9L cell line are shown. The Figure 22 figure shows to the cellular membrane after incubation with poloxamer 185 solutions. It was clearly observed that no significant change of the membrane occurred in the range of concentrations used.

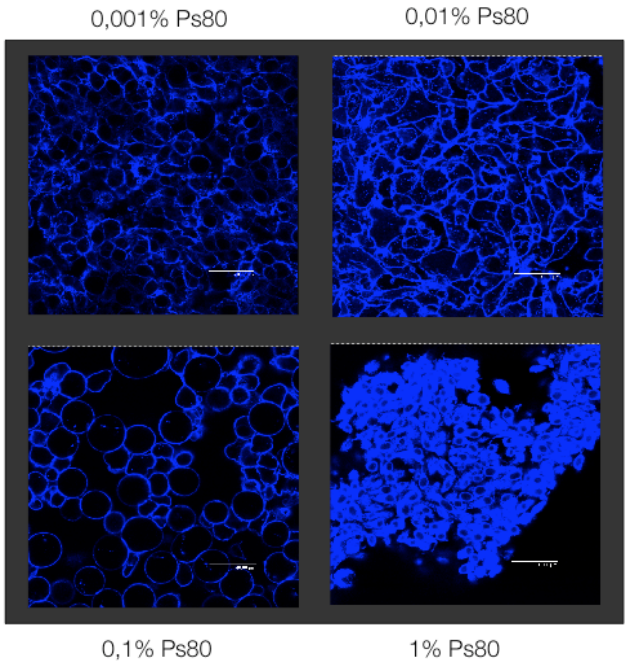

Figure 23. Integrity of membrane cellular of GS-9L cell line after incubation for 24 h with different concentrations of polysorbate 80.

As shown the Figure 23, some modification of the membrane was detected at 0.01 % concentration of polysorbate 80. At up to 0.1 % concentration changes of the cellular form were perfectly evident because of loss of the cellular membrane integrity. The cells swelled changing into a spheric form, and at 1 % concentration the cells integrity totally vanished, showing important toxic effects on the cells.

It was therefore concluded that the poloxamer 185 did not display any effect on the cellular membrane or any toxic effect on the cells at the range of concentration tested. However, polysorbate 80 showed rather significant changes in the membrane in the same range of concentrations, and even toxic effects at higher concentrations.

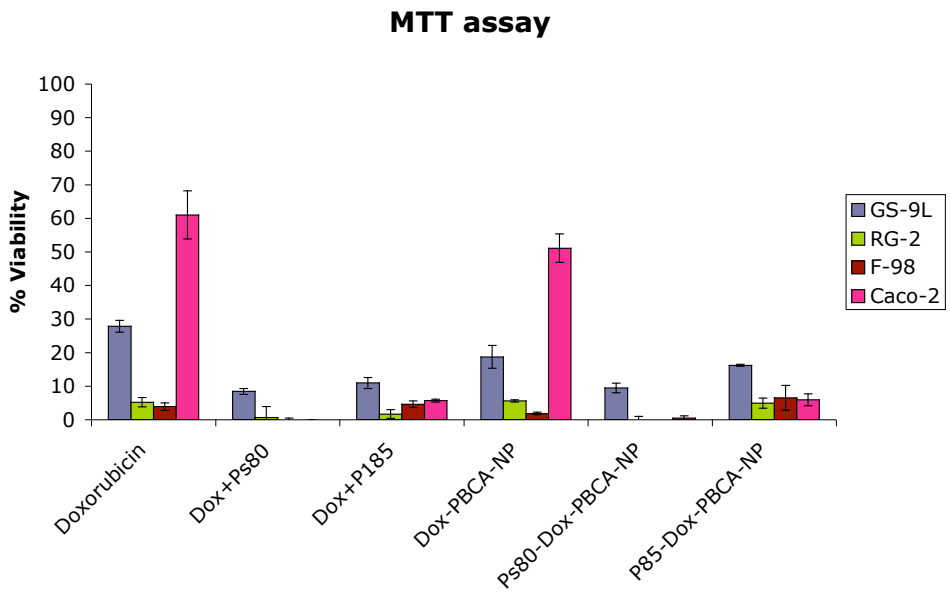

Figure 24. Viability of GS-9L, RG-2, F-98, and Caco-2 cell lines after incubation with different doxorubicin formulations for 24 h.

When it was noticed that poloxamer 185 considerably improved the uptake of doxorubicin the question arose if poloxamer 185 also was able to enhance the activity of doxorubicin. The advantage of a high intracellular accumulation of doxorubicin would be an increase in its activity as a cytotoxic drug. To prove this, the viability of the resistant-GS-9L cell line and the doxorubicin sensitive-RG-2 and F-98 cell lines was measured after incubation with poloxamer 185 doxorubicin and polysorbate 80 doxorubicin formulations as well as a doxorubicin solution. The Caco-2 cell line was used as control cell line. The MTT assay was employed for this analysis. The percentage of viable cells observed after 24 h incubation with the different samples is represented in Figure 24. The value 100 % corresponds to untreated cells.

The doxorubicin in solution and Dox-PBCA-NP showed a high efficacy against the RG-2 and F-98 cells and a very low efficacy against the Caco-2 cells and GS-9L showing high viability values after the treatment with both formulations. However, a considerable decrease in the viability by the presence of poloxamer 185 and polysorbate 80 occured in both cell lines but to a lesser degree in the GS-9L cells. This effect is attributable to the high P-gp expression that both cell lines present on the surface.

Unexpectedly, the lowest values of viability were found after incubation with polysorbate 80 formulations. UnexpectIdly because the low uptake of doxorubicin in the presence of polysorbate 80 shown by CLSM and FACS analysis contradicts the low values of viability with the identical polysorbate formulations. Moreover, as the viability values indicate the polysorbate formulations seem to be more effective than the poloxamer 185 formulations, which again contradict the high entry of doxorubicin in the presence of poloxamer 185 in the formulations (see above). The apparent effectivity of polysorbate on the viability of the cells was observable in all cell lines tested. According the MTT assay, all four cell lines also showed lower viability in presence of polysorbate formulations than in the presence of poloxamer 185 formulations.

4.6 Effects of poloxamer 185 and polysorbate 80 on the Rhodamine 123 accumulation

Using Rhodamine 123 (Rho-123), a P-gp substrate, Batrakova et al. demonstrated that Pluronic® block copolymers could enhance Caco-2 cell accumulation of Rho-123 at concentrations below the CMC of the poloxamers (9). In contrast, at concentrations above CMC, the poloxamers were found to cause an increased efflux of Rho-123 from Caco-2 cells (9). Miller et al. (8) were first to report the concentration-dependent effects of poloxamer 185 on Rhodamine 123 accumulation in BBMCE monolayers.

Therefore, our interest was to analyse this concentration-dependent effect of poloxamer 185 and polysorbate 80 on the accumulation of Rho-123 in GS-9L, RG-2 and F-98 glioma cell lines. Rho-123 is fluorescent and therefore its cell accumulation also can be measured by flow cytometer. The concentration-dependent effect of poloxamer 185 and polysorbate 80 also was compared to the effect of verapamil, a known P-gp substrate.

