Development of nanocarriers for targeted drug delivery to the placenta

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Natalie Cureton
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ABSTRACT

Name of the University: The University of Manchester
Full title: Miss Natalie Cureton
Degree Title: PhD
Thesis Title: Development of nanocarriers for targeted drug delivery to the placenta
Date: September 2016

Pregnancy complications such as fetal growth restriction (FGR) are often attributed to poor uteroplacental blood flow, but the risk of systemic side-effects hinders therapeutic intervention. We have utilised novel placental-specific homing peptides to overcome this and have conjugated these to biocompatible liposomes.

Peptide-conjugated liposomes were found to selectively bind to the outer syncytiotrophoblast layer of the human placenta and to the uteroplacental vasculature and labyrinth region of the mouse placenta.

The novel vasodilator SE175 was selected as a nitric oxide donor with a favourable stability and release profile, to encapsulate in peptide-conjugated liposomes in an attempt to restore impaired uteroplacental blood flow in a mouse model of FGR, the endothelial nitric oxide synthase knockout mouse.

Liposomes containing SE175 or PBS were prepared by lipid film hydration and targeting peptides coupled to the liposomal surface. Vehicle control, free SE175, PBS- or SE175-containing liposomes were intravenously injected on embryonic (E) days 11.5, 13.5, 15.5 and 17.5. Animals were sacrificed at E18.5 and fetal and placental weights recorded.

Targeted delivery of SE175 significantly increased fetal weight compared to vehicle control but no other treatment groups, whilst significantly decreasing placental weight, indicating improved placental efficiency. Treatment was well tolerated, having no impact on litter size or resorptions. Targeted delivery of SE175, but no other treatment group, reduced a marker of lipid peroxidation in the placenta, indicating a reduction in oxidative stress.

These data suggest that selective delivery of SE175 to the uteroplacental vasculature in peptide decorated liposomes may represent a novel treatment for FGR.
DECLARATION

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### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ACH</td>
<td>Acetylcholine</td>
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<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ASPA</td>
<td>Animal (Scientific Procedures) Act 1986</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine 3',5'-monophosphate</td>
</tr>
<tr>
<td>CTB</td>
<td>Cytotrophoblast</td>
</tr>
<tr>
<td>DAPI</td>
<td>4’,6-diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DSPC</td>
<td>1,2-distearoyl-sn-glycero-3-phosphocholine</td>
</tr>
<tr>
<td>DSPE-PEG</td>
<td>1,2-diastearoyl-sn-glycero-3-phosphoethanolamine-N-[PEG]</td>
</tr>
<tr>
<td>E</td>
<td>Embryonic day</td>
</tr>
<tr>
<td>eNOS</td>
<td>Endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>eNOS&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Endothelial nitric oxide synthase knock-out mouse</td>
</tr>
<tr>
<td>F:P</td>
<td>Fetal:placental weight ratio</td>
</tr>
<tr>
<td>FGR</td>
<td>Fetal growth restriction</td>
</tr>
<tr>
<td>g</td>
<td>Grams</td>
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<tr>
<td>GC</td>
<td>Guanylate cyclase</td>
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<tr>
<td>KO</td>
<td>Knock-out</td>
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<tr>
<td>KPSS</td>
<td>High potassium salt solution</td>
</tr>
<tr>
<td>LU</td>
<td>Lumen</td>
</tr>
<tr>
<td>mPEG</td>
<td>Polyethylene glycol maleimide</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PE</td>
<td>Phenylephrine</td>
</tr>
<tr>
<td>PEC</td>
<td>Pre-eclampsia</td>
</tr>
<tr>
<td>PEG</td>
<td>Polyethylene glycol</td>
</tr>
<tr>
<td>PSS</td>
<td>Physiological salt solution</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>Sflt-1</td>
<td>Soluble fms-like tyrosine-1</td>
</tr>
<tr>
<td>SGA</td>
<td>Small for gestation age</td>
</tr>
<tr>
<td>sGC</td>
<td>Soluble guanylate cyclase</td>
</tr>
<tr>
<td>SNP</td>
<td>Sodium nitroprusside</td>
</tr>
<tr>
<td>ST</td>
<td>Syncytiotrophoblast</td>
</tr>
</tbody>
</table>
VS Villous stroma
WT Wild type

Amino acid abbreviations:
A = alanine
E = glutamic acid
G = glycine
K = lysine
L = leucine
N = asparagine
P = proline
Q = glutamine
R = arginine
S = serine
T = threonine
V = valine
W = tryptophan
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Chapter 1 Introduction
1.1 Introduction

Around 10% of pregnancies are affected by complications, two of the more common conditions being pre-eclampsia and fetal growth restriction (Huppertz, 2011).

