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Gentamicin-Modified Nanocarriers For Placental Targeted Drug Delivery To Treat Pregnancy-Related Complications

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GENTAMICIN-MODIFIED NANOCARRIERS FOR PLACENTAL TARGETED DRUG DELIVERY TO TREAT PREGNANCY-RELATED COMPLICATIONS

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^{1}H NMR	proton nuclear magnetic resonance
1xHBSS	1x Hank's Buffered Saline Solution
AHA-Boc	6-(Boc-amino)hexanoic acid
BOC	Di-tert-butyl dicarbonate
Bis-MPA	2,2-bis(hydroxymethyl)propionic acid
BSA	Bovine serum albumin
CHOL	Cholesterol
CY5.5	Cyanine 5.5
Da	Dalton
DCM	Dichloromethane
DHB	2,5-dihydoxybenzoic acid
DI water	deionized water
DIPEA	N,N-Diisopropylethylamine
DLS	dynamic light scattering
DMAP	4-(Dimethylamino)pyridine
DMEM	Dulbecco's Modified Eagle Medium 1x high glucose
DMF	N,N-Dimethylformamide
DMSO	Methylsulfoxide
DNCs	dendrimer nanocarriers
DSPE	1,2-Distearoyl-sn-glycero-3-phosphoethanolamine
EDC	N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide

EDTA	Ethylenediaminetetraacetic acid
EE	Encapsulation efficiency
eGFP	enhanced Green Fluorescent Protein
EMEM	Eagle's Minimum Essential Medium
Ex/Em	Excitation/Emission
FACS	flow associated cell sorting (flow cytometry)
FBS	Fetal bovine serum
FITC	fluorescin isothiocynate
G4NH2	Generation 4 amine-terminated PAMAM dendrimer
GD	Gestational day
GM	Gentamicin
G4OH	Generation 4 polyester dendrimer
HCl	Hydrochloric acid
HD	Hydrodynamic diameter
HepG2	Hepatocellular carcinoma
HSPC	Hydrogenated soybean Phosphatidylcholine
ICC	Immunocytochemistry
IUGR	Intrauterine Growth Restriction
IV	intravenous
IVIS	in vivo imaging system
LA	lauryl alcohol
LS	light scattering
MALDI-TOF	Matrix-Assisted Laser Desorption Ionization Time-of-Flight

MeOH	methanol
MFI	Median Fluorescence Intensity
mRNA	messenger RNA
MWCO	Molecular Weight Cut Off
NHS	N-Hydroxysuccinimide
PAMAM	poly(amidoamine)
PBS	Phosphate buffer saline
PE	preeclampsia
PEG	polyethylene glycol
PNCs	polymeric nanocarriers
SA	succinic anhydride
SD	Standard deviation
SEM	scanning electron microscopy
siRNA	short interfering RNA
SynT	syncytiotrophoblast
TEER	transepithelial electrical resistance
TV	tail vein
ULV	unilamellar liposomes

CHAPTER 1

INTRODUCTION

1.1 Overview

Nanomedicine has recently gained popularity as a medical application of nanotechnology in order to aid in the development of medical treatment, diagnosis, monitoring, and control of biological systems.(Moghimi et al., 2005) Drug delivery using nanocarriers has many potential advantages as for example more efficient formulation, reduced side effects, higher bioavailability, longer circulation and improved pharmacokinetics and biodistribution(Mirza and Siddiqui, 2014). Nanocarriers can be devised from natural or synthetic, organic or inorganic materials such as dendrimers, liposomes, gold nanoparticles, protein-based carriers, and viral-derived nanoparticles(Mirza and Siddiqui, 2014; Moghimi et al., 2005). Many important advances in nanotechnology have been already translated into medical research; several are FDA approved and commercially available for treatment of different types of diseases such as cancer,(Miele et al., 2009; Muggia, 2001; Wagner et al., 2006) hepatitis,(Zeuzem et al., 2000) fungal infections,(Bawa, 2008) pain and influenza(Faraji and Wipf, 2009). Despite the progress accomplished in a wide spectrum of diseases in the area of nanomedicine, there are no clinical applications of nanocarriers to prevent or treat pregnancy-related complications, which begs the question: can we use nanomedicine for the treatment of fetal and/or maternal complications? In this introductory chapter I review the human placental tissue and its contribution to pregnancy related complications; as well as a detailed comparison to mouse

placenta, an analogous in vivo model. I also review the use of nanocarriers as drug delivery tools to the placenta, focusing on the platforms we used: liposomes and dendrimers and how we aim to utilize them to enhance the delivery of therapeutics to the placenta in order to treat pregnancy related complications that are caused by placental malfunction.

Pregnancy-related complications are common, affecting more than 20% (approximately 26 million) of all pregnancies.(Duley, 2009) Some of those complications include preeclampsia (PE), intrauterine growth restriction (IUGR), gestational diabetes, fetal hypothyroidism, bacterial and viral infections(McDonagh et al., 2004; Racicot and Mor, 2017; Silasi et al., 2015). Many of such complications result from compromised functions of the placenta, some of which can lead to maternal and/or fetal mortality if not controlled(Thornburg and Marshall, 2015). Administration of medications in the maternal circulation can treat some diseases of pregnancy. Maternal steroids, for instance, are used to accelerate fetal lung maturation(Roberts et al., 2017). Cardiac drugs can also be used similarly to treat fetal arrhythmias(Oudijk et al., 2000). Levothyroxine can be invasively injected to the amniotic fluid, although being invasive and a less desirable route, to treat fetal hypothyroidism(Gulraze et al., 2013). On the other hand, no effective treatments exist to treat several other complications of the pregnancy, including PE and IUGR.(Romo et al., 2009) In the case of PE, for instance, labor is the best option to mitigate the disease,(McCoy and Baldwin, 2009) however, that is not always a possibility. Therefore, hypertension medications are currently used to lower high blood

pressure associated with PE(Roberts and Gammill, 2005). Anticonvulsant medications are used if PE is severe(Sibai, 2004; Zuspan, 1966). Life style changes such as bed rest is also often required. To understand many of those diseases and be able to devise a strategy to approach them, it is essential to understand the anatomy and physiology of the placenta as well as the pathogenesis of such diseases.

1.2 Understanding the Placenta

1.2.1 Functions:

Placenta is a specialized organ that is present only during pregnancy. At term, the human placental weight is approximately 500 g, has a diameter of 15–20 cm and thickness of 2–3 cm.(Griffiths and Campbell, 2014) It functions by separating two completely distinct circulations: maternal and fetal. It serves several functions that contribute to the synchronization between pregnant mothers, the fetus and the placenta itself.(John and Hemberger, 2012) It is responsible for gas exchange between the two circulations and providing nutrients from the maternal circulation to the fetal one as well as eliminating the waste from the fetal circulation.(Nelson, 2015) The placental tissue also serves as a barrier protecting the fetus from maternal infections and diseases, as well as certain potentially harmful molecules. It is also a major site for metabolism. It can metabolize several drugs and biologics.(Donnelly and Campling, 2014) Moreover, the placenta is also a major endocrine organ that secrets more than 100 peptide and steroid hormones to the fetal or maternal circulations in order to

modulate different functions such as fetal growth, metabolism and parturition.(Burton and Jauniaux, 2015; Gude et al., 2004)

1.2.2 Development and Anatomy:

Placentation starts after implantation of the blastocyst (a newly developed fertilized egg), where trophoblast cells begin proliferating and migrating to the decidua part of the endometrium.(Wooding and Flint, 1994) Part of the proliferating trophoblast starts fusing together to form multinucleated syncytiotrophoblast (SynT), which grow invaginations in the endometrium(Carter, 2007). Such invaginations make up independent functional vascular units called cotyledons. The villous trees located within the cotyledons separate maternal circulation from fetal circulation. The villus is composed of four layers: the SynT
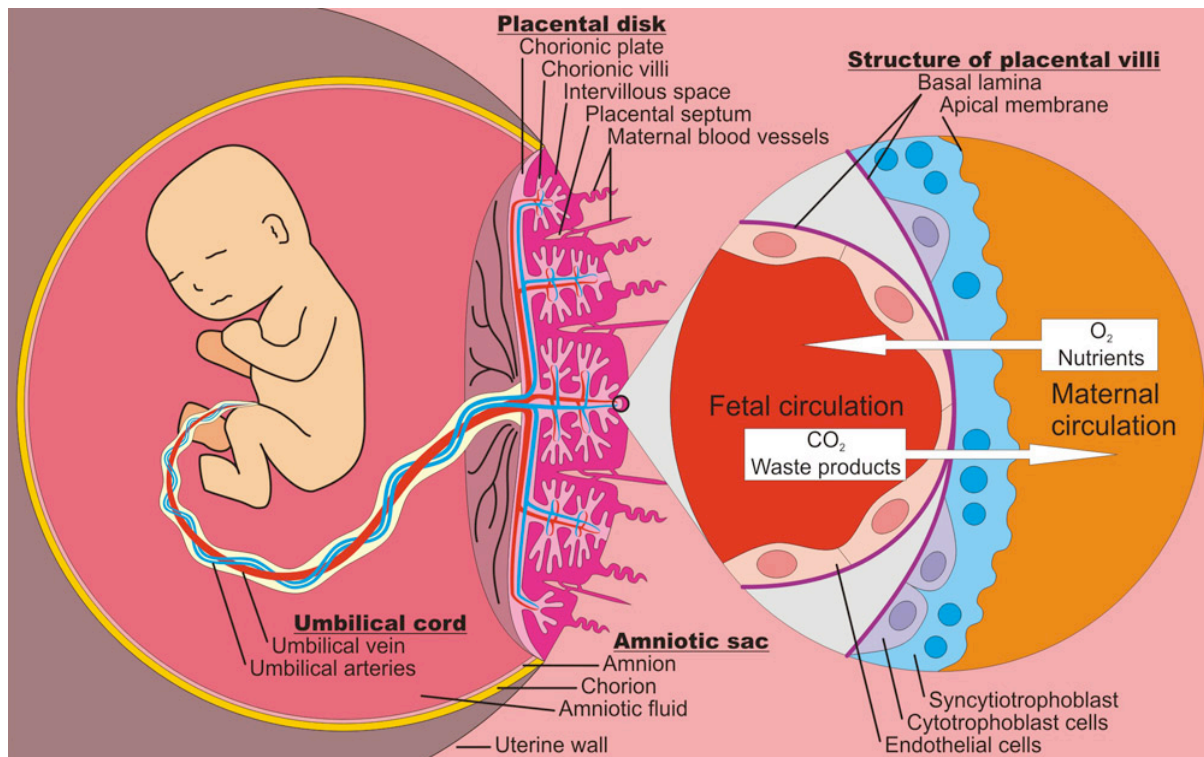
Figure 1.1 Schematic drawing of a full-term placenta with a close-up to the placental villi showing the SynT layer. http://pimeonline.blogspot.com

layer, cytotrophoblast cells, connective tissue of the villus and the endothelium of the fetal blood vessels arranged from the maternal to the fetal side, respectively. Such arrangement continues for the first 20 weeks of gestational period. However, after that, the cytotrophoblast layer disappears leaving the placental barrier with the remaining three layers, placing maternal and fetal circulations about 2 to 4 um in proximity as illustrated in **Figure 1.1**. This arrangement makes the SynT layer to become the main barrier at the maternal-fetal interface.

The SynT layer is a multinucleated epithelial layer that is in direct contact with the maternal blood and is covered on the apical (maternal) side with branched microvilli that maximize the surface area for gas and nutrient/waste exchange. The blood vessels that ramify through the villous stroma carry embryonic/fetal blood, which serves as portal to fetal tissues(Robbins et al., 2010). All these arrangements are the fetal part of the placenta, which is known as the chorionic plate. The maternal part, on the other hand, is called the basal plate. The basal plate contains the endometrium and it provides the endometrial blood vessels that would pour into the space between the chorionic plate and the basal plate; this space is known as the intervillous space. The maternal side is arranged as basal plate, the decidua basalis and the endometrium from fetal to maternal side, respectively. **Figure 1.1** provides an illustration of the arrangements of different parts of the human placenta.

1.2.3 Human VS Rodent Placenta:

The placenta in both humans and rodents serves the main purpose of providing the interface between the mother and the fetus. It is through this

interface all the functions mentioned above are accomplished. While there are many differences between the two species, there are many similarities in terms of placental development, anatomy and functions as illustrated in **Figure 1.2** (Krishnan et al., 2013). From the maternal to the fetal side, the arrangement is as follows: **First region** is the decidual basalis, which is the maternal portion of the placenta that is in direct contact with the myometrium. **Second region** is the *junctional zone* (*jz*, also know as *basal zone*) in mice and basal plate (bp, also known as *cytotrophoblastic shell*) in humans.
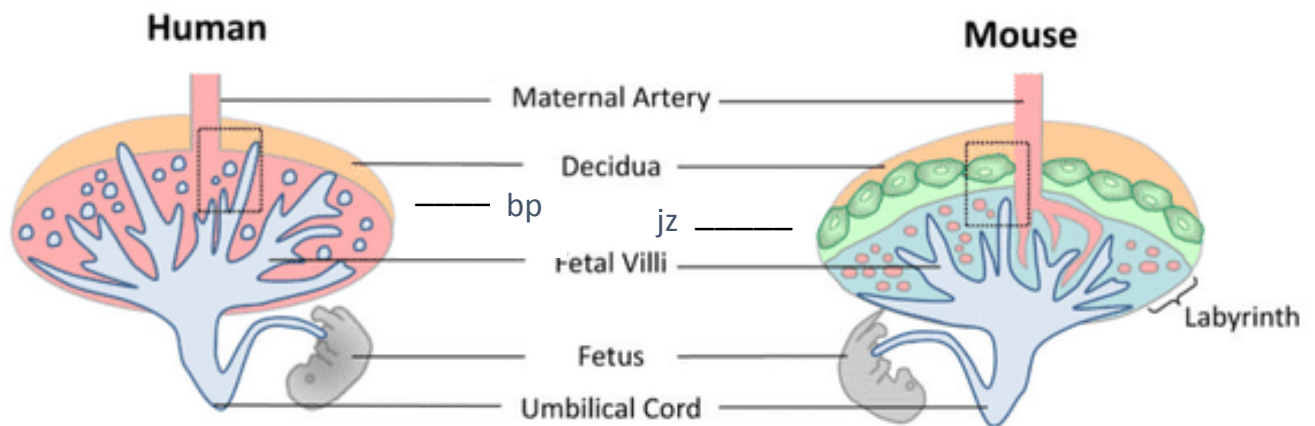
Figure 1.2 Illustration of similarities between human and mouse placentas showing the analogous regions between the two species.(Krishnan et al., 2013)

Third region is known as the *fetal placenta* in humans and *labyrinth* in mice. This region is the fetal part of the placenta and it develops from the zygote. It is within the chorionic plate and is the region at which the villous trees (chorionic villi) bathe in maternal blood within the intervillous space, as shown in **1.3** (Kulvietis et al., 2011). Within the core of the chorionic villi are the fetal blood vessels that make up the umbilical arteries and veins.

The human fetal placenta is functionally analogous the mouse labyrinth despite slight structural differences (Georgiades et al., 2002). In mice, the labyrinth consists of 3 layers of cells, and is therefore known as trichorial placenta. On the other hand, fetal placenta has one layer and is known as monochorial placenta. The three layers of the labyrinth are arranged as Layer I, (cytotrophoblast) Layer II, and III SynT from maternal to fetal side, respectively(Georgiades et al., 2002). It is important to emphasize that both Layers I and II are in contact with the maternal blood. In the human fetal placenta, which is analogous to mice labyrinth, only one SynT is present.
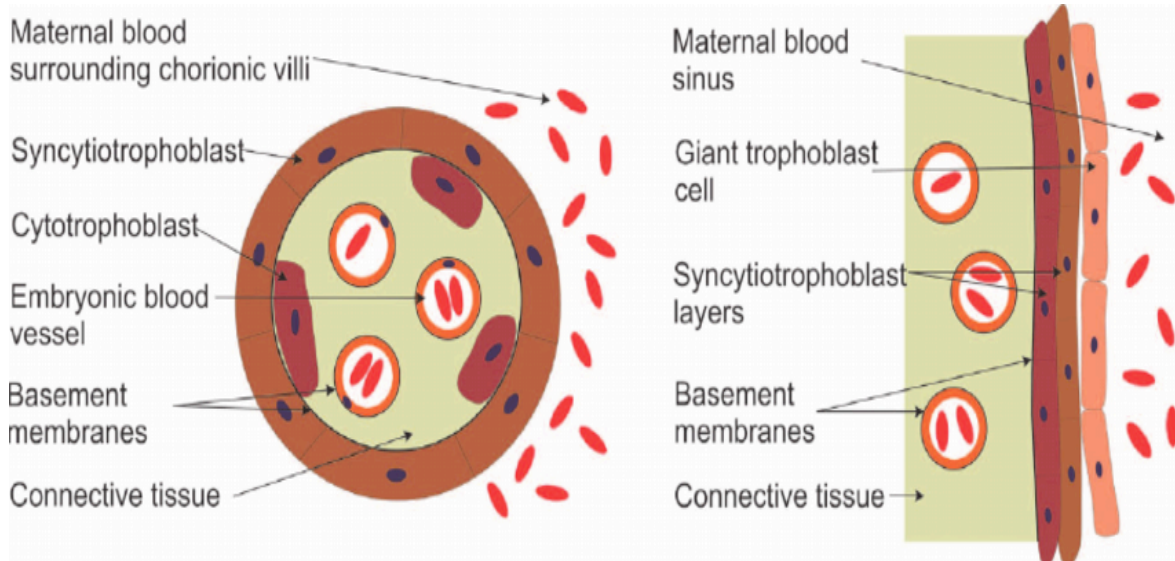
Figure 1.3 Comparative representation of human (third trimester) and rodent (GD18.5) placentas showing their differences. Left, human placenta showing a single layer (SynT) separating maternal and fetal circulations. Right, rodent placenta showing three layers (2 SynT layers and one giant trophoblast)

1.2.4 Surface Receptors in the SynT Layer and Aminoglycosides as a Targeting Moiety to SynT

The SynT layer, as explained above, determines the possibility of uptake of materials present in the maternal circulation and potentially transport to the fetal circulation. Some molecules can pass freely via simple diffusion such as gasses. Other molecules can pass via facilitated diffusion such as glucose. Several other biomolecules including amino acids can only be taken up through active transport (Jansson, 2001). Many other substances including riboflavin, folic acid, cobalt and immunoglobulin G (IgG) are taken up via receptor-mediated endocytosis. Hence, many receptors are expressed on the maternal side of the SynT layer including megalin receptors,(Akour et al., 2015a; Akour et al., 2013; Akour et al., 2015b) transferrin receptors,(Bergamaschi et al., 1990) porcine growth hormone (pGH) and human growth hormone (GH) receptors (Velegrakis et al., 2017).

Megalin receptors are also expressed on the apical surface of renal proximal tubular epithelial cells and mainly responsible for the reabsorption of proteins and vitamins (Akour et al., 2015b; Verroust et al., 2002; Verroust and Christensen, 2002) via clathrin-dependent RME.(Verroust et al., 2002; Verroust and Christensen, 2002) They are also expressed on the maternal side of the placenta and responsible for uptake of vitamins, lipids, and hormones. Megalin is thus a valuable target for ligand-modified nanocarriers to actively deliver drugs and biologics to the placenta for the treatment of diseases of the pregnancy. Such receptors have been shown to be expressed on the SynT layer of the placenta of human and mice, as well as on human choriocarcinoma (BeWo) cells, an *in vitro* placental cell model (Akour et al., 2015b; Lybbert et al., 2016; Saito et

al., 1994; Storm et al., 2016).

Recent studies have shown Megalin to be receptors for gentamicin (GM), an antimicrobial aminoglycoside (AG) that is produced naturally by bacteria,(Weinstein et al., 1963) and such megalin-based endocytic processes to follow Michaelis-Menten kinetics (Akour et al., 2015b). AGs are also known to have low protein binding (Mehrotra et al., 2004). AG may thus serve as potential targeting agents that modify the surface of drug containing nanocarriers so as to improve targeting of the maternal-fetal interface.

1.3 Nanocarriers for Placental Drug Delivery:

Various platforms can be used as nanocarriers in drug containing nanomedicines including liposomes, dendrimers, polymeric micelles, gold nanoparticles, linear polymers (synthetic or natural), protein-based carriers, viral-derived nanoparticles and many others (Mirza and Siddiqui, 2014; Moghimi et al., 2005). In this dissertation, two different nanocarrier platforms (liposomes and dendrimers) were used and therefore turn our focus on those nanocarriers, which have different strengths as they relate to drug delivery and targeting to the placenta and their translational potential.

Liposomes are the most widely used nanocarriers in drug containing nanomedicine in the clinic and under clinical trials, and thus have well known toxicity profiles, which is paramount for applications involving pregnant mothers. Biodegradable dendrimers on the other hand provide an opportunity for improved release profiles and thus PK and biodistribution of conjugated drug products, and also provide for an opportunity to deliver high payloads and tailoring of surface

properties to control their interaction with different physiological environments including the blood and the cytosol.

1.3.1 Liposomes: are spherical lipid bilayer vesicles that can be used as vehicles to carry hydrophilic and lipophilic small molecular weight drugs and biologics within their hydrophilic core or hydrophobic lipid bilayer (Bozzuto and Molinari, 2015a). The physicochemical properties of liposomes can be manipulated so as to optimize their function. Size, for instance, can vary depending on the method of preparation and can range from 20 nm to >1000 nm (Akbarzadeh et al., 2013a; Bozzuto and Molinari, 2015a; Pattni et al., 2015) Surface charge can also be modulated and is usually determined by the type lipids used (Miller et al., 1998b) The length and saturation of the lipid tails affect the transition temperature of the lipid itself leading to different release profiles and stability (Wolfram et al., 2014). Thus, the type of lipid is usually selected prudently. Many different molecules can be added to the surface of the lipids that eventually form liposomes, including masking agents such as polyethylene glycol (PEG) (decrease serum protein binding), (Papahadjopoulos et al., 1991) imaging agents such as fluorescent dyes including cyanine and FITC (Gao et al., 2018; Portnoy et al., 2011), and/or targeting moieties such as peptides, (Huang et al., 2008; Yang et al., 2014) carbohydrates (Zhang et al., 2010), antibodies (Heath et al., 1980; Heath et al., 1983) or other functional groups (Sercombe et al., 2015). Moreover, several different drugs and biologics, can be encapsulated within the liposomes including peptides, nucleic acids and small molecular weight

drugs (Tiwari et al., 2012) as schematically illustrated in **Figure 1.4** (Robson et al., 2018).
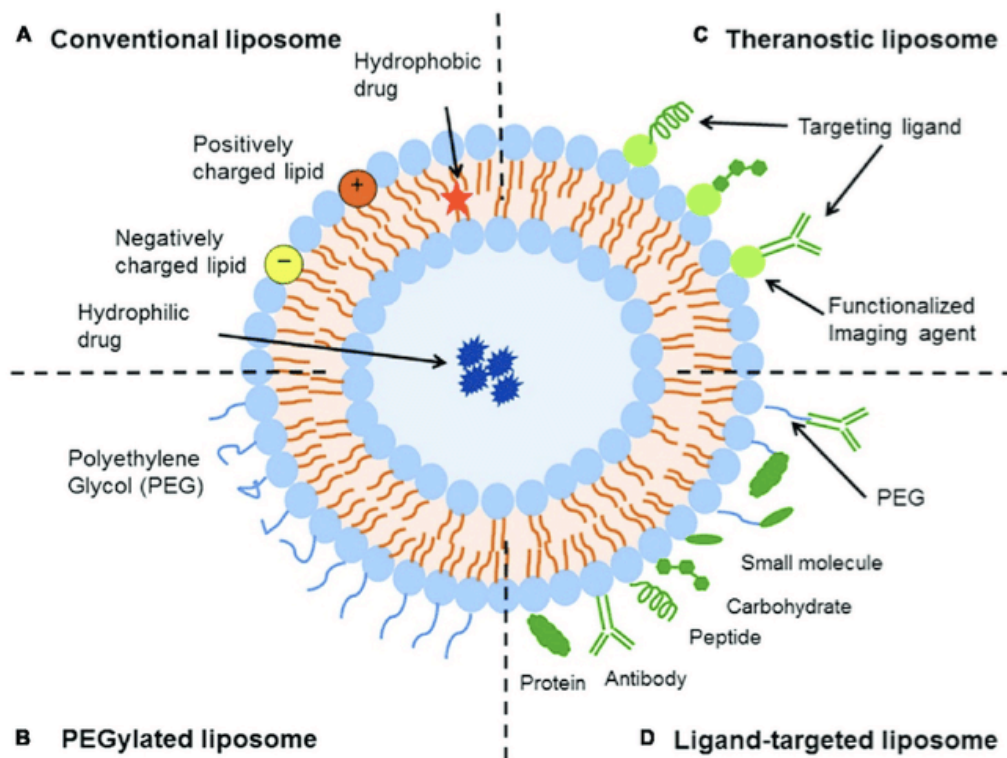
Figure 1.4 Illustration of a liposome (lipid bilayer vesicle) with versatile properties such as size, surface charge, surface modifications and capacity to carry small molecular weight hydrophilic and lipophilic drugs and biologics.