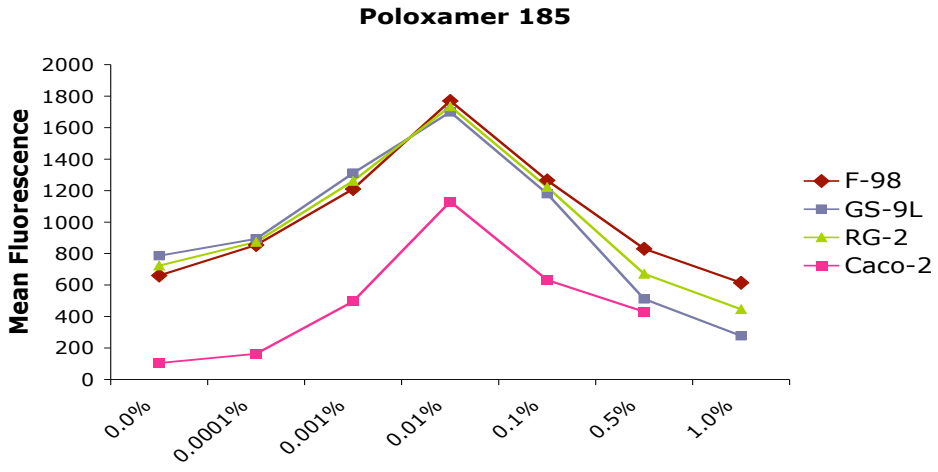

Figure 25. Rho-123 uptake in GS-9L, F-98, RG-2, and Caco-2 cell lines by FACS analysis. The effect of poloxamer 185 on the Rho-123 uptake was measured at different concentrations.

The concentration-dependent effect of the different poloxamer 185 concentrations on Rho-123 cellular accumulation is represented in Fig. 25. To estimate the ability of poloxamer to inhibit the Pgp efflux transport in the cells different poloxamer 185 solutions were produced. As seen in the figure, one pattern observed in all cell lines is that the Rho-123 cellular accumulation reaches maximal levels at or near the CMC and decreases at concentrations above CMC.

Indeed, the increases in the Rho-123 uptake at low concentrations of poloxamer 185 from 0.0001 % to 0.01 %, coincide with the inhibition of the P-gp efflux in the BBMEC monolayers in Batrakova´s experiments (25). A maximal Rho-123 accumulation was observed at 0.01 % poloxamer 185, which is below the CMC (= 0.03 %) of this polymer. This result is consistent with the earlier reports suggesting that the unimers of poloxamer block copolymers are responsible for the inhibition of P-gp in the cells (32, 21, 22). At higher concentrations of poloxamer 185 (0.1 % - 1 %), the Rho-123 levels decrease. This effect of high poloxamer 185 concentrations is believed to be due to incorporation of the Rho-123 in the poloxamer micelles resulting in the decrease in the amounts of the free Rho-123 available for diffusion into the cells (21). The four cell lines in the present study were exposed to a maximal uptake of Rho-123 at 0.01 % poloxamer 185. However, Caco-2 cell lines showed the lowest levels in the uptake of Rho-123 since this cell line displays the highest P-gp expression. Surprisingly, the GS-9L cell line, the next in the range of higher P-gp expression, showed a comparable Rho-123 uptake to RG-2 and F-98 cell lines.

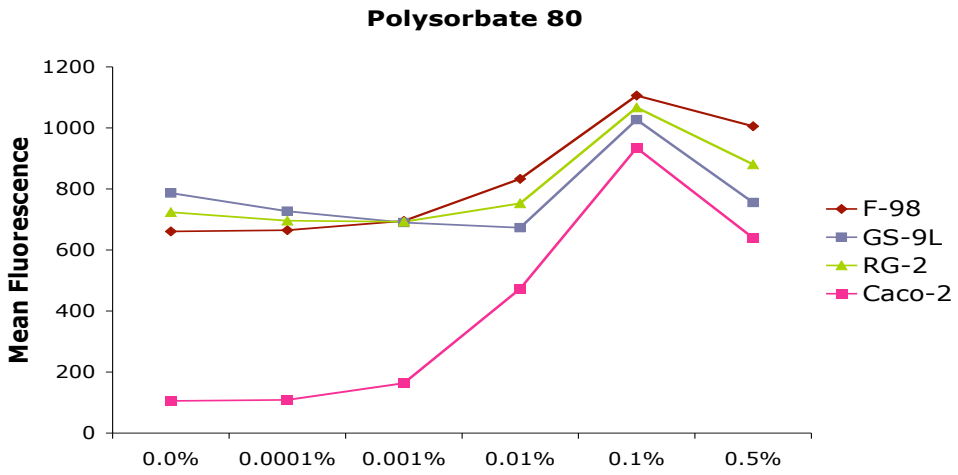

Figure 26. Rho-123 uptake in GS-9L, F-98, RG-2, and Caco-2 cell lines by FACS analysis. The effect of polysorbate 80 on the Rho-123 uptake was measured at different concentrations

In the case of polysorbate (Figure 26), the accumulation of Rho-123 also increased in a concentration-dependent manner. However, this effect was not seen at low concentrations the Rho-123 in contrast to poloxamer 185. Additionally, the uptake of Rho-123 after polysorbate 80 was considerable lower than with poloxamer 185. Above a polysorbate concentration of about 0.001 % (0.01 % in the case of the GS-9L cell line) a significant fluorescence increase occurred peaking at 0.1 % in all cell lines. Above this concentration the uptake of Rho-123 started to diminish.

The three glioma cell lines achieved similar Rho-123 concentrations whereas the Caco-2 cell line again showed the lowest values of fluorescence, as with poloxamer 185.

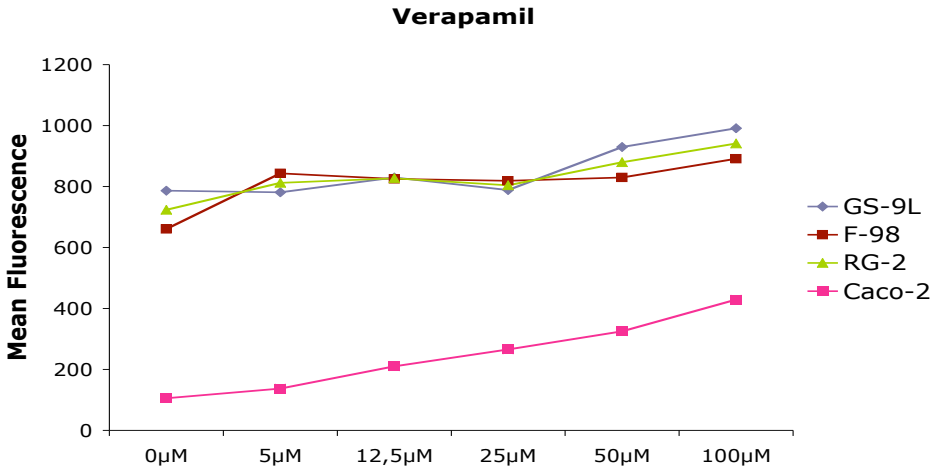

Figure 27. Rho-123 uptake in GS-9L, F-98, RG-2, and Caco-2 cell lines by FACS analysis. The effect of Verapamil on the Rho-123 uptake was measured at different concentrations

No concentration-dependent effect was visible with Verapamil. The three glioma cell lines again yielded the highest mean fluorescence values, whereas Caco-2 cell line showed the lowest Rho-123 uptake.

Unexpectedly, the accumulation of Rho-123 again was not significantly different between the resistant GS-9L cell line and the two sensitive cell lines, RG-2 and F-98, as in the case of doxorubicin.

5 Discussion

The potential of poly(butyl cyanoacrylate) nanoparticles to be used as a doxorubicin carrier was evaluated in an *in vitro* cytotoxic study, by comparing the cytotoxicity of doxorubicin in solution and doxorubicin-loaded poly(butyl cyanoacrylate) nanoparticles (Dox-PBCA-NP). For this purpose, the GS-9L, F-98 and RG-2 rat glioma cell lines were employed as glioma models.