Despite the high proportion of women presenting with complications, there are currently no medical interventions available, with the only treatment being pre-term delivery (Thornton, 2003). The exact causes of both pre-eclampsia and FGR are not fully understood, however it is commonly accepted that these conditions occur as a result of placental abnormalities (Huppertz, 2011, Roberts and Escudero, 2012). With this in mind, developing a treatment for either complication would need to address these abnormalities, with the aim of improving them in order to provide a better prognosis.

A major setback in developing a treatment for placental complications is off target side effects that occur following systemic delivery of drugs. Recent efforts have been made to develop technology for targeted delivery of a variety of therapeutics, ensuring that any off target side effects are kept to a minimum. Targeting drugs to the placenta directly would therefore enable treatment with a therapeutic that would otherwise be unsuitable for systemic delivery.

A recent study from our laboratory has identified a number of placental-specific homing peptides that actively and selectively bind to the mouse placenta following intravenous delivery (King et al., 2016).

The research to be carried out across the course of this PhD aims to capitalise on this discovery, using the placental homing peptides as a starting point to develop a targeted drug delivery system for the treatment of pre-eclampsia and FGR.
1.2 The Structure and Function of the Human Placenta

1.2.1 Structure and function of the placenta in uncomplicated pregnancies

The placenta is essential for the healthy growth and maintenance of the fetus, acting as an interface across which efficient exchange of essential nutrients, gases and waste products can occur. In this sense the placenta acts as the major organs of the fetus during pregnancy, e.g. the lungs, kidneys, liver and gastro-intestinal tract. As well as providing a surface for exchange, the placenta also has a protective role; it acts as a barrier to ensure that nothing that could be harmful to the fetus, for example microbes, passes to the fetus.

Formation of the placenta occurs after implantation of a fertilised embryo has occurred. Once fertilised, the embryo undergoes cell divisions to form the blastocyst, consisting of an inner cell mass surrounded by a layer called the trophectoderm, the precursor to trophoblast cells. When the blastocyst is ready to implant into the uterus, it loses its outer shell, known as the zona pellucida, and orientates itself such that the inner cell mass is closest to the uterus. At this point, attachment of the outer trophectoderm to the uterus occurs and the process of implantation begins (Wang and Dey, 2006).

The outer trophectoderm of the blastocyst forms the trophoblast, which attaches and invades the uterine wall. Trophoblast differentiation may then commence, with the trophoblast differentiating down several lineages to form various regions of the placenta, described in more detail below. The extraembryonic mesoderm develops to form the villous stroma and the vasculature of the villous placenta, and the inner cell mass of the blastocyst develops to become the fetus (Wang and Dey, 2006).

The trophoblast layer has a protective role by acting to form a barrier between the maternal and the fetal blood supplies. This barrier function not only prevents crossing of substances to the fetus that may be harmful, but also protects the fetus from maternal immune attack by keeping the maternal and fetal blood circulations separate.

The trophoblast layer surrounds tree-like projections called chorionic villi, and these villi extend from the fetal side of the placenta, also known as the chorionic
plate. Some villi form an attachment with the uterine wall, anchoring the villi and providing structural support, whereas others float freely in the intervillous space through which maternal blood can circulate. The chorionic villi extend and branch creating a vast surface area over which exchange can occur (Figure 1.2). Fetal vessels originating from the umbilical cord branch into the chorionic villi thereby providing a blood supply to the developing villi to support exchange; deoxygenated fetal blood is brought to the villi by the fetal artery, which then branches to form the capillaries of the villi; this blood is replenished with nutrients and oxygen and returned to the fetus via the fetal vein.