1.3.2 Dendrimer Nanocarriers (DNCs): are hyper-branched synthetic polymers made structured in a tree-like shape as illustrated in **Figure 1.5.** Each added layer of branches is considered a generation starting with the core, generation 1 (G1), G2, G3, G4 and so on. As the generation grows, so does the molecular weight and size. Because of the synthetic approach used to synthesize such systems, they are the most monodisperse polymers available in our toolbox (Biswas et al., 2012; Cheng and Xu, 2008; Nanjwade et al., 2009).

They have unique properties including small nanoscale size (a few nanometers) (Kolhe et al., 2006), high monodispersity, and a large number of functional groups that can be used to conjugate the drug payload through liable bonds, or other ligands so as to modulate their interaction with the physiological environment (Duncan and Izzo, 2005; Yellepeddi et al., 2011), as well as other probes that allow us to track such systems in vitro and in vivo (Wijagkanalan et al., 2011; Zhu et al., 2010). Dendrimers can be prepared from a large variety of chemistries including polyamidoamine (PAMAM), polypropyleneimine (PPI), poly-L- lysine (PLL), poly(glycerol-co-succinic acid), melamine, poly(glycerol), 2,2-bis(hydroxymethyl)propionic acid (Bis-MPA), poly(ethylene glycol) (PEG), and others (Kesharwani and Iyer, 2015). Biodegradable polyester dendrimers are very promising given they reduced toxicity profiles (Heyder et al., 2017; Kolhe et al., 2006; Wijagkanalan et al., 2011) as the polyester bonds that connect the branches lead to the slow disintegration of the polymer into fragments that can be easily eliminated from the physiological environment (in a predictable way)(Heyder et al., 2017) after delivery the payload to the intended tissue.
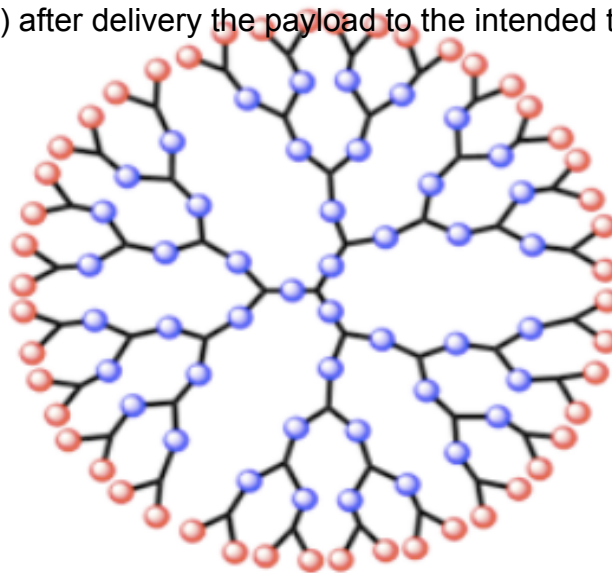
Figure 1.5. Illustration of a fourth generation (G4) dendrimer containing 48 surface groups that can be used for conjugating different molecules such as the drug cargo, targeting moieties,

1.3.3 Nanocarriers for Placental Drug Delivery (literature Review)

There have been several in vivo pre-clinical and ex vivo studies that discuss the use of nanocarries for the placental drug delivery (Bajoria et al., 1997a; Bajoria et al., 2013; Menjoge et al., 2011; Soininen et al., 2015; Valero et al., 2018; Valero et al., 2017; Yu et al., 2017). The effect of certain physical properties of (non-targeting) liposome including size (Bajoria and Contractor, 1997), surface charge,(Bajoria et al., 2013; Miller et al., 1998a) and physical properties of the lipids (Grazia Calvagno et al., 2007) on the transport across the maternal-fetal interface has been investigated. It was observed that small (70nm) unilamellar liposomes showed enhanced uptake of a fluorescent dye in perfused human term placenta compared to larger liposomes (>300nm), as measured fluorometrically (Bajoria and Contractor, 1997). In terms of the surface charge of liposomes, it was determined that anionic liposomes have increased placental uptake of a encapsulated carboxyfluorescein in $vitro$ (trophoblast cells) that was quantified fluorometrically (Bajoria et al., 1997b). Another study by the same group was done in perfused human term placenta in order to deliver thyroxine (T_4) to the fetus by encapsulating it in anionic small unilamellar liposomes where results showed enhanced delivery compared to free T_4, which was metabolized to reverse triiodothyronine (rT_3) (Bajoria et al., 1997a). This placental uptake on anionic small unilamellar liposomes were also demonstrated in in $vivo$ models (Tuzel-Kox et al., 1995). On the other hand, cationic liposomes have been shown to reduce drug uptake. A study showed that encapsulating warfarin, a drug that passes the placenta freely, in cationic liposomes significantly reduced its

passage to the fetus in perfused human term placenta (Bajoria et al., 2013). Moreover, indomethacin (IND), a prostaglandin production inhibitor that reduces uterine contractions and can pass the placenta freely causing fetal toxicity, was encapsulated in cationic multilamellar liposomes in order to reduce transplacental crossing. Results showed that cationic liposomes reduced the passage to the fetus 7.6 folds compared to free IND (Refuerzo et al., 2015). All these studies demonstrate that manipulating liposomal properties can serve different purposes leading to variable end results.

Dendrimers have also been investigated for transplacental transfer abilities. One study investigated the placental uptake of PAMAM dendrimers in comparison with antipyrine, a freely diffusible molecule, and showed that the former exhibited a significantly lower transfer from maternal to the fetal side across the perfused human placenta, demonstrating the possibility of delivering drugs to the mother but not the fetus (Menjoge et al., 2011). Another group used PAMAM dendrimers to stabilize and deliver siRNA to the placenta of preeclamptic rat model in order to reduce the secretion of sFlt1, an antiangiogenic factor that is excessively secreted from the placenta, causing preeclamptic symptoms. Results showed that employing PAMAM dendrimer significantly reduced the secretion of the sFlt1 and led to improved pregnancy outcomes in the preeclamptic rats (Yu et al., 2017).

More recently, modification of nanocarriers with moieties capable of targeting the placenta has been in a timed pregnant mouse model. Liposomes encapsulated with carboxyfluorescein or insulin-like growth factor 2 were

incubated with tumor-homing peptides after liposome preparation were administered to timed-pregnant mice on GD 11.5, 13.5, 15.5, and 17.5 and compared to plain liposomes. Targeting liposomes significantly increased placental weight and improved fetal weight (King et al., 2016b); however, with studies involving nanocarriers, more specifically liposomes, it is imperative to show the release profiles of the encapsulated materials to eliminate bias that can be introduced via different release profiles between the targeting and control liposomes. Moreover, the study shows quantification only based on fluorescence localization of the carboxyfluorescein within the placental tissue. Furthermore, targeted liposomes to the uterus have been employed to reduce preterm birth in an in vivo (mouse) model of pregnancy. Indomethacin, a tocolytic agent that can reduce uterine contractions but can cross the placenta and cause fetal complications, was encapsulated in liposomes that were decorated with oxytocin receptor antagonist to reduce placental passage and target the uterus. Results showed inhibition of uterus contractility in ex vivo model and increasing drug concentration in the uterus in vivo (pregnant mice), maintaining the preterm birth rate (Refuerzo et al., 2016). Another study also designed liposomes with conjugated antibodies that bind the extracellular domain of the oxytocin and encapsulated with contraction-blocking or contraction-enhancing agents and tested them in an in vitro model as well as timed-pregnant mice. In vitro results showed the blocking and enhancing activities while in vivo results showed significant accumulation of the targeted liposomes within the uterus of the pregnant mice (Paul et al., 2017).

More recent applications of targeted nanocarriers using lipid-PLGA nanoparticles incubated with targeting peptides have also shown positive results in terms of placental targeting (Zhang et al., 2018). Such nanoparticles were modified with synthetic placental CSA-binding peptide (plCSA-BP) that can bind to chondroitin sulfate A (CSA), a sulfated polysaccharide expressed on the trophoblast layer. In vivo results show significant accumulation within the placental tissue. The study also reported that (plCSA-BP) caused dramatic placental and fetal developmental impairment. While targeted drug delivery to the placenta can be beneficial, it is essential to consider the fate of the nanocarrier and the safety of the fetus as well as the mother. This is illustrated in several other studies that use nanocarriers that can impose toxicity to the fetus as well as some maternal tissues.

1.4 Hypothesis and Objectives

We hypothesize that GM conjugation to lipid and dendrimer nanocarriers will promote their uptake in the SynT layer of in vitro and in vivo placental models. The rationale for this work is that GM is a substrate for megalin, a surface receptor in the SynT involved in clathrin-medicated processes, and thus its presence (and presentation) on the surface of the nanocarriers will lead to specific interactions with the SynT layer, and thus accumulation of the cargo encapsulated/conjugated by the nanocarriers. We propose to test our hypothesis by preforming the work prosed in the following two aims:

Aim #01: Synthesize GM-modified lipid conjugates and investigate the uptake of corresponding liposomal formulations in an in vitro (BeWo polarized

monolayer) and *in vivo* (timed pregnant Balb/c) model of the SynT layer. We hypothesize that the conjugation of GM on the PEG modified lipids (instead of directly to the lipids) that will form the liposomes will be available to interact with the megalin receptors, and that an increase in the density of the targeting moiety on the liposomal surface will further promote its internalization. We further hypothesize that the presence of the low binding GM on the surface of PEGylated liposomes will lead to stable nanocarriers in vivo, and thus promote the targeting of the megalin receptors on the apical side of the SynT layer.

In Chapter 2, we report the details of this objective. Briefly, we report the chemical synthesis of the lipid to the targeting moiety, gentamicin. We also report the characterization using Matrix-Assisted Laser Desorption and Ionization (MALDI) and Nuclear Magnetic Resonance (^{1}H NMR) and the purification using extraction and dialysis. We describe the preparation and characterization of liposomes. Next is a detailed description of the in vitro and in vivo studies with explanation of the biodistribution analysis both quantitatively (tissue extract and semi-quantitatively (IVIS in vivo imaging system).

Aim #02: Synthesize GM-modified PEGylated polyester dendrimers and investigate the uptake of corresponding dendrimer formulations in an in vitro (BeWo polarized monolayer) and *in vivo* (timed pregnant Balb/c) model of the SynT layer. For the *in vivo* study, we first investigate the pharmacokinetics (PK) of the PEGylated GM-dendrimers in order to determine the time point at which the biodistribution study is performed. Following that, the biodistribution is executed at the determined time point. Concentrations in maternal tissues

(placentas, kidneys, and liver) as well as fetuses are analyzed using both *In Vivo* Imaging System (IVIS) and tissue extract are reported.

In Chapter 3, we explain the details of this objective. Briefly, we discuss the chemical modification of the dendrimers, starting with partial conversion of the dendrimer terminal –OH groups to –NH$_2$, followed by the conjugation of Cyanine 5.5 (Cy5.5) and finally the conjugation of HO-PEG-OCH$_3$ (control) or PEG-GM (targeting modification- **in progress**). Throughout this process, purification and characterization using MALDI and ^{1}H NMR are reported for every step. Thereafter, we report the PK study with time points (0.25, 0.5, 1, 8, 24 and 48 h). Plasma concentration vs. time curves are plotted and evaluated for AUC, $t_{1/2}$, and K_{el}. Finally, biodistribution will be evaluated based on tissue extract and IVIS imaging (similar protocols to those in Chapter 2).

Different from liposomes, dendrimers have the disadvantage of not having a well-known toxicity profile in humans. However, they allow for the conjugation of the therapeutic cargo through designer bonds that allow for more detailed controlled of spatial and temporal release of the cargo and are thus also proposed here.

1.5 Relevance and Innovation

This work is innovative in several aspects. We are the first group to propose the use of AGs (in this case GM) as a targeting moiety to the placenta. In particular, this is the first time that GM- modified lipids are synthesized and the ability to target models of the placenta in vitro and in vivo demonstrated. Such novel methods of targeting the placenta for the delivery of drug and biologics are

significant as they provide for a nanotherapy platform for the potential treatment of diseases of the pregnancy.

CHAPTER 2

Megalin-targeting Liposomes for Placental Drug Delivery

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ABSTRACT

Every year, complications during pregnancy affect more than 26 million women. Some of those diseases are associated with significant morbidity and mortality, as is the case of preeclampsia, the main cause of maternal deaths globally. The ability to improve the delivery of drugs to the placenta upon administration to the mother may offer new opportunities in the treatment of diseases of the pregnancy. The objective of this study was to develop megalin-targeting liposome nanocarriers for placental drug delivery. Megalin is a transmembrane protein involved in clathrin-mediated endocytic processes, and is expressed in the syncytiotrophoblast (SynT), an epithelial layer at maternal-fetal interface. Targeting megalin thus offers an opportunity for the liposomes to hitchhike into the SynT, thus enriching the concentration of any associated therapeutic cargo in the placental tissue. PEGylated (2 KDa) lipids were further modified with gentamicin (GM), a substrate to megalin receptors, and used to prepare placental-targeting liposomes. The rate and extent of uptake of a fluorescent probe encapsulated within the liposomes into an *in vitro* model of the SynT (polarized BeWo monolayers) was assessed by flow cytometry. Targeting liposomes containing 5 mol% GM-modified lipids enhanced the uptake of the probe by 1.5 fold compared to the non-targeting control. An increase in mol% of the modified lipid resulted in further enhancement in uptake 2 fold compared to the control. In a competition assay, inhibition of the megalin receptors resulted in a significant reduction in uptake of the fluorescence probe encapsulated in GM-modified liposomes compared to the uptake without free inhibitor ($p < 0.0001$), indicating the involvement in the receptor in the internalization of the liposomes. The ability of the targeting liposomes to enhance accumulation of a fluorescence probe

was also assessed in an *in vivo* placental model - timed-pregnant Balb/c mice at gestational day 18.5. The targeting liposomes increased the accumulation of a conjugated fluorescence probe in the placenta with a total accumulation of 2.8% of the initial dose, which corresponds to an increase of 94 fold compared to the free probe (p<0.0001), and 2-4 fold compared to the non-targeting control liposomes (<0.0001), as measured by both tissue extraction and *ex vivo* imaging. Taken together, these results demonstrate that megalin-targeted liposomes may offer an opportunity to enhance the delivery of therapeutics to the placenta for the treatment of diseases of the pregnancy.

2.1 INTRODUCTION

More than 130 million newborns are delivered every year all over the world.(Keelan et al., 2015a) Over 20% of those pregnancies experience at least one complication (Duley, 2009). Some pregnancy-related complications lead to high maternal and fetal morbidity and mortality, including cases of preeclampsia (Roberts and Gammill, 2005), intrauterine growth restriction (IUGR),(Romo et al., 2009) and bacterial (McDonagh et al., 2004) and viral(Racicot and Mor, 2017; Silasi et al., 2015) infections. Preeclampsia affects about 8% of all pregnancies and accounts for 10-15% of all maternal deaths globally(Duley, 2009). IUGR, affects approximately 7-15% of all pregnancies(Yamashita et al., 2011) and is commonly associated with premature delivery (Garite et al., 2004; Goldenberg et al., 2008). Many pregnancy-related complications originate due to a compromised placenta, which is, therefore, a valuable target for the delivery of small molecule drugs and biologics (Al-Enazy et al., 2017; Audus, 1999).

The placenta is the tissue that governs the exchange of nutrients and waste between the mother and the fetus (Griffiths and Campbell, 2014) It contains independent functional vascular units called cotyledons. The villous trees located within the cotyledons separate maternal circulation from fetal circulation. The syncytiotrophoblast (SynT) is a multinucleated epithelial layer in the villous trees that is in direct contact with the maternal blood and is covered on the apical (maternal) side with branched microvilli that maximizes the surface area for gas and nutrient/waste exchange. The maternal side of the SynT layer has been shown to express megalin receptors (Akour et al., 2015a; Akour et al., 2013), which are involved in clathrin-mediated endocytic processes (Orlando et al., 1998; Saito et al., 1994). Megalin is thus a potential target for ligand-modified drug nanocarriers for the active targeting and delivery of therapeutic molecules to the placenta.

Our team has previously shown that the uptake of gentamicin (GM) by polarized human choriocarcinoma (BeWo) cells, an vitro model of the SynT (Li et al., 2013; Poulsen et al., 2009), is mediated by megalin receptors.(Akour et al., 2015b) These natural aminoglycosides (Clarot et al., 2004) have low plasma protein binding affinity(Mehrotra et al., 2004) and have been extensively used in the clinic to treat infections in both the expecting mother and fetus (Dashe and Gilstrap III, 1997), and thus have known cytotoxic profiles. Such characteristics make GM stand out as candidate targeting ligands to megalin.

Liposomes have been extensively employed in the clinic as nanocarriers in drug products containing nanomedicines (Pattni et al., 2015) for the treatment

of various diseases including malaria, influenza and different types of cancers.(Bozzuto and Molinari, 2015b) Targeted delivery with liposomes can be active or passive. While passive delivery depends on the unique physiology of the targeted tissue (Šentjurc et al., 1999), active delivery involves surface modification with a ligand, which can be a small molecule or a biomacromolecule (Forssen and Willis, 1998; Tagalakis et al., 2011). Several ligands have been conjugated to liposomes for active delivery such as folic acid, transferrin and mannose.(Pattni et al., 2015) Such surface-modified liposomes have been shown to enhance tissue specificity of the nanomedicine (Derycke et al., 2004; Gabizon et al., 2004; Lee and Low, 1995; Pattni et al., 2015; Song et al., 2008; Zong et al., 2014) and consequently the therapeutic index of the encapsulated cargo.

Liposomes have also been studied pre-clinically in *in vivo* models of pregnancy to evaluate their potential for the delivery of drugs to the placenta (Bajoria et al., 1997b) and are of particular importance for diseases of the pregnancy given their known toxicity profiles (Parnham and Wetzig, 1993). Physicochemical properties of liposomes have been exploited in order to manipulate placental uptake. Small unilamellar liposomes were shown to increase placental uptake (Tuzel-Kox et al., 1995) while multilamellar liposomes decreased placental uptake (Refuerzo et al., 2015). Anionic liposomes also exhibited increased placental uptake compared to a cationic counterpart (Tuzel-Kox et al., 1995). Recently, a tumor-homing peptide was shown to accumulate in the placenta of a mouse model of pregnancy (King et al., 2016a)

Carboxyfluorescein encapsulated in PEGylated liposomes accumulated in the placenta of pregnant mice and at a higher extent (as qualitatively assessed by confocal microscopy) when the liposomes were pre-incubated with the tumor-homing peptides (King et al., 2016a).

Given the challenges and opportunities described above, the objective of this work was to develop a megalin-targeting liposome that may serve as a platform for active delivery of therapeutics to the placenta. We synthesized GM-modified lipids and prepared and characterized targeting liposomes with such lipids. We assessed their interaction with both *in vitro* and *in vivo* models of the placenta. Targeting liposomes were shown to enhance the uptake of an encapsulated fluorescence probe in polarized BeWo cells (*in vitro* model of the placenta) in a megalin dependent way. These liposomes were also seen to accumulate in the placental tissue in an *in vivo* pregnancy model (timed pregnant Balb/c mice) at significantly greater concentrations than controls.

2.2 MATERIALS AND METHODS

2.2.1 Materials. Hydrogenated Soy Phosphatidylcholine (HSPC) and N-(Carbonyl-methoxypolyethyleneglycol 2000)-1,2-distearoyl-sn-glycero-3-phosphoethanolamine, sodium salt (DSPE-PEG2K) were purchased from NOF America (White Plains, NY). 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000] (DSPE-PEG2K-NH2) was purchased from Laysan Bio Inc. (Arab, AL). Gentamicin (GM), cholesterol (CHOL), chloroform and ethanol were purchased from VWR Analytical. Fluorescein isothiocyanate (FITC) was purchased from Thermo Fisher Scientific. Cyanine 5.5 was

purchased from Lumiprobe (Hunt Valley, MD). Eagle's Minimum Essential Medium (EMEM) and Trypsin-EDTA (1X) were purchased from Corning. DMEM/F12 was purchased from Thermo Fisher and fetal bovine serum (FBS, USDA Origin) was purchased from Serum Source International (Charlotte, NC). Tissue culture flasks, non-essential amino acids (NEAA, 100X), Transwell inserts (polyester membrane, 0.33 cm^2, 1 μm pore size) were purchased from VWR International. Phosphate buffer saline (PBS, 1X, pH=7.4), Hank's Balanced Salt Solution (HBSS, 1X, pH=7.4), citrate buffer (pH=4.5) and tissue digesting buffer were prepared in our laboratories. Di-*tert*-butyl dicarbonate (BOC), (dimethylamino) pyridine,4- [4-(dimethylamino)pyridine, 99%] (DMAP), cesium fluoride (CsF) (N,N-dimethylformamide), anhydrous, 99.8+% (DMF) and hydrogen chloride, 4M in 1,4-dioxane, 99% were purchased from Alfa Aesar. Succinic anhydride (SA), [N-Hydroxysuccinimide (NHS) and (N-(3-dimethylaminopropyl)-N`-ethylcarbodiimide hydrochloride) (EDC) were purchased from Sigma Aldrich.

