



Ganglioside nanomicelles for anticancer drug delivery

TDK thesis

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List of abbreviations.

BBB	blood-brain barrier
c.m.c.	critical micellar concentration
DLS	dynamic light scattering
DMSO	dimethyl sulfoxide
DOX	doxorubicin
FDA	Food and Drug Administration
GSL	glycosphingolipid
PTX	paclitaxel
SOR	sorafenib
TMZ	temozolomide
UIPAC	International Union of Pure and Applied Chemistry
UV-vis	ultraviolet-visible

1. Introduction

Gangliosides are glycosphingolipids which contain a ceramide lipid tail and a glycan headgroup with one or more sialic acid residues. They are necessary for the maintenance of the central nervous system, as well as for the protection and strengthening of neural injuries. Externally administered gangliosides may cross the blood-brain barrier (BBB), making them an important area of research in the clinical treatment of neurodegenerative diseases such as Alzheimer's, Parkinson's disease and cancer.

In this study, GM1 nanomicelles were formed and investigated under various conditions. Physical and chemical examination of prepared GM1 ganglioside nanomicelles was carried out at the Functional Nanoparticles Research Group, Institute of Materials and Environmental Chemistry, Research Centre for Natural Sciences located at the University of Pannonia. Moreover, gangliosides are promising drug carrier materials, thus my other aim was microencapsulation of anticancer drugs. The drug concentration was measured by UV-vis spectrophotometry.

Doxorubicin hydrochloride, sorafenib, paclitaxel and temozolomide anticancer drugs were entrapped by GM1. Due to the low solubility of paclitaxel and sorafenib in water, they were dissolved in DMSO in order to solubilize them in the aqueous GM1 solution, while doxorubicin hydrochloride and temozolomide could be directly dissolved in the GM1 solution. The size and zeta potential of nanomicelles were investigated by dynamic light scattering (DLS) and laser Doppler micro-electrophoresis, respectively. The entrapped doxorubicin and paclitaxel anticancer drugs did not change substantially the size distribution of GM1. To

verify drug presence in GM1 micelles, the solutions were dialysed to remove the free drug. The doxorubicin and paclitaxel encapsulation efficiencies of GM1 nanomicelles were found to be 94.2 % and 83.0 %, respectively. S/TEM imaging of the drug-loaded micelles by a FEI Talos F200XG2 scanning/transmission electron microscope also supported the results obtained by DLS method.

2. Literature overview

Numerous glycosphingolipids (GSL) found in the nervous system and elsewhere are implicated in the metabolic and pathological alterations associated with Parkinson's disease. Gangliosides are identified as cancer-associated antigens and are overexpressed in cancer tumours [1]. They have attracted the attention of several researchers due to its prominent role in the etiology, cancer treatment and potential treatment of Parkinson's disease [2].

2.1. Gangliosides

Gangliosides are a subclass of GSL distinguished by the presence of one or more sialic acid residues in its carbohydrate moiety [3]. The lipid part of gangliosides shared by all sphingolipids is called ceramide. Ceramide is a lipid molecule composed of two long alkyl chains, also known as sphingosine, linked to a fatty acid via an amide bond. Stearic acid covers up to more than 90 percent of the total fatty acid content in nervous tissue, where gangliosides are 10 times more prevalent than in any other tissue [4]. Sialic acid is a sugar that separates gangliosides from neutral GSL-s and sulfatides. The mother-molecule sialic acid, neuraminic acid, is systematically termed 5-amino-3,5-dideoxy-D-glycero-D-galacto-non-2-ulonic acid, because it does not exist in free form in nature due to its quick cyclization to create an internal Schiff base [5].

Svennerholm defined ganglioside nomenclature as comprising the number of sialic acid residues (indicated by the initials M, D, T, and Q for mono, di, tri, and tetra respectively), the number of sugar groups (represented by: “five minus the chain length”) and the number of sialic acid residues on the inner galactose group (none = 0, a = 1, b = 2 or c = 3) [3], [6]. The GM1 ganglioside name structure is shown in Figure 1.

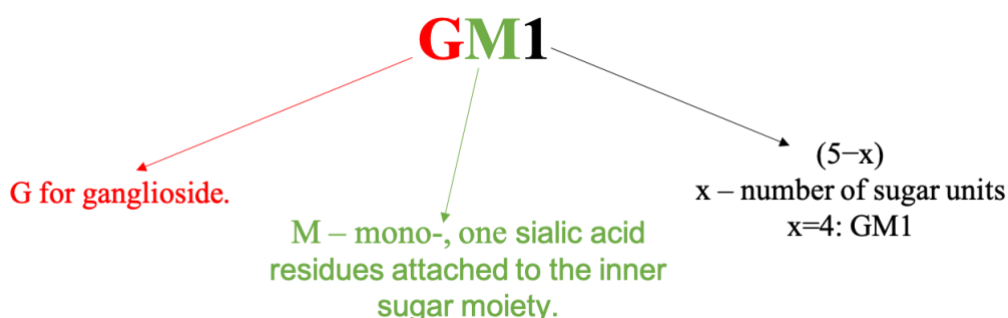
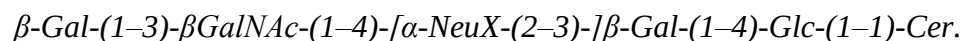


Figure 1. Ganglioside name structure.

2.2. GM1 ganglioside

GM1 is a monosialo-glycosphingolipid which belongs to the gangliotetrahexosyl group with formula:



Where *Gal*, *GalNAc*, *Glc* and *Cer* are for galactose, galactosamine, glucose and ceramide respectively. “*NeuX*” in this formula signifies any sialic acid structure, according to the IUPAC [7], [8].

Monosialic GM1 ganglioside, shown in Figure 2, is mostly found in the plasma membrane, where two hydrocarbon chains anchor ganglioside as a part of ceramide moiety [9], [10]. Such gangliosides are amphipathic molecules with a negatively charged head group which is modified with carbohydrate groups that differ amongst more than 200 recognized ganglioside species.

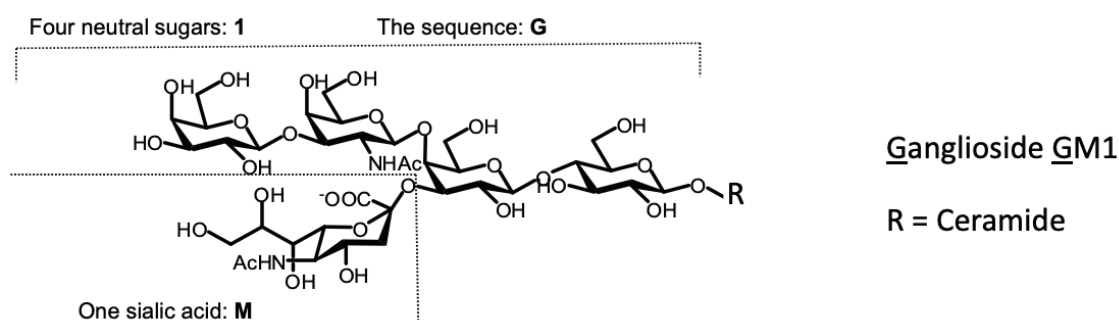


Figure 2. Schematic structure of GM1 ganglioside [7].

GM1 is soluble in water [11], where it clumps due to its hydrophobic nature. The broad hydrophilic head determines the shape of the GM1 monomer. It forms tiny ellipsoidal micelles, sometimes termed toroidal-like micelles. Experimental and theoretical determinations place the critical micellar concentration (c.m.c.) of GM1 micelles in a range of 10^{-8} – 10^{-9} M. Free monomers in equilibrium with aggregates cannot exceed the c.m.c. value at any concentration of GM1 [7].

Ganglioside GM1 was found to be able to recover the damaged nervous system. However, the ability of GM1 to cross the blood-brain barrier (BBB) is limited by its amphiphilic nature, thus it has restricted clinical translation potential.

In cancer treatment the expression levels of GM1 are primarily controlled by glycosyltransferases. Generally, cancer cell signalling and development are coupled with glycosylation alterations, including alterations in the surface-expressed gangliosides [12].

2.3. Self-assembled micelles – anticancer drug carriers loaded into GM1 micelles

Monosialoganglioside GM1 has been widely studied in clinical researches as anticancer drug transporter. Self-assembled micelles are block copolymer nano-carriers, which contain active ingredient. Polymer

micelles frequently act as carriers for hydrophobic chemicals, thus enhancing the solubility of drug. When the medicine is delivered to the target area, for example tumour, the micelles are able to disassemble into smaller molecular structures [14]. However, most therapeutic medications cannot be delivered to brain tumour tissue due to the BBB, thus this is one of the most difficult parts of treating such disorders. Self-assembled micelles with the ability to pass the BBB may be the solution of this problem, but further experiments are required to confirm their applicability [13].

2.3.1. Doxorubicin

Doxorubicin (DOX) is a white crystalline hydrophilic powder, with the chemical name (1S,3S)-3-Glycoloyl-3,5,12-trihydroxy-10-methoxy-6,11-dioxo-1,2,3,4,6,11-hexahydrotetracycen-1-yl 3-amino-2,3,6-trideoxy- α -L-lyxo-hexopyranoside corresponding to the molecular formula $C_{27}H_{29}NO_{11}$ and relative molecular mass of 543.5 g/mol [15]. **DOX** was one of the anthracyclines isolated from the pigment-producing *Streptomyces peucetius* in early 1960s [16]. It is recognized as one of the most powerful Food and Drug Administration (FDA) - approved chemotherapy medicines, demonstrating significant therapeutic potential. Since several decades, it has been well accepted that the capacity to battle rapidly dividing cells and slow disease development is limited only by its toxicity to noncancerous cells in the human body [17]. **DOX** has both aglyconic and carbohydrate moieties (Figure 3) [16].