5.1 Cytotoxicity study of doxorubicin and doxorubicin formulations.

At first, the growth inhibitory effect of doxorubicin alone was tested in the different glioma cell lines at doxorubicin concentrations ranging from 0.1 µg/ml to 5 µg/ml. A concentration-dependent decrease in the viability of the three cell lines was clearly observable already at lower concentrations of doxorubicin. However, the IC_{50} of the three cell lines, GS-9L, RG-2, and F98, was different in the three assays, MTT, LDH, and ATP, which measure different biochemical parameters as indicators for cell death (see below).
On the other hand, the IC_{50} values showed a concordance between the three assays, indicating clearly that the GS-9L cells display the highest resistance towards free doxorubicin, visible in all three assays. RG-2 exhibited the lowest resistance in the LDH assay, whereas the F-98 showed the lowest resistance in the ATP assay. No significant difference between RG-2 and F-98 appeared in the MTT test.

As described as in the Results section, the cytotoxic effect of the poly(butyl cyanoacrylate) polymer was assessed by incubating the cell lines with different concentrations of unloaded PBCA-NP. The maximal concentration of polymer used, 15 µg/ml, did not induce any significant cytotoxic effects in all three different assays (MTT, LDH, and ATP). Therefore, it was assured that this concentration of unloaded nanoparticles, which was not exceeded in the following experiments did not exert any toxic effect on the cell lines tested. Thus, the decrease of the viability showed in the following treatment of the cells with Dox-PBCA-NP was only due to the cytotoxic activity of doxorubicin adsorbed onto the nanoparticles, indicating a high efficacy of the loaded nanoparticles.

The cytotoxicity of doxorubicin compared to doxorubicin adsorbed on PBCA-NP was analyzed only using the MTT, and LDH assays because the ATP assay was not available any more.

Comparing the results between the uncoated and surfactant-coated Dox-PBCA-NP and doxorubicin solution, in general at equivalent concentrations of doxorubicin a higher decrease in viability of the cells was observed after incubation with nanoparticle-bound doxorubicin than after incubation with doxorubicin in solution, demonstrating an enhanced efficacy against the cancer cells enabled by the nanoparticles. A pronounced decrease of the viability clearly occurred also with the nanoparticles at lower concentrations of doxorubicin. However, as previously indicated, the coating of the nanoparticles with the surfactants rendered the nanoparticle surface more hydrophilic, which in turn obstructed their interaction with the cellular membranes. This effect is comparable to the so-called stealth effect: in *in vivo* experiments (97, 98) the capacity of poloxamine 908-coated nanoparticles and poloxamer 188-coated nanoparticles was already shown to prevent or retard the opsonization by macrophages because of the steric and the hydrophilic modification. As a result, the nanoparticles exhibited long-circulating properties. However they were still not able to increase the brain concentration of nanoparticles-bound drugs, because they were unable to overcome the efflux mechanism present in the membranes of the brain endothelial cells. On the other hand, further *in vivo* experiment demonstrated the ability of nanoparticles coated with polysorbate 80 to successfully overcome these mechanisms reaching the brain, which means that polysorbate 80 was able to interact with the cellular membrane. In our results, with the exception of polysorbate 80, no distinct difference in efficiency was observable between uncoated and coated Dox-PBCA-NP.
The influence of the different surfactant coatings of the unloaded PBCA-NP on the viability of the three cell lines was not relevant, except for the coating with polysorbate 80 in the RG-2 cells in the LDH assay. The different coatings of the Dox-PBCA-NP in general did not show any significant enhancement of the cytotoxicity of doxorubicin. Only some cases, the polysorbate 80-coated Dox-PBCA-NP exhibited a marked dose-dependent effect.

5.2 The use of viability assays to determine the cytotoxic effects

The results in Figure 9 and Figure 10 as well as those mentioned above with doxorubicin solution alone (Fig. 7) demonstrated that the viability of the three cell lines differed according to the assay used.

The sensitivity of these cytotoxicity assays used differed depending on the difference in mechanisms measured by these assays. Briefly, in the MTT assay, tetrazolium (MTT) salt is added to the media and upon internalizing into the cells is reduced to a blue product by the mitochondria dehydrogenase into the mitochondria. In the LDH assay, the amount of LDH a cytosplamic enzyme is determined, which is released after cellular membrane damage from the cytosol into the extracellular media. This enzyme is quantified by its property to catalyze the reduction of lactate to a formazan. In the ATP assays, the amount of the metabolic ATP contained in the cells is used to give the conversion from luciferin to oxiluciferin with the corresponding emittance of light, which corresponds to the amount of ATP, and therefore, to the number of viable cells.

Fischer D. et al., 2003 already carried out a comparative *in vitro* study where the cytotoxicity of different substances was analysed looking for diverse toxic effects on the cells. They searched for specific intracellular effects on the mitochondrial and metabolic activity (MTT assay) as well as for membrane damage by the release of lactate dehydrogenase (LDH assay), finding a correlation between the results obtained in both assays. In contrast, in our study, we looked for cellular survival. Therefore, the cells were subjected to the same conditions during the entire incubation procedure, following a standard protocol for the three assays to determine the cellular survival with regard to their mitochondrial activity, their LDH activity, and the amount of ATP in these cells. Consequently, it may be stated that the decrease of the viability given as a decrease of the mitochondrial functionality differed to the viability given as a decrease in the LDH activity or as an ATP decrease.

Weyermann J. et al. (2005) compared four cytotoxic detection assays in one cellular system using three agents, Triton X-100, chloroquine, and sodium azide, and they concluded that the sensitivity of the cytotoxic assay used differed depending on the different mechanism, which leads to cell death. They also found differences in the viability of the treated cells depending on the test agent. Correspondingly, in our study, the sensitivity of the cytotoxic assay used also differed depending on the different mechanisms. Only one cytotoxic agent, doxorubicin, was analysed in the three cytotoxic assays in three different cell lines, thereby the differences in the sensitivity could be additionally highlighted by the variability of the cell lines.

The different sensitivity of the different parameters measured by the three assays, the mitochondrial activity (MTT assay), the LDH activity (LDH assay) and the ATP content (ATP assay) of the surviving cells was responsible for the variability of the results in the cellular viability after the drug exposure between these assays.

5.3 Multidrug resistance in the used *in vitro* glioma model

The results of the present study also showed that the three glioma cell lines additionally responded differently to the doxorubicin action, presumably due to a different drug uptake into these cells. Previous extensive studies have identified mechanisms through which tumour cells escape the cytotoxic effects of a variety of chemotherapeutic drugs. The cellular resistance to chemotherapy is multifactorial and may be affected by the cell cycle stage, proliferation status, biochemical mechanisms (such as detoxification), cellular drug transport (influx, efflux, and retention), and DNA replication and repair mechanisms. The resistance to multiple drugs, referred to as multidrug resistance (MDR) is a major impediment to the successful treatment of various human cancers. MDR is cell resistance not to single substance but to numerous substances characterized by different mechanisms of action and by different chemical structures. In some types of tumours, multidrug resistance is inherited, while in others it is acquired.

In this study, we focused on the cellular multidrug resistance caused by a decrease of doxorubicin accumulation by the tumour cells. The mechanism of this drug efflux from the cells is mediated by the activity of transpoter proteins, i.e., P-glycoprotein (P-gp), transporter of the MRP family, and some others proteins.

Since the present cytotoxic study showed a variable resistance to doxorubicin in the tested glioma cell lines and since doxorubicin is a P-gp substrate, it was decided to analyze this resistance investigating the P-gp expression in these cell lines, GS-9L, RG-2, and F-98. Indeed, the P-gp expression of the three cell lines was different, showing the highest P-gp expression in the GS9L cells, and, correspondingly, a lower doxorubicin uptake and lower doxorubicin cytotoxicity in this cell line. Therefore, the variable degree at the protein expression level in the GS-9L, RG-2, and F-98 cell lines was responsible for the different resistance. With a higher P-gp expression, the uptake of doxorubicin into the cells was decreased as it was shown by FACS analysis, increasing the number of surviving cells after a doxorubicin exposure.