2.2.2 Synthesis and Characterization of Targeting Lipids.

The synthesis of the targeting lipid DSPE-PEG2K-GM was performed by conjugating GM to the DSPE-PEG2K-NH2 as shown in Scheme 1. First, the amine groups of GM were protected by addition of di-*tert*-butyl dicarbonate (BOC) – Scheme 1a. Briefly, GM (0.0795 mmol) and sodium carbonate (0.7726 mmol) were dissolved in DI H$_2$O. BOC (0.5023 mmol) was dissolved in 1 mL acetonitrile and added drop-wise and the reaction was stirred for 24 h. The reaction mixture was diluted with 10 mL DI H$_2$O and then extracted with ethyl

acetate (3 × 10 mL). The organic layers were combined and then dried over anhydrous sodium sulfate for 30 min. The sodium sulfate was filtered off and the organic solvent was removed by rotary evaporation and further dried under high vacuum for 6 h to obtain BOC-protected GM product (GM-BOC). The GM-BOC (0.075 mmol) was then dissolved in a 6 mL anhydrous ethyl acetate and reacted with succinic anhydride (SA, 0.15 mmol) in the presence of 4-dimethylaminopyridine (DMAP, 0.15 mmol) and cesium fluoride (CsF, 0.03 mmol) for 24 h to yield BOC-protected gentamicin terminated with a carboxyl group (-COOH), GM-BOC-SA. The reaction mixture was diluted with 5 mL ethyl acetate and purified by extraction against DI H_2O (3 × 7 mL) and dried as explained above. The product (0.037 mmol) was then dissolved in 10 mL anhydrous DMF and reacted with EDC and NHS to activate the -COOH followed by the addition of DSPE-PEG2K-NH2 (0.026 mmol) for 24 h to form DSPE-PEG2K-GM-BOC – Scheme 1b. After the completion of the reaction, the DMF was removed by vacuum for 6 h. The deprotection of BOC from the DSPE-PEG-GM-BOC product was performed by dissolving it in 5 mL 4.0 M HCl in dioxane for 30 min followed by removal of the solvent by a flow of nitrogen to yield the gentamicin-terminated, PEGylated lipid DSPE-PEG2K-GM. Lastly, the final product was purified by dissolving DSPE-PEG2K-GM in DMSO, adding it to a dialysis tube (MWCO 1000 Da) and dialyzing against DMSO for 24 h followed by dialysis against deionized water for 24 h, with periodical exchange of the solvents. The final product was lyophilized and obtained as powder. The DSPE-PEG2K-GM was characterized by matrix-assisted laser desorption/ionization Time-of-Flight spectroscopy

(MALDI-TOF, Voyager-DE PRO, JBI SCIENTIFIC) and proton nuclear magnetic resonance spectroscopy (^{1}H NMR, Bruker avance iii 400 mHz).

2.2.3 *In vitro* Studies

2.2.3.1 Liposome preparation.

Liposomes were prepared following the thin-film hydration method (Akbarzadeh et al., 2013b; Laouini et al., 2012), and using sonication to produce small unilamellar liposomes (ULV). Different molar ratios of HSPC, CHOL, and DSPE-PEG2K or DSPE-PEG2K-GM were dissolved in chloroform in a round bottom flask. The mixture was then vortexed to ensure homogeneity. Chloroform was removed by rotary evaporation at 40 ^{o}C followed by high vacuum overnight to ensure the removal of the residual organic solvent. The lipid film was then hydrated with 3 mL of PBS to form liposomes. The liposome suspension was sonicated for 10 min over ice with 30% power intensity using the OmniRuptor 250 Ultrasonic Homogenizer to obtain the optimal size, followed by centrifugation at 141,000 rcf$_{max}$ (Beckman Coulter XL-100K Optima Ultracentrifuge, SW41 Rotor) for 2 h at 4 ^{o}C to pelletize them.

2.2.3.2 FITC loading in Liposomes.

The liposome pellet was then reconstituted with 3 mL of citrate buffer (pH= 5.5) containing 0.1 mg/mL of FITC and incubated overnight at 4 ^{o}C to actively load FITC within the liposomes following a previously established method (Fritze et al., 2006). After overnight incubation, the liposomes were centrifuged again for 2 h at 4 ^{o}C. The supernatant was collected and FITC concentration was assessed, providing an indirect measure of the encapsulation efficiency (Indirect

EE). EE was also assessed directly (Direct EE) by reconstituting the liposomes in PBS, adding a fraction of that sample to ethanol (rupturing liposomes) and subsequently quantifying the amount of FITC (Ohnishi et al., 2013; Panwar et al., 2010).

$$\textbf{Direct EE} = \frac{|\text{Encapsulated FITC Concentration}|}{|\text{Total FITC Concentration}|}$$

$$\textbf{Indirect EE} = \frac{|\text{Total FITC Concentration}| - |\text{FITC Concentration in Supernatant}|}{|\text{Total FITC Concentration}|}$$

2.2.3.3 Liposome Characterization.

Three different formulations were investigated in the *in vitro* studies: GM-targeting liposomes containing HSPC:CHOL:DSPE-PEG2K-GM with molar ratios of (5.5**:**4**:**0.5) named *5PEG2K-5GM* and (5.5**:**3.5**:**1) named *10PEG2K-10GM.* The third group acting as the control lacking the GM-targeting moiety contained HSPC:CHOL:DSPE-PEG2K with molar ratio of (5.5**:**3.5**:**0.5) and was named *5PEG2K-0GM*. The hydrodynamic diamter (HD) and zeta potential (ζ), a measure of the surface charge of the liposomes, were characterized by light scattering (LS) using a Malvern Zetasizer Nano-ZS (Malvern, UK).

2.2.3.4 *In vitro* FITC Release. After determining the Direct EE, FITC release from liposomes was evaluated under sink conditions. Briefly, a 3 mL aliquot of liposomes containing encapsulated FITC was sealed in a dialysis tube with 8,000 MWCO. The tube was then dialyzed against a 40 mL PBS (10 mM, pH 7.4) at 37 $^{\circ}$C in the dark. The FITC released from the liposomes into surrounding PBS was

analyzed by taking 0.1 mL samples from the dialysate at intervals 0, 15 min 30 min, and every hour from 1-6 h, and measuring the fluorescence of FITC (Excitation: 490 nm, Emission: 525 nm) using Microplate Reader (BioTek Instruments, Inc. Winooski, VA, USA). The samples were taken in triplicate and returned to the dialysate buffer after the measurements were completed. The amount of FITC was calculated based on an established standard curve. Release profiles were obtained from three independent liposome formulations with consistent EE.

2.2.3.5 Preparation and characterization of the *in vitro* Placental Model

2.2.3.5.1 Cell Culture.

Human Choriocarcinoma (BeWo) cells (which express megalin) were cultured with DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin−streptomycin and incubated at 37 ^{o}C with 5% CO_2. Human hepatocellular carcinoma (HepG2) cells (used as a negative control as they do not express megalin) were cultured with EMEM supplemented with 10% (FBS) and 1% penicillin−streptomycin and incubated at 37 ^{o}C with 5% CO_2

2.2.3.5.2 BeWo Monolayer Formation and Polarization.

The polarization of the BeWo cells was assessed indirectly by measuring the transepithelial electrical resistance (TEER) as a function of time. Briefly, BeWo cells were seeded into 24-transwell plate inserts with a pore size of 1 μm at a 150,000-cells/cm^2 density. Medium was changed in both apical and basolateral side every other day. TEER values were then recorded everyday for 9 days post-seeding using EvomX meter (World Precision Instruments Inc) and

reported as a function of time. Background resistance was determined in blank inserts.

2.2.3.5.3 Tight Junctions in the BeWo Monolayer.

The monolayer formation and polarization of the BeWo cells was also verified using immunocytochemistry (ICC) by assessing the formation of tight junctions between the cells. BeWo cells were seeded into 24-transwell plate inserts with a pore size of 1 μm at a 150,000-cells/cm^2 density and allowed to grow for 6 days post-seeding. Cell monolayers were fixed on Transwell insert membranes using 4% paraformaldehyde for 20 minutes. After fixation, nonspecific binding was blocked by 2% BSA in PBS. Monolayer membrane was then incubated with primary rabbit ZO-1/TJP1 antibody (Invitrogen) for one hour at room temperature followed by one hour incubation with donkey anti-rabbit antibodies (Life Technologies). Nuclei staining was accomplished by incubation with Hoechst 33342 (Thermo fisher Scientific) for 20 minutes. Monolayers were washed 3 times for 5 minutes after each of the previous steps. Lastly, the membrane was cut, mounted on a slide and visualized using confocal laser scanning microscope (Zeiss LSM 700)

2.2.3.5.4 BeWo Monolayer Cross-Section.

Cross-sections of the cell monolayers grown on inserts were processed at the VCU Microscopy Core Facility. Briefly, BeWo cells were grown on the inserts at the same conditions as mentioned above. On the sixth day post-seeding the BeWo cell monolayer was fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) overnight. Samples were then dehydrated using a serious of

ethanol concentrations followed by Toluidine Blue/Azure II/ methylene blue staining. Finally, samples were cut into 1-µm slices using an ultramicrotome and visualized using the light microscope.

2.2.3.5.5 Liposomal Targeting *in vitro*.

Internalization of FITC encapsulated in liposomes was determined by flow cytometry using Cytoflex Flow Cytometer (Beckman Coulter) following a method previously described by our laboratories.(Bielski et al., 2015; Zhong et al., 2016a) Briefly, BeWo cells were grown into confluent, polarized monolayers in a 96-transwell plate by seeding 150,000-cells/cm^2 and allowed to grow for 6 days as described earlier. On day 6, each well was washed twice and incubated in sterile Hank's Balanced Salt Solution HBSS buffer for 30 minutes followed by the addition of the non-targeting and targeting liposomal formulations (***5PEG2k-0GM, 5PEG2k-5GM***, ***10PEG2k-10GM***) each containing 250 ng of encapsulated FITC. At time points 0.25, 0.5, 1, 2, 3, 4, 5, 6 h, after contacting with the liposomes, the cell monolayers were washed thrice with cold sterile HBSS (1X, pH 7.4), incubated with trypsin-EDTA, and resuspended in 0.5 mL HBSS. Samples were run in the flow cytometer where the median fluorescence intensity (MFI) of FITC was recorded to analyze the rate and extent of cellular internalization of the FTIC encapsulated in the liposome systems as a function of GM density and time. Similar experiments were performed in HepG2 cell monolayers, which serve as a control cell line that does not express megalin receptors. Finally, Competition studies were performed in BeWo monolayers using similar procedure as described above, and comparing FITC internalization in the presence and

absence of (2 mM) free GM, which was incubated with the cell monolayers 20 minutes prior to the liposomal treatments. The GM concentration of 2 mM was chosen based on cell viability assay – BeWo cells are 100% viable at that concentration, as shown in **Figure S2.3**.

2.2.4 *In Vivo* Experiments.

2.2.4.1 Synthesis of Cy5.5-modified PEGylated Lipids: DSPE-PEG2K-CY.

Cyanine5.5 (Cy5.5-COOH) was conjugated to DSPE-PEG2K-NH2 for the preparation of liposomes used for the *in vivo* studies - **Scheme S1**. Briefly, the Cy5.5-COOH was activated by reacting with NHS and EDC (both 1.5 molar eq.) in dry DMSO. The activated Cy5.5 was then reacted with DSPE-PEG2K-NH2 at 0.8 molar eq., followed by addition of the products to a dialysis tube (MWCO 1000 Da) and dialyzing against DMSO for 24 h followed by dialysis against deionized water for 24 h. The final product, DSPE-PEG2K-CY, was lyophilized and obtained as powder and characterized by (MALDI-TOF) - **Figure S2.1**.

2.2.4.2 Preparation of Targeting Liposomes for *in vivo* studies.

For the *in vivo* studies, lipoid-conjugated Cy5.5 (DSPE-PEG2K-CY) was used as a tracer instead of encapsulated FITC, thus allowing us to overcome issues associated with tissue auto-fluorescence that prevents accurate biodistribution assays with FITC. The same liposomal preparation method mentioned earlier was used in this case, except for the FITC encapsulation steps. Briefly, HSPC, CHOL, DSPE-PEG2K or DSPE-PEG2K-GM and DSPE-PEG2K-CY were mixed in chloroform in the following molar ratio: 5.33**:**4**:**0.5**:**0.17, respectively. Chloroform was removed and the lipid film was hydrated and

sonicated. The HD and ζ of the liposomes were determined as described earlier. Non-targeting (control) **10PEG2K-0GM-CY** and targeting liposomes **10PEG2K-10GM-CY,** both with 10mol% PEGylated lipids were investigated *in vivo*.

2.2.4.3 Timed pregnancy.

All animal experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee at Virginia Commonwealth University (IACUC approved protocol # AD10001841). Eight-week-old female Balb/c mice (20-22 g) were housed under a 12 h light/dark cycle and allowed food and water ad libitum. After mating, female mice were inspected daily to observe the presence of a mating plug. Upon detection of the mating plug, gestational day 0.5 (GD 0.5) was assigned. Due to Balb/c strain having higher chance of false positive mating plugs, we further assessed pregnancy by tracking weights of the pregnant females, as discussed previously(Heyne et al., 2015). Biodistribution / targeting experiments were carried out at GD18.5, at which placental barrier is analogous to human third trimester placenta(Georgiades et al., 2002). Female mice were divided in 4 treatment groups as follows: Group 1: PBS; Group 2: free Cy5.5; Group 3: non-targeting liposomes (**10PEG2K-0GM-CY**); and Group 4: targeting liposomes (**10PEG2K-10GM-CY**). On GD 18.5, mice were administered the formulations retro-orbitally with 0.70 mg/Kg Cy5.5 equivalent. Four hours post treatment, mice were euthanized, followed by laparotomy; fetuses and maternal tissues (placentas and kidneys) were collected for analysis. We chose 4 h exposure time based on previous liposomal studies.(Refuerzo et al., 2015; Refuerzo et al., 2016; Tuzel-Kox et al., 1995)

2.2.4.4 Biodistribution: ex vivo imaging and tissue quantification.

The ability of the liposomes to target the placenta was indirectly assessed by measuring the concentration of Cy5.5 in tissue. *Ex vivo* Cy5.5 fluorescence intensity of each tissue was measured using IVIS Imaging. After tissue excision, they were washed in PBS (pH. 7.4) and placed in the IVIS chamber for measurement at 680/710 nm. For each mouse, the average intensity of five placentas, five fetuses, and two kidneys were assessed, and this was repeated for n=4 pregnant mice. For the tissue extraction studies, the total dose of Cy5.5 was also directly determined in relevant tissues, for a total of n = 4 pregnant mice for each group.   In this case, calibration curves were constructed by spiking known amounts of Cy5.5 in placenta (3 placentas pooled together) and fetus (1 fetus), and Cy5.5 assessed per gram tissue. Similar placental pooling was done when assessing biodistribution. Each tissue (or pooled tissue) was washed in PBS (pH.7.4), placed in known volume of digesting buffer (0.5 M phosphate buffer) and mechanically broken down using tissue homogenizer to create a uniform suspension. Tissue suspension was allowed to shake/rotate for 72h at 4°C. Subsequently, each tissue homogenate was centrifuged for 10 min at 14000 g at 4°C. Supernatant was collected and fluorescence intensity was measured (excitation/emission 680/710 nm) using microplate reader (BioTek Synergy HT). Cy5.5 concentration was measured based on established calibration and expressed as % of initial dose (% ID) per gram tissue. Absolute concentrations are reported in *Supporting Information*. This procedure follows previously

established methods in our laboratories for tissue quantification of fluorescent probes (Zhong et al., 2016b).

2.2.5 Statistical Analysis.

All data are presented as mean ± standard deviation (SD). Each measurement had a minimum of four independent repeats (n ≥ 4) in which the mean and SD were subsequently calculated. GraphPad Prism 7 software was utilized to perform two-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. Means were considered statistically significant if $p < 0.05$. P-values were categorized as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0005$.

2.3 RESULTS AND DISCUSSION

2.3.1 Ligand Design and Synthesis of the Targeting Lipid.

2.3.1.1 Ligand Design.

There are several reasons for selecting gentamicin (GM) as the ligand to develop placental-targeting liposomes. First, GM is a substrate of megalin, which is a surface receptor in the apical (maternal) side of the SynT layer (Akour et al., 2013). This is relevant as megalin is known to be involved in clathrin-mediated endocytosis processes in the human placenta (Orlando et al., 1998; Saito et al., 1994), and is also expressed in the *in vivo* (mouse) and *in vitro* (polarized BeWo cell monolayers) models of pregnancy used in this work (Akour et al., 2015b; Saito et al., 1994). GM also has unique physicochemical properties as a targeting agent(Oroojalian et al., 2017) as it has low plasma protein binding affinity (Nagai and Takano, 2004; Pechere and Dugal, 1979) and is expected, therefore, to

confer enhanced stability to liposomes when in systemic circulation, thus further promoting their placental targeting ability. Moreover, GM is currently used in the clinic to treat maternal and fetal infections during pregnancy (Ward and Theiler, 2008) and thus has a known toxicity profile.

2.3.1.2 Synthesis of the Targeting Lipids.

The DPSE-PEG2K-GM targeting lipids were conjugated as illustrated in Scheme 1. GM is composed of three major components known as C1, C1a and C2 (Gberindyer et al., 2017; Seidl and Nerad, 1988; Sun and Liu). These major components have slightly different molecular weights: 472, 486, 500 Da, respectively – see Scheme 1a. GM has 5 amine groups, which we believe are involved in its uptake into the SynT by megalin. Therefore, we conjugated GM to the lipid using a hydroxyl (-OH) group instead.
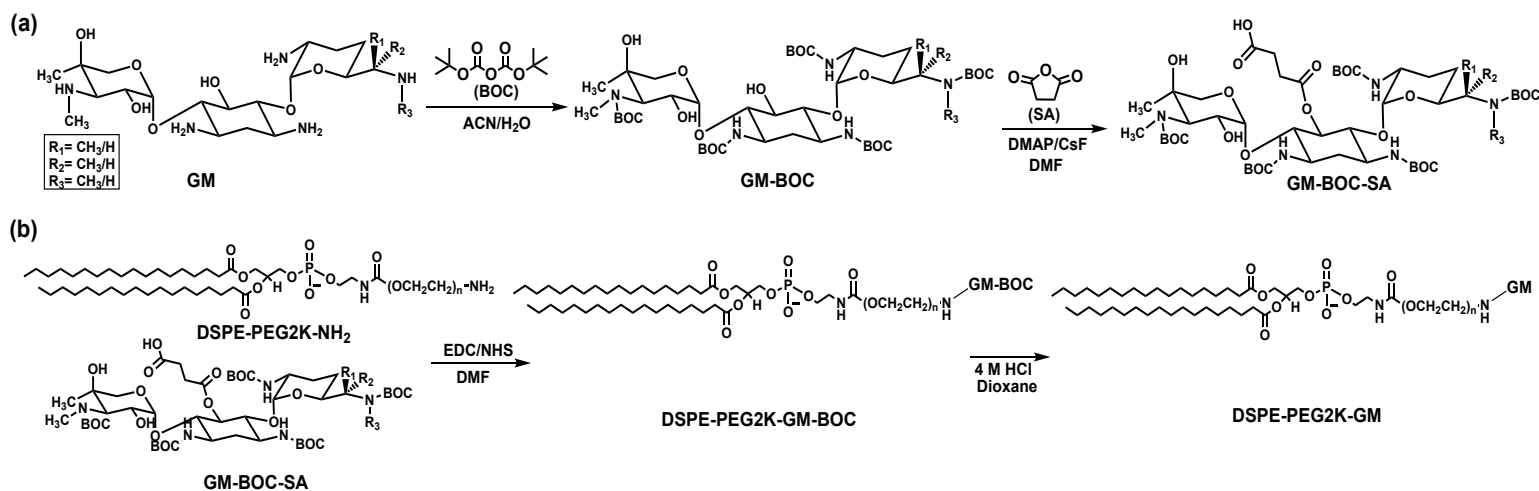
GM=Gentamicin
BOC=Di-tert-butyl dicarbonate
SA= Succinic Anhydride
DSPE-PEG2K-NH₂= 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]

Scheme 2.1: Schematic of the chemical synthesis of the targeting lipid DSPE-PEG2K GM.
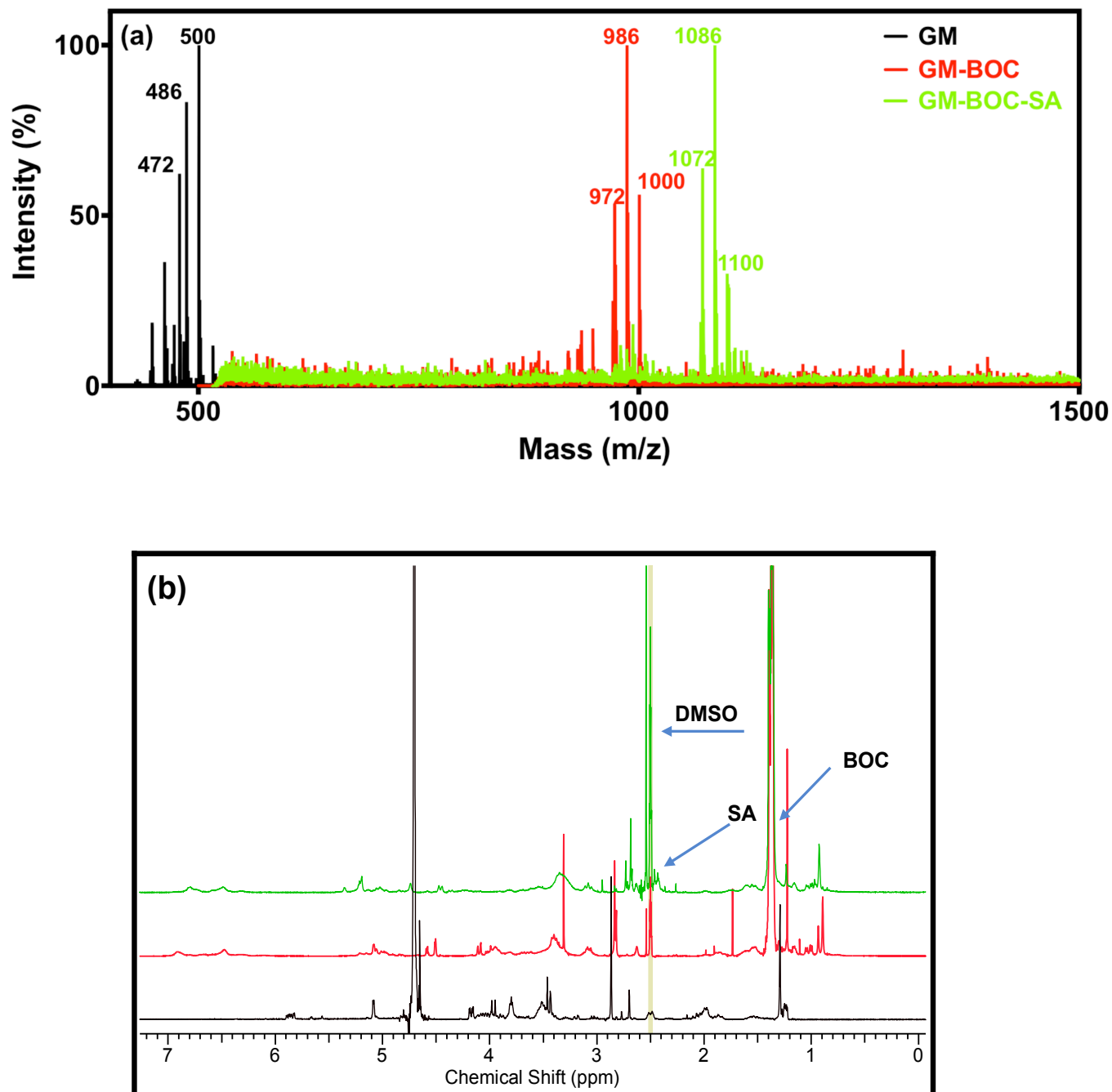
Figure 2.1 (a) MALDI-ToF spectra showing the main three peaks of gentamicin (GM, 478, 486, 500Da), BOC-protected gentamicin (GM-BOC, 972, 986, 1000Da) and the succinic anhydride (SA) modified protected gentamicin (GM-BOC-SA, 1072, 1086,1100Da). **(b)** ^{1}H NMR spectra showing the successful modification of gentamicin (GM, black) with BOC (GM-BOC, in red) and SA (GM.BOC-SA, green).

The NH2 groups of GM were first protected with BOC (MW: 101 Da leading to GM-BOC products of mass corresponding to 972, 986, 1000 Da, respectively. After protection, a carboxyl functionality (-COOH) was added to GM-BOC via conjugation of succinic acid to the more reactive–OH, to form GM-BOC-SA, with expected masses of 1072, 1086, 1100 Da, respectively. MALDI-ToF spectra for the various GM conjugates are shown in **Figure 2.1a**.