The placenta is fully formed by around 12 weeks of gestation; this is when maternal blood begins to flow into the intervillous space. Early on in the development of the placenta, the trophoblast differentiates to form a bilayer composed of the syncytiotrophoblast layer and the cytotrophoblast layer. The outer layer is formed of syncytiotrophoblast; this is a continuous multinucleated cell layer which is formed by the fusion of the underlying mononuclear cytotrophoblasts. The locus of exchange between the maternal and fetal sides is known as the vasculo-syncytial membrane (VSM). The interhaemal barrier is comprised of syncytiotrophoblast, the basement membrane of the syncytiotrophoblast, the stroma of the villi, the basement membrane of the capillary and the endothelium of the capillary. The VSM is formed by the close contact of fetal capillaries and the syncytiotrophoblast, leading to the fusing of the two basement membranes; fusing causes the membrane that separates the maternal and fetal circulations to be 2-3\(\mu\)m thick, allowing a high efficiency of exchange to be achieved (Jones and Fox, 1991, Kurt Benirschke, 2012, Wang and Dey, 2006).

As well as providing a barrier, the placenta also contains active pumps that will return certain molecules back into the mother’s circulation if they do enter the syncytium. The ABC (ATP-binding cassette superfamily) act as the main efflux transporters in the placenta (Evseenko et al., 2006) and includes such proteins as MRP2, which is present in the syncytiotrophoblast and is responsible for efflux of anionic compounds, such as xenobiotics and endogenous steroids (Kruh and Belinsky, 2003, Young et al., 2003).

The maternal blood supply is optimised to bring oxygenated, nutrient-rich blood to the placenta, in order to provide a gradient for exchange of molecules with the
fetal blood. The maternal uterine spiral arteries undergo remodelling during the first 20 weeks of gestation in order to provide a high flow, low resistance supply of blood to the placenta. The maternal blood flows from the spiral arteries into the intervillous space; chorionic villi are bathed in maternal blood, and the maternal and fetal circulations are close enough to permit exchange of materials, whilst the trophoblast layer prevents the bloods from mixing, thereby keeping the fetus protected (Figure 1.1) (Huppertz, 2008, Georgiades et al., 2002, Audus, 1999).

Figure 1.1. Human placenta showing the fetal and maternal sides, the chorionic villi surrounded by the trophoblast layer and the blood flow through the placenta
1.3 Pregnancy-related Complications

1.3.1 Pregnancy-related Complications – Disease

Two of the most serious pregnancy-related complications are pre-eclampsia and fetal growth restriction (FGR), also commonly referred to as intrauterine growth restriction (IUGR). Each occurs in approximately 5% of pregnancies (Cox et al., 2009). Symptoms of pre-eclampsia include maternal hypertension, proteinuria and oedema. The Oxford Handbook of Midwifery states that pre-eclampsia is defined by ‘the onset of hypertension after 20 weeks gestation where BP ≥140/190 mmHg is detected on two occasions, at least 6 hours apart, but without evidence of end organ damage, and where proteinuria of at least 0.3g/ml total is detected in a 24hour sample’ (Whitehead, 2007). Growth restriction of the fetus is common in pre-eclampsia and can lead to babies being born small for gestational age (SGA), which is deemed normal, or babies being born with FGR, which is deemed pathological and is associated with poor outcomes. Pre-eclampsia can progress to eclampsia, with the appearance of tonic-clonic seizures that are life-threatening to both the mother and the baby (Whitehead, 2007, Ahmed et al., 2010, Krauss et al., 2004, Roberts and Escudero, 2012).

In FGR, the fetus is not able to grow to its genetically determined size; the most common definition for diagnosis is a baby that weighs below the fifth centile for its gestational age, and has an abnormal maternal uterine artery Doppler, indicative of aberrant blood flow through the uteroplacental unit (Chen et al., 2011a).