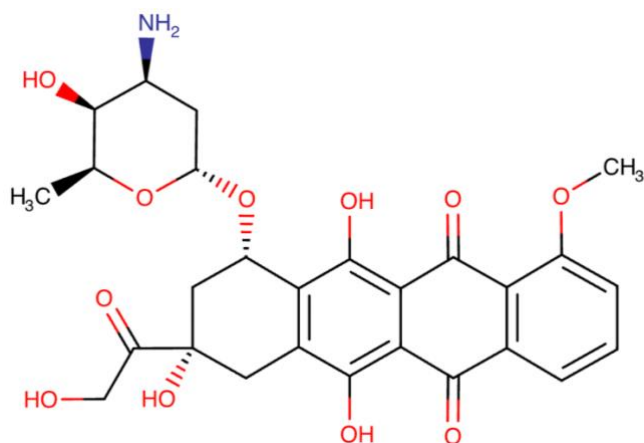


Figure 3. Chemical structure of doxorubicin (DOX) [14].

2.3.2. Sorafenib

Sorafenib (SOR) appears as white, hydrophobic, crystalline powder. The chemical name of **SOR** is 4-(4-(((4-Chloro-3-(trifluoromethyl)phenyl)amino)carbonyl)amino)phenoxy)-N-methyl-2-pyridinecarboxamide corresponding to the molecular formula $C_{21}H_{16}ClF_3N_4O_3$ (Figure 4) and relative molecular mass of 464.825 g/mol [18]. This anticancer drug is very soluble in dimethylformamide, although

almost insoluble in water and acetonitrile [19]. **SOR** is a multi-kinase inhibitor that has been approved by the FDA for the treatment of advanced hepatocellular and renal cell carcinomas.

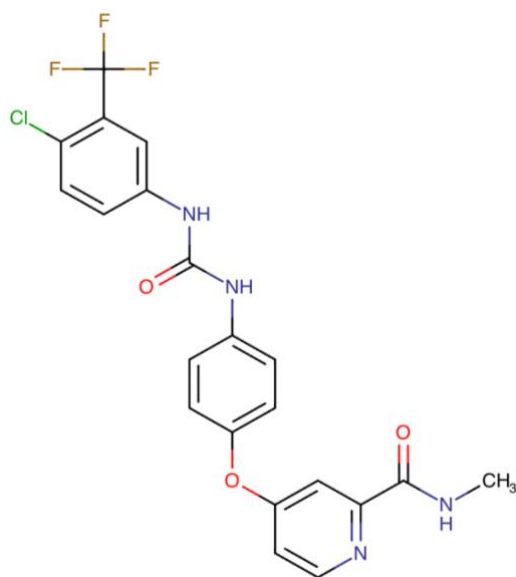


Figure 4. Chemical structure of sorafenib (SOR) [18].

In addition, preclinical studies have demonstrated that **SOR** possesses powerful anticancer effects in several tumour cell lines, including breast cancer, melanoma, thyroid and colorectal carcinomas [20]. It belongs to a class of drugs with limited water solubility under physiological conditions and rapid metabolism. In other words, its bioavailability is extremely low. In recent years, a variety of nanocarriers have been proposed to improve its therapeutic profile [19].

2.3.3. Temozolomide

Temozolomide (TMZ) is a white non-hygroscopic powder which is partly soluble in water and acetonitrile [21]. The chemical name of **TMZ** is 3,4-Dihydro-3-methyl-4-oxoimidazo(5,1-D)-1,2,3,5-tetrazine-8-carboxamide, which corresponds to the formula C₆H₆N₆O₂ (Figure 5) and relative molecular mass of 194.151 g/mol [22].

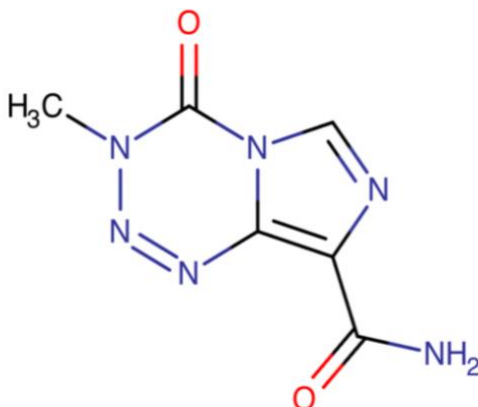


Figure 5. Chemical structure of temozolomide (TMZ) [21].

TMZ is a very toxic drug, however anticancer nanocarriers are seen as prospective transporters capable of mitigating this disadvantage, enhancing its penetration through the BBB and decreasing metabolic rate [14].

2.3.4. Paclitaxel

Paclitaxel (PTX) is a naturally occurring diterpenoid pseudoalkaloid, shown in Figure 6. Originally it is isolated from the bark of *Taxus brevifolia* with a chemical name 5beta,20-Epoxy-1,2-alpha,4,7beta,10beta,13alpha-hexahydroxytax-11-en-9-one 4,10-diacetate 2-benzoate 13-ester with (2R,3S)-N-benzoyl-3-phenylisoserine, molecular formula $C_{47}H_{51}NO_{14}$ corresponding to molecular weight of 853.906 g/mol [23], [24].

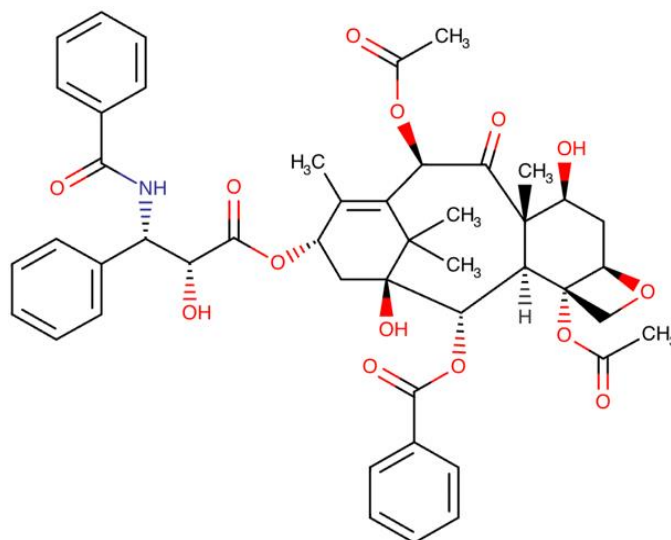


Figure 6. Chemical structure of paclitaxel (PTX) [24].

PTX is a white crystalline powder which is insoluble in water. The most significant advantage of nanocarriers for **PTX** delivery is that nanoparticles can increase the solubility of drugs, and due to their small size and high permeability, they facilitate drug delivery to the tumour [23].

3. Aims

Monosialoganglioside GM1 has been widely studied in clinical researches as anticancer drug transporter. The ability of GM1 to cross the BBB is limited due to its amphiphilic nature, thus it has restricted clinical translation potential. One of the aims of my research work is to examine the chemical and physical stability of nanomicelles formed from GM1 gangliosides. Another novelty of this research is that the used GM1 has been synthesized from its components by the *Carbocode GmbH* in contrast with the studies available in the scientific literatures, which investigated GM1 gained from the brain of animals. The synthetic GM1 has the potential to obtain permission for human use, while the one from animals have not had a chance to obtain it.

Self-assembled micelles carrying anticancer drugs and capable of passing the BBB might be the breakthrough treatment for cancer, however further developments are necessary. Once nanomicelles have been produced, the question how their properties relate to the corresponding parameters is always important to reply.

Another aim of my research is the microencapsulation of anticancer drugs used against brain tumours with gangliosides to create nanomicelles for drug delivery. Doxorubicin and sorafenib were selected as model drugs to study the GM1 interaction with a hydrophilic and a lipophilic drug, while paclitaxel and temozolomide were chosen as potential drugs with different mechanism of action against brain tumours. As well as examination of physical, chemical, drug release and cytotoxicity properties of prepared drug nanocarriers.

4. Methods, materials and tools

GM1 was kindly provided by *Carbocode GmbH* (Konstanz, Germany). Sorafenib (free base) and doxorubicin hydrochloride were purchased from *Active Biochem* (Hong Kong, PRC). NaCl and DMSO were obtained from *Sigma Aldrich* (Saint Louis, MO, USA). Temozolomide and paclitaxel were provided by *Chemgood LLC* (Glen Allen, VA, USA).

4.1. Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) measurements exploit the interaction of light and particles. If the size of the particle is smaller than the wavelength, the scattered light propagates in all directions. However, because of Brownian motion, the number of scattering centres in the observed region of space is constantly changing, and therefore the intensity of the of scattered light is changing as well. The device measures the time correlation function of the intensity of light scattered by free particles.

4.1.1. *Nanomicelle size measurement*

Malvern Zetasizer Nano-ZS device (Malvern, UK), the principle of which is based on the dynamic light scattering, works under the light source, which is a He-Ne laser with a wavelength of 633 nm.

Nanomicelle size determination was carried out with Zetasizer in the disposable cell (DTS0012) in the room temperature using DTS nano or Zetasizer Ultra-Pro ZS Xplorer softwares running under Windows OS shown in Figure 7.

A typical setup consists of the Zetasizer instrument (1) and a PC with the Zetasizer software installed (2). The sample is placed in a cell (plastic cuvette) (3) and loaded into the cell area of device (4).



Figure 7. Malvern Zetasizer Nano ZS device and the DTS0012 cell [25],[26].

The average hydrodynamic diameter in nm and the polydispersity index (PDI) of the samples were determined. The PDI is a dimensionless number that describes the particle size distribution. It characterizes the degree of size monodispersity, or polydispersity, of a given sample. PDI measured by DLS Malvern Zetasizer Nano-ZS device determines the PDI between 0 and 1. A low PDI value is a representative of the monodispersity of the sample. If $PDI > 0.3$ the sample is considered to be polydisperse. To standardise the measurements, I measured the sample according so-called measurement template, which predefined all measurement settings. These predefined measurement setting were adjusted according to the properties of the material to be measured, i.e. GM1 (absorbance = 0, RI (refractive index) = 1.4) and the dispersion medium, i.e. water (RI = 1.33). The measurement consists of several sub-measurements (runs), which allows filtering of the data. At the end of data collection, the quality of all sub-measurements is evaluated by previously mentioned [softwares](#). The number of sub-measurements is freely selectable – more measurements lead to more accurate results.