These chemical modifications were followed, as well, using ^{1}H NMR (**Figure 2.1b**). When compared to the spectrum of the free drug, it is possible to observe the appearance of new peaks due to the chemical modifications discussed. Firstly, the introduction of BOC groups resulted in the appearance of a strong peak in the range 1.37-1.40 ppm (-CH_3). Additionally, the new peaks in the region 6.33-6.92 ppm correspond to the hydrogens present in the -N*H*-Boc group, confirming the protection of all amine groups. The reaction with succinic anhydride lead to new peaks in the range of 2.43-2.47 ppm (very close to the residual solvent peak), which correspond to the new -CH_2 groups conjugated to the molecule. Besides those, a broad peak is present at 12.18 ppm, corresponding to the -COO*H* group. GM-BOC-SA was subsequently conjugated to DSPE-PEG2K-NH2 forming an amide bond as shown in Scheme 1b. The DSPE-PEG2K-GM-BOC (MW: ~3861 Da), was lastly deprotected (BOC groups removed), yielding the targeting lipid DSPE-PEG2K-GM, with an m/z of ca. 3500Da. MALDI-ToF spectra are shown in **Figure 2.2**.
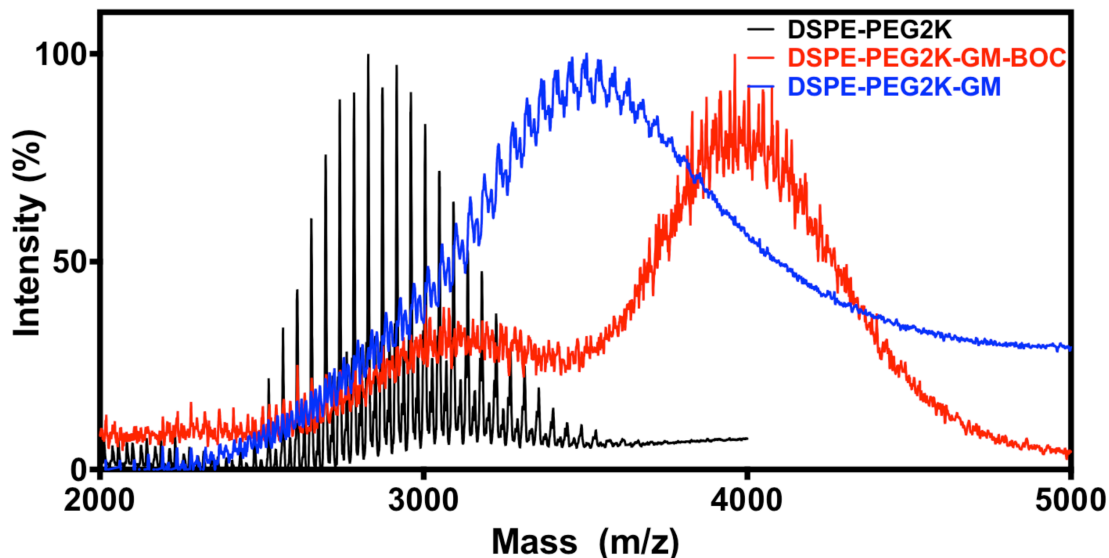
Figure 2.2 MALDI-ToF results showing DSPE-PEG2K (2792Da, black), conjugated DSPE-PEG2K to SA-modified, protected gentamicin (DSPE-PEG2K-GM-BOC, 3961Da, red) and the deprotected final product, the targeting lipid DSPE-PEG2K-GM (3500Da, blue).

2.3.2 *In vitro* studies.

2.3.2.1 FITC-loaded Liposome Preparation and Characterization.

Three different liposomes were prepared for the *in vitro* studies: PEGylated liposome with 5 mol% PEGylated lipid (i) without GM modification (without targeting ligands) (*5PEG2K-0GM*) and (ii) with targeting ligands (*5PEG2K-5GM*), and PEGylated liposome with a higher mole fraction of PEGylated lipids (10 mol%) that were modified with the targeting ligands (*5PEG2k-10GM*). The size (hydrodynamic diameter, HD in nm) and surface charge (zeta potential, ζ in mV) of the various formulations are summarized in **Table 2.1**.

Table 2.1: Hydrodynamic Diameter (HD), Zeta Potential (ζ), FITC Encapsulation Efficiency (EE) and percent release at 6h (pH 7.4) for the three different liposomal formulations used in the *in vitro* studies. SD = standard deviation, n = 4.

Liposome	HD ± SD (nm)	ζ (mV)	EE (%)	% Release at 6h (pH 7.4)
5PEG2K-0GM	105 ± 12	- 4.4 ± 2.3	86.5 ± 7.2%	28.7 ± 1.5%
5PEG2K-5GM	112 ± 18	- 4.3 ± 2.5	84.4 ± 3.3%	28.2 ± 1.6%
10PEG2K-10GM	117 ± 19	- 3.7 ± 3.3	85.2 ± 8.3%	27.0 ± 1.6%

5PEG2K-0GM = liposomes prepared with 5mol% of DSPE-PEG2K lipid.
5PEG2K-5GM = liposomes prepared with 5mol% of DSPE-PEG2K-GM lipid.
10PEG2K-10GM = liposomes prepared with 10mol% of DSPE-PEG2K-GM lipid.

Non-targeting liposome controls **5PEG2K-0GM** showed an average HD of 105 ± 12 nm, with the targeting liposomes **5PEG2K-5GM** and **5PEG2k-10GM** having a similar (not statistically different from the non-targeting control) HD of 112 ± 18 and 117 ± 19 nm, respectively. The size of liposomes is a critical attribute(Juliano and Stamp, 1975) and impacts their behavior including cellular uptake.(Bajoria and Contractor, 1997) The preparation of liposomes with similar sizes thus helps remove confounding effects that may arise due to size differences.

The zeta potential (ζ) of all liposomes is slightly negative and very close (no statistically significant difference) among the three formulations with **5PEG2k-0GM** -4.4 ± 2.3 mV, **5PEG2K-5GM** -4.3 ± 2.5 mV and **10PEG2K-10GM** -3.7 ± 3.3 mV, as measured in PBS.   These negative ζ happen in spite of the

presence of GM that has 5 protonatable amines each. This is important as cationic liposomes have been shown to significantly reduce placental uptake of liposomes when compared to anionic counterparts (Bajoria et al., 2013).

The encapsulation efficiency (EE %), drug loading and release profiles were also consistent among the three systems. The encapsulation of FITC was $86.5 \pm 7.2\%$, $84.4 \pm 3.3\%$ and $85.2 \pm 8.3\%$ of the starting FITC amount for **5PEG2K-0GM, 5PEG2K-5GM** and **10PEG2K-10GM**, respectively, as summarized in **Table 2.1**. The loading efficiency of FITC was ca. 7 wt./wt.% drug-to-lipid ratio. Although it was possible to achieve higher loading efficiencies, this amount was sufficient to work with in terms of fluorometric analysis.

The release profiles are shown in **Figure 2.3** and were seen to be consistent among the different formulations throughout the first 6 hours with 28.7%, 28.2% and 27.0% being released at the end of 6h incubation at pH 7.4. The fact that the three systems follow the same release profile helps remove any confounding effect that may arise regarding rate and extent of uptake of FITC in the monolayers due to liposomal (encapsulated FITC) vs. free FITC (released FITC) in the medium as will be discussed next. Overall, the characteristics of the three systems were not impacted by the presence of GM on the liposomes, or by the different mol % of the targeting lipid containing PEG 2000Da. In fact, the small ratios of GM used to prepare the liposomes may explain the consistency in profiles for size, zeta potential, drug loading, encapsulation and release among the three formulations.
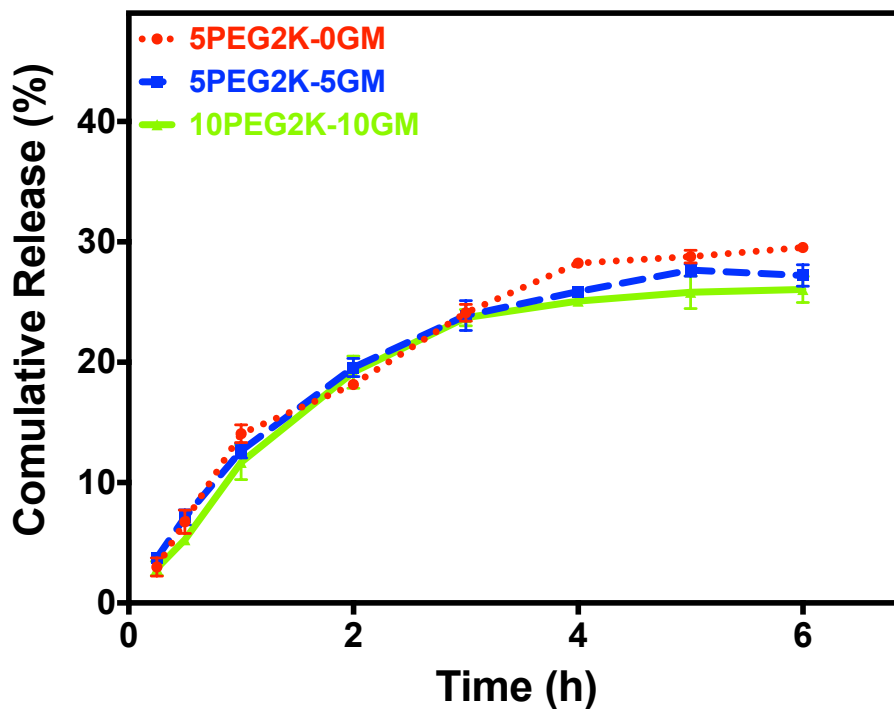
Figure 2.3 Cumulative amount of FITC released from actively-loaded, non-targeting (5PEG2K-0GM, red dotted line) and targeting (5PEG2K-5GM – blue dashed line, and 10PEG2K-10GM green line), liposomes at pH 7.4 over 6 hours - incubated at 37 ^{0}C. Data represents mean ± SD (n = 4). 5PEG2K-0GM = liposomes with 5 mol% DSPE-PEG2K lipid; 5PEG2K-5GM = liposomes with 5 mol% DSPE-PEG2K-GM lipid; 10PEG2K-10GM = liposomes with 10 mol% DSPE-PEG2K-GM lipid

2.3.2.2 The *in vitro* Placental Model.

BeWo cells have been shown to express megalin receptors, and when polarized monolayers are formed, BeWo cells have been shown to be involved in the uptake of gentamicin in a megalin-mediated manner (Akour et al., 2015b). Establishing polarized monolayer was thus essential for this cell line in order to be used as a model for the syncytiotrophoblast (SynT) layer. TEER was recorded to assess the integrity of the monolayer. **Figure 2.4a** shows the increase in TEER values over a period of nine days post cell seeding. TEER values plateau at 40-50 $\Omega\,cm^2$ around days 5-6. Optimal micrographs of tin sections of

prepared system (**Figure 2.4b**) show the formation of monolayers, compared to multilayers formed on day 7 and beyond - **Figure S2.2b**. BeWo monolayer polarization was confirmed by staining of the tight junction ZO1 proteins - **Figure 2.4c**. Monolayers were initially characterized on days 5, 6, 7 and 8. Day 6 was chosen to carry out the FITC internalization studies based on the plateauing of TEER values, formation of tight junctions and the presence of a monolayer (vs. multilayer on later days).
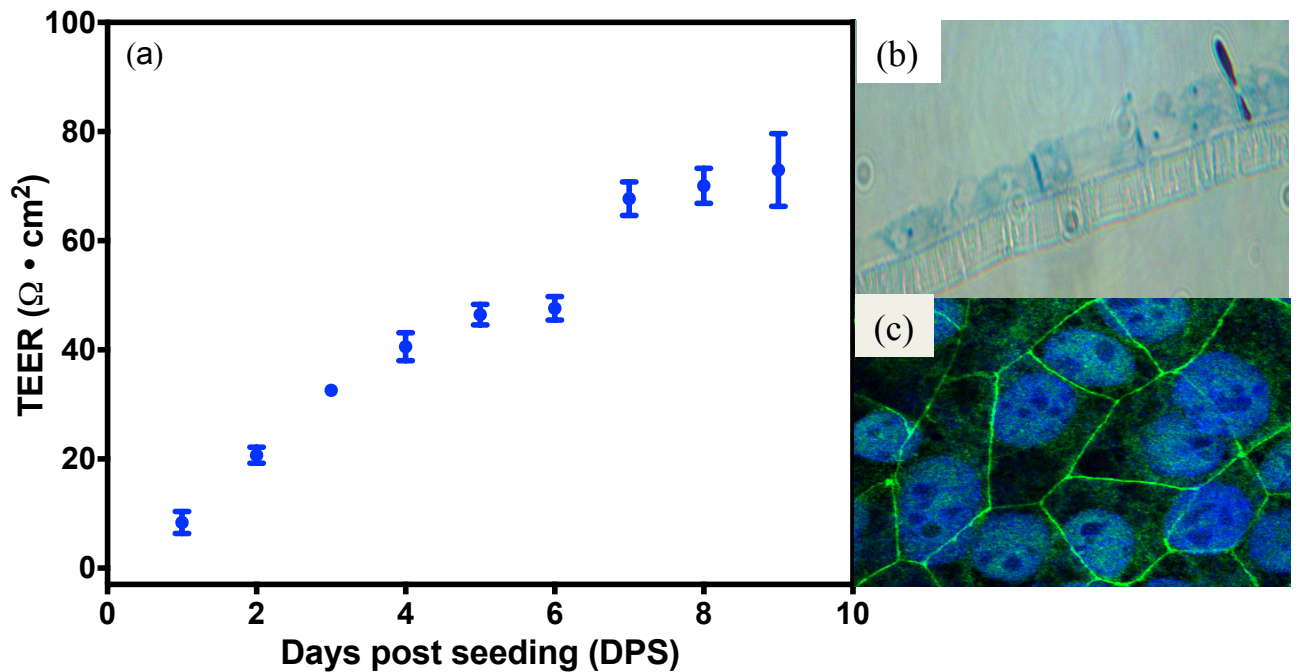
Figure 2.4: (a) Transepithelial electrical resistance (TEER) of BeWo monolayers cultured on 0.33 cm^2 Transwell® inserts as a function of time. Data represents mean ± SD (n = 4). (b) 40X optical image showing a cross-section of the BeWo monolayer on the 6th day post seeding (DPS). (c) ZO-1 staining of a confluent BeWo monolayer obtained on the 6th DPS, indicating tight junction formation.

2.3.2.3 Targeting the *in vitro* placental model.

2.3.2.3.1   Uptake   in   BoWo   Monolayers.

The uptake of FITC encapsulated in the different liposomal formulations by

BeWo polarized monolayers and a control monolayer (HepG2, which does not express megalin) was evaluated as a function of time using flow cytometry. Formulations containing two levels of GM at **5PEG2K-5GM** and **10PEG2K-10GM**, and the negative control (**5PEG2K-0GM**) were studied.

As shown in **Figure 2.5**, the extent and rate of internalization of liposomes were significantly enhanced upon the addition of the targeting agent - **5PEG2K-5GM** ($p < 0.0001$) and **10PEG2K-10GM** ($p < 0.0001$) at 6 h, compared to the control **5PEG2K-0GM**. During the first time point (15 min), there was no significant increase of uptake among compared to the control. At time = 0.5 h, the formulation with 10mol% targeting lipid **10PEG2K-10GM** showed a significant increase ($p = 0.0362$) compared to control; however, the uptake profile for **5PEG2K-5GM** remained not significantly different compared to control. Such uptake profile is not uncommon for the early time points as previously shown for liposomal systems with different targeting moieties (Hong et al., 2009; Zidan and Aldawsari, 2015).

After 1 h, uptake of **5PEG2K-5GM** and **10PEG2K-10GM** liposomes was significantly enhanced ($p = 0.0357$ and $p < 0.0001$ respectively) compared to the control group. At time = 2 h and longer contact times, both systems sustained significantly enhanced FITC uptake ($p < 0.0001$). Increasing the mol% of targeting lipid (compared **10PEG2K-10GM** and **5PEG2K-5GM**) also led to enhancement in FITC uptake at all times, except 0.25h.
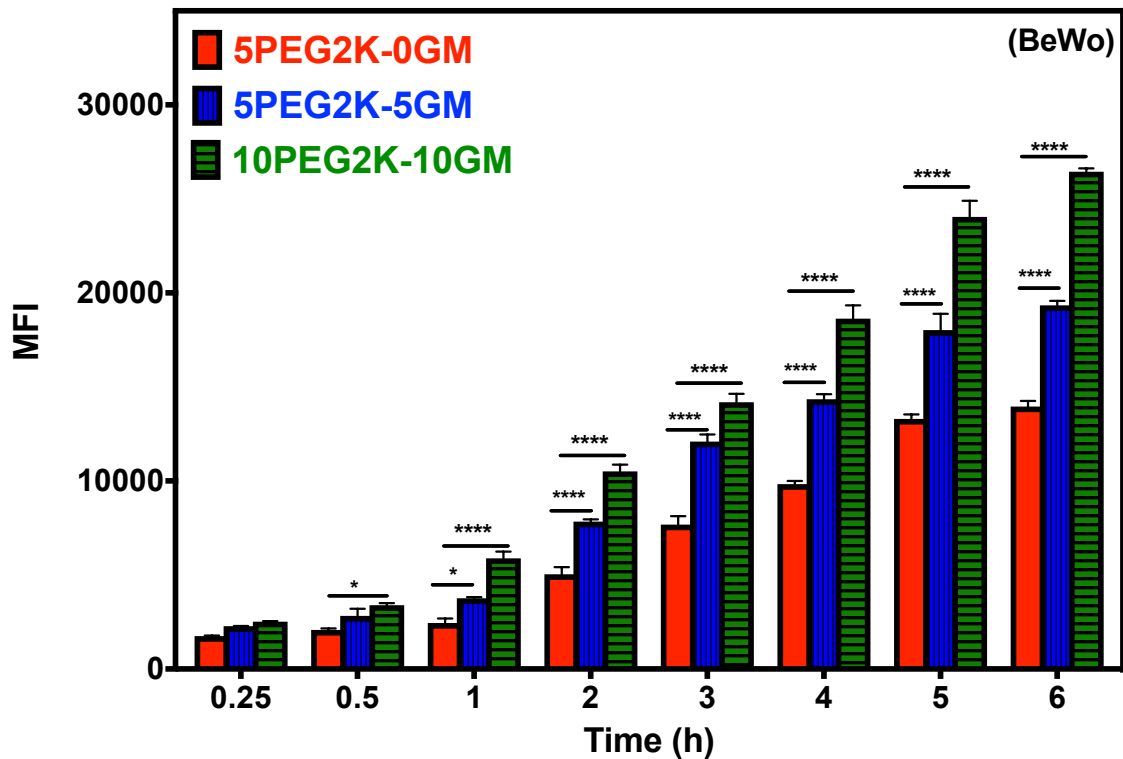
Figure 2.5: Cellular internalization of FITC on polarized BeWo cells (6th day post seeding) determined by flow cytometry, as a function of time post contacting the monolayer with FITC-encapsulated liposomes. The median fluorescence intensity (MFI) data represents mean ± SD (n = 4), with at least 6,000 events (singlets). 5PEG2K-0GM = liposomes with 5 mol% DSPE-PEG2K lipid; 5PEG2K-5GM = liposomes with 5 mol% DSPE-PEG2K-GM lipid; 10PEG2K-10GM = liposomes with 10 mol% DSPE-PEG2K-GM lipid.

2.3.2.3.2 Uptake in Hepatocellular Carcinoma (HepG2) Monolayers.

Uptake of FITC encapsulated in *5PEG2k-0GM* and *5PEG2K-5GM* was also studied in hepatocellular carcinoma (HepG2) monoalyers. HepG2 was used as negative control, as it does not express megalin receptors (Akour et al., 2015b). **Figure 2.6** shows the cellular uptake of FITC at 0.25, 3 and 6 h. There is no significant difference between the two systems at the three time points (p > 0.999, p = 0.872 and p = 0.4528 respectively). These results support the idea

that increases in uptake of FITC in BeWo cells seen for the liposomes with GM-modified lipids in **Figure 2.5** are due to specific interactions between GM and the megalin receptors, which we expect to lead to enhanced internalization of FITC via fluid phase endocytosis.
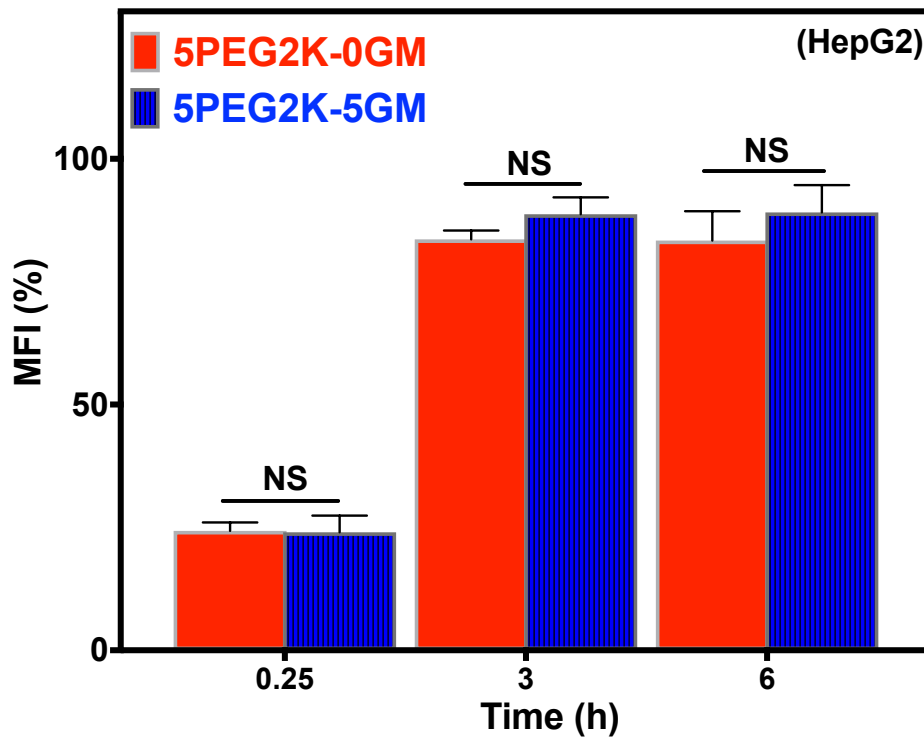
Figure 2.6: Normalized cellular Internalization of FITC on HepG2 cell monolayers determined by flow cytometry, as a function of time post contacting the monolayer with FITC-encapsulated liposomes. The median fluorescence intensity (MFI) data represents mean ± SD (n = 4) with at least 6,000 events (singlets). 5PEG2K-0GM = liposomes with 5 mol% DSPE-PEG2K lipid; 5PEG2K-5GM = liposomes with 5 mol% DSPE-PEG2K-GM lipid.

Increases in FITC uptake with time for the targeting liposome systems (**Figure 2.6**) are expected even for monolayers that do not express megalin due to the fact that liposomes are known to be internalized via different endocytic mechanisms (Duzgune et al., 1999; Friend et al., 1996; Straubinger et al., 1983).

This includes those that are not receptor mediated as e.g. macropinocytosis (Swanson and King, 2018) and is also due to the internalization of free FITC that is being released from the liposomes as a function of time.