A major cause of most cases of pre-eclampsia and FGR is a poorly functioning placenta, caused by structural and/or functional abnormalities (Huppertz, 2011, Roberts and Escudero, 2012). The placenta must have a vast surface area for efficient exchange to occur. In a normal placenta, this large surface area is achieved by the highly branched structure of the chorionic villi; however in complicated pregnancies the villi may be underdeveloped, in particular the terminal villi, leading to a reduced surface area for exchange and therefore a decreased overall exchange efficiency. It is also a possibility that the vasculature within the chorionic villi may be underdeveloped, leading to villi that are not well perfused; this would lead to decreased fetal blood flow to the terminal villi and

Another cause of FGR is a reduction in maternal blood flow through the uterine arteries that supply the placenta. The blood flow must be of high volume in order to support the growth of the fetus by transporting nutrients and oxygen to the placenta for exchange at a sufficient rate, but low resistance to avoid damaging the placenta surface with high shear forces. In normal pregnancy, this is achieved by the process of uterine spiral artery remodelling; this modifies the spiral arteries such that they have a wider diameter and are lacking in smooth muscle, thereby creating the high flow, low resistance conditions that are required for optimal delivery of blood to the placenta. The loss of vascular smooth muscle cells also mean that the spiral arteries are unable to constrict, thereby ensuring a constant low pressure, non-pulsatile blood supply.

In pre-eclampsia, spiral artery remodelling is impaired, leading to arteries retaining their original diameter and still containing vascular smooth muscle cells and elastic fibres. This leads to a situation where the volume of blood flowing through the placenta is reduced and it is delivered at a higher pressure; a high pressure, low flow system is the opposite of what is desirable. This means that there is an insufficient volume of blood reaching the placenta, which causes a reduction in the amount of nutrients and oxygen that can be delivered for exchange (Burton et al., 2009). The increase in blood flow velocity may damage the surface of the placenta causing increased shedding of STB fragments, altered patterns of placental development, placental lesions and in severe cases, placental abruption. There is also evidence to support that this damage may occur in some types of FGR (Roberts and Escudero, 2012, Sankaran and Kyle, 2009, Saleh and Dkhil, 2008, Kingdom and Kaufmann, 1999).

Reduced efficiency of gaseous exchange may lead to a placenta that is hypoxic. In turn, hypoxia or hypoxia-reperfusion injury leads to the placenta producing and releasing specific factors. These include anti-angiogenic factors such as soluble fms-like tyrosine kinase-1 (sFlt1), which binds to circulating vascular endothelial growth factor (VEGF) thereby preventing its ability to promote angiogenesis, and pro-inflammatory cytokines such as interleukin-1 (IL-1). These factors circulate in the maternal blood and may contribute to the maternal clinical signs
or symptoms of pre-eclampsia, such as endothelial cell activation, hypertension and proteinuria (Benyo et al., 1997, Laresgoiti-Servitje and Gomez-Lopez, 2012).

Sub-optimal nutrient exchange, either due to reduced transport caused by reduced surface area, reduced expression of nutrient transporters or reduced inflow of maternal blood, may lead to malnutrition of the fetus (Desforges and Sibley, 2010). In some cases of FGR, in an attempt to adapt to this lack of nutrition, the growing fetus reduces its rate of metabolism and production and release of growth-promoting hormones, for example growth factors such as insulin like growth factors 1 and 2 (IGF-I and IGF-II). These alterations cause the fetus to have a reduced growth trajectory, which reduces its requirement for nutrients; hence, the fetus is able to match its demands for nutrients with the available supply, but the end result may be FGR (Saleh and Dkhil, 2008, Arroyo and Winn, 2008, Huppertz, 2011). Adaptations to a sub-optimal nutrient supply have been reported to be gender dependant, with male fetuses failing to respond to adverse conditions, whereas female fetuses adapt their growth to match the lowered nutrient supply; this results in female fetuses being smaller but having better survival, as opposed to male fetuses who are larger but have greater morbidity and mortality (Clifton, 2010, Clifton, 2005). The anti-oxidant tempol, a superoxide dismutase mimetic, has been shown to improve fetal growth in a mouse model of FGR, the endothelial nitic oxide synthase knock-out mouse, but treatment did not improve uterine artery function of placental transport (Stanley et al., 2012).