4.1.2. Zeta potential

Different processes (e.g. protonation) can form electric charges on the surface of colloidal particles. These particles with a charge in solution move towards the pole with the opposite charge to their charge under the influence of an external electric field. This is the phenomenon of electrophoresis. During the measurements, particles moving in the electric field between the electrodes of the electrophoresis cell change the frequency of the light according to the magnitude and direction of their velocity (Doppler effect).

The determination of zeta potential for the state of charge of some samples was also carried out by a Malvern Zetasizer device after the determination of the hydrodynamic diameter. For measurements at $> +/-$

500mV V, special measuring cells containing gold-coated platinum electrodes and Universal 'dip' cell kit were used as shown in Figure 8.



Figure 8. DTS1070 cell and ZEN1002 Universal 'dip' cell kit [\[26\]](#).

4.2. S/TEM

Size and morphology of particular micelles were also imaged in the Nanolab by Péter Pekker at the University of Pannonia by a *FEI Talos F200XG2* scanning/transmission electron microscope (S/TEM, Thermo Fischer Scientific, Waltham, MA, USA) operated at 200 keV after staining GM1 micelles with phosphotungstic acid.

4.3. Entrapment of anticancer drugs

There are several methods for the delivery of anticancer drugs. An interesting approach is the microencapsulation of active chemicals with components that might cross the BBB, for example with gangliosides. In such a case anticancer drug encapsulated with gangliosides is a type of converted original drug, in which the drug molecules are entrapped inside nanoscale ganglioside micelles.

4.3.1. *Doxorubicin and sorafenib*

50 mg/ml GM1 solution was prepared in 0.1M NaCl, since it was experienced in preliminary investigations that the micelle formation occurs in the presence of sufficient salt. In case of MilliQ water, the GM1 was successfully dissolved in water, although in the lack of micelle formation the drugs could not be entrapped. **DOX** was entrapped with ganglioside nanomicelles using a 50 mg/ml GM1 micelle solution and stored in the refrigerator for 24 hours before the experiments, in order to form GM1 micelles. **SOR** anticancer drug carriers were prepared in a similar way to **DOX** using the same GM1 solution, but due to the hydrophobic properties of **SOR**, DMSO was also added to solubilize the drug. Both of the drugs were added in 10% m/m related to GM1 weight, i.e. 5 mg/ml.

5.1 mg **DOX** was stirred with the added micelle solution (1.02 ml) for 1 hour on a magnetic stirrer until a clear solution was obtained. The size and zeta potential of nanomicelles were then measured using the methods above.

5.1 mg **SOR** was dissolved in 0.051 ml of DMSO by shaking and then stirred with 0.969 ml of 50 mg/ml GM1 micelle solution for one hour. At the time of addition, opacity was observed, which did not change with stirring. However, neither aggregation nor sedimentation was observed. When sorafenib seemed to be dissolved, the micelle size and zeta potential measurements were determined.

4.3.2. *Temozolomide and paclitaxel*

The chemical and physical properties of **TMZ** were examined in 100 mg/ml GM1-MilliQ water and 50 mg/ml GM1-NaCl (0.1 M) solutions.

TMZ-GM1 solution was created by dissolving 5.0 mg **TMZ** in 1 ml 50 mg/ml GM1-MilliQ water solution. Moreover, further experiments showed that **TMZ** could be dissolved in 0.1 M NaCl as well. 2.5 mg/ml **TMZ** solution was prepared in 0.84 ml of 50 mg/ml GM1-NaCl solution.

All the measurements were conducted at room temperature. The size distribution of **TMZ** entrapped by GM1 ganglioside was measured by Malvern Zetasizer Nano-ZS device.

The solubility properties of **PTX** in DMSO and acetonitrile, as well as **PTX** behaviour in 50 mg/ml GM1-MilliQ water and 50 mg/ml GM1-NaCl (0.1 M) solutions were examined in addition.

PTX anticancer drug was completely dissolved in the presence of both DMSO and acetonitrile in different concentrations at room temperature. PTX-DMSO solution was mixed with the 0.96 ml of 50 mg/ml GM1-MilliQ solution. Size distribution was measured as it was mentioned previously. For further experiments I tested the co-encapsulation of **TMZ** and **PTX** anticancer drugs with GM1 gangliosides and investigate their chemical and physical properties.

4.3.3. *Anticancer drug complexes with GM1*

To increase the anticancer drugs efficiency in the system the combination of two or more different drugs may be useful tool to avoid drug resistance. The co-entrapment of drugs with gangliosides was used to enhance drug stability.

I investigated the co-entrapment of **DOX** and **SOR** in GM1-NaCl (0.1 M) solution. GM1-SOR micelles resulted in micron-sized complexes, hence centrifugation was used to separate them. The sample was centrifugated at 40,000 rpm for 20 minutes at 25 ° C on an *Optima MAX XP ultracentrifuge*. DOX-loaded GM1 micelles could be separated from the free drug and other components only using dialysis. The size distribution, S/TEM microscopy and UV-vis spectrophotometry measurements were conducted after dialysis.

Another object of the research was the co-encapsulation of **TMZ** and **PTX** drugs which can be effective agents against brain tumours. GM1-TMZ and PTX-DMSO solutions were prepared separately and mixed. **TMZ** solution was created in the presence of 50 mg/ml GM1-MilliQ water solution. Subsequently size distribution, dialysis, zeta potential and UV-vis spectrophotometry measurements were performed.

Similar procedure was performed using acetonitrile as solvent of **PTX** to eliminate the disturbing effect of DMSO during the analysis of drug content. 0.2 ml 25 mg/ml **PTX** in acetonitrile was added to GM1-TMZ-NaCl solution containing 50 mg/ml GM1 and 2.5 mg/ml **TMZ**.

To co-encapsulate **TMZ** and **PTX** drugs in the presence of 0.1 M NaCl, similar measurement was performed, with the only difference that was dissolved in DMSO. 50 mg/ml PTX-DMSO sample could be completely dissolved in 2.5 mg/ml GM1-TMZ-NaCl solution. Afterwards some size distribution, dialysis and UV-vis spectrophotometry measurements were conducted.

4.4. Dialysis

To ensure that the drugs were present in the GM1 micelles and to remove DMSO or acetonitrile from drug complexes, the solutions were dialyzed (Figure 9) overnight using a 3.5 kDa cutoff membrane against ~100-fold volume MilliQ water or 0.1 M NaCl. After 4 hours, the dialysis medium was replaced with new one of the same volume.

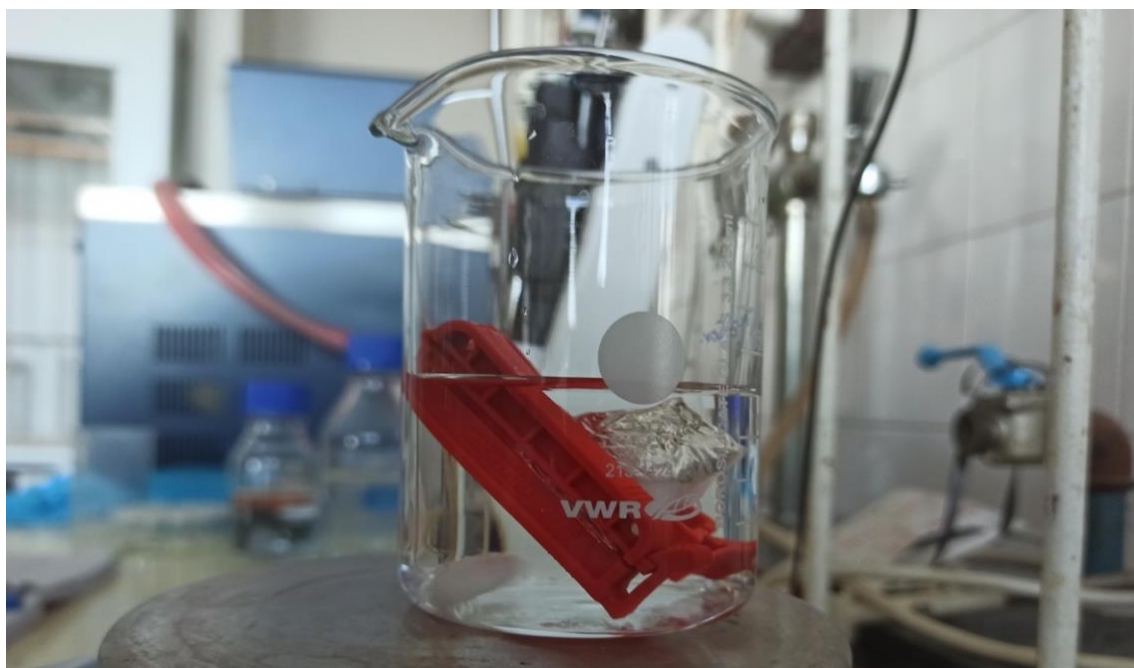


Figure 9. Membrane dialysis.

After the dialysis of GM1 complexes, the active agents were analysed for drug encapsulation efficiency in both internal and external dialysis media with UV-vis spectrophotometry of the samples.

4.5. UV-vis spectrophotometry

The **DOX** and **SOR** spectrophotometric measurements were performed with *Shimadzu UV1800* UV-VIS two-beam spectrophotometer at 25°C using quartz cuvettes with an optical path length of 1 cm. The results were obtained with *UVProbe* software running on Windows OS. **DOX** concentration was determined in the dialysis membrane by UV-vis spectrophotometry at its absorbance maximum of 486 nm.