2.3.2.3.3 Competitive Inhibition of Megalin Receptors.

Competitive inhibition studies were carried out by saturating the megalin receptors with free GM before contacting the targeting and non-targeting liposome formulations with the polarized BeWo monolayers. Saturation was accomplished by adding 2 mM free GM in HBSS media 15 to the cell monolayer minutes prior to the addition of liposomal formulations. This molar concentration was selected based on the fact that BeWo cells retain 100% viability under such conditions – see Supporting Information - **Figure S2.3**. The uptake of **5PEG2K-5GM** was seen to be significantly higher than the control liposome (p<0.0001) in the absence of free GM (GM -) as seen in **Figure 2.7** (as also shown in **Figure 2.5**); however, in the presence of 2 mM free GM (GM +), the uptake of **5PEG2K-5GM** was significantly reduced and seen not to be statistically different compared with non-targeting control **5PEG2k-0GM** (p = 0.0599).

Put together, the cellular internalization experiments described in this work support the idea that GM-modified liposomes are internalized in polarized BeWo monolayers via megalin-mediated endocytosis, and that the ability to target megalin leads to enhancement in the rate and extent of internalization of FITC, the encapsulated probe.
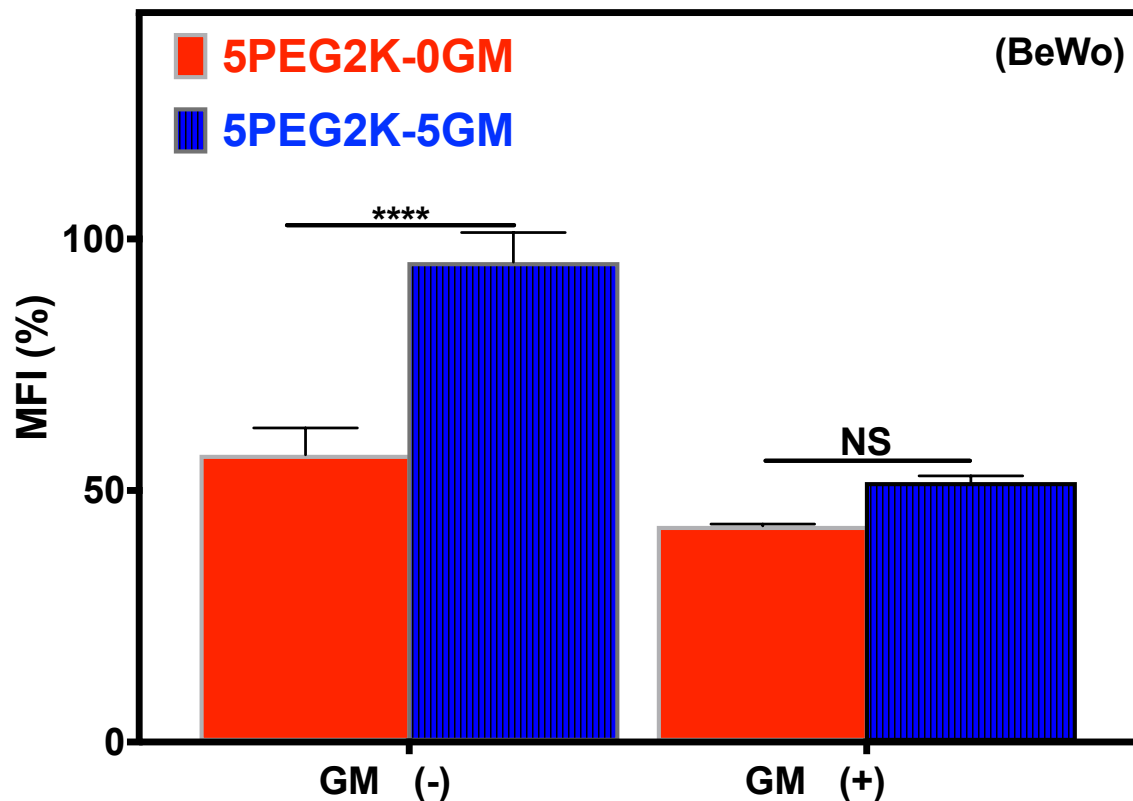
Figure 2.7 Normalized cellular Internalization of FITC on polarized BeWo cells (6th day post seeding) in the absence and presence of free gentamicin (Free GM; 2mM) determined by flow cytometry at 6 h post contacting the monolayer with FITC-encapsulated liposomes. The median fluorescence intensity (MFI) data represents mean ± SD (n = 4) with at least 6,000 events (singlets). 5PEG2K-0GM = liposomes with 5 mol% DSPE-PEG2K lipid; 5PEG2K-5GM = liposomes with 5 mol% DSPE-PEG2K-GM lipid; 10PEG2K-10GM = liposomes with 10 mol% DSPE-PEG2K-GM lipid.

These results are of importance as they represent a novel strategy to potentially target the placenta and enhance the delivery of drugs and biologics to treat a variety of diseases of pregnancy. The liposomes with 10mol% targeting lipids (and the respective control) were thus selected to carry out the *in vivo* studies.

2.3.3 *In Vivo* Studies.

2.3.3.1 Synthesis and Characterization of Cy5.5-modified lipids (DSPE-PEG2K-CY).

Synthesis of the Cy5.5-conjugated lipid was achieved in a one-step reaction – Scheme S1. DSPE-PEG2K-NH2 (MW ca. 2790 Da) was reacted with Cy.5.5-COOH (MW 619 Da) via an amidation reaction to yield DSPE-PEG2K-CY (3400 Da), which was characterized by MALDI-ToF as shown in **Figure S2.1**.

2.3.3.2 Liposomal Formulations for *in vivo* Studies.

Liposomal formulations for *in vivo* studies were prepared with the Cy5.5 probe (DSPE-PEG2K-CY lipids) to avoid any potential sensitivity issues with FITC due to tissue background auto-fluorescence. The liposomal formulations for *in vivo* studies with the DSPE-PEG2K-CY lipid exhibited similar HD and ζ as the formulations with encapsulated FITC, as shown in Table S1, demonstrating that the presence of small amounts of the CY-lipid conjugate do not significantly impact the characteristics of the liposomes. The non-targeting liposomes (***10PEG2K-0GM-CY***) and the targeting (***10PEG2K-10GM-CY***) liposomes prepared with 1.7mol% of DSPE-PEG2K-CY and 10 mol% of PEGylated lipid (without or with GM targeting) had HDs of 103 nm and 114 nm, and ζ of -5.4 and -3.4 mV, respectively.

2.3.3.3 Targeting the *in vivo* placental model.

The *in vivo* biodistribution of Cy5.5 in pregnant mice was evaluated via an in vivo imaging system (Xenogen IVIS Spectrum), and also by tissue extraction and quantification of the fluorescence of Cy5.5. The total flux of Cy5.5 of the excised placental, fetal, and maternal kidney tissues for the treatment groups

including free Cy5.5, and the targeting and non-targeting liposomal formulations are shown in **Figure 2.8a**. Images of those tissues are shown in Figure **2.8b**, including the PBS controls.

The results indicate that the total flux from the Cy5.5 probe in the placenta is significantly enhanced (p=0.009) by formulation of Cy5.5 in the PEGylated liposomes compared to free Cy5.5. It is likely that the prolonged circulation times of PEGylated liposomes(Kooijmans et al., 2016) combined with a SynT layer that has a high surface area and is highly irrigated by maternal blood(Georgiades et al., 2002) promotes the contact of the probe with the placenta when in liposome formulation, leading to its enhanced accumulation. Other studies have shown that physicochemical properties of PEGylated liposomes were manipulated to enhance delivery of placental limited-uptake drugs to the placenta(Bajoria and Contractor, 1997; Bajoria et al., 1997a; Bajoria et al., 2013; Valero et al., 2018). The presence of the targeting moiety in the ***10PEG2K-10GM-CY*** liposome formulations further promotes (p<0.0001) the accumulation of Cy5.5, leading to a 3.7 fold increase in flux in the placental tissue when compared to the non-targeting control liposomes. The results show that the presence of the targeting ligand GM not only leads to the preparation of liposomes with similar characteristics in terms of HD and ζ to the non-targeting PEGylated controls, but also seems to indicate that it does not negatively impact the *in vivo* stability of the formulation, as GM has low plasma protein binding(Pechere and Dugal, 1979).

For fetal tissues, IVIS imaging showed no significant accumulation of either free Cy5.5 (p=0.3599) or Cy5.5 formulated in the liposomes (p 0.9917 and

0.9149, for non-targeting and targeting, respectively) compared to the control fetal tissue (PBS administration) (n=6) or amongst each other. On the other hand, kidneys also show an increase in uptake of Cy5.5 formulated in liposome form compared to free Cy5.5, similar to placental tissues. Targeting further increased the uptake of Cy5.5 by 3.9 fold (p <0.0001) as expected as this tissue also expressed megalin receptors.(Campeiro et al., 2019; Faber et al., 2006) Considering an average of 5 placentas, the flux ratio (all placentae to both kidneys) is ca. 2X, indicating a significantly higher placental accumulation for the targeting liposomes.
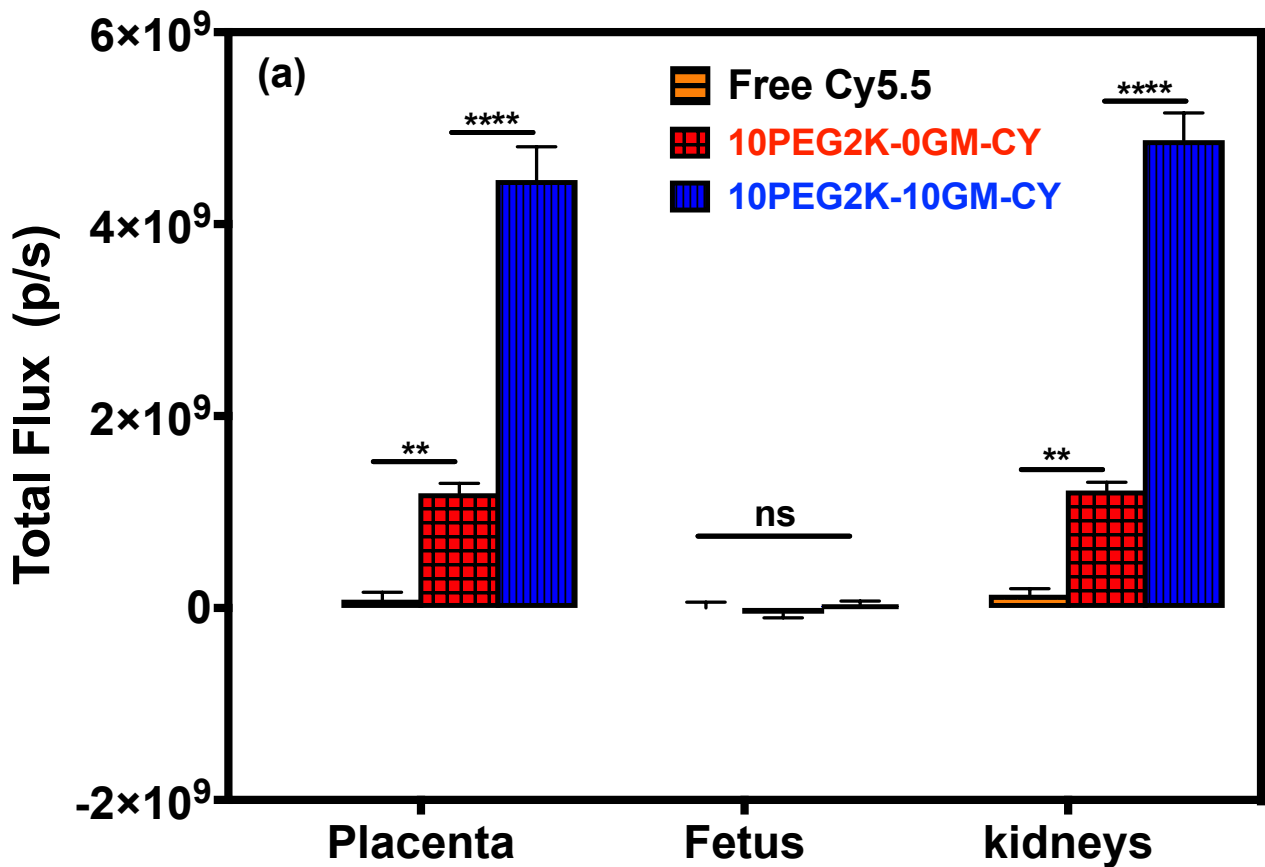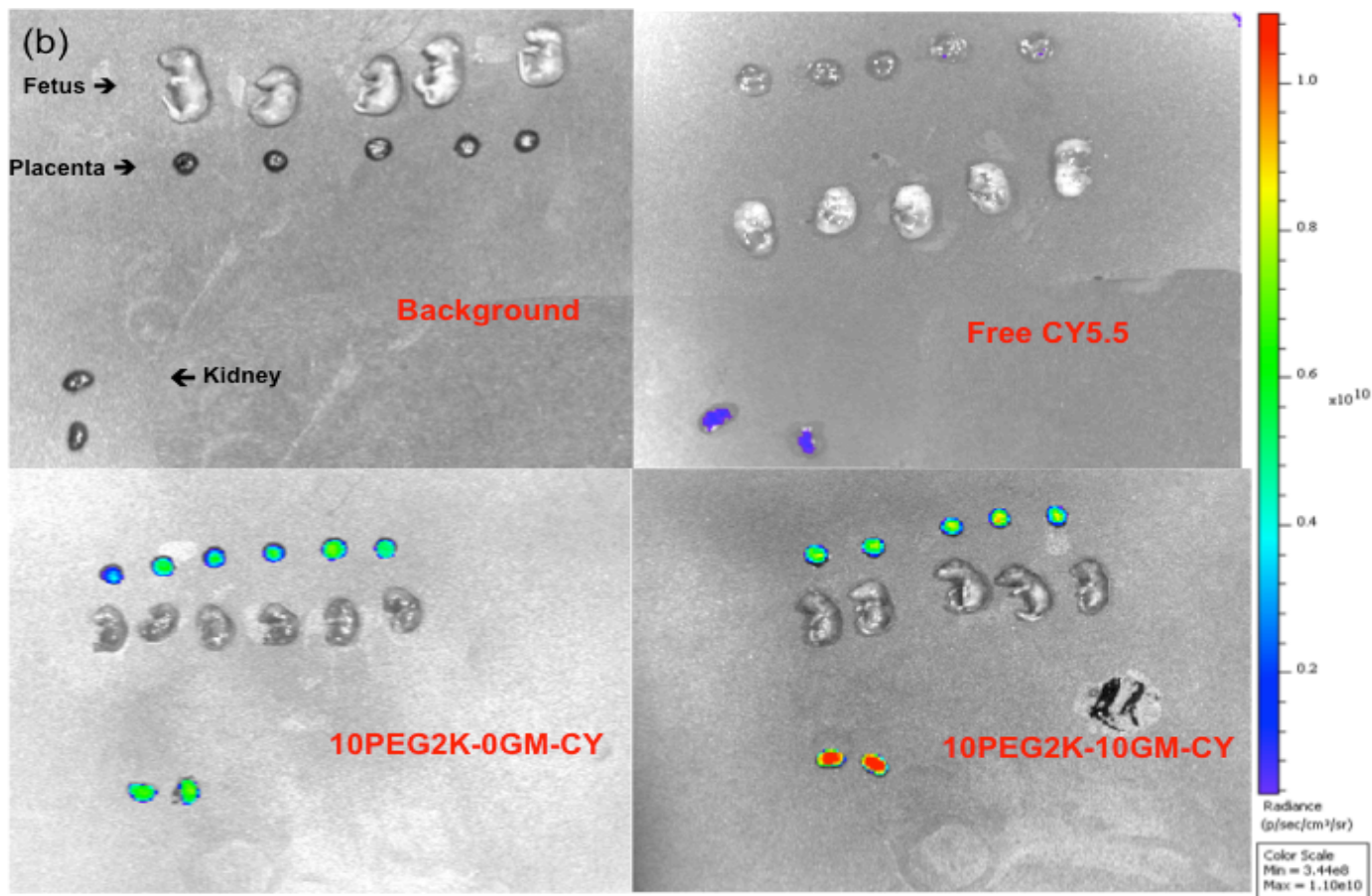
Figure 2.8: (a) Total flux of Cy5.5 for free Cy5.5, 10PEG2K-0GM-CY and 10PEG2K-10GM-CY liposomes measured ex vivo by IVIS imaging showing placentas, fetuses and kidneys 4 hr post treatment. Results shown have been subtracted from background which are fluxes of tissues exposed to PBS control only; **(b)** representative images of the placental and fetal tissues, as well as kidneys for the various treatment groups. 10PEG2K-0GM-CY = non-targeting liposomes with 10 mol% DSPE-PEG2K lipid and 1.7 mol % DSPE-PEG2K-CY lipid; 10PEG2K-10GM-CY = targeting liposomes with 10 mol% DSPE-PEG2K-GM lipid and 1.7 mol % DSPE-PEG2K-CY lipid.

The distribution of Cy5.5 in placental and fetal tissue was also assessed by tissue extraction (n=4). **Figure 2.9** shows the fluorometric analysis based on an established calibration curve, with the inset indicating the % accumulation in the placenta relative the initial dose of 25ug of Cy5.5.

Average concentration of Cy5.5 from **10PEG2K-10GM-CY** targeting liposomal formulations was ca. 88 fold greater than free Cy5.5. Mirroring the IVIS results, the targeting liposomes led to ca. 2 fold increase the accumulation of Cy5.5 compared to the non-targeting **10PEG2K-0GM-CY** liposomal formulation, with the following accumulated amounts in the placenta: **10PEG2K-10GM-CY**: 1405 $\pm$ 10 ng/g (2.81% total dose); **10PEG2K0GM-Cy**, 685 $\pm$ 5 ng/g (1.34% total dose); free Cy5.5, 128 $\pm$ 3 ng/g (0.03% total dose); $p<0.0001$.
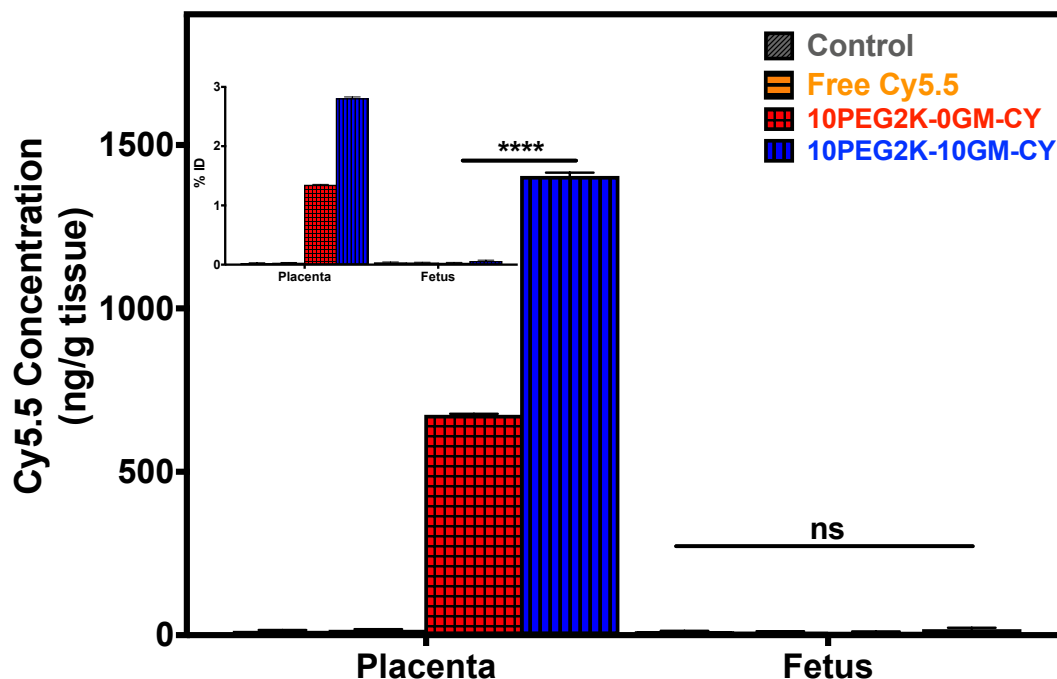
Figure 2.9: (a) Quantitative analysis (ng/g tissue) of Cy5.5 for control (no exposure to Cy5.5), free Cy5.5, 10PEG2K-0GM-Cy and 10PEG2K-10GM-Cy liposomes measured from homogenized tissues 4 hr post treatment; (b) same results plotted as % initial dose (%ID) – total of 25ug in tissue. Cy5.5 assessed fluorometrically by microplate reader and concentrations obtained based on established calibration curves on spiked tissues. 10PEG2K-10GM-CY = targeting liposomes with 10 mol% DSPE-PEG2K-GM lipid and 1.7 mol % DSPE-PEG2K-CY lipid.

Fetal analysis shows no significant accumulation of Cy5.5 (no statistical difference from PBS control) for any of the treatments groups (**10PEG2K-10GM-CY**, 14 ng/g; **10PEG2K-0GM-CY** 17 ng/g; free Cy5.5, 25 ng/g). While the current placental-targeting liposomal formulations do not show significant increases in the fetus in mice, it is important to reemphasize the anatomical differences between placenta of humans and rodents. Having three layers (two SynT layers and a third trophoblast layer)(Georgiades et al., 2002) at GD18.5, as opposed to one SynT layer in human third trimester placenta (Georgiades et al., 2002), mice placenta may represent a stricter barrier than that of humans, thus showing less transplacental transport of certain compounds (Kulvietis et al., 2011).

2.4 CONCLUSION

In this study we synthesized gentamicin (GM)-modified PEGylated lipids and demonstrated that the corresponding liposomal formulations prepared with these lipids promote the uptake of associated fluorescent probes in both *in vitro* (polarized BeWo monolayers) and *in vivo* (timed pregnant mice) placental models. GM is a substrate of megalin (Akour et al., 2013), a transmembrane protein in the apical side of the syncytiotrophoblast (SynT) layer of the maternal-fetal interface (human and murine placentae and BeWo cells), and the nanocarriers prepared here were designed to hitchhike those clathrin-mediated processes that megalin is involved in. Targeting liposomes promoted the uptake of the associated fluorescent probe in polarized BeWo monolayers to a significantly greater extent than the non-targeting PEGylated analogs, and the extent of uptake increased as the density of targeting lipids in the liposomes

increased. We also observed that competition for megalin receptors resulted in a decrease in uptake of the associated probe in targeting liposomes to the same level of non-targeting liposomes, further supporting the role of megalin endocytic processes in the uptake. In *in vivo* biodistribution experiments (IVIS and direct quantification by tissue extraction), formulation of the probe as conjugate to lipids resulted in significant enhancement in uptake in the placental tissue compared to the free probe. This is likely due to improved circulation of the conjugated probe, while no measureable amounts of the probe were found to transport into the fetal tissue. Addition of targeting lipids to the liposomal formulation further enhanced the uptake of the conjugated probe, leading to 2-4 fold higher uptake in the placenta compared to the non-targeting liposomes, while the probe is still not seen into fetal tissue. The proposed GM-targeting liposomal nanoformulations may serve as a platform for the delivery of small molecule therapeutics and biomacromolecules to support the treatment of diseases of pregnancy.

2.5 ACKNOWLEDGMENTS

and Cancer Mouse Models Core Laboratory Shared Resources, supported, in part, with funding from NIH-NCI Cancer Center Support Grant P30 CA016059."