Poor uteroplacental perfusion has been shown to increase placental oxidative stress, resulting in a dysfunctional placenta and a greater systemic inflammatory response in cases of FGR and pre-eclampsia (Burton and Jauniaux, 2011, Burton and Jauniaux, 2004). Oxidative stress in the placenta has been shown to cause reduced protein translation of signalling proteins, such as AKT and mTOR which are important in mediating nutrient transport. It has also been shown to lead to lipid peroxidation and the production of hydroxynonenal, a compound associated with activation of apoptosis (Burton and Jauniaux, 2011, Cindrova-Davies et al., 2007). Attempts to improve placenta dysfunction with anti-oxidants have been investigated. Trials of vitamins C and E in humans demonstrated that supplementation does not reduce onset of pre-eclampsia, but does increase the
rates of SGA fetuses and cause an increased risk of fetal loss and pre-term rupture of membranes (Xu et al., 2010, Poston et al., 2006).

In spite of the ability to recognise the complications that arise in pregnancy, little in terms of preventative or curative treatments are available. FGR babies will, by their very nature, be of a low birth weight, and even though this can be detected by ultrasound in mid-pregnancy, there are currently no treatments available to enhance growth; only close monitoring can be carried out in order to track growth progression. In cases where FGR is deemed to be putting the health of the fetus at high risk, a decision may be taken to induce preterm delivery; this delivery is generally through a caesarean section to reduce the stresses to the fetus.

Exactly what action is to be taken once pre-eclampsia has been diagnosed depends on how advanced the pregnancy is and how large the baby is estimated to be. Currently, the only curative treatment for pre-eclampsia is delivery of the baby and removal of the placenta, which depending on the stage of pregnancy may be detrimental to the fetal survival. Late onset pre-eclampsia, from around 34 weeks of gestation onwards, will usually be managed by induction of labour. For treatment of early onset pre-eclampsia, before 34 weeks gestation, induction of labour is likely to be unsuitable due to the prematurity of the fetus and the risk of neonatal morbidity or mortality. In this case, close monitoring in a hospital environment along with steroid injections to aid fetal lung maturation is more appropriate, with labour being induced once the fetus has reached a sufficient weight to ensure good odds of survival (Sankaran and Kyle, 2009, Thornton, 2003, Bamfo and Odibo, 2011, Sibai, 2006). However, if the mother’s health continues to deteriorate, as frequently occurs in pre-eclampsia, the baby will be delivered despite having a poor prognosis.

Neonates of low birth weight have an inherently higher risk of morbidity and mortality. Short term complications can include hypoglycaemia, hypothermia and asphyxia (Chan et al., 2010). In addition, long term complications, referred to as developmental programming, can be observed in childhood but tend to be more evident during adolescence and beyond, and connections between low birth weight and poor adolescent and mid-life health have been discovered as a result
of many retrospective studies (Barker, 1990, Calkins and Devaskar, 2011). Common complications can include increased risk of neurobehavioral disorders, such as attention deficit hyperactivity disorder (ADHD) and also an increased risk of childhood asthma (Vucetic et al., 2010). It has also been observed that there is an increased risk of metabolic disorders, such as obesity and type II diabetes, and cardiovascular disorders, including heart disease and hypertension. These complications are thought to be attributed to a number of factors: insulin resistance is believed to play a major role in development of type II diabetes, but is also predictive of ischaemic heart disease and hypertension (Ong and Dunger, 2002). Maternal diet is also an important factor in the risk of developing adulthood disease. Modification of protein intake in the pregnant rat throughout gestation has demonstrated that even modest protein restriction leads to fetuses that develop hypertension in adulthood (Langley and Jackson, 1994); further studies have shown that alterations in the expression of the renin-angiotensin system and renal sodium transporters contribute to dysregulated sodium homeostasis and the resultant onset of hypertension (Sahajpal and Ashton, 2003, Ashton et al., 2007).

Also said to play a major role in long term complications is post-natal catch-up growth, in which the low birth weight neonate puts on weight at a faster rate than a normal birth weight neonate. This period of catch up growth correlates with long term programming of metabolic and hormonal systems and body composition; altered body composition introduces higher levels of adipose tissue, which in turn leads to higher incidence of obesity, therefore predicting other complications such as coronary heart disease and atherosclerosis (Torche and Echevarria, 2011, Neitzke et al., 2011, Varvarigou, 2010, Pallotto and Kilbride, 2006, Ong and Dunger, 2002).