TMZ and **PTX** spectrophotometric measurements were performed with the *Thermo Scientific Multiscan SkyHigh Microplate* UV-VIS spectrophotometer at the room temperature. The fundamental difference between two mentioned spectrophotometers is that the second one can assess up to 96 sample during one measurement, as shown in Figure 10.

The applied wavelength range for the experiments with **TMZ** and **PTX** was from 200 nm to 400 nm due to the drugs maximum absorbance values which are 330 and 230 nm respectively. At the beginning the empty plate was measured to eliminate the sample contamination and for accurate evaluation of absorbance values. The UV-vis spectrophotometry was achieved with calibration in the applied solutions.

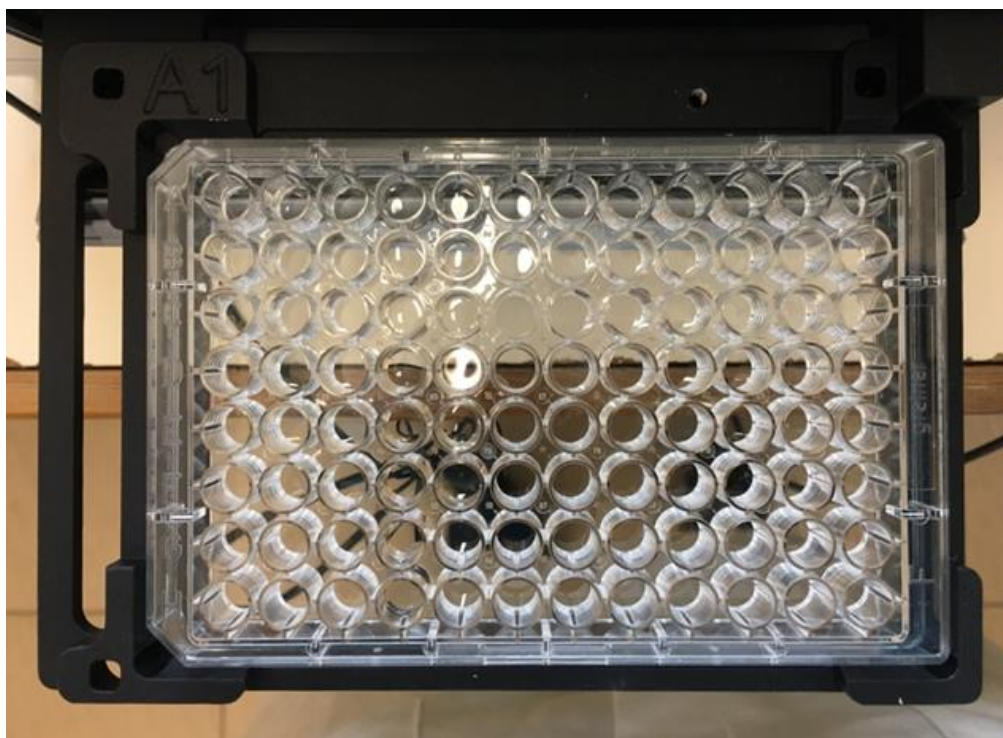


Figure 10. *Thermo Scientific Multiscan SkyHigh UV-vis spectrophotometer microplate.*

5. Results and discussion

5.1. GM1 nanomicelle examination

50 mg/ml GM1 solution was prepared in 0.1M NaCl, since it was experienced in preliminary investigations that the micelle formation occurs in the presence of sufficient salt. Monomodal size distribution was obtained for GM1 nanomicelles as shown in Figure 11.

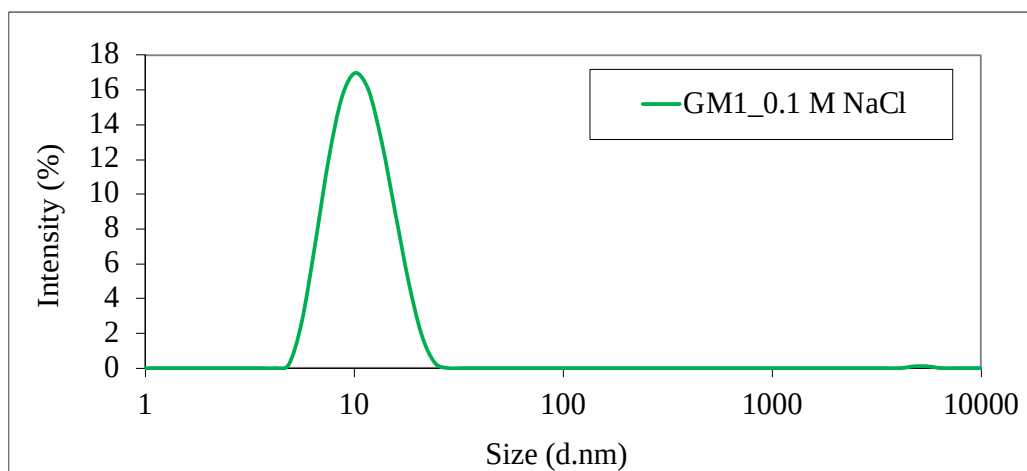


Figure 11. Size distribution of 50 mg/ml GM1 micelles by intensity in 0.1 M NaCl solution.

The average hydrodynamic diameter of the produced GM1 ganglioside nanomicelles was determined to be **10.0 nm**, which is in good agreement with the literature data [27]. The PDI in the investigated model was **0.116**, indicating that the size distribution of nanomicelles is monodisperse ($PdI < 0.2$). Thus, the desired size ganglioside nanomicelles with a narrow size distribution were successfully synthesized.

A 10x dilution with MilliQ water was prepared to reduce the NaCl concentration from 100 mM to 10 mM. The micelle size increased to **24.6 nm** and the PDI to **0.278** in this case, and a second peak appeared in the micron size range (Figure 12). This indicates that the salt concentration significantly affects micelle formation.

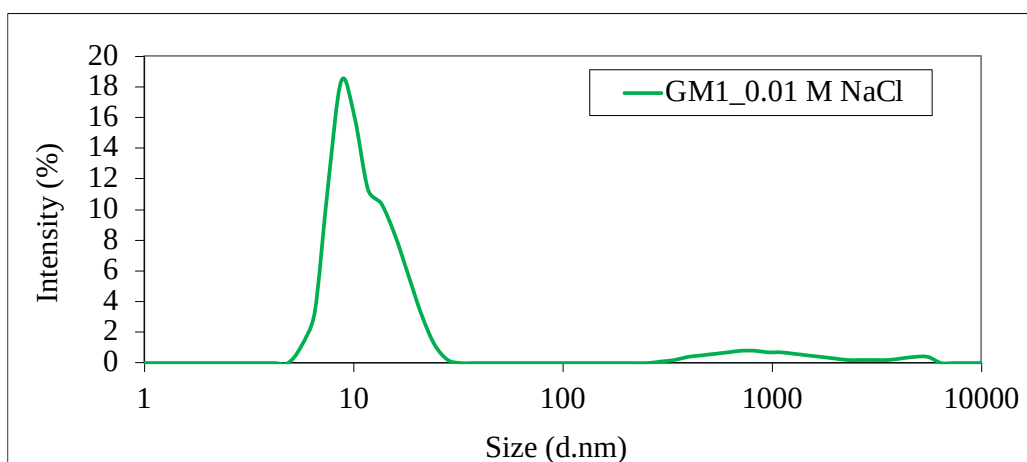


Figure 12. Size distribution of 5 mg/ml GM1 micelles by intensity in 0.01 M NaCl solution.

At a concentration of 50 mM salt, **10.25 nm** and **0.180** PDI were obtained. A negligible second peak was visible at this concentration, nevertheless, the size distribution was monodisperse (Figure 13).

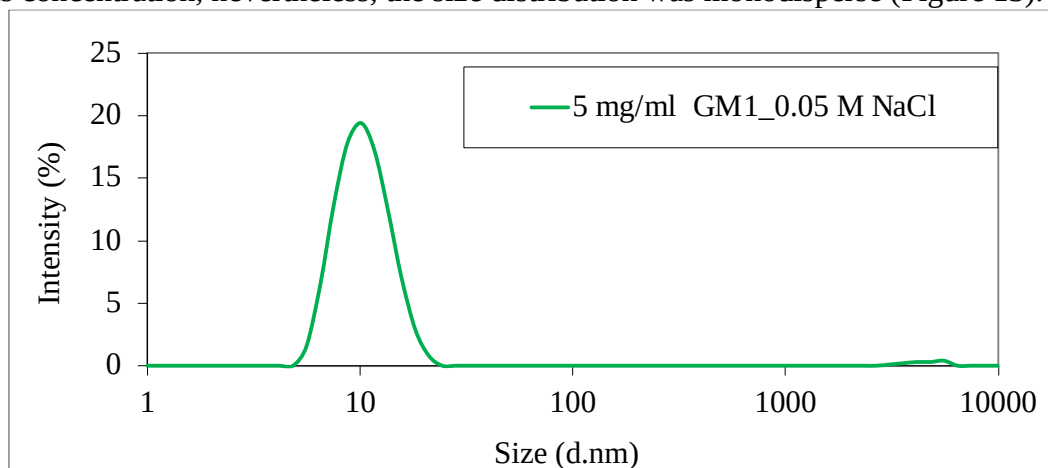


Figure 13. Size distribution of 5 mg/ml GM1 micelles by intensity in 0.05 M NaCl solution.

S/TEM imaging showed similar size of GM1 nanomicelles (Figure 14) to that found by DLS (Figure 13). It can be concluded that 50 mM NaCl concentration was sufficient to prepare homogeneous and monomodal nanomicelles of GM1 ganglioside.