The analysis of the P-gp expression in the glioma cell lines, which corroborated the results obtained by the doxorubicin cytotoxicity analysis. A Western blot confirmed the high expression of the P-gp in the cellular membrane of the GS-9L cell line, corresponded with the high resistance towards the doxorubicin action by these cells. The weaker expression of P-gp in the RG-2 and F-98 cell lines corresponded to the high sensitivity that both cell lines exhibited towards treatment with doxorubicin. The P-gp-related sensitivity or insensitivity of the three cell lines was reflected in all three cytotoxicity assays the MTT, the LDH, and ATP assays coincided

in the different resistance displayed by the cell lines.

5.4 The use of poloxamer 185 and polysorbate 80 to overcome the multidrug resistance of glioma cell lines

One of the major failures in cancer therapy is the simultaneous cellular resistance to multiple drugs presented by the cell tumours. The mechanisms by which tumour cells become resistant to multiple chemotherapeutic drugs are still poorly understood. However the numerous strategies to overcome these resistance mechanisms resulted in encouraging results (6, 59, 86, 87). One of these strategies was the employment of various types of nanoparticles (59, 60).
As previously described, the expression of the P-glycoprotein on the cellular membrane of the glioma cell lines was responsible for the avoidance of the doxorubicin entry into the cells and therefore the blocking out of the drug action. The P-gp inhibitors block the P-gp-mediated tranport of the drug out of the cells and consequently, facilite its intracellular accumulation and consequently, increasing its efficacy.
A number of studies demonstrated a rather effective P-gp inhibition activity of the pluronic block copolymers surfactant (5, 6, 8, 9). However, one of the most outstanding known poloxamers as P-gp inhibitor is poloxamer 185. Poloxamer 185 posses the structural characteristics required for maximal impact on drug efflux transporter activity (89). Previous work reported that poloxamer 185 increases accumulation of the MRP (multidrug resistance related protein) substrates vincristine and doxorubicin (70).

Since in the above cytotoxicity study polysorbate 80 showed an improvement of the doxorubicin action and since it has been also described as a P-gp inhibitor, it was also used as a second P-gp inhibitor in addition and comparison to the poloxamer 185.
The treatment of the cells with poloxamer 185 and polysorbate 80 doxorubicin-formulations led to different results. The expected inhibition of the efflux of doxorubicin in many cases was clearly seen after treatment with poloxamer 185, which was especially significant in cell lines with a high P-gp-expression, such as GS-9L. In the cell lines with a low P-gp expression, such as RG-2 and F-98, the effect of poloxamer 185 on the transport of doxorubicin was slight to nil. This demonstrated that the presence of poloxamer 185 had an important effect on the transport of doxorubicin into the glioma cells with a significant P-gp expression, i.e. doxorubicin resistant cells. Poloxamer 185 not only enhanced the transport of doxorubicin solution into the cells but

also that of nanoparticles-bound drug, although to a lower degree.

On the other hand, the polysorbate 80 formulations influenced the doxorubicin internalization very little and in some cases even a lower transport than for the doxorubicin in solution was observed. This effect was observable in all the cell lines, although to a different degree. For instance, the RG-2 cell line seems to be affected lesser by the polysorbate 80 action than the F-98 cell lines in which the accumulation of doxorubicin was hardly perceptible after the treatment with polysorbate 80-doxorubicin formulations, as shown by CLSM.

As said above, the capacity of poloxamer 185 to increase the intracellular doxorubicin in P-gp-expressing cells was clearly shown in the GS-9L cell line. In contrast, in the low P-gp expressing cell lines, RG-2 and F-98, no significant difference in the transport of doxorubicin by the presence of poloxamer 185 was visible. Given that in these cell lines the doxorubicin did not find any resistance to its internalization due to the absence of a high P-gp expression on the membrane, doxorubicin enters the cell by passive diffusion. On the other hand, the resistance to doxorubicin internalization displayed by the GS-9L cells due to their P-gp expression was overcome by the effect of poloxamer 185, blocking the extrusion out the cells of the doxorubicin, and increasing its intracellular accumulation.

The different results obtained after the treatment with the two surfactants, poloxamer 185 and polysorbate 80, led us to look into possible mechanism that could lead to these differences.

In the literature there are differences in the surfactant action on the cellular membranes. Therefore the effects of poloxamer 185 and polysorbate 80 solutions on the cellular membrane were studied in the present investigation, using confocal microscopy. The cellular membranes of GS-9L cells were observed after treatment with different concentrations of poloxamer 185 and polysorbate 80 solutions. The changes in the membrane were rather visible in the case of the polysorbate 80, leading to stark toxic effects on the cells at a 1 % concentration. In contrast to poloxamer 185, no visible change was observable at the same concentrations and after the same incubation conditions.

For this reason, the influence of polysorbate 80 on doxorubicin uptake and accumulation may be a direct effect on the cellular membrane, leading to a loss of the integrity at higher concentrations and, therefore, to an uncontrolled transport in and out of the cells. That could explain the low intracellular internalization of doxorubicin after polysorbate 80 incubation in the cells.

In first studies, Batrakova et al (8, 22) concluded that the inhibition of P-gp by poloxamer 185 (Pluronic® P85) involves two effects: one, a decreasing interaction or affinity of P-gp with respect to both ATP and the drug molecules; second, a depletion of intracellular ATP (energy depletion) in the endothelial cells of the brain microvessel.

This fact assures that poloxamer 185 has a direct effect on the functionality of P-gp rather than on the cellular membrane, which may explain the intact membrane after the incubation with poloxamer 185 (Fig. 22).

The capacity of poloxamer 185 to increase the internalization of doxorubicin into the cells may be used to improve the doxorubicin action on the tumour cells, decreasing the viability of the tumour cells after poloxamer 185-doxorubicin formulation treatment. Hence, cell survival after treatment with poloxamer 185- and polysorbate 80-doxorubicin formulation was studied.
The analysis carried out in the glioma cell lines as well as in the Caco-2 cell line suggested that the formulations containing polysorbate decreased the viability of the all cell lines in a greater manner than the poloxamer 185 formulations. On the first glance, this is surprising because poloxamer 185 improved the doxorubicin internalization into the cells, thereby the activity of doxorubicin should be also improved. Moreover, the decrease of the viability after the treatment with polysorbate formulation is not corresponding with the low accumulation of doxorubicin as previously showed. On the other hand, the low accumulation of doxorubicin in the cells after treatment with the polysorbate 80-containing formulations should not decrease the cell viability to a great extend.
However, the study of the membrane integrity after treatment with polysorbate 80 indicated a significant deteriorated membrane, and even more toxic effects on the cells at higher concentration. So it can be concluded that the effect of polysorbate 80 formulations on the viability of the cells is greater because it is the combination of the two toxic effects: doxorubicin action and polysorbate 80 cellular membrane damage (Fig. 23).

5.5 The concentration-dependent effect of poloxamer 185 and polysorbate 80 on the P-gp inhibitory function.

One important factor for overcoming the drug resistance by poloxamer 185 is the concentration. Therefore, the effect of poloxamer 185 concentration on the accumulation of Rho123 in the three glioma cell lines, GS-9L, RG-2 and F-98 cell lines was determined. The concentration effects of poloxamer 185 also were compared with the concentration effects of polysorbate 80 and verapamil, a known P-gp substrate.
In the range of concentrations used a concentration-dependent increase in the uptake of Rho123 was observed in all cell lines, reaching a maximal value at 0.01 % concentration to the

CMC poloxamer 185 showing its maximal effect and decreasing at concentrations above the CMC. This result is consistent with previous reports suggesting that the unimers of poloxamer 185 (single chains of block copolymers) are responsible for the inhibition of P-gp efflux transport out of the cells (22, 25, 29). The decrease of Rho123 at higher concentrations above of the CMC of poloxamer 185 is believed to be due to incorporation of the drug into poloxamer 185 micelles, which in turn decreases the amount of free drug available to diffuse into the cells.