In spite of the prevalence of pregnancy-related complications and the evidence to show their impact on neonatal outcome, adolescent development and adult disease, there is still an ongoing issue of the lack of therapeutics available to treat such complications. There is a consistent lack of research into treatments for complications of pregnancy, and this in part may be related to perceived risks, issues and opinions of drug therapy during pregnancy.
1.3.2 Pregnancy-related Complications –Therapeutic Intervention

Since the thalidomide disaster in the 1960s, attitudes and opinions towards prescribing and taking therapeutic drugs during pregnancy have changed dramatically. The use of thalidomide to treat symptoms associated with morning sickness led to babies being born with limb abnormalities due to its previously undetected teratogenic effects. When thalidomide was tested prior to its release to market, it was deemed safe for use during pregnancy; only the FDA (USA) disapproved of the release of thalidomide as they felt that more testing was required.

After this, more stringent testing of drugs being brought to market was introduced; all drugs must now be tested for adverse teratogenic, reproductive and developmental effects on offspring. However, this extra testing and additional control over drugs has done little to ease fears of the risks involved with taking therapeutics during pregnancy (Webster and Freeman, 2001).

Recent research into the perception of the teratogenic risk of common medicines, for example aspirin, has shown that misconceptions of the true teratogenic risk of drugs deemed to be safe during pregnancy, are held not only by pregnant women, but also by those who should be aware of the true risks, such as general practitioners and post-medical students. This misconception could lead to under prescribing, and a lack of trust in what medicines have been prescribed during pregnancy, even when the therapeutic is safe; this could lead to unnecessary suffering by pregnant women or prescriptions not being adhered to, which may in turn put the woman and her baby at a higher risk of adverse outcomes (Sanz et al., 2001, Koren and Levichek, 2002, Damase-Michel et al., 2008).

With such an attitude existing towards prescribing and taking therapeutics during pregnancy, little has been done in terms of research and development of novel therapeutics directed at providing treatment of pregnancy-related complications; this has lead to a deficiency of drugs for improving maternal and fetal health (Fisk and Atun, 2008). With around ten percent of all pregnancies being affected by either pre-eclampsia or FGR, research into the treatment of such complications is warranted. Furthermore, considering that pregnancy-related complications have a detrimental effect on neonatal health in the short term and can also lead to longer-term complications during adolescence and adulthood, the cost to the
NHS is much greater than that required to treat these complications when they arise (Goldenberg and Jobe, 2001, Fisk and Atun, 2009).

This lack of treatments for pregnancy-related complications (Fisk et al., 2011) is worrying, especially with less frequent and better understood disease states receiving more funding to research therapies; one example of this is diabetes, the approximate prevalence of which worldwide is 2.8% (Wild et al., 2004) compared to a prevalence of 10% for pre-eclampsia and FGR. The Gates Foundation has provided funding to aid research (Global Health Strategy Overview by The Bill and Melinda Gates Foundation 2010) to reduce the numbers of mothers and infants that die during and immediately after birth; however this research does not focus on prevention, but rather developing tools and instruments that help to manage and detect causes of maternal and neonatal death.

As FGR and pre-eclampsia are primarily caused by a poorly developed or poorly functioning placenta, a successful strategy would be administer a therapeutic to treat the placenta, but without causing any adverse effects to the mother or fetus. An approach such as this would require selective targeting of the appropriate drug to the placenta.

### 1.4 Drug Targeting

#### 1.4.1 Non-specific targeting

Targeting of drugs to specific organs or tissues ensures that the therapeutic is able to efficiently reach the desired location whilst minimising accumulation in other tissues in the body; this dramatically reduces side effects of therapies and also means that lower doses may be administered, as the majority of the dose will reach the desired target.