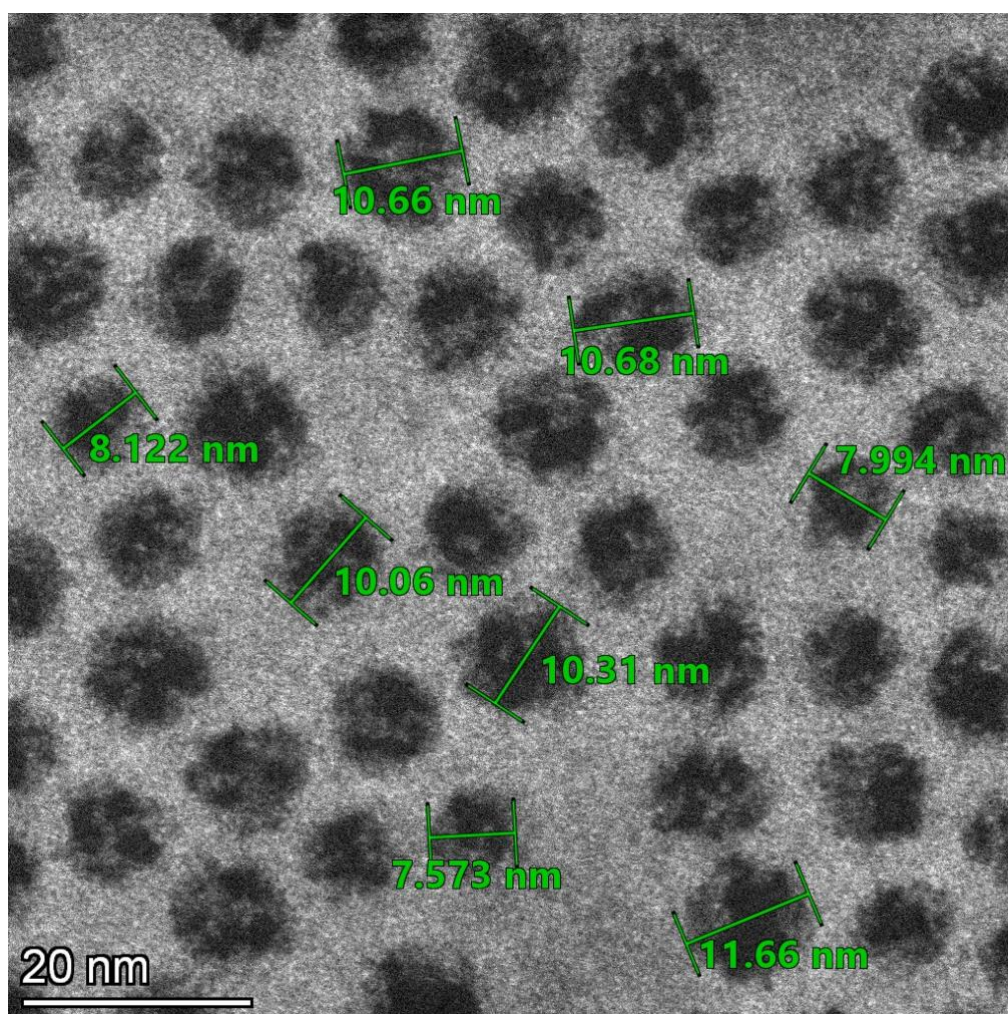


Figure 14. S/TEM image of GM1 nanomicelles.

5.2. Entrapment of doxorubicin and sorafenib model drugs

Following the microencapsulation of anticancer drugs doxorubicin and sorafenib, the physical changes in nanomicelle properties were analysed.

5.2.1. *Size distribution via DLS*

The addition of 5 mg **DOX** to 50 mg/ml GM1 solution, a micelle size of **10.6 nm** and a slightly polydisperse distribution with **0.209** PdI were obtained, along with the appearance of a small second peak in the micron size range. The size distribution did not change significantly from that of pure GM1 nanomicelles (Figure 15).

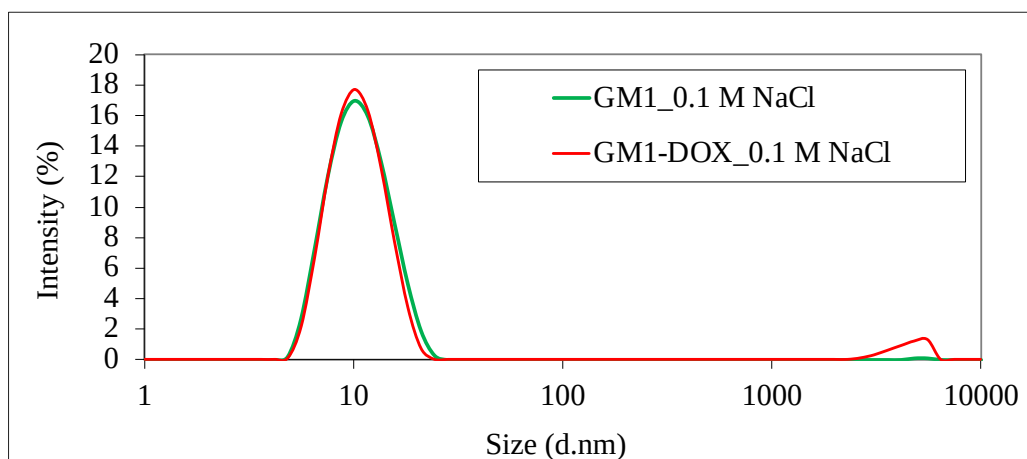


Figure 15. Size distribution of GM1 micelles at a concentration of 50 mg/ml in 0.1 M NaCl (red line) and the same including 5 mg/ml DOX (green line).

Adding 5 mg/mL **SOR** to a 50 mg/mL GM1 solution increased the average micelle size to **2702 nm** and the PdI to **0.818**. These data also showed the presence of extremely polydisperse sorafenib-containing micelles, as seen in Figure 16.

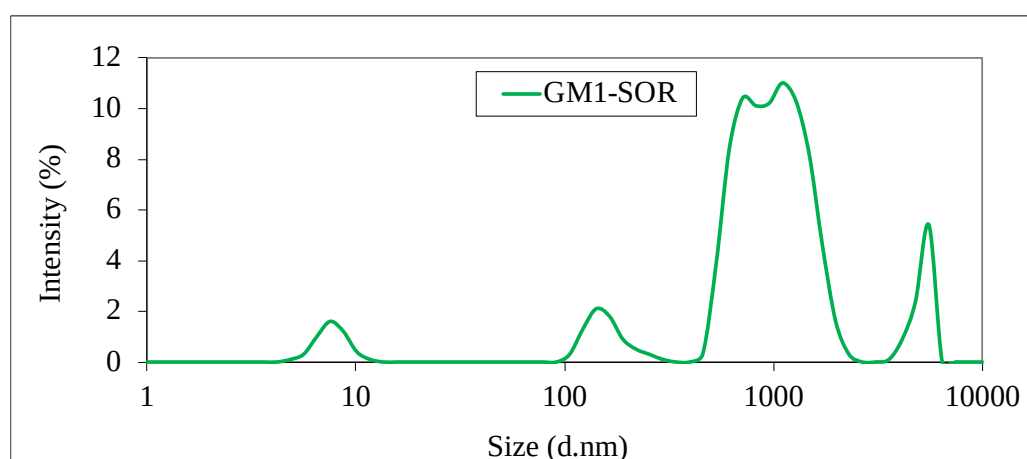


Figure 16. Size distribution of GM1 micelles with SOR at a GM1 concentration of 50 mg/ml and 5 mg/ml SOR.

The above-mentioned **SOR** measurement was repeated twice on subsequent days, and the average sizes were **2637 nm** and **2264 nm** with **0.650** and **0.680** PdI values respectively. Over time, a decrease can be observed for both parameters; nevertheless, it did not provide monomodal nanomicelles. After centrifuging the sample, it was found that the pellet partially immediately redispersed (Figure 17), indicating the fraction with low size.

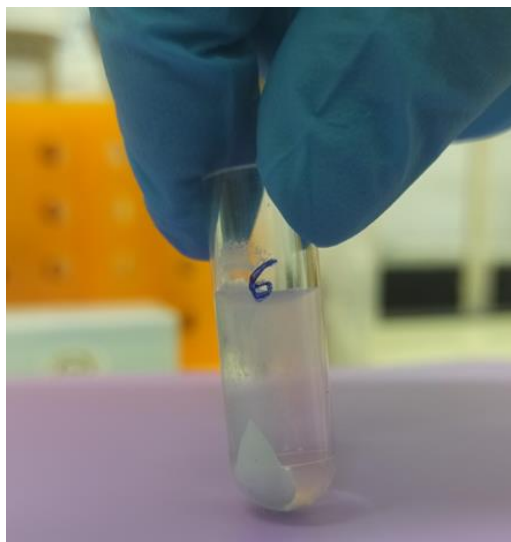


Figure 17. GM1-SOR solution after ultracentrifugation.

The redispersed sample in MilliQ was therefore dialyzed to remove the DMSO from the system. 1 ml sample was dialysed against a SnakeSkin (3.5 kDa) dialysis membrane with 100 ml distilled water as the external phase. Dialysis was maintained overnight, ca. for 24 hours, with magnetic stirring (120 rpm).

Addition of doxorubicin enhanced the size even more (Figure 18) to an average diameter of **3462 nm**; however, a monomodal size distribution was resulted and the PdI was reduced to **0.346**.