In the case of polysorbate, the accumulation of Rho123 also increased in a concentration-dependent manner. Polysorbate exerted the greatest effect on the Rho123 uptake at 0.1 % for each cell line. Above 0.1 % the uptake of Rho123 also started to diminish. This decrease, however, may not only be due to micelle incorporation but also may be due to the toxic effects by polysorbate at high concentrations as indicated by the study of the integrity of the cellular membrane, where at 0.1 % these effects appeared and at 1 % toxic effects were totally evident.

Thus, the concentration-dependent effect of poloxamer 185 may justify the fact that in the GS-9L cells after the treatment with P185-Dox-PBCA-NP did not yield a high enhancement of the doxorubicin accumulation as it was observed after the treatment with doxorubicin+P185. Taken into consideration that both formulations contain the same concentration of poloxamer 185, in the case of P185-Dox-PBCA-NP formulation poloxamer 185 acted as coating that could decrease the concentration of free unimers able to inhibit the P-gp-mediated transport of doxorubicin, whereas in the case of doxorubicin+P185, the concentration of free unimers would be higher and consequently nearer to 0.01% poloxamer 185, at which concentration poloxamer exerts its highest effect.

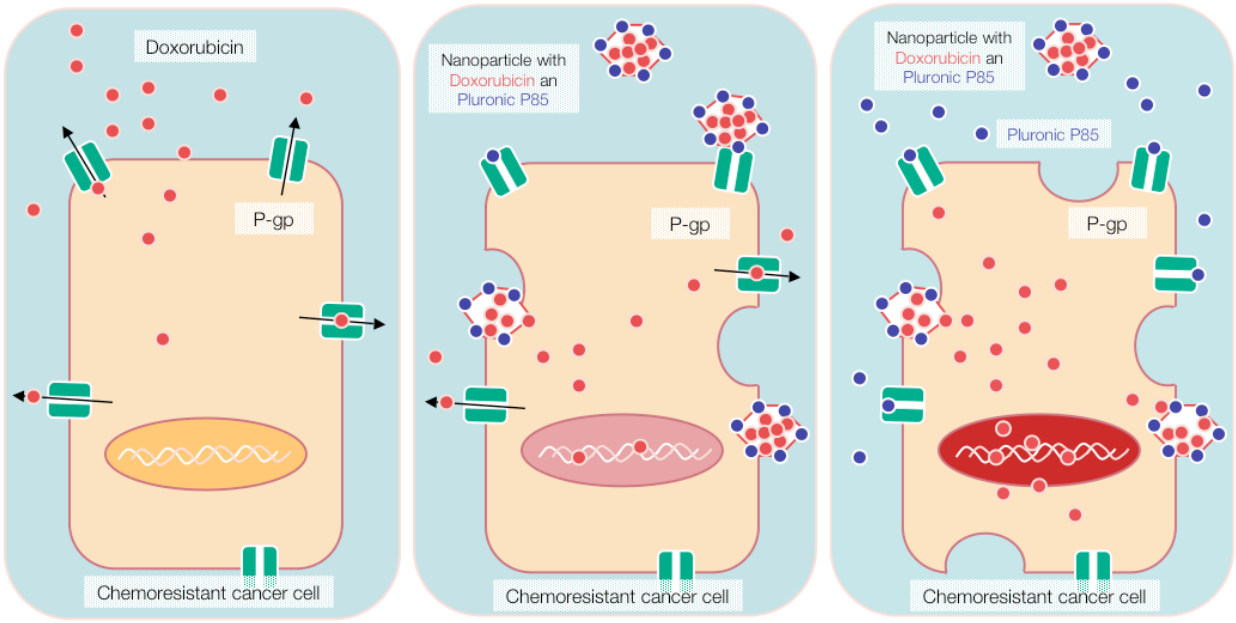

Figure 28. Mode of action of coated nanoparticles

In conclusion, poloxamer 185 noteworthy enhanced the transport of the anthracycline, doxorubicin, in doxorubicin-resistant cell lines, such as the GS-9L and the Caco-2 by overcoming the P-gp-mediated efflux. However this enhancement in the doxorubicin transport did not occur in doxorubicin-sensitive cell lines, such as the RG-2 and the F-98. Moreover, after incubation with different concentrations of poloxamer 185, the cellular membrane of GS9L did not show any visible alteration. Therefore, a selective interaction with P-gp rather than an unspecific membrane alteration provoked the increase in the doxorubicin transport in the doxorubicin-resistant cells. Batrakova et al. demonstrated using the hydrophobic membrane probe DPH, that poloxamer 185 induced changes in the microviscosity of the cell membranes in BBMEC and they also reported that similar changes were observed in the cancer cells treated with Pluronic® block copolymers (100).

Therefore, it is suggested that poloxamer 185 may have a double effect: through the ATP depletion and membrane fluidization, and that it seems very important that Pluronic® is bound to the membranes during the entire procedure. The removal of poloxamer 185 from the medium suggested that in the absence of the interaction of the poloxamer 185 with the P-gp- containing membranes the efflux system was inhibited insufficiently.

However, contrary to poloxamer 185, in the case of polysorbate 80 did not show a clear selective interaction with the P-gp. The entry of doxorubicin into the cells doxorubicin-resistant

cells did not increase after the treatment with the polysorbate 80 formulations, whereas with Rho-123 a concentration dependent increase occurred after incubation at certain concentrations of polysorbate 80. The stark changes in the integrity of the cellular membrane observed after treatment with polysorbate 80 solutions indicated an important interaction of the surfactant with the membrane and even more a loss of the membrane integrity at 1 % concentration.

Summary

The use of doxorubicin is a promising approach for the treatment of glioma tumours since it is a very effective neoplastic agent. However, one problem faced by the use of doxorubicin for the treatment of brain tumours is the fact that doxorubicin is a substrate of efflux pump proteins, such as P-glycoprotein (P-gp), which is located on the luminal side of the brain capillary endothelium and in many tumour cells. Therefore, these transporters are responsible for the multidrug resistance (MDR) in the case of brain tumours, pumping substances like doxorubicin out of the cell, and blocking their transport into the tumour cell. A demonstrated strategy to enhance the doxorubicin delivery into the brain is the use of nanoparticles.

The present in vitro study analyzed the transport of different doxorubicin formulations in three glioma models, the GS-9L, the RG-2 and the F-98 glioma cell lines. It was shown, that the treatment of doxorubicin bound to poly(butyl cyanoacrylate) nanoparticles decreased the viability of the three glioma cell lines significantly in comparison to doxorubicin in solution, indicating an improvement of the nanoparticles-bound doxorubicin transport into the cells.
The modification of the nanoparticles surface with different surfactants even may enhance the delivery of the drug into the cells. Searching for an improvement of the doxorubicin internalization, the nanoparticles surface was modified using polysorbate 80, poloxamer 188, and poloxamine 908 surfactants. Poloxamer 188 and polaxamine 908-modified nanoparticles did not yield a significant enhancement of the doxorubicin internalization. In contrast, the treatment with polysorbate 80-modified nanoparticles led in some cases to a significant decrease of cancer cell viability.
The cytotoxicity of doxorubicin formulations was analyzed using three cytotoxicity detection assays: the MTT, the LDH, and the ATP assays. These three assays showed a significant variability in the results of viability. The different sensitivity of the different parameters measured by the three assays, the mitochondrial activity (MTT assay), the LDH activity (LDH assay) and the ATP content (ATP assay) of the surviving cells was responsible for the variability of the results in the cellular viability after the drug exposure between these assays. Moreover, the differences in the sensitivity of the three assays could be additionally highlighted by the variability of the three cell lines used.