CHAPTER 3

Polyester Dendrimer for Placental Drug Delivery in Mice

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3.1 INTRODUCTION

There have been significant recent advances in the use of drug containing nanomedicines (Kesharwani and Iyer, 2015). There are several advantages in using nanotechnology for drug delivery including the ability to control the pharmacokinetics and biodistribution of the therapeutic cargo (Pattni et al., 2015), reduction of side effects (Lim et al., 2012), enhancing sustained release(Natarajan et al., 2014), and providing new strategies for the delivery of poorly soluble drugs (Gao et al., 2002). Several fields have benefited from nanomedicine such as oncology, ophthalmology, cardiology and microbiology (Doane and Burda, 2012; Shi et al., 2010). Moreover, numerous drug containing nanomedicines have been approved by the FDA, including Doxil ®, a treatment of many different types of cancer. More recently, nanotechnology approaches have been proposed to tackle diseases of pregnancy (Refuerzo et al., 2016; Yu et al., 2017), which are very prominent and contribute to many maternal and fetal health problems with significant morbidity and mortality including preeclampsia (Roberts and Gammill, 2005). Some in vitro and in vivo model studies have shown promising results with regards to the delivery of therapeutics to the placenta in order to improve the health of the growing fetus (Refuerzo et al., 2016; Valero et al., 2018; Valero et al., 2017); however, there is a still a long way to go in terms of developing drug containing nanomedicines for the treatment of diseases of pregnancy. There are many considerations when designing nanocarriers that can target the placenta to treat such disease including the potential toxicity that can be imposed to the mother or the fetus. Thus, it is

essential to consider the fate of such nanocarriers and whether there is a collateral damage associated with their use. In this chapter we discuss the potential of polyester dendrimers as a platform for the delivery of therapeutics to the placenta. While our end objective is to develop gentamicin (GM)-modified dendrimers for targeting megalin in the SynT, as we did for liposomes, here we report initial studies of PEGylgated polyester dendrimers (their chemistry and characterization) and the design and initial steps in the preparation of GM-targeting dendrimers. We discuss PK results in non-pregnant animals to make preliminary determination of optimum times in which to study the biodistribution in the placenta and fetus of such carriers, which are our future experiments. We also have planed for the future in vitro uptake studies once the targeting dendrimer has been synthesized and characterized.

3.2 MATERIALS AND METHODS

3.2.1. Materials.

Gentamicin (GM), chloroform and ethanol (EtOH) were purchased from VWR Analytical. Cyanine 5.5 (Cy5.5) was purchased from Lumiprobe (Hunt Valley, MD). Eagle's Minimum Essential Medium (EMEM) and Trypsin-EDTA (1X) were purchased from Corning. DMEM/F12 was purchased from Thermo Fisher and fetal Phosphate buffer saline (PBS, 1X, pH=7.4), Hank's Balanced Salt Solution (HBSS, 1X, pH=7.4) were prepared in our laboratories. Di-*tert*-butyl dicarbonate (BOC), 4-(Dimethylamino)pyridine (DMAP, 99%), cesium fluoride (CsF), N,N-dimethylformamide anhydrous (DMF, 99.8+%) and hydrogen chloride, 4M in 1,4-dioxane (99%) were purchased from Alfa Aesar. Succinic anhydride

(SA), n-hydroxysuccinimide (NHS) and N-(3-Dimethylaminopropyl)-N`-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma Aldrich. BOC-6-aminohexanoic acid was purchased from Alfa Aesar.

3.2.2. Synthesis and Characterization of Gentamicin-PEG1000Da conjugated, Cy5.5-modified, generation 4, polyester dendrimers (G4OH-PEG1K-GM).

Previously we successfully synthesized 3 systems of non-PEGylated generation 4 polyester dendrimer (G4OH) by conjugating FITC (G4OH-FITC) and GM (G4OH-GM) directly on the surface of the dendrimer. MALDI-ToF and ^{1}H NMR characterization is shown in **Figure S 3.1.** Due to water solubility issues and potential issues related to the proximity of the targeting agent to the dendrimer surface, in the end we decided to change the route of the synthesis by including PEG as solubility enhancer, and also as a flexible spacer to conjugate the targeting GM to the dendrimer surface. In this new approach, we first conjugated a near infrared dye Cy5.5, which is commonly used in the *in vivo* studies and avoid tissue background issues as it happens with FITC.

In this new approach we used G4OH was obtained from previous synthesis in our laboratories (Heyder et al., 2017). A fraction of terminal –OH groups of the dendrimer were first modified to –NH$_2$. Briefly, G4OH dendrimer was reacted with BOC-6-aminohexanoic acid in the presence of DMAP and EDC followed by deprotection in 4M HCl in dioxane to yield partially –NH$_2$-terminated dendrimer, where 15 groups out of 48 terminal groups were converted. The

product was reacted with Cy5.5 in the presence of NHS and EDC to yield **G4OH-NH2-Cy5.5** (Intermediate product).

G4OH-NH2-Cy5.5 and HOOC-PEG1000 were reacted via an amidation reaction to give the final non-targeting control G4OH-NH2-CY5.5-PEG (named **CY-G4OH-PEG1K**. This product was purified by dialyzing against DMSO for 24 hrs followed and against water for 8 hrs with continuous replacement of the outer medium. Final product was lyophilized and stored as powder.

For the targeting dendrimer, the intermediate product **G4OH-NH2-Cy5.5** was reacted with bifunctional HOOC-PEG1000-NH2 with its amines protected giving **Cy5.5-G4OH-NH2-PEG-NH2-BOC** followed by deprotection in 4M HCl in dioxane. **Cy5.5-G4OH-NH2-PEG-NH2** was then reacted with the previously synthesized **GM-BOC-SA**, as described in Chapter 2. The final product (work in progress) will be deprotected to give the final product, the PEGylated targeting dendrimer **CY-G4OH-PEG1K-GM.**

Characterization of each step of the synthesis was performed using MALDI-TOF and ^{1}H-NMR. The hydrodynamic diameter (HD in nm) and surface charge (zeta potential – ζ in mV) of the dendrimer were characterized by Light Scattering using Malvern Zetasizer Nano-ZS (Malvern, UK).

3.2.3. In Vivo Experiments.

While our final goal is to measure the biodistribution of the targeting dendrimer and the control in the placenta (and fetal tissue), among other maternal tissues, because the targeting dendrimer was not finished yet, here we report only the pharmacokinetic studies performed in non-pregnant mice. These

PK results will help us understand the best time after administration of the dendrimers at which to collect initial biodistribution results. It will also help us determine the stability of the polyester dendrimer in the in vivo model.

3.2.3.1 Pharmacokinetic Study in non-pregnant mice

The formulations were administered through a retro-orbital injection of 10 µg Cy5.5 eq. dose /mouse (20-22 g). Blood was collected at 0.25h, 0.5h, 1h, 8h, 24h and 48h via retro-orbital blood collection method for one time point followed by a terminal point for the following point. Blood was mixed with EDTA in ependorf tubes to prevent clotting. Blood samples were centrifuged for 15 minutes at 5000 RPM and plasma was collected. Plasma concentration of Cy5.5 was determined fluorometrically using a microplate reader (SynergyH1, Biotek), based on a pre-determined calibration curve in plasma. Plasma concentration vs. time curves were plotted and AUC, $t_{1/2}$, and K_{el} determined using GraphPad Prism 7.

3.2.4 Statistical Analysis. All data is presented as a mean ± standard deviation. Each measurement had a minimum of three independent trials (n ≥ 3) in which the mean and standard deviation were subsequently calculated. When necessary, GraphPad Prism 7 software was utilized to perform two-way analysis of variance (ANOVA) followed by Tukey's Multiple Comparison Test. Means were considered statistically significant if $p < 0.05$. P-values were categorized as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0005$

3.3 RESULTS AND DISCUSSION

3.3.1 Dendrimer Modification: hydroxyl terminated, generation 4, polyester

dendrimers (G4OH) were synthesized in our laboratories as descried

earlier(Heyder et al., 2017). G4OH was modified with –NH2 shown in the

Schematic 3.1.
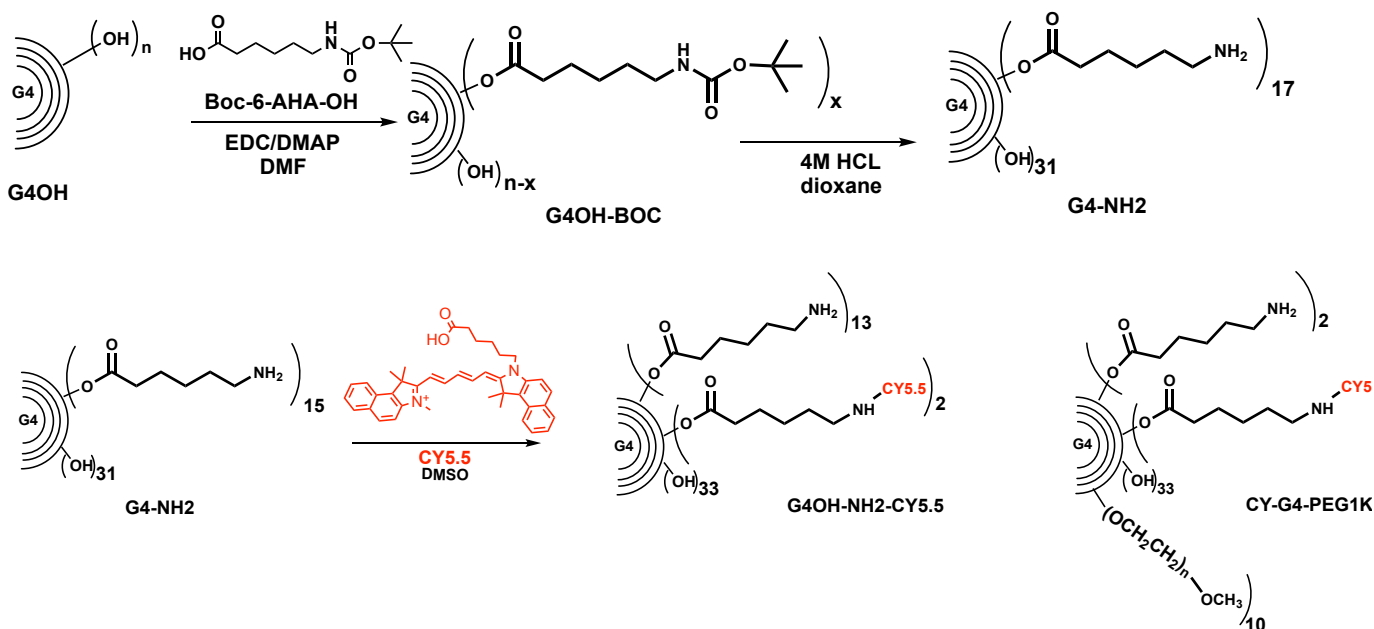
Scheme 3.1 Schematic overview of the partial conversion of –OH to NH2, followed by conjugation of Cy5.5 then mPEG1K

MALDI-ToF (Voyager-DE/JBI SCIENTIFIC) and ^{1}H-NMR results show –

Figure 3.1S and 3.2S in Supplemental Information. Results show that 15 out of

48 terminal groups were successfully modified to –NH2, as indicated by a shift in

the mass of the G4OH from 5353 Da to 7412 Da (**Figure S3.2)**, with each -NH2

functional group representing a mass of 130 Da. ^{1}H NMR results show that the

reaction with boc-protected amino hexanoic acid, and subsequently deprotection,

led to the appearance of new peaks related to the -CH$_2$ groups from the amino

acid (1.31, 1.51, 1.57, 2.28, 2.74 ppm). Those peaks were used to assign the

number of conjugated linkers, which is calculated as 15 (**Figure S 3.4 a, b**).

Cy5.5 (583 Da) was subsequently conjugated to the G4OH-NH2 dendrimers via an amide bond. The successful addition of Cy5.5 was observed in the MALDI-ToF (**Figure S3.3** – Supplemental Information) through the appearance of a peak at 8698 Da. The conjugation of the dye was confirmed by ^{1}H NMR by the appearance of peaks in the 7.5-8.5 ppm range (related to the rings and double bonds of the structure) and the number of conjugates was determined through the integration of the peak at 6.35 ppm. This peak was selected due to the fact that it is very isolated from other peaks and no overlap was observed. The integration of it showed the conjugation of 1.8 molecules of dye per dendrimer **(Figure S3.4 c)**.

Following the conjugation of Cy5.5, the product was divided to two batches for further reaction: control dendrimer (PEGylated and no GM), and targeting dendrimer (PEGyalated with GM-modified PEG). For the control dendrimers, HOOC-PEG-OCH$_3$ (mPEG, 1160 Da) was conjugated via amidation reaction. MALDI-ToF shows an increase of the mass to 18500 indicating the addition of 8 PEG 1KDa **(Figure S3.5)**. This was confirmed by ^{1}H NMR by the appearance of a very intense peak at 3.51 ppm, which is correspondent to the -CH$_2$ units of the polymeric chain. To quantify the number of polymer chains connected, we used the integration of the terminal methyl group (-CH$_3$) of mPEG at 3.24 ppm. It was determined that 10.2 PEG chains were conjugated per dendrimer **(Figure S3.4 d)**. Additionally, in the spectrum of this final structure it is possible to observe the peaks of Cy5.5 showcasing that the dye was not affected by the reaction between dendrimer and mPEG.

For the targeting dendrimer, after HOOC-PEG-NH2-BOC was conjugated to the dendrimer, MALDI-ToF results show a shift in peaks from 8698 to 20000 Da **(Figure S3.6)**. Deprotection and conjugation of GM is in progress. A summary of the characteristics for the various dendrimer cognates intermediates and the final targeting and control dendrimers is shown in **Table 3.1**. The characteristics include number of –NH2 surface groups, number PEG1K and PEG1K-GM, and number of Cy5.5, as measured by MALDI-ToF (M) and ^{1}H-NMR (N).

Table 3.1 Molecular weight, number of Cy5.5, mPEG, and GM-PEG as shown by MALDI-ToF (M) and 1h NMR (N), the hydrodynamic diameter (HD) and surface charge (zeta potential, ζ) of the G4 polyester dendrimer conjugates.

G4OH	MW (Da)	#CY 5.5 M	N	mPEG M	N	PEG-GM M	N	HD (nm)	ζ (mV)
CY-G4OH-PEG1K	170900	2	1.8	8	10	0	0	7 ± 3	-15.4
CY-G4OH-PEG1K-GM		2	1.8	0	0	TBD		TBD	TBD

3.3.2 Pharmacokinetics (PK)

The plot of plasma concentration of Cy 5.5 as a function of time is shown in **Figure 3.1**.PK analysis for the PEGylated polyester dendrimer CY-G4OH-PEG1K shows ($t_{1/2}$) at 4.1 h, with elimination rate constant (Ke) of 0.169 h^{-1}.

Mean residence time was 4.8 h while total systemic clearance (Cltot) was 0.0059 ml/min. Area under the curve (AUC) was 17066305.04 ng/ml*min.

Based on this analysis, we will perform the biodistribution study at that time point 4 h. At this time point, we will perform our biodistribution study as 50% of the dendrimer has been distributed/eliminated. Similar studies will be performed for the targeting dendrimer. Once those experiments have been performed, we will measure biodistribution in pregnant animals.
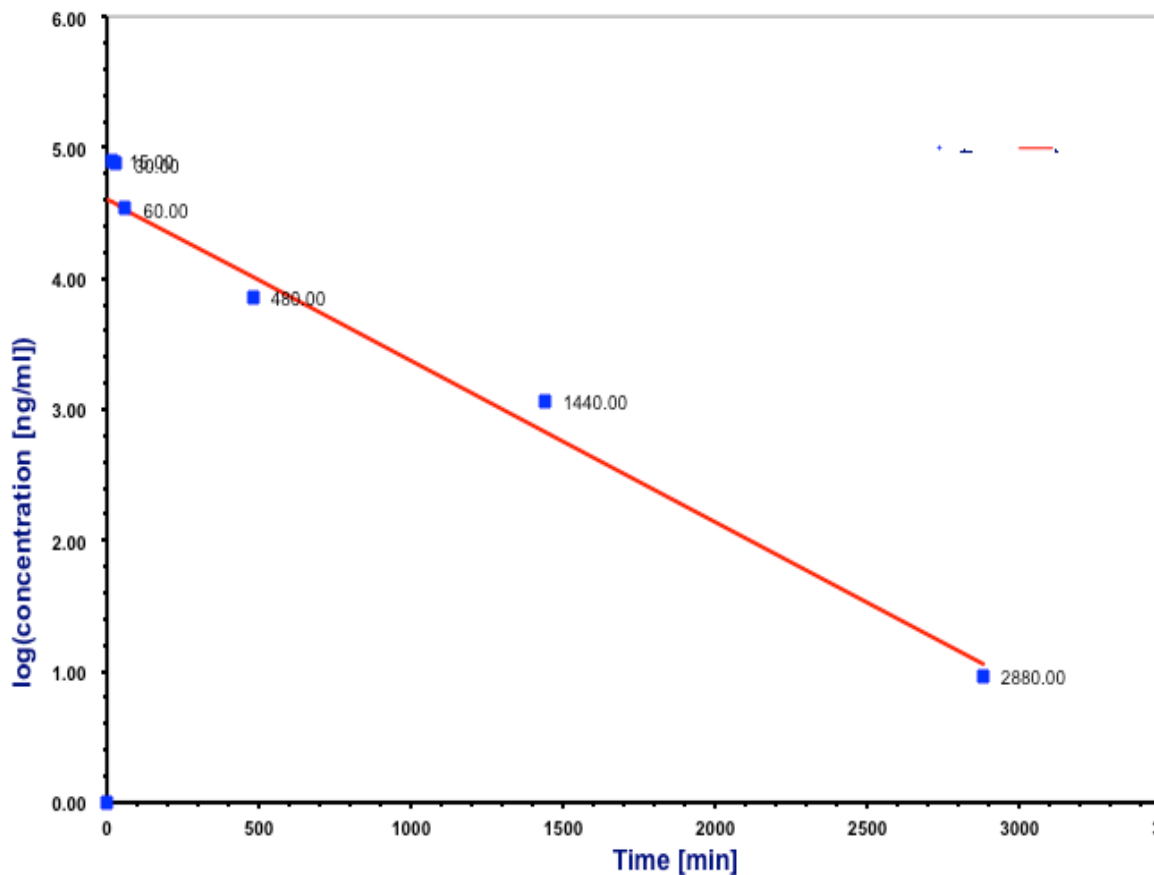
Figure 3.1 Plasma concentration (C_p) analysis as a function of time after retro-orbital administration of CY-G4OH-PEG1K. Concentrations are reported in log values (ng/ml)

3.4 CONCLUSION

Polyester dendrimers offer a great potential nanoplatform for the controlled delivery of therapeutics to the placenta. While their toxicity profile has not been studied in nearly as much detail as liposomes (at this time less translational potential), it offers an opportunity to control the release in a more predictable way, potential way to target fetal circulation and thus treat diseases of the fetus, and it is yet biodegradable and can also be excreted. In this Chapter we discuss the design, synthesis and modification of control polyester dendrimer conjugates and most of the steps towards the preparation of the targeting dendrimers. We also report some preliminary data on the pharmacokinetics of the non-targeting PEGylated polyester dendrimer. Their half-life were determined to be 4.1h, which compares with PAMAM with no PEG or low PEG density, as expected, as the density of PEG in this case is limited. These dendrimers are also almost completely eliminated after 24 hrs, which may be beneficial in terms of eliminating long-term toxicity issues.

This work is in progress and we are in the process of synthesizing the targeting dendrimer and assessing their pharmacokinetics. Subsequently, similar methods of *in vitro* uptake study and *in vivo* biodistribution will be executed based on what was reported in Chapter 2. We expect to finish these experiments shortly, as the timed pregnant mice reach the GD 18 within the coming weeks.

CHAPTER 4

CONCLUSIONS AND FUTURE WORK

Pregnancy-related complications are the major threat for the expecting mother and/or fetus because of the wide range of consequences that are associated with them. Preeclampsia, for instance, can lead to severe consequences for both the mother and fetus especially if not managed. It can lead to maternal and fetal morbidity and mortality (Jim and Karumanchi, 2017; Roberts and Gammill, 2005). IUGR is also another common disease of pregnancy that affects the growing fetus and is associated with preterm birth, which is also a major problem associated with pregnancy, contributing to approximately one million deaths annually (Goldenberg et al., 2008; Simmons et al., 2010). Unfortunately, there are no prescribed treatments to any of such complications; however, in some cases, a few medications are given to control the symptoms associated with those diseases. For example while preeclampsia does not have a treatment (besides delivering the babby), the high blood pressure associated with it is controlled by hypertension medications. Preterm birth may also be controlled using agents that can stop the uterine muscle contractions leading to delayed labor; however, preventing premature birth by simply stopping the uterine muscle contractions may not necessarily provide neonatal positive outcomes. Therefore, serious efforts with regards to developing treatments in obstetrics and gynecology are needed. Along with that, the development of formulations that can safely and efficaciously deliver the cargo is

critical so that the health of the mother and fetus is minimally affected by the treatment (Keelan et al., 2015b).

Our efforts to establish a nanoplatform that can be utilized to deliver therapeutic molecules in a targeted manner to the placenta, which is a major organ whose dysfunctions lead to many of the mentioned complications, are expected to support the development of such safe and effective formulations for treating diseases of the pregnancy. More specifically, we designed targeting nanocarriers that can bind megalin receptors expressed on the maternal side of the placental-fetal interface, the SynT later. We explored our strategies using existing well-established in vitro and in vivo models where megalin is also expressed.

In this work, we established a 2D in vitro placental model consisting of polarized monolayers of BeWo cells by culturing them and allowing them to grow as a function of time to confluent monolayers that form tight junctions. Because the SynT layer expresses certain receptors on the maternal side (apical side), the polarization of the BeWo monolayers was essential in order to mimic such conditions. We executed that by first seeding BeWo cells to porous membranes and culturing them for 9 days. Throughout the cell growth, we assessed the TEER values everyday. TEER values plateaued around 5-6 days, which is a surrogate for the monolayer polarization. We also confirmed the monolayer formation on day 6 by constructing cross-sections that show monolayers, and polarization by staining the tight junctions to confirm the barrier properties of the monolayers. Those results compared well / better then existing literature.

Future work regarding this model is to establish a more complex 3D model that can include more parameters and therefore be more representative of the placental barrier. The 3D model can be developed with BeWo cells on one side of the porous membrane, and fetal endothelial cells on the other side, representing maternal to fetal circulation. Moreover, a microfluidic system would enhance the flow properties on the apical side representing the maternal blood. Such model would make it more relevant not only to evaluate placental uptake of targeting nanocarriers but also transplacental crossing of agents from the maternal to the fetal circulations. It may also improve phenotype of the in vitro model.

On the other side, we successfully designed liposomes that can target the megalin receptors expressed on the apical side of the *in vitro* model of the SynT and enhance the uptake. DSPE-PEG-NH$_2$ was linked with the GM without modification of its amine groups. Using different molar ratios of this targeting lipid (5% or 10%), we were able to prepare different liposomal formulations and encapsulate FITC. We used 5% molar ratio of DSPE-PEG without the GM as a control. All the three systems showed consistency in terms of hydrodynamic size, surface charge, and encapsulation and release profiles, which were carefully assessed and were critical to the ability to measure the targeting potential of the nanocarriers. Our results showed enhanced uptake for both targeting systems compared to the control. We also concluded that higher density of DSPE-PEG-GM further enhances the uptake compared to the (5%) confirming that the uptake is megalin-mediated endocytosis. Furthermore, we tested the 5%

targeting liposomes with the non-targeting liposomes under different conditions. First, we tested the liposomal uptake in the presence and absence of 2 mM free GM as a method to saturate megalin receptors. Our results showed significant uptake reduction of the targeting liposomes in the presence of the free GM, further confirming liposomes are taken up via receptor-mediated endocytosis. Second, we tested the uptake of the same formulations on a different cell line (HepG2) that is known not to express megalin receptors. Results showed no significant difference between the two systems further confirming our hypothesis that modification nanocarriers with GM will results in enhanced uptake in megalin-expressing cells.