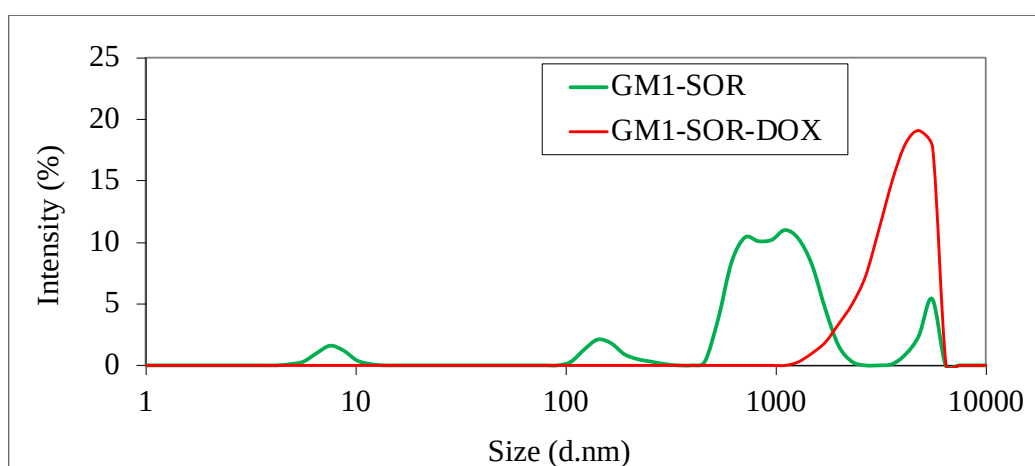


Figure 18. Size distribution of GM1-SOR solution at a GM1 concentration 50 mg/ml and 5 mg/ml **SOR** in 0.1 M NaCl (red line) and the same including also 5 mg/ml doxorubicin (green line).

S/TEM images of sorafenib-loaded GM1 micelles revealed aggregates, supporting the DLS results (Figure 19).

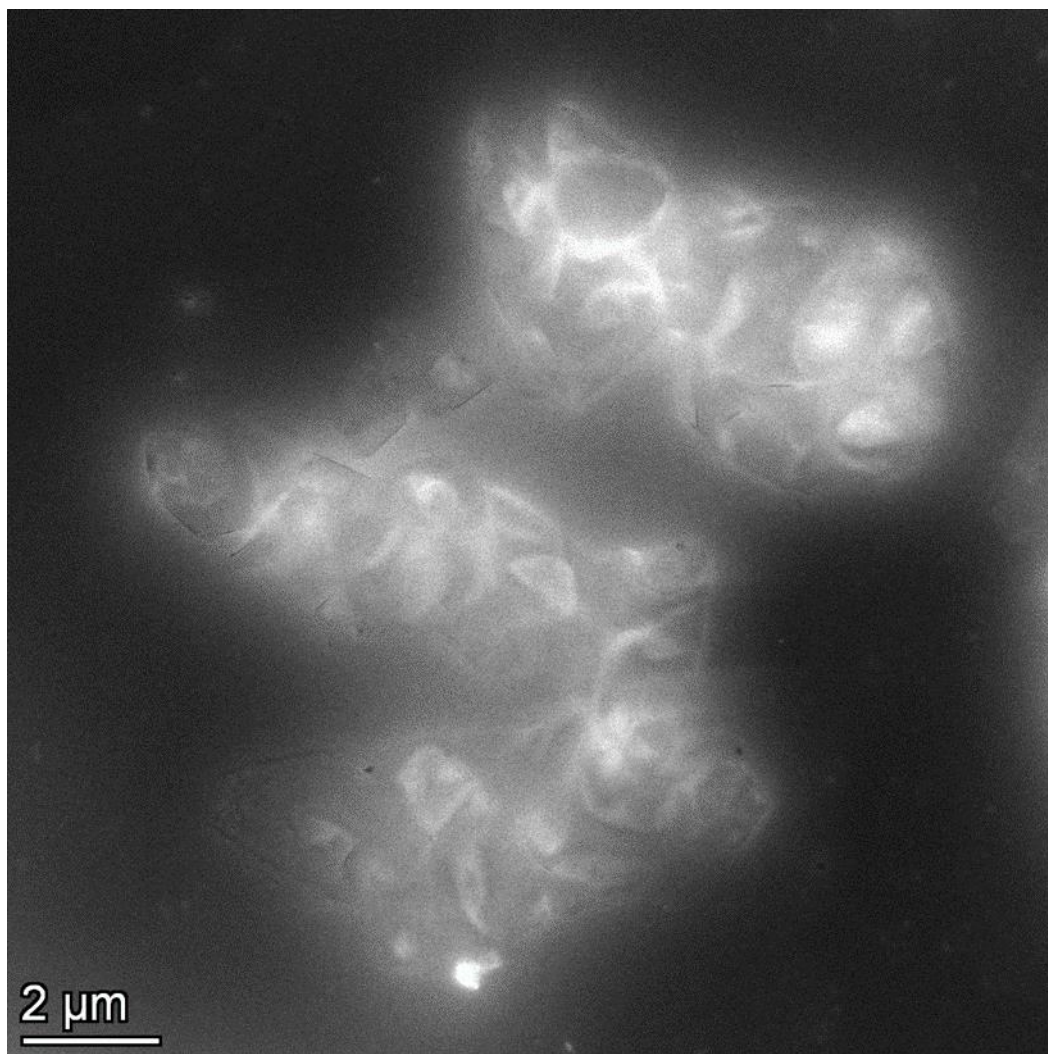


Figure 19. S/TEM image of GM1-SOR micelles.

5.2.2. Zeta potential determination

The GM1 zeta potential was measured in the Zeta Dip Cell and the disposable measuring cell at 50 mg/ml concentration in 0.1 M NaCl. The zeta potential was -9 mV.

To reduce the NaCl concentration from 100 mM to 10 mM, the solution was diluted with MilliQ ten times. In this case, there was no micelle production, indicating that the lower concentration of NaCl was not sufficient for the micelle formation.

In order to obtain appropriate conditions for the zeta potential measurements, the salt concentration was increased to 50 mM. The zeta potential decreased to -12 mV. The encapsulated **DOX** anticancer drug carrier had a zeta potential of -13.8 mV. Due to the polydispersity of sorafenib-loaded micelles, zeta potential was not detectable. These zeta potential values did not indicate charge stabilization as expected, since the nanomicelles trigger steric stabilization.

5.2.3. UV-vis spectrophotometry of doxorubicin

The concentration of microencapsulated **DOX** was determined using *Shimadzu UV1800 spectrophotometer* and *UVProbe software* at the absorbance maximum of 486 nm after dialysis.

GM1-DOX calibration was prepared in MilliQ within the detectable linear calibration range (5-40 µg/ml). The calibration curve is shown in Figure 20.

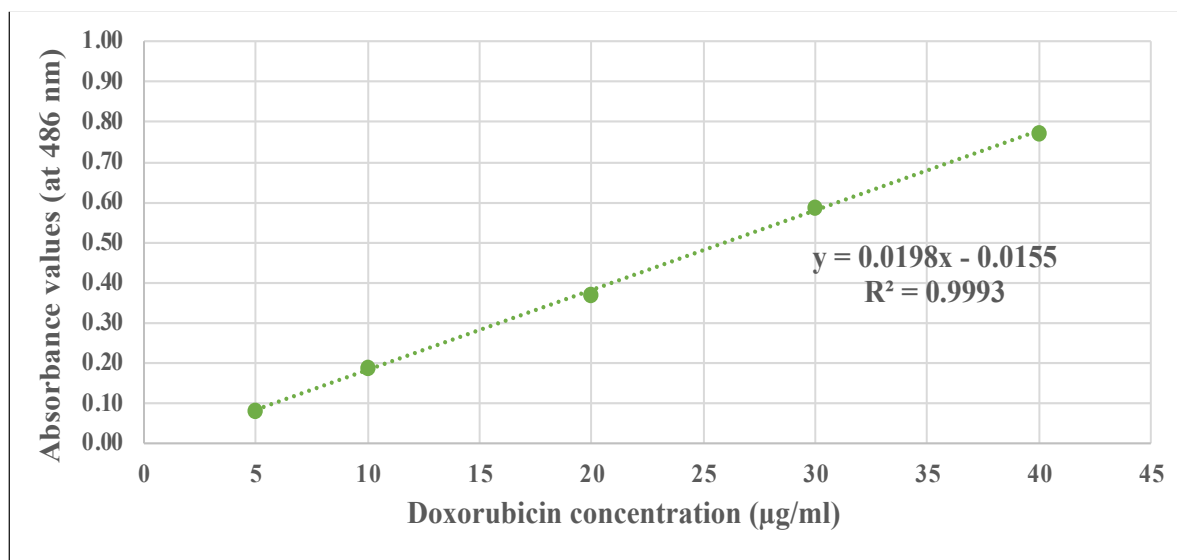


Figure 20. GM1-DOX calibration.

From the measured absorbance, the **DOX** concentration in the dialysis membrane was calculated. The initial drug concentration was 5 mg/ml which decreased to 4.7 mg/ml during dialysis also considering the dilution. This means 4.7 mg/ml **DOX** was retained by GM1 micelles in the dialysis tube.

Regarding to this it is possible to determine the anticancer **DOX** drug encapsulation efficiency using the following calculations

$$\text{DOX encapsulation efficiency} = \frac{\text{DOX corrected by dialysis dilution}}{\text{DOX initial concentration}} * 100\%$$

From this equation a high encapsulation efficiency of 94.2% can be calculated for doxorubicin by GM1 gangliosides.

Due to the unacceptable size of GM1-SOR and GM1-DOX-SOR, the **SOR** encapsulation efficiency was not investigated.

5.3. Entrapment of temozolomide and paclitaxel

Microencapsulation of temozolomide and paclitaxel, which are potential drugs against brain tumours, have also been studied by GM1 nanomicelles.

5.3.1. Size distribution by DLS and zeta potential measurements

Firstly, the size of temozolomide in the presence of 100 mg/ml GM1-MilliQ solution was investigated. Adding 5 mg **TMZ** to 100 mg/ml GM1-MilliQ water solution, an average micelle diameter of **45.1 nm** and a polydisperse size distribution with **0.273** PDI were obtained, along with the appearance of few smaller peaks (Figure 21). Even though the solution looked completely homogeneous, micelle formation was excluded considering the size distribution, which was similar to that of GM1 solution in MilliQ without NaCl.