Moreover, the results of the present study also showed that the three glioma cell lines responded differently to the doxorubicin action, presumably due to a different drug uptake into

these cells. Previous extensive studies have identified mechanisms through which tumour cells escape the cytotoxic effects of a variety of chemotherapeutic drugs. The cellular resistance to chemotherapy is multifactorial and may be affected by the cell cycle stage, proliferation status, biochemical mechanisms (such as detoxification), cellular drug transport (influx, efflux, and retention), and DNA replication and by repair mechanisms. The resistance to multiple drugs, referred to as multidrug resistance (MDR) is a major impediment to the successful treatment of various human cancers. MDR is cell resistance not to single substance but to numerous substances characterized by different mechanisms of action and by different chemical structures. In some types of tumours, multidrug resistance is inherited, while in others it is acquired.

In this study, we focused on the cellular multidrug resistance causing a decrease of doxorubicin accumulation by the tumour cells. The mechanism of this drug efflux from the cells is mediated by the activity of transpoter proteins, i.e., P-glycoprotein (P-gp), transporter of the MRP family, and some others proteins. Thus, the different responses to doxorubicin shown were due to the fact that the transport of this drug into the glioma cells was subjected to the action of the P-glycoprotein expressed on their cellular membrane. A higher level of the P-gp expression correlated with a weaker response towards the doxorubicin treatment. The GS-9L cell line showed a significant higher level of P-gp expression than the F-98 and RG-2 cell lines, and consequently, the GS-9L cell line presented the highest resistance to doxorubicin with the highest viability after doxorubicin treatment.

As above mentioned, one of the major failures in cancer therapy is the simultaneous cellular resistance to multiple drugs presented by the cell tumours. The mechanisms by which tumour cells become resistant to multiple chemotherapeutic drugs are still poorly understood. However, the numerous strategies to overcome these resistance mechanisms resulted in encouraging results. One of these strategies was the employment of nanoparticles coated with different surfactants, where one of the components may function by overcoming these resistance mechanisms.

As previously described, the expression of the P-glycoprotein on the cellular membrane of the glioma cell lines was responsible for the avoidance of the doxorubicin entry into the cells and, therefore, the blocking out of the drug action. The P-gp inhibitors block the P-gp-mediated transport of the drug out of the cells and consequently, facilite its intracellular accumulation thereby, increasing its efficacy.

A number of studies demonstrated a rather effective inhibition of P-gp activity by the pluronic block copolymers surfactant (5, 6, 8, 9). In this group of surfactants, one of the most

outstanding poloxamer known as P-gp inhibitor is poloxamer 185. Poloxamer 185 posses the structural characteristics required for maximal impact on drug efflux transporter activity (89). Previous work reported that poloxamer 185 increases accumulation of the MRP (multidrug resistance related protein) substrates vincristine and doxorubicin (70).

For this purpose, the use of the P-gp inhibitor, poloxamine 185 was studied. The use of poloxamer 185 resulted in an enhancement of the uptake as well as of the accumulation of doxorubicin into the cells. The effect of poloxamer 185 on the doxorubicin uptake was especially pronounced in the case of doxorubicin-resistance cells, such as the GS-9L cell line. In some cases, the presence of the nanoparticles formulation further improved the uptake.

The use of a P-gp inhibitor in combination with chemotherapeutic agents may improve their efficacy. Because of the wide spectrum of substances acting as P-gp inhibitors, in many cases the exact inhibitory mechanisms still remain unclear. The non-ionic surfactant polysorbate 80 has been shown to increase the permeability of certain transport markers in Caco-2 cells as well as improving the permeation of proteins susceptible to P-gp-mediated efflux. Poloxamer (Pluronics®) copolymers have also been shown to enhance the diffusion of compounds such as doxorubicin across model lipid bilayers. These investigations suggest that surfactant interactions with membranes leading to increased membrane permeability may play an important role in enhancing the transmembrane diffusion of a P-gp substrate and its intracellular accumulation, independently of the inhibitory effects of these surfactants on P-gp. However, in our previous experiments, it was observed that the uptake of doxorubicin was increased by poloxamer 185 in cells with a higher P-gp expression level, whereas polysorbate 80 even led to a reduction in the intracellular accumulation of doxorubicin in the doxorubicin sensitive-cell lines RG-2 and F-98. The evaluation of a described potential P-gp inhibitor, polysorbate 80, did not show an important improvement in doxorubicin uptake in the P-gp-expressing cell line, GS-9L. On the other hand, the polysorbate 80-Dox-PBCA nanoparticles formulation decreased the viability of the glioma cells to greater extend than the poloxamer185-Dox-PBCA nanoparticles. Although, the P-gp inhibition was undoubtedly higher in the presence of poloxamer 185, polysorbate 80 showed a significant effect on the disruption of the cellular membrane, resulting in an important cellular viability decrease. It appears that poloxamer 185, on the other hand, exhibits a direct effect on the functionality of the P-gp protein, which would be of great importance for the sensitization of resistant cancer cells.

The concentration of poloxamer 185 had a very important influence on the inhibitory effect on the P-gp-mediated transport mechanism. Therefore, the effect of poloxamer 185 concentration on the accumulation of Rhodamine 123, a known P-gp substrate, in the three glioma cell

lines, GS-9L, RG-2, and F-98 was determined. The concentration effect of poloxamer 185 also was compared with the concentration effects of polysorbate 80 and Verapamil, a known P-gp substrate. The accumulation of Rhodamine-123 increased in a range of concentration from 0.001 % to 0.01 %, whereas at 0.1 % poloxamer 185 the accumulation significantly decreased. A maximal Rho-123 accumulation was reached at 0.01 % poloxamer 185. In the case of polysorbate, the accumulation of Rho123 also increased in a concentration-dependent manner. Polysorbate exerted the greatest effect on the Rho123 uptake at 0.1 % for each cell line. Above 0.1 % the uptake of Rho123 also started to diminish. This decrease, however, may not only be due to micelle incorporation but also may be due to the toxic effects by polysorbate at high concentrations as indicated by the study of the integrity of the cellular membrane, where at 0.1 % these effects appeared and at 1 % toxic effects were totally evident.

In conclusion, poloxamer 185 noteworthy enhanced the transport of the anthracycline, doxorubicin, in doxorubicin-resistant cell lines, such as the GS-9L and the Caco-2 by overcoming the P-gp-mediated efflux. However this enhancement in the doxorubicin transport did not occur in doxorubicin-sensitive cell lines, such as the RG-2 and the F-98. Moreover, after incubation with different concentrations of poloxamer 185, the cellular membrane of GS9L did not show any visible alteration. Therefore, a selective interaction with P-gp rather than an unspecific membrane alteration provoked the increase in the doxorubicin transport in the doxorubicin-resistant cells. Batrakova et al. demonstrated using the hydrophobic membrane probe DPH, that poloxamer 185 induced changes in the microviscosity of the cell membranes in BBMEC and they also reported that similar changes were observed in the cancer cells treated with Pluronic® block copolymers (100). This author (8, 22) also demonstrated that the inhibition of P-gp by poloxamer 185 (Pluronic® P85) involves two effects: one, a decreasing interaction or affinity of P-gp with respect to both ATP and the drug molecules; second, a depletion of intracellular ATP (energy depletion) in the endothelial cells of the brain microvessel.
This fact assures that poloxamer 185 has a direct effect on the functionality of P-gp rather than on the cellular membrane, which may explain the intact membrane after the incubation with poloxamer 185 (Fig. 22).