After concluding the positive results from the *in vitro* study, we successfully established an *in vivo* model using timed-pregnant female Balb/c mice. Liposomal formulations were altered to contain a near infrared dye. Cy5.5 was then conjugated to DSPE-PEG-NH$_2$ to replace FITC Encapsulation in order to establish a more robust strategy to assess the targeting / tissue biodistribution as the probe is not encapsulated but conjugated to the lipid, thus allowing us to directly assess the uptake of the nanocarrier/lipis instead of free probe that may be released in systemic circulation. We developed and implemented a new animal protocol for sourcing timed-pregnant mice. Targeting studies were performed on gestational day (GD) 18.5 as it represents the third trimester, as the placental barrier of mice and humans is functionally analogous at that time point of pregnancy (Georgiades et al., 2002). We performed biodistribution analysis at 4h post treatment based on previous studies performed with similar

PEGylated liposomes. Results showed enhanced targeting efficiency to the placental tissue for the GM liposomes compared to both the non-targeting liposomes and free drug model (Cy5.5) (P<00001). Kidneys also showed enhanced uptake as was expected due to the presence of megalin receptors in that tissue. Finally, fetal concentration showed no significant uptake between the two liposomal systems and the free Cy5.5, which was a positive outcome as at this point we sought to avoid reaching fetal circulation. However, more studies are required to probe the kinetics of biodistribution of the nanocarriers. Also, there are different scenarios here. One may be interested in preventing the nanocarriers to reach the fetal circulation for safety reasons, but further consideration as to the potential of nanocarriers reaching the fetal circulation to treat specific fetal diseases should be considered as for example targeting neural inflammation. This work shows the capability of GM-modified nanocarriers to target megalin receptor expressing cells and deliver therapeutics to the placenta. Further investigation of the fetal and maternal concentrations of payloads (not only carrier) are needed, including small molecules and biologics to understand their true potential. Other areas to explore here including varying the PEG length, density (and targeting density), and the use of other targeting ligands that may further enhance uptake in the SynT.

In conclusion, this work has established a viable nanoplatform for the targeted delivery of therapeutics to the placenta for the treatment of placental-based pregnancy complications.

There are many other interesting future studies/ directions for this formulation. From my perspective, one of the most attractive would be encapsulating short interfering RNA (siRNA) for the treatment of preeclampsia. siRNA is unstable in blood and easily degraded, thus, its encapsulation would add several advantages including, stability, longer circulation, sustained release and targeted delivery.

Preeclampsia is a common disease of pregnancy and a leading cause of maternal deaths. In the recent years, it has been linked to an imbalance of angiogenic factors leading to less angiogenesis in the placenta and consequently preeclamptic symptoms including high blood pressure. More specifically, soluble fms-like tyrosine kinase-1 (sFlt-1 or sVEGFR-1) has been reported to be excessively expressed in placental tissue. Therefore, targeted delivery of siRNA to the placental tissue in order to knock down the expression of the corresponding genes would be a valuable direction to consider.

Extending this work to ex vivo studies of perfused human placenta and more relevant animal models, including non-human primates would be also helpful to test the approach in more relevant models. Some studies have used such model to assess the transplacental crossing of nanocarriers. PAMAM dendrimers, for instance showed a low rate of transfer from maternal to the fetal side across the perfused human placenta where others have used similar *ex vivo* model to show that the transplacental crossing of warfarin can be minimized when encapsulated in cationic liposomes (Bajoria et al., 2013).

Dendrimers nanocarriers (DNCs) were also used in the project in order to evaluate the GM targeting using a different platform than liposomes. We believe because of their size and stability, megalin-targeting dendrimers may be able to not only hitchhike through the megalin-mediated endocytosis, but also transport to the fetal circulation, and that would be a major breakthrough. Dendrimers are significantly smaller than liposomes and provide for the ability to carry a higher payload and implement triggered release mechanisms. While we are about to execute the experiments with the targeting dendrimers, we did not have time to report here the outcome of those experiments. We show, however, that we were able to modify polyester dendrimers with two Cy5.5 molecules. Cy5.5 is a near infrared dye that is important in tracking the distribution of the nanocarrier. We also show that the CY-Dendrimer was successfully conjugated with 10 PEG1000 molecules, and we are currently working on the conjugation of PEG with the GM targeting ligand. Here PK studies will help us decide optimum time to get the preliminary biodistribution studies. Besides that, the protocols for the in vitro and in vivo studies have already been established and we should be able to execute the studies as soon as the chemistry is done. In the mean time, we will start by measuring the biodistribution of the PEGylated dendrimers with Cy5.5. PEGylation alone is expected to improve partitioning to the placenta compared to free probe as we observed with the liposomes due to improved systemic circulation and extensive irrigation and surface area of the SynT.

APPENDIX

Scheme S2.1: Schematic of the chemical synthesis of Cy5.5-modified DSPE-2KPEG lipid (DSPE-PEG2K-CY).
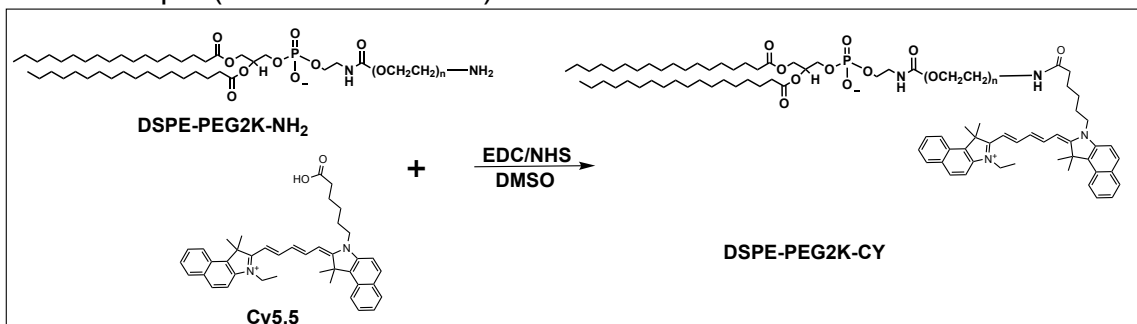
Cy5.5 =Cyanine 5.5
DSPE-PEG2K-NH$_2$= 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[amino(polyethylene glycol)-2000]
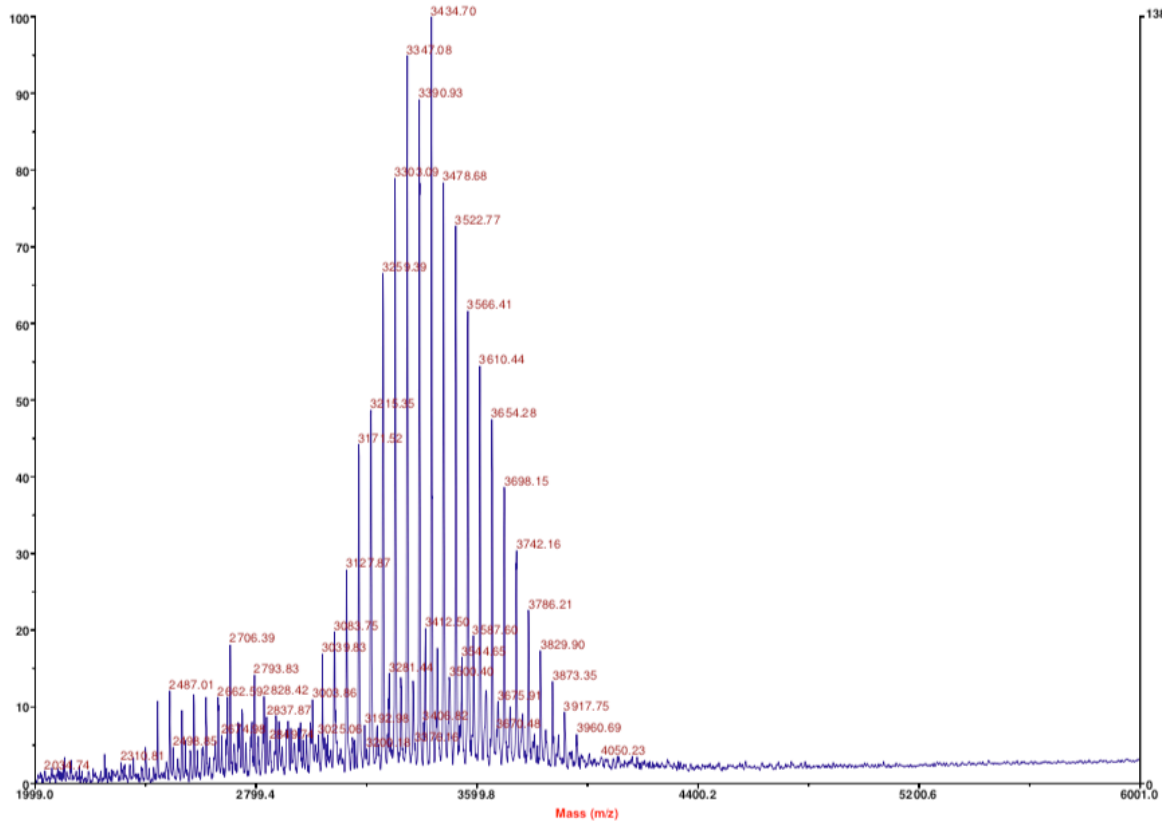
Figure S2.1: MALDI-ToF showing the peak shifts for DSPE-PEG2K-CY compared to DSPE-PEG2K (ca. 2790 Da, Figure 2). Cy5.5 with 619 Da.
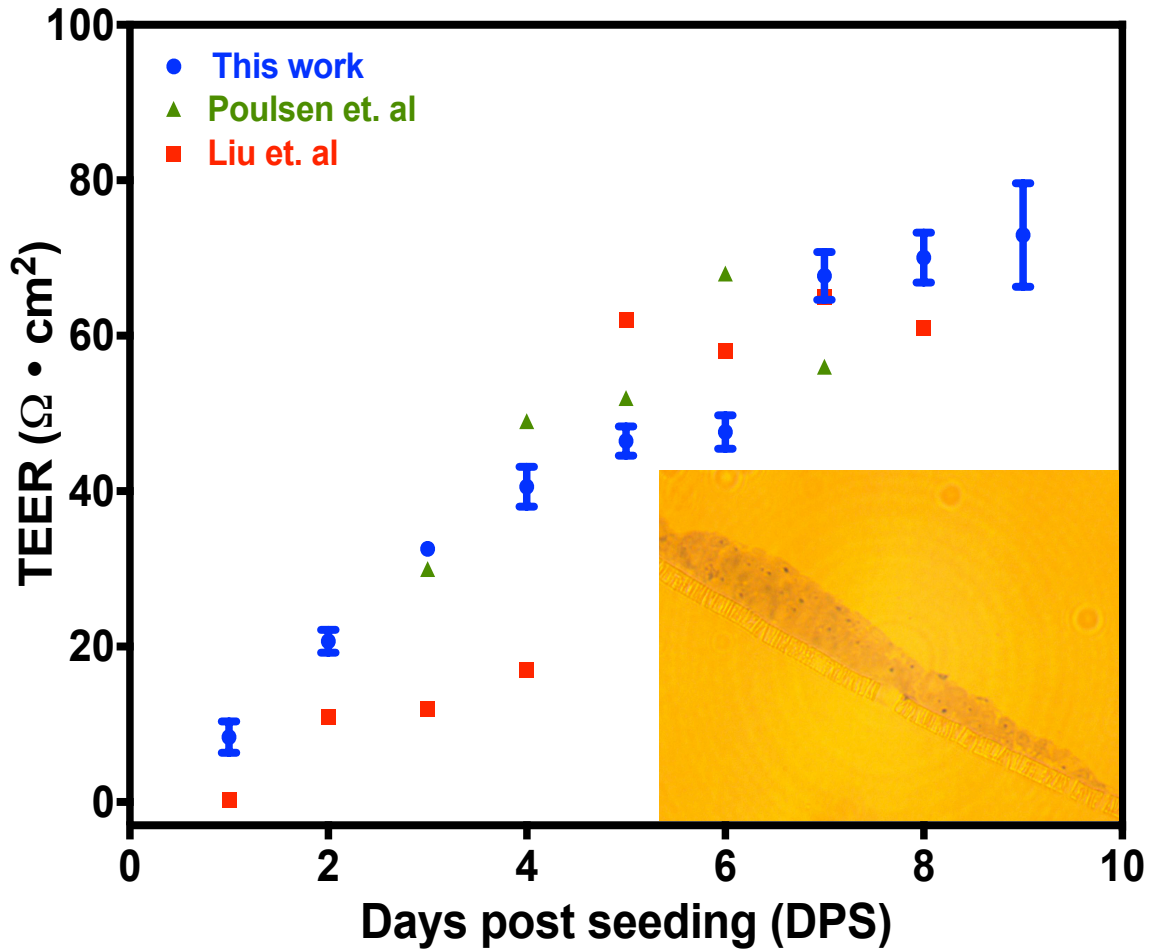
Figure S2.2. (a) Increase in transepithelial electrical resistance (TEER) of BeWo monolayers as a function of time. The figure shows the increasing TEER values of this study compared to other previously reported work(Hong et al., 2009; Poulsen et al., 2009). Note a shift in TEER at day 7. Based on cross section micrographs this corresponds to the formation of multilayers **(b)**. Our work is all at day 6 when BeWo is in monolayer form and polarized (b) as multilayer formation after day 6. Our work is performed when BeWo is polarized and in monolayer form – at day 6. .
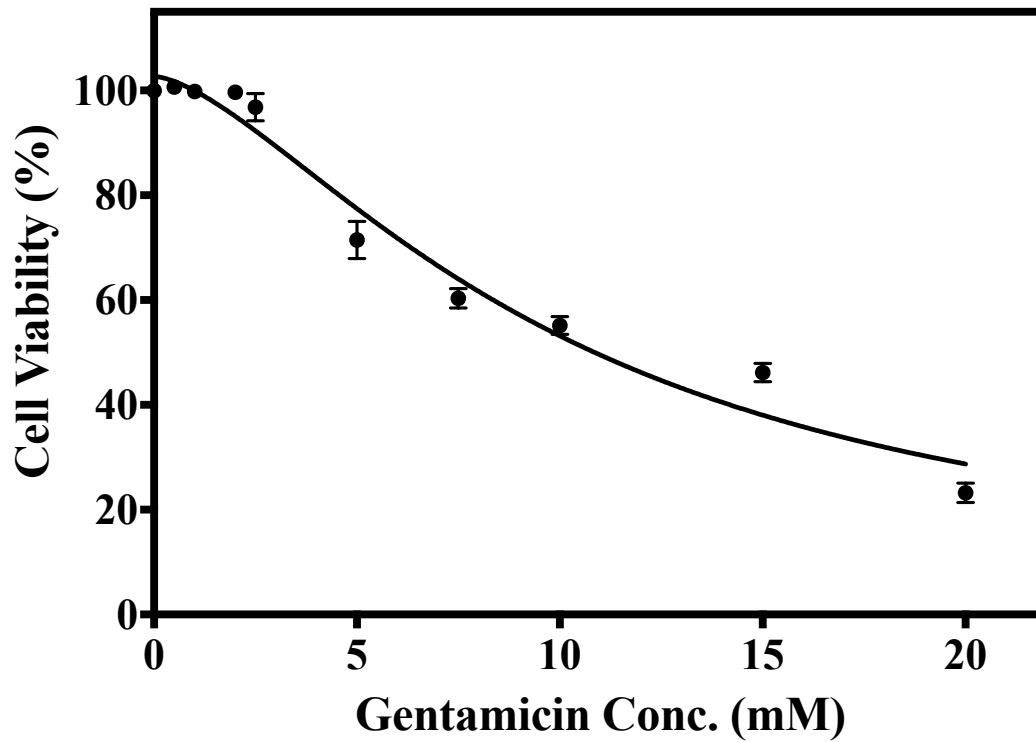
Figure S2.3. Cell Viability of BeWo Cell Monolayer after treatment with free

gentamicin (n=3). IC50 =10.09 mM.

Table S2.1. Hydrodynamic Diameter (HD) and Zeta Potential (ζ) of the *In Vivo* Liposomal Formulations. SD = standard deviation, n = 4.

Liposomes	HD ± SD (nm)	ζ (mV)
10PEG2K-0GM-CY	**103 ± 3**	**- 5.4 ± 2.3**
10PEG2K-10GM-CY	**114 ± 4**	**- 3.4 ± 1.3**
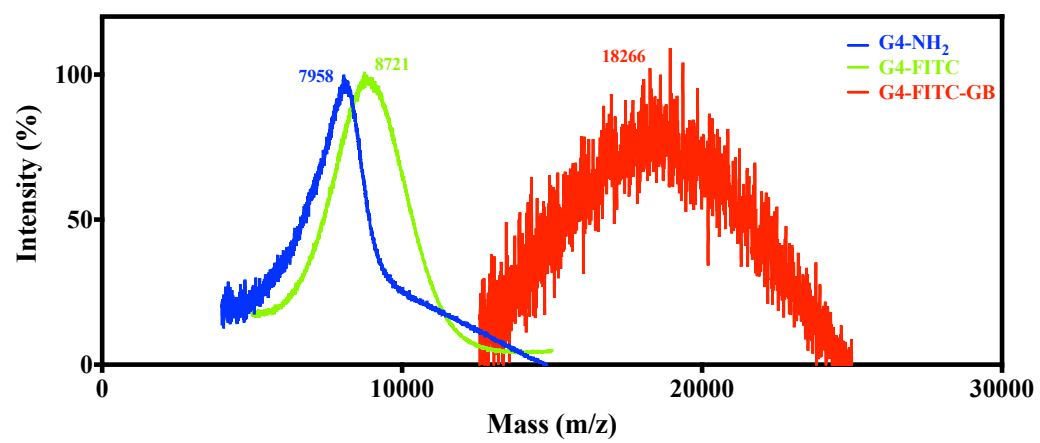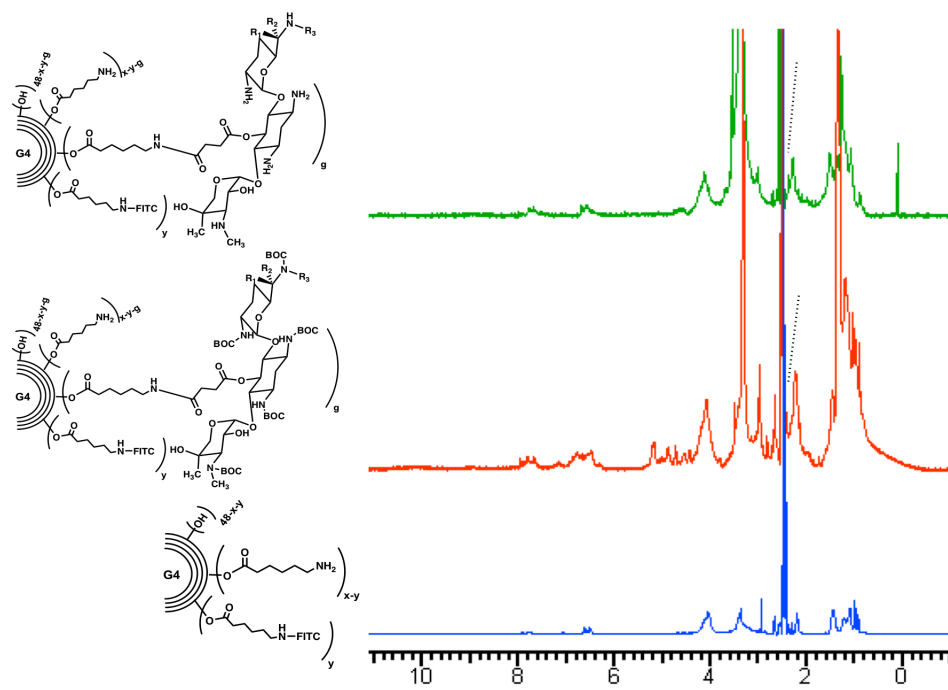
Figure S3.1 MALDI ToF and ^{1}H NMR showing successful modification of polyester dendrimer with FITC and GM.
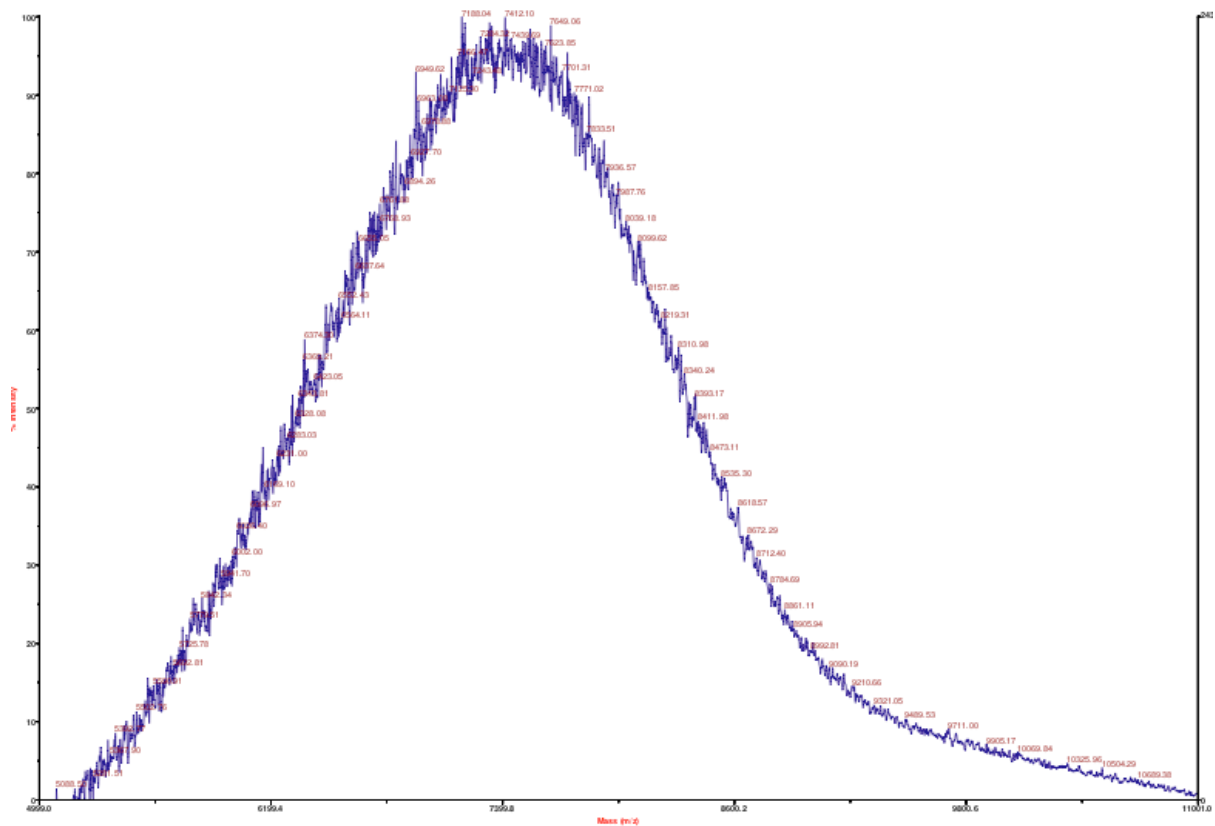
Figure S3.2 MALDI-ToF peak showing the successful partial conversion of **–OH** to **–NH2** (15), **7498** Da
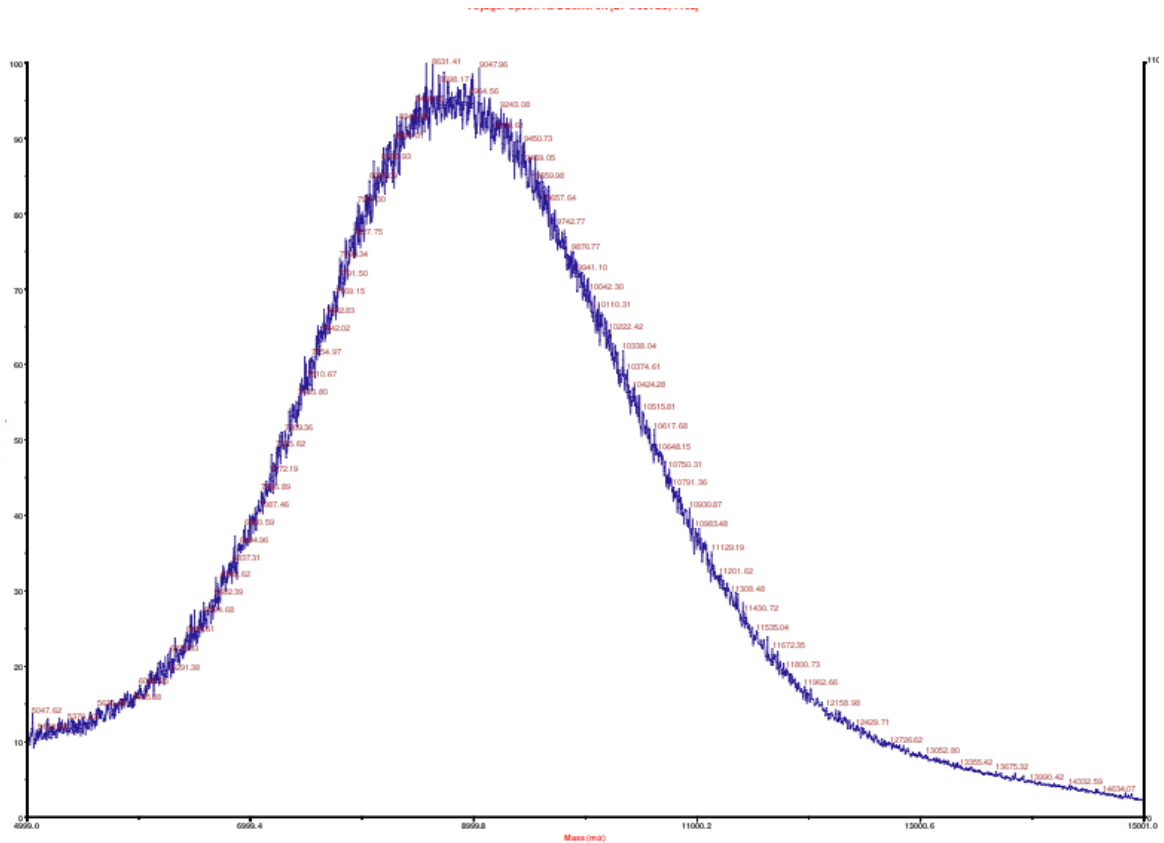
Figure S3.3 MADLI-ToF results showing the peak of G4OH-CY at **8600** Da
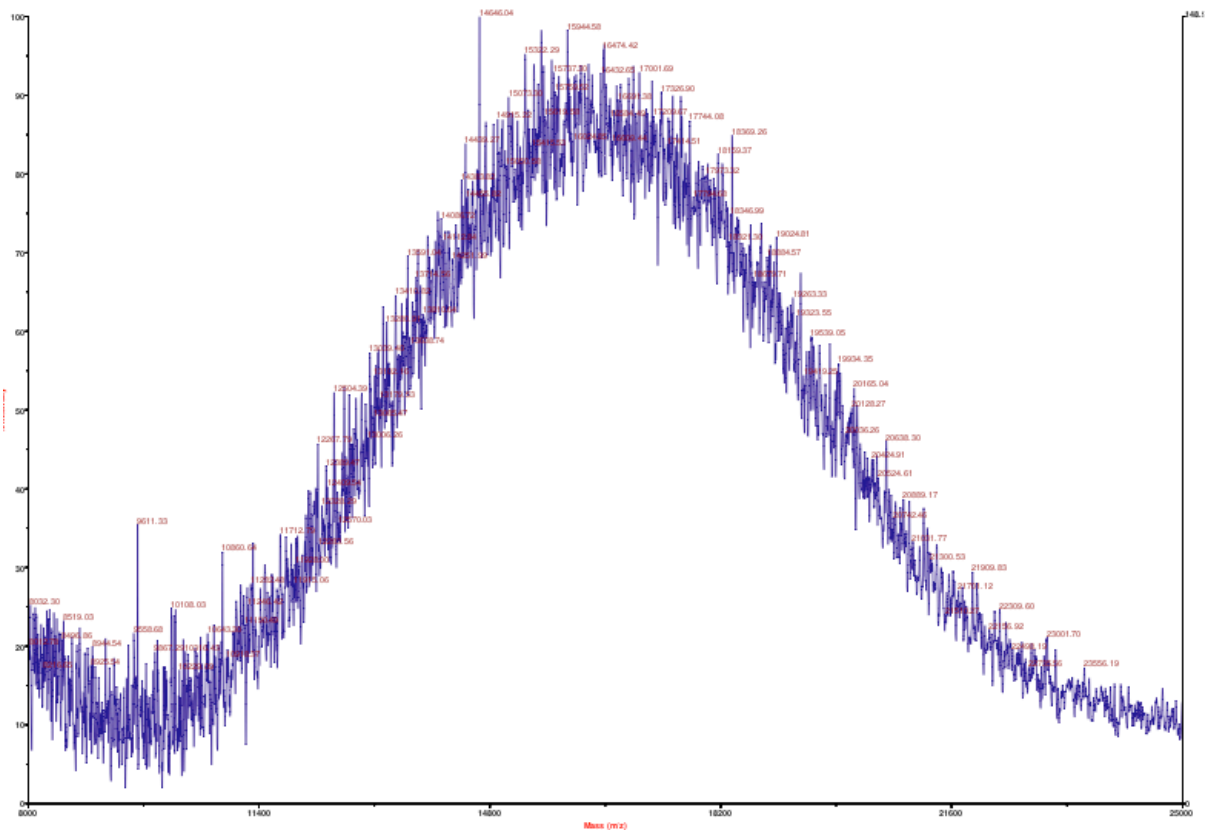
Figure S3.4 MADLI-ToF results showing the peak of G4OH-CY conjugation of mPEG1K at **17000** Da