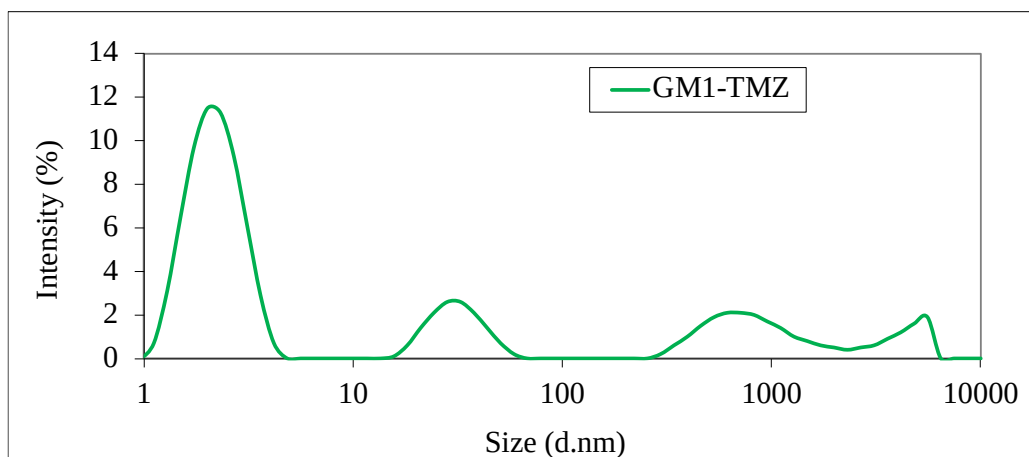


Figure 21. Size distribution of GM1 micelles at a concentration of 100 mg/ml in MilliQ water including 50 mg TMZ.

Hence, the size distribution of GM1-**TMZ** in the presence of 0.1 M NaCl was tested as well using *Thermo Scientific Multiscan SkyHigh* UV-vis spectrophotometer. Adding 2.5 mg **TMZ** to 1 ml 50 mg/ml GM1-NaCl solution decreased the average hydrodynamic diameter of the ganglioside nanomicelles to **9.9 nm** and the PDI value to **0.11** (Figure 22).

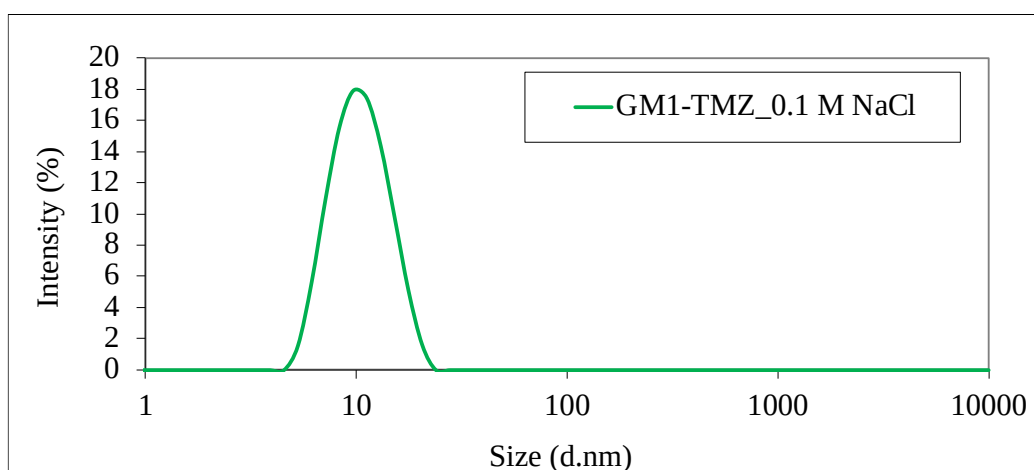


Figure 22. Size distribution of GM1 micelles at a concentration of 50 mg/ml in 0.1 M NaCl solution including 2.5 mg TMZ.

The PDI value of GM1-NaCl-TMZ solution indicated that the size distribution of the nanomicelles was monodisperse (PDI<0.2). The physical properties of the GM1 nanomicelles didn't change by adding **TMZ**. The drug was completely dissolved rapidly with stirring.

Due to the fact that paclitaxel is insoluble in water, 100 μ l 50 mg/ml PTX-DMSO solution was prepared to dissolve the drug. Subsequently the sample was microencapsulated by mixing it with 1 ml 50 mg/ml GM1-MilliQ solution. The solution was dialyzed overnight and the size distribution was measured afterwards. The measured hydrodynamic diameter of **6472 nm** and **0.236** PDI value were registered (Figure 23). It seemed that due to the lack of salt, micelles could not be formed.

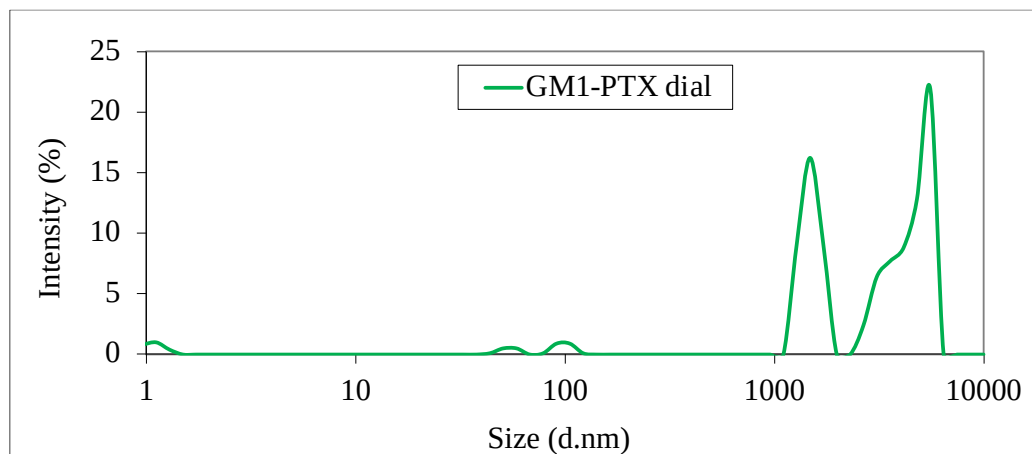


Figure 23. Size distribution of GM1 micelles at a concentration of 50 mg/ml in MilliQ water including 100 μ l 50 mg/ml PTX-DMSO solution.

Referring to the fact that both **TMZ** and **PTX** microencapsulated by GM1 gangliosides might create drug complexes, further experiments were conducted to increase the anticancer drugs efficiency.

5 mg **PTX** in 0.1 ml DMSO was added to 1 ml 2.5 mg/ml TMZ-GM1 solution in order to co-encapsulate the two drugs by GM1.

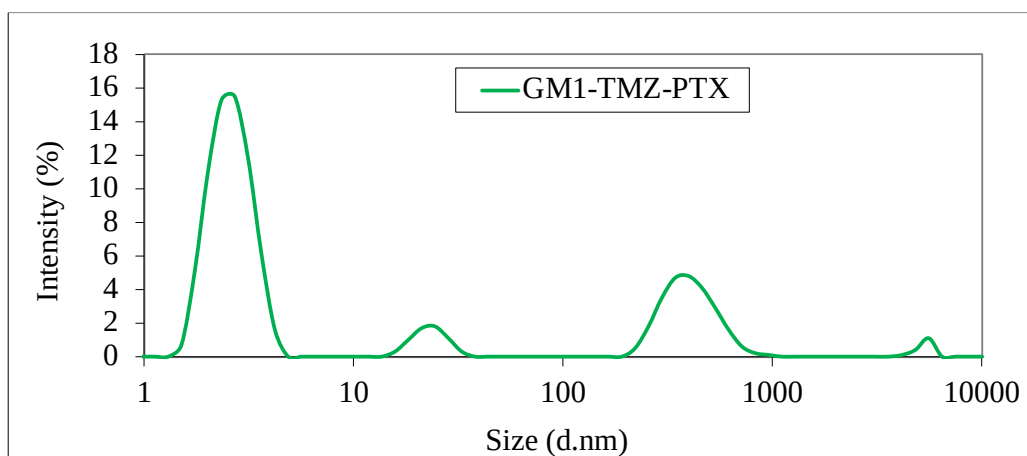


Figure 24. Size distribution of GM1 micelles at a concentration of 2.5 mg/ml TMZ-GM1 solution in 0.1 M NaCl solution including 100 μ l 50 mg/ml PTX-DMSO solution.

Figure 24 shows that nanomicelles did not form. Thus, according to these results, the desired complex must be created in different concentrations or in the presence of other solvents.

The zeta potential was -18.3 mV, which shows some surface charge of the system, however, in the lack of nanomicelles the solution was not evaluated to be useful for further experiments with this composition.

To promote micelle formation, 0.1 M NaCl was included in the solution above. The ability of both **TMZ** and **PTX** microencapsulated with GM1 to create drug-loaded nanomicelles was investigated in two ways. In the first case 5 mg **PTX** in 0.1 ml acetonitrile solution was added to 1 ml of 2.5 mg/ml TMZ-GM1 solution in the presence of 0.1 M NaCl. However, this sample was inappropriate for further measurements due to the low solubility and precipitation of NaCl in acetonitrile.

In the second case 5 mg **PTX** dissolved in 0.1 ml DMSO solution was added to 1 ml of 2.5 mg/ml TMZ-GM1-NaCl.

The average hydrodynamic diameter of GM1 ganglioside nanomicelles in the presence of **TMZ** and **PTX** drugs was determined to be **11.1 nm** (Figure 25). The PDI value in the investigated composition was **0.231** indicating that the size distribution of nanomicelles is slightly polydisperse (PDI>0.2) due to the second peak in the micron size range. However, it could be stated that according to the above-mentioned results the desired size ganglioside nanomicelles with a narrow size distribution were successfully created. The experiment was followed by dialysis and UV-vis spectrophotometry measurements.