Therefore, it is suggested that poloxamer 185 may have a double effect: through the ATP depletion and membrane fluidization, and that it seems very important that Pluronic® is bound to the membranes during the entire procedure. The removal of poloxamer 185 from the medium suggested that in the absence of the interaction of the poloxamer 185 with the P-gp- containing

membranes the efflux system was inhibited insufficiently.
However, contrary to poloxamer 185, in the case of polysorbate 80 did not show a clear selective interaction with the P-gp. The entry of doxorubicin into the cells doxorubicin-resistant cells did not increase after the treatment with the polysorbate 80 formulations, whereas with Rho-123 a concentration dependent increase occurred after incubation at certain concentrations of polysorbate 80. The stark changes in the integrity of the cellular membrane observed after treatment with polysorbate 80 solutions indicated an important interaction of the surfactant with the membrane and even more a loss of the membrane integrity at 1 % concentration.

In conclusion, the association of poloxamer 185 with doxorubicin in solution and the poloxamer 185 coated-nanoparticle formulation of doxorubicin allowed overcoming the P-gp-mediated transport. Important requirements for that were the concentration of poloxamer 185 and the direct effect of poloxamer 185 displayed on the P-gp activity.

Zusammenfassung

Die Verwendung von Doxorubicin stellt einen wichtigen therapeutischen Ansatz in der chemotherapeutischen Behandlung von Tumoren gliomalen Ursprungs dar, da sich Doxorubicin bereits als sehr effektiver neoplastischer Wirkstoff erwiesen hat. Es besteht jedoch das Problem, dass Doxorubicin durch Multidrug-Resistenzproteine, wie P-gp sowohl aus den Endothelzellen der Blut-Hirn Schranke als auch aus Gerhirntumorzellen entfernt wird. Diese sind in der Membran dieser Zellen lokalisiert und pumpen Doxorubicin und andere Substrate in einem ATP-abhängigen Prozess aus der Zelle. Aus diesem Grund ist P-gp für die Multidrug-Resistenz bei Gehirntumoren verantwortlich, indem es Substanzen wie Doxorubicin aus den Zellen pumpt oder ihren Eintritt in das Zellinnere blockiert. Eine Möglichkeit die Doxorubicin-Applikation im Gehirn zu verbessern ist die Verwendung von Nanopartikeln.

Die vorliegende Arbeit befasste sich mit dem Transport verschiedener Anwendungsformen von Doxorubicin in drei Glioma-Modelle, den GS-9L, den RG-2 und die F-98 Gliomazellen. Hierbei konnte gezeigt werden, dass die Wirkung von Doxorubicin durch die Bindung an Poly(butyl-cyanoakrylat)-Nanopartikel im Vergleich zu freiem Doxorubicin in Lösung signifikant gesteigert werden konnte. So konnte die Anzahl an über lebenden Zellen nach einer Behandlung der Glioma Zelllinien GS-9L, RG-2 und F-98 mit Doxorubicin-beladenen Nanopartikeln signifikant erniedrigt werden, was für eine erhöhte effektive Doxorubicin-Konzentration in den Zellen spricht.

Die Modifikation der Oberfläche der Nanopartikel mit unterschiedlichen Surfaktantien könnte das Einbringen von Doxorubicin in die Zielzellen zusätzlich erhöhen. Bei der Suche nach einer verbesserten Doxorubicin-Internalisation wurde die Oberfläche der Nanopartikel mit Tensiden wie Polysorbat 80, Poloxamer 188 und Poloxamin 908 modifiziert. Die mit Poloxamer 188 und Polaxamin 908 modifizierten Nanopartikel zeigten keine signifikante Verbesserung der Doxorubicin-Internalisation in die Zellen. Im Gegensatz dazu konnte eine signifikante Reduktion der lebenden Krebszellen durch die Behandlung mit Polysorbat 80-modifizierten Nanopartikeln beobachtet werden.

Die Zytotoxizität der verschiednen Doxorubicin-Anwendungsformen wurde in drei verschiedenen Zytotoxizität-Analyseversuchen untersucht: den MTT-, LDH- und dem ATP-Tests. In den unterschiedlichen Versuchen werden verschiedene Parameter gemessen, die mitochondriale Aktivität (MTT-Test), die Laktatdehydrogenase Aktivität (LDH-Test) und der ATP

Gehalt der Zellen (ATP-Test). Die verschiedenen Parameter besitzen eine unterschiedliche Sensitivität, wodurch sich die Variabilität der Versuchergebnisse erklären lässt. Diese unterschiedliche Sensitivität wurde ferner in den Ergebnissen der drei verwendeten Zelllinien deutlich.

Die vorgelegten Ergebnisse zeigen auch, dass die verschiedenen Zelllinien unterschiedlich auf die Doxorubicin Behandlung reagierten, welches wahrscheinlich mit einer unterschiedlichen Wirkstoffaufnahme in Verbindung steht. Intensive Studien konnten Mechanismen identifizieren, mit welchen Krebszellen die zytotoxischen Effekte einer Vielzahl von chemotherapeutischen Wirkstoffen umgehen. Die zelluläre Resistenz gegenüber Chemotherapeutika besitzt eine Vielzahl von Faktoren und könnte durch den Zellzyklusstatus, den Status der Proliferation, biochemischen Mechanismen (wie z.B. Entgiftung), zellulärer Wirkstoff Transport (Influx, Efflux und Retention) und DNA-Replikation sowie Reparaturmechanismen beeinflusst werden. Die Resistenz gegenüber verschiedenen Wirkstoffen, welche als Multidrug Resistenz (MDR) bezeichnet wird, ist ein wichtiger Faktor in der erfolgreichen Behandlung von menschlichen Tumoren. MDR bedeutet nicht die Resistenz gegenüber einem einzelnen Wirkstoff sondern schließt eine Vielzahl von Wirkstoffen ein, die sich sowohl in ihrer chemischen Substanzklasse als auch in ihrem Wirkungsmechanismus unterscheiden können. In manchen Tumoren ist die MDR bereits vorhanden, in anderen kann sie durch Mutationen erlangt werden.

In der vorliegenden Studie war der zelluläre MDR-Mechanismus, welcher eine reduzierte Doxorubicin-Akkumulation in den Krebszellen zur Folge hatte, Fokus der Untersuchungen.
Der Mechanismus des Wirkstoffexports aus den Zellen wird von Transporterproteinen, wie dem P-Glykoprotein (P-gp), Transportern der MDR-Proteinfamilie und anderen Proteinen, vermittelt. Die unterschiedlichen Wirkungen von Doxorubicin in den verschiedenen Zelllinien war darauf zurückzuführen, dass Doxorubicin als Substrat von P-gp, welches sich in der Zellmembran der Zellen befindet, aus den Gliomazellen transportiert wird. Hierbei korrelierte eine erhöhte P-gp Expression mit einer schwächeren Doxorubicin-Wirkung. Die Zelllinie GS-9L zeigte eine signifikant höhere P-pg-Expression als die F-98- und RG-2-Zellen. Konsequenterweise wurde in dieser Zelllinie die höchste Doxorubicin-Resistenz ermittelt mit den höchsten Werten an überlebenden Zellen nach Doxorubicin-Behandlung.
Da der Transport von Doxorubicin aus den Glioma-Zellen mit der Aktivität des P-gp-Proteins korreliert, konnte durch die Verwendung des P-gp-Inhibitors Poloxamer 185 eine verbesserte Aufnahme und Akkumulation von Doxorubicin in den Zellen erreicht werden. Dieser Effekt der verbesserten Doxorubicin-Aufnahme konnte besonders gut in den Doxorubicin-resistenten

GS-9L Zellen beobachtet werden. In einigen Fällen konnte auch eine verbesserte Aufnahme durch die Verwendung der Nanopartikel beobachtet werden.