(a)

(b)

(c)
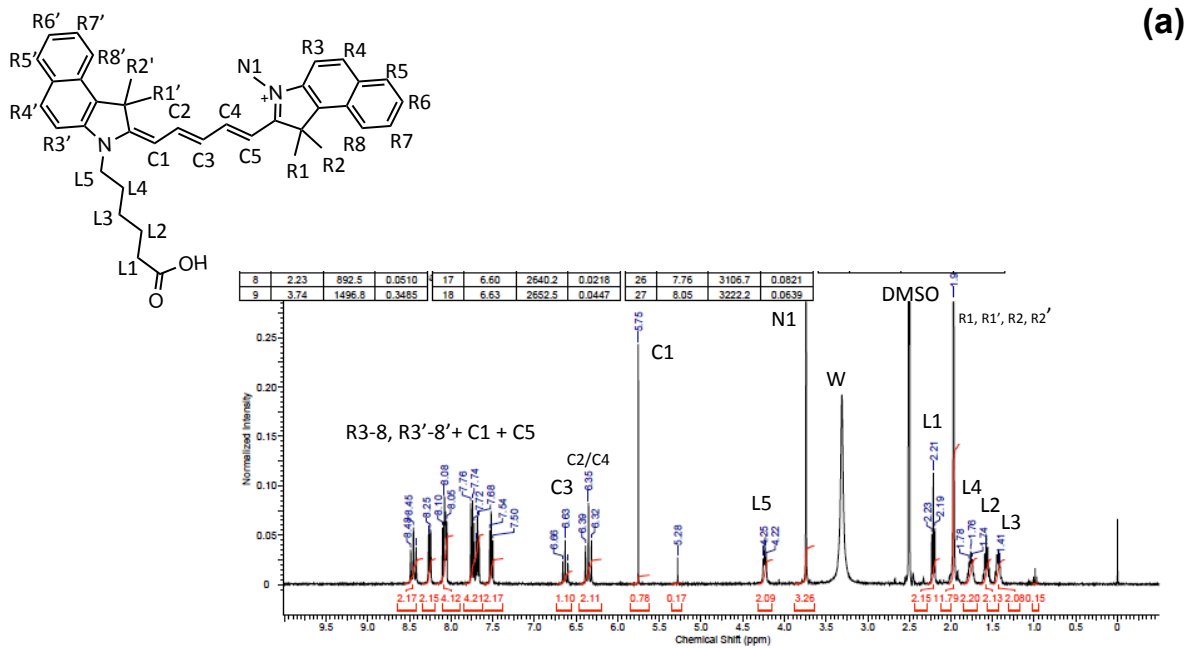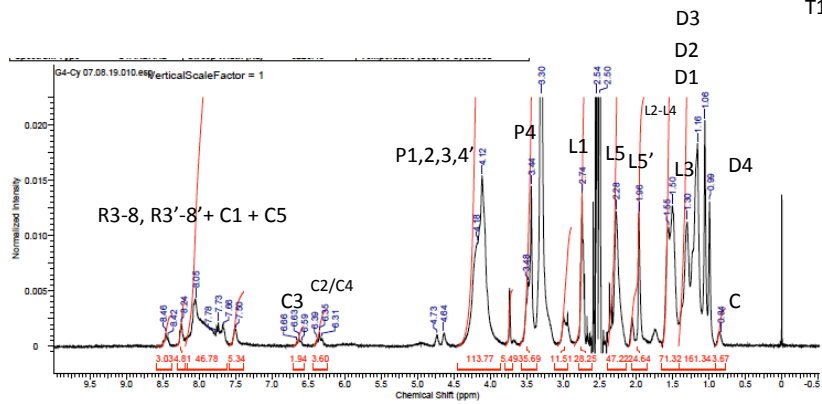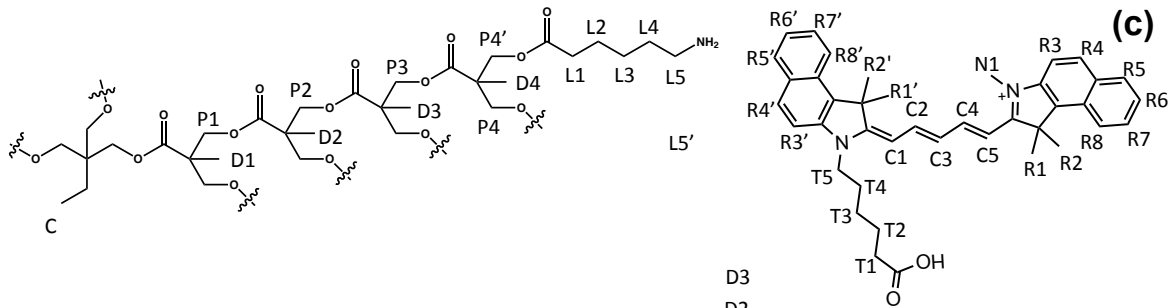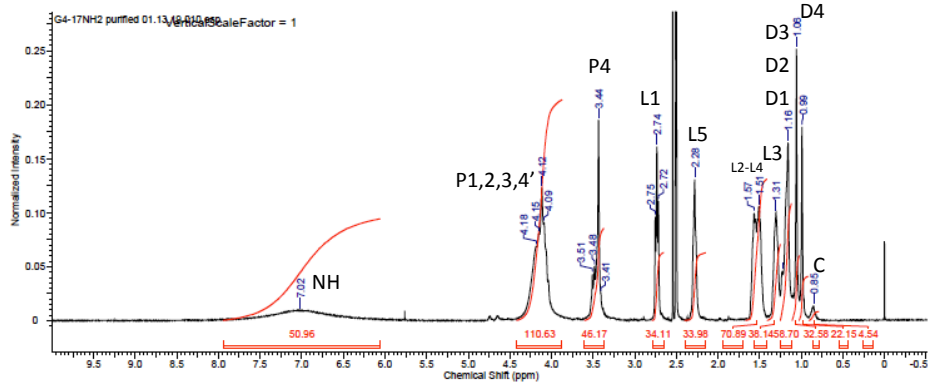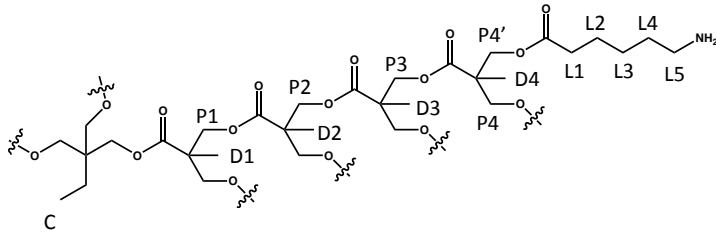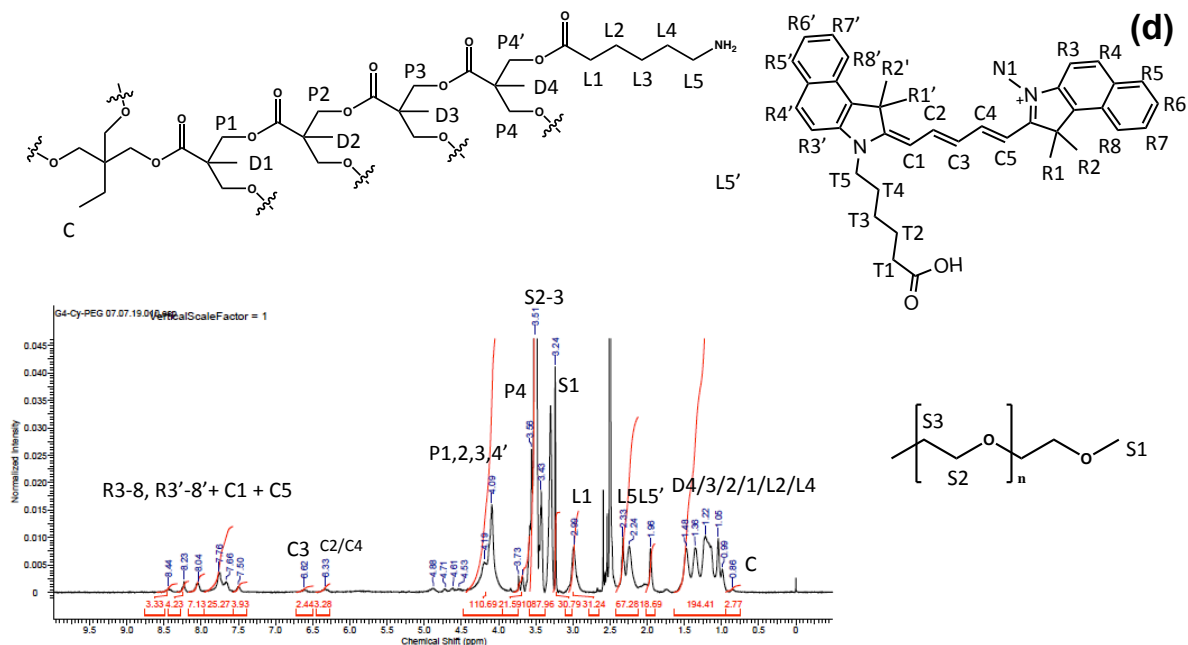
Figure S3.5 Detailed 1H NMR spectra of (a) Cy5.5, (b) dendrimer, (c) G4OH-CY, and (d) G4OH-CY-mPEG1000.

^{1}H NMR Explanation

A fourth generation polyester dendrimer was first modified with a protected amino acid (linker) in order to add terminal amino groups to the dendrimer. This amino groups would have a higher nucleophilicity and would increase the efficiency of the reaction between the dendrimer and the dye (Cy5.5). The reaction with boc-protected amino hexanoic acid, and subsequently deprotection, led to the appearance of new peaks related to the -CH$_2$ groups from the amino acid (1.31, 1.51, 1.57, 2.28, 2.74 ppm). Those peaks were used to assign the number of conjugated linkers, what as calculated as 17. After this first modification, the macromolecule was reacted with Cy5.5

forming an amide bond between the linker and the dye. The conjugation of the dye was checked by the appearance of peaks in the 7.5-8.5 ppm range (related to the rings and double bonds of the structure) and the number of conjugates was determined through the integration of the peak at 6.35 ppm. This peak was selected due to the fact that it is very isolated from other peaks and no overlap was observed. The integration of it showed the conjugation of 1.8 molecules of dye per dendrimer.

After the obtainment of the labeled dendrimer, it was reacted with mPEGCOOH (1,000 Da) and the purified macromolecule was analyzed through ^{1}H NMR. The conjugation of mPEG resulted in the appearance of a very intense peak at 3.51 ppm, which is correspondent to the -CH$_2$ units of the polymeric chain. To quantify the number of polymer chains connected, it was used the integration of the terminal methyl group (-CH$_3$) of mPEG at 3.24 ppm. It was determined that was conjugated 10.2 PEG chains per dendrimer. Additionally, in the spectrum of this final structure it is possible to observe the peaks of Cy5.5 showcasing that the dye was not affected by the reaction between dendrimer and mPEG.
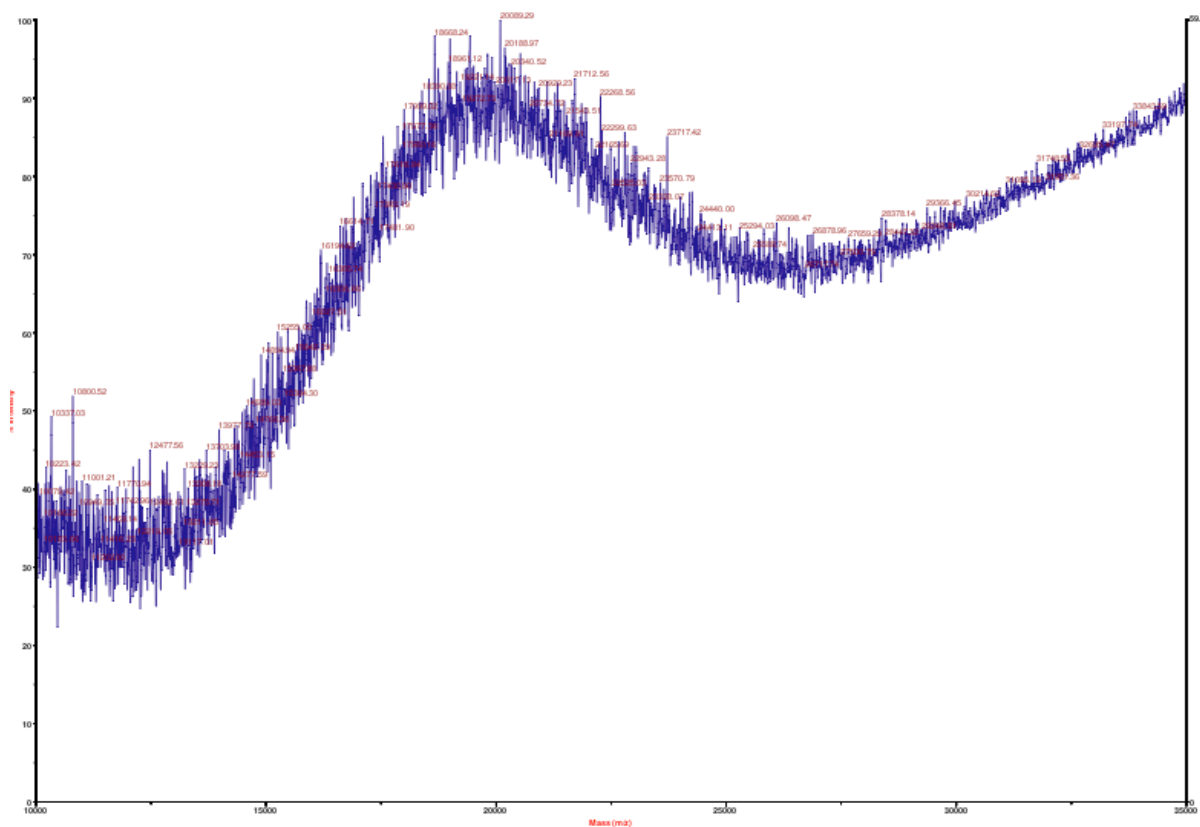
Figure S3.6 MALDI-ToF peak **(200089 Da)** showing the successful conjugation of HOOC-PEG-NH2-BOC to the G4-CY.

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ABSTRACT

GENTAMICIN-MODIFIED NANOCARRIERS FOR PLACENTAL TARGETED DRUG DELIVERY TO TREAT PREGNANCY-RELATED COMPLICATIONS
by

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Diseases of pregnancy are the leading cause of maternal and neonatal morbidity and mortality affecting more than 20% of all pregnancies annually. Those diseases include preeclampsia, preterm labor, and intrauterine growth restriction, many of which are caused by placental dysfunctions. Placenta is a specialized organ that creates a maternal-fetal interface. It provides many functions that contribute to the fetal development. Unfortunately, there is currently no treatment for such diseases. Our work focuses on the development of a novel nanoplatform that can be used to target the placenta so as to deliver therapeutics, which can be encapsulated or conjugated to the nanoformulations.

In this work, we have established and characterized an *in vitro* placental model based on BeWo cell monolayers that mimic the placental barrier, the syncytiotrophoblast. Next, we synthesized lipid conjugates with gentamicin, as a targeting moiety that is a substrate to megalin receptors, which are expressed on the maternal (apical) side of the syncytiotrophoblast. Using those targeting lipids, we successfully prepared and characterized targeting liposomes nanocarriers and showed that they are more significantly taken up by the BeWo polarized

monolayers compared to liposomes lacking the targeting lipid. Furthermore, we tested our liposomal formulations under different conditions: 1) saturated megalin receptors 2) a different cell line (HepG2) that does not express megalin. Results reinforced the ability of the liposomes to target the *in vitro* syncytiotrophoblast model and that the internalization mechanism is megalin-mediated endocytosis. Subsequently, we established an *in vivo* model of the syncytiotrophoblast using timed-pregnant Balb/c mice. On gestational day 18.5, we administered the liposomal formulations and assessed the biodistribution in placentas, kidneys, and fetuses. Placental analysis showed significant accumulation of the targeting liposomes compared to the control non-targeting liposomes and the free Cy5.5 (drug model). Moreover, kidneys showed significant accumulation, which was expected because those organs also express megalin receptors. Fetal tissues showed no significant difference between the three groups suggesting that the fetus should experience no toxicity due to limited exposure to the nanocarriers. This conclusion confirms our hypothesis and satisfies our goal of successfully establishing a nanoplatform for the targeted delivery of therapeutics to the placenta.

AUTOBIOGRAPHICAL STATEMENT

Ali Alfaifi received his Bachelor of Science in 2011 from Wayne State University majoring in biological sciences. Beginning of 2012, Ali joined the biomedical engineering program at Wayne State University and earned his Master of Science degree in 2013. After graduation, he went back to his home country, Saudi Arabia, and worked in King Fahd Center for Medical Research. In 2015, Ali went back to Wayne State University to continue his PhD in the biomedical engineering department under the guidance of Prof. Sandro R. P da Rocha. Ali performed most of his PhD research in the Center for Pharmaceutical Engineering and Sciences in the Nanomedicine Group, under Prof. da Rocha a faculty in the Department of Pharmaceutics in School of Pharmacy at Virginia Commonwealth University, with support also from Dr. Mahendra Kadvia in Biomedical Engineering at WSU. During his PhD, Ali attended several conferences including Nanomedicine and Drug Delivery Symposium (NanoDDS) 2017 and 2018 and American Association for Pharmaceutical Scientists (AAPS) PharmSci 360, 2018 where he had an opportunity to disseminate the work being reported here.