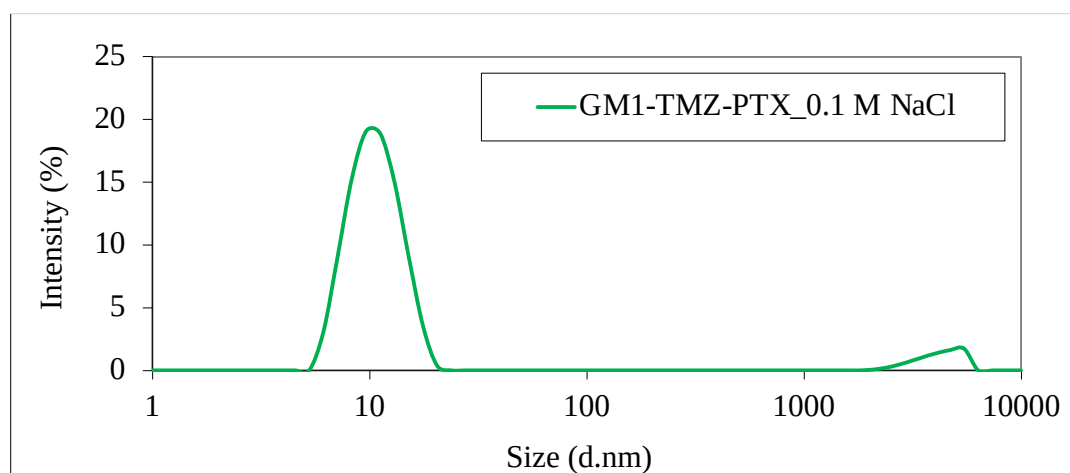


Figure 25. Size distribution of GM1 micelles at a concentration of 2.5 mg/ml TMZ-GM1 in the presence of MilliQ water including 1 μ l 5 mg/ μ l PTX-DMSO solution.

5.3.2. UV-vis spectrophotometry

The formation of GM1 nanomicelles with **PTX** and/or **TMZ** was investigated by UV-vis after dialysis to remove the free drug.

The concentrations of microencapsulated **TMZ** and **PTX** were determined using the *Thermo Scientific Multiscan SkyHigh* Microplate spectrophotometer and *Microsoft Excel* software in the dialysis membrane at the absorbance maximum of 330 and 240 nm, respectively.

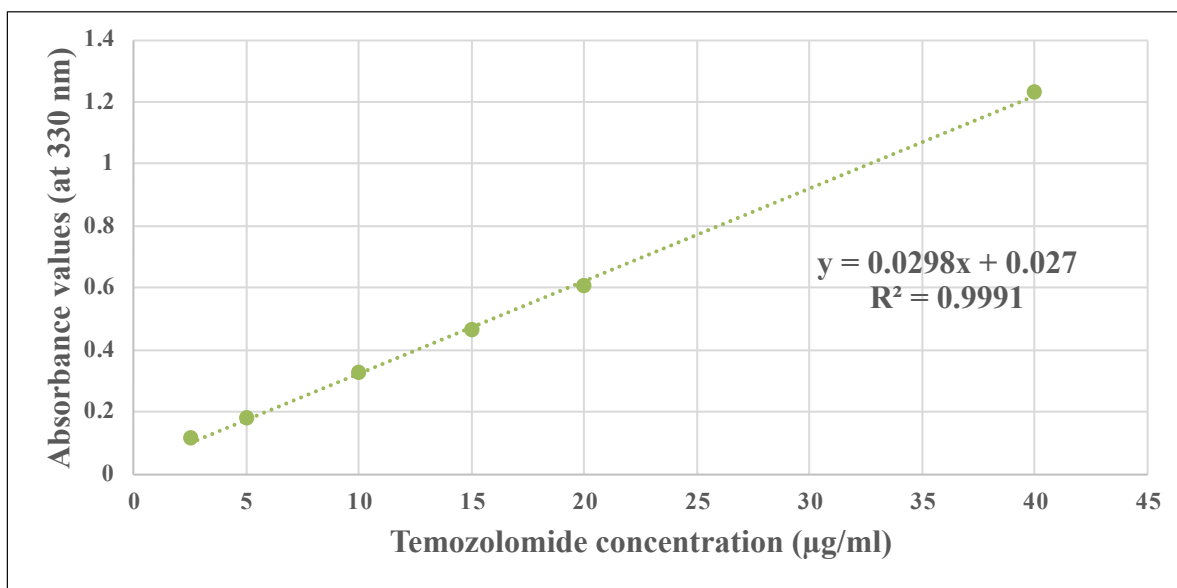


Figure 26. Calibration curve of TMZ present in pure 0.1 M NaCl.

TMZ calibration shown in Figure 26 was prepared within the detectable linear calibration range (2.5 – 40 µg/ml). The internal dialysed phase of 2.5 mg/ml TMZ-GM1-PTX-DMSO solution in pure 0.1 M NaCl including 0.1 ml 50 mg/ml PTX-DMSO [solution](#), the 10-fold and 100-fold dilution of it and both external dialysis phases were measured as well.

The **TMZ** concentration in the dialysis membrane was calculated from the measured absorbance values. The initial drug weight was 2.5 mg, which decreased to 2.52 µg during dialysis considering the dilution.

This indicates that **TMZ** was not entrapped by ganglioside micelles.

PTX calibration was prepared in acetonitrile within the detectable linear calibration range from 0 µg/ml to 40 µg/ml. Further calculations for the **PTX** encapsulation efficiency with GM1 nanomicelles were completed from the calibration curve shown in Figure 27.

The internal dialysed phase of 100-fold diluted GM1-TMZ-PTX was measured with spectrophotometer to calculate the **PTX** encapsulation efficiency. The **PTX** amount in the dialysis membrane was calculated to be 4.15 mg, while the initial drug amount was 5.0 mg, thus the **PTX** drug encapsulation efficiency with GM1 nanomicelles was 83.0%.

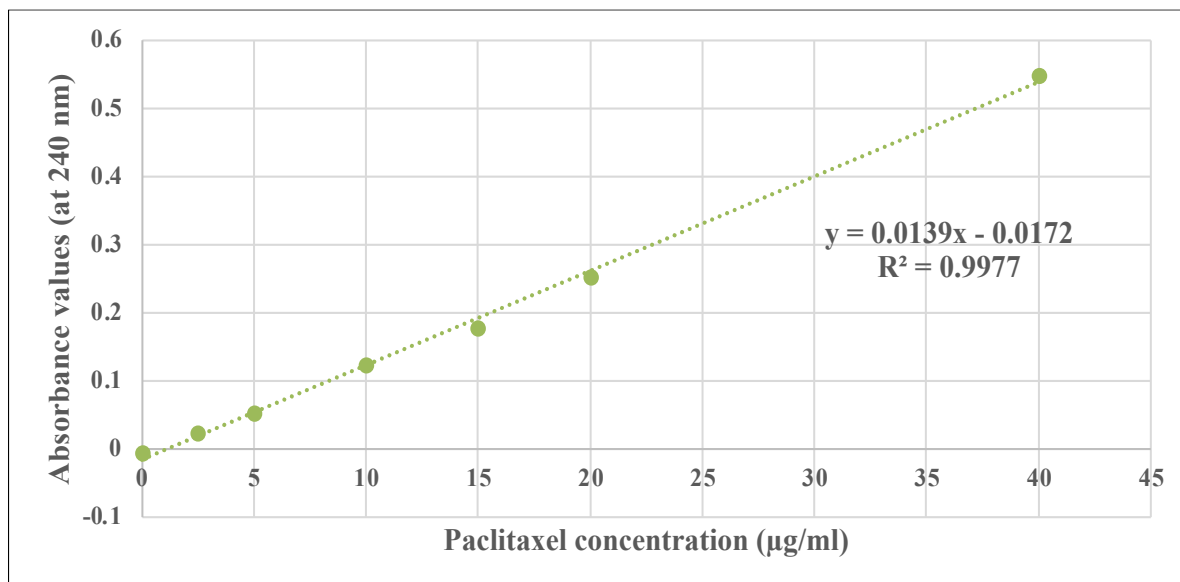


Figure 27. Calibration curve of PTX present in acetonitrile.

6. Summary

The structure of the GM1 ganglioside, as well as its physical and chemical characteristics in response to salt concentration were investigated. Diverse size distributions and zeta potentials of GM1 at various salt concentrations were measured to determine this relation. According to the wide range of results, the GM1 micelle stability was highly dependent on the solution salt concentration.

Most of the cancer treatments, according to the literature, are unable to pass the BBB, while GM1 gangliosides have been demonstrated in several studies to be capable to pass the BBB. The task of my research was to microencapsulate doxorubicin and sorafenib model anticancer drugs, as well as temozolomide and paclitaxel drugs against brain tumours in self-assembled micelles capable of crossing the BBB. The physical properties of encapsulated anticancer drug carriers and their drug encapsulation efficiencies were also investigated.

The addition of doxorubicin had no significant effect on the monodisperse size distribution of GM1 nanomicelles. To ensure that the doxorubicin was present in the GM1 micelles, the solution was dialyzed by a membrane of 3.5 kDa cutoff. The doxorubicin encapsulation efficiency with GM1 was determined to be 94.2 %.

Sorafenib in DMSO solution significantly enhanced micelle size in the presence or absence of doxorubicin. S/TEM pictures also revealed a significant increase in micelle size. The size of GM1-sorafenib aggregates is insufficient for further experiments, since they probably cannot cross the BBB.

The paclitaxel encapsulation efficiency with GM1 in acetonitrile was determined to be 83.0 %. Temozolomide and paclitaxel co-encapsulation was investigated; however, the gangliosides were unable to encapsulate the temozolomide, and the drug crossed the membrane after dialysis.

Based on these results, it may be beneficial to attempt to covalently bind temozolomide with a carboxyl-to-amine reactive cross-linker in order the GM1 nanomicelles include it.

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