Wie oben dargestellt wurde, ist die Hauptursache einer nichtwirksamen Krebstherapie die simultane Resistenz der Tumorzelle gegenüber verschiedenen Wirkstoffen. Der Mechanismus, wodurch diese MDR erreicht wird, ist immer noch nicht ausreichend erforscht. Allerdings haben bereits verschiedene Strategien die MDR zu überwinden ermutigende Erfolge erzielt. Eine dieser Strategien war die Behandlung mit Nanopartikeln, deren Oberfläche mit verschiedenen Tensiden modifiziert war, wobei eines dieser Tenside dazu betragen kann, die Wirkstoffresistenz zu überwinden.
Wie bereits beschrieben, war die Expression des P-gp Proteins in der Zellmembran dafür verantwortlich, dass Doxorubicin nicht in die Krebszellen gelangen konnte, um seine Funktion zu erfüllen. Mit Hilfe des P-gp-Inhibitors gelingt es die Funktion des Proteins zu blockieren, wodurch die Doxorubicin-Anreicherung in den Gliomazellen und eine damit verbundene Wirkungssteigerung möglich wird.

Eine Reihe von Studien konnten einer recht effiziente Inhibition der P-gp Aktivität durch “pluronic block copolymers surfactant” (5,6,8,9). In dieser Gruppe von Tensiden ist das Poloxamer, welches am besten als P-gp Inhibitor beschrieben ist, Poloxamer 185. Poloxamer 185 besitzt die strukturellen Eigenschaften, um einen maximalen Einfluss auf die Medikament Efflux Transportaktivität zu besitzen.
A number of studies demonstrated a rather effective P-gp inhibition activity of the pluronic block copolymers surfactant (5, 6, 8, 9). However, one of the most outstanding known poloxamers as P-gp inhibitor is poloxamer 185. Poloxamer 185 posses the structural characteristics required for maximal impact on drug efflux transporter activity (89). Previous work reported that poloxamer 185 increases accumulation of the MRP (multidrug resistance related protein) substrates vincristine and doxorubicin (70).

Aus diesem Grund wurde die Verwendung eines P-gp-Inhibitors, Poloxamin 185, untersucht. Die Verwendung von Poloxamin 185 führte zu einer erhöhten Doxorubicin Aufnahme und Akkumulation in den Gliomazellen. Der Effekt der Doxorubicin-Aufnahme war besonders in Doxorubicin-resistenten Zellen, wie GS-9L-Zellen, erhöht. In einigen Fällen konnte die Doxorubicin-Aufnahme durch die Oberflächen-Beschaffenheit der Nanopartikel weiter erhöht werden.

Auch in der vorliegenden Arbeit führte die Verwendung von P-gp-Inhibitoren in Kombination mit chemotherapeutischen Agentien zu ermutigenden Resultaten. Trotzdem bleibt durch das grosse Spektrum an Substanzen, die in der Lage sind das P-gp Protein zu inhibieren, der exakte Mechanismus der Inhibition weiterhin ungeklärt. Das nicht-ionische Tensid Polysorbat 80 konnte sowohl die Permeabilität von bestimmten Transportmarkern in Caco-2-Zellen erhöhen, als auch die Permeabilität von Proteinen, die ein Substrat von P-gp-vermitteltem Export sind. Für Poloxamer (Pluronics®) Copolymere konnte ebenfalls gezeigt werden, dass sie die Diffusion von Wirkstoffen wie Doxorubicin über Modellen von Doppellipidschichen erhöhen. Diese Entdeckungen lassen vermuten, dass die Tensid-Interaktionen mit Membranen die Membranpermeabilität erhöhen und auf diese Weise eine wichtiger Faktor in der Erhöhung der Transmembrandiffusion eines P-gp Substrates und dessen Akkumulation in den Zielzellen sind, unabhängig von der Inhibition des P-gp Proteins durch diese Tenside. In unseren bisherigen Versuchen wurde beobachtet, dass die Aufnahme von Doxorubicin durch Poloxamer 185 in Zellen mit starker P-gp Expression erhöht, wo hingegen durch die Behandlung der Doxorubicin-sensitiven Zellen RG-2 und F-98 mit Polysorbat 80 die Doxorubicin Akkumulation sogar vermindert wurde. Ferner konnte die Verwendung von Polysorbat 80 die Aufnahme von Doxorubicin in GS-9L Zellen nicht erhöhen. Allerdings wurde die Zahl an lebenden Glioma Zellen durch die Behandlung mit Polysorbat 80-Dox-PBCA Nanopartikeln stärker reduziert als durch die Behandlung mit Poloxamer 185-Dox-PBCA Nanopartikeln. Obwohl der Effekt der P-gp Inhibition in Gegenwart von Poloxamer 185 höher war, zeigte Polysorbat 80 einen starken Effekt auf die Zellmembran, wodurch die Anzahl der lebenden Zellen stark vermindert werden konnte. Poloxamer 185 scheint hingegen einen direkten Einfluss auf die Funktionalität von P-gp auszuüben, wodurch es von großer Bedeutung für die Sensitivierung bei der Chemotherapie von resistenten Krebszellen sein könnte.

Die Höhe der Poloxamer 185-Konzentration ist hierbei der entscheidende Parameter um einen inhibitorischen Effekt auf den P-gp-vermittelten Transportmechanismus zu erzreichen.

Die Akkumulation von Rhodamin-123 (Rho-123), einem bekannten P-gp Substrat, konnte durch die Zugabe von 0,001 % bis 0,01 % stark erhöht werden, wobei ab einer Poloxamer 185-Konzentration von 0,1 % sich die Rho-123 Akkumulation wieder signifikant reduzierte. Die maximale Rho-123 Akkumulation wurde bei einer Konzentration von 0,01 % Poloxamer 185 erreicht.

Zusammenfassend lässt sich sagen, dass die gleichzeitige Applikation von Poloxamer 185 mit Doxorubin in Lösung und die Poloxamer-umhüllten, Doxorubizin-beladenen Nanopartikel den P-

pg-vermittelten Transport verhindern konnten. Eine wichtige Vorraussetzung hierfür ist die Poloxamer 185 Konzentration und die dadurch erzielten Effekte auf die P-gp Aktivität.

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Eidesstattliche Erklärung

Hiermit versichere ich, dass ich die vorliegende Arbeit selbstständig verfasst habe und dass keine weiteren Quellen und Hilfsmittel als die hier aufgeführten verwendet worden sind.
Die Arbeit hat in gleicher Weise noch keiner Prüfungsbehörde vorgelegen.

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Curriculum Vitae

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Education

since January 2002	PhD student at the Parmaceutical Technology Department of the University of Frankfurt in the group of Prof. Dr. Jörg Kreuter; title of the thesis:

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01.10.00-01.07.01	Erasmus-Student in the group of P.C. Schmidt at the departament of Pharmaceutical Technology of the University of Tübingen, Germany.
01.10.95-01.09.01	Study of Pharmacy at the Alcala de Henares University in Madrid, Spain
01.10.92-01.09.95	Study of Pharmacy at the University of Salamanca in Salamanca, Spain
03.06.1992 Spain	"Abitur" at the Institute de Lucia de Medrano, Salamanca,

Languages

Spanish:	native
English:	fluent
German:	fluent
French:	basic knowledge

Other abilities

Handling of Apple and Microsoft operating systems, MS Office

Illustration und Picture processing with Adobe Photoshop and Illustrator

Personal activities and interests

Sports: Swimming, running

Hobbies: reading, traveling, listening to music

Publications and Posters

Poster Presentation in International Meeting on, Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology 2004, Nürnberg
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Congresses and Conferences

04.2002 International Meeting on Pharmaceutics, Biopharmaceutics and Pharmaceutical Technology, Florence, Italy.

05.2002 4. Blut-Hirn-Schranke - Expertentreffen, Bad Herrenalb, Germany

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