Passive drug targeting takes advantage of the body's natural physiological and biochemical mechanisms in order to ensure that accumulation of a desired therapeutic is achieved in a particular location. A notable example of this is the liposomal chemotherapeutic Doxil®. Doxil® nano-liposomes have been developed to target solid tumours by taking advantage of enhanced permeability and retention (EPR) of tumour tissues; in essence tumours have 'leaky vasculature' that allows liposomes to exit the circulation at their intended target site. This passive targeting approach has enabled Doxil® to be a successful treatment of cancers such as metastatic breast cancer (Barenholz, 2012).
Encapsulation of drugs in nanoparticles can cause non-specific accumulation in the liver and spleen. This phenomenon has been exploited for the delivery of therapeutics such as disease-modifying anti-rheumatic drugs. Research has been undertaken to assess the targeting abilities of Actarit, which acts on enlarged spleens that are seen in rheumatoid arthritis, following its encapsulation into solid lipid nanoparticles to assess targeting abilities. A significant increase in accumulation in the spleen was observed using this formulation, along with a significant decrease in renal distribution, showing potential for a passive targeted drug delivery system (Ye et al., 2008). Non-specific targeting has proven to have advantages in delivery of therapeutics to some tissues; however there are limitations for which organs can be targeted. For a more efficient delivery of therapeutics, a specific means of targeting must be utilised.

1.4.2 Tissue-specific targeting

Targeting of drugs to specific tissues has been made possible by the discovery that individual tissues display their own distinct markers; these markers can then be considered as tissue-specific receptors.

Tissue-specific targeting requires a targeting moiety that is able to actively and selectively bind to markers or receptors that are highly expressed on certain tissue types. A number of moieties have been investigated for their targeting abilities including proteins, antibodies and antibody fragments, and nucleic acids (Choi et al., 2010).

Antibody fragments have been utilised in the development of immunoliposomes, which exploit the targeting abilities of the fragments in order to deliver drugs to a specific site. Research has been carried out using fragment antigen binding regions of antibodies for membrane type-1 matrix metalloproteinase (MT1-MMP), which is found to be highly expressed in angiogenic tumour endothelium, attached to liposomal doxorubicin. Mice that were treated with targeted liposomal doxorubicin were found to exhibit a significant suppression in tumour growth as compared to non-targeting liposomal doxorubicin, showing the potential for specific targeting in the future of chemotherapeutics (Hatakeyama et al., 2007).
Other moieties that have been utilised for specific targeting are peptides. It is possible to screen vast peptide libraries via the use of phage-display, in order to determine which specific peptide sequences bind to each tissue type. Over the past 20 years, this strategy has identified tissue-specific homing peptides that selectively bind to a number of healthy and diseased tissue types; these peptides can then be used to facilitate tissue-specific drug delivery (Ruoslhti et al., 2010, Rajotte et al., 1998).

Delivery of therapeutic macromolecules, such as proteins and nucleic acids, by the use of peptide-conjugated liposomes has also been examined. Conventional delivery of therapeutic macromolecules is often hindered by the inefficiency of cellular uptake of such macromolecules. A study to determine the ability of peptide-modified liposomes, in particular following conjugation of the cell-penetrating peptide e.g. Pep-1 (KETWWETWWTEWSQPKKKRKV), to deliver therapeutic macromolecules was employed by Kang et al. It was found that Pep-1-conjugated liposomes were able to deliver encapsulated contents efficiently into the cytoplasm in vitro, showing the potential for this formulation to be used for intracellular delivery in vivo (Kang et al., 2011, Vives et al., 2008).

Peptide libraries have been screened against different tumour types in order to identify tumour-specific homing peptides. For solid tumours to survive, they must induce local angiogenesis to enable the continually growing tumour to be supplied with nutrients and oxygen. As tumours and tumour vasculature represent a unique tissue type that is distinct from every other organ, they express cell-surface markers that are not present, or are present at a much lower level, in normal, non-cancerous tissues, and act as receptors for homing peptides (Lee et al., 2007). Peptides with specific amino acid sequences, for example the RGD motif, have been identified as tumour homing peptides. It has been postulated that discovering unique peptides that actively and selectively targets tumours may be easier than for other tissues, primarily due to their distinct tissue types (Ruoslhti, 2002).

Much like tumours, the placenta is a highly specialised tissue that expresses unique surface markers. This differentiation from normal tissues makes the placenta an ideal candidate for targeted drug delivery by the use of homing peptides. Recent work from our laboratory, undertaken in collaboration with Prof.
Ruoslahti (and currently unpublished) has used phage screening (Teesalu et al., 2012) to identify a number of novel placental-specific homing peptides that were found to bind selectively to the mouse placenta upon intravenous administration, indicating that specific placental targeting can be achieved.

1.5 Use of Liposomes for Drug Targeting

1.5.1 Liposomes as a drug delivery system

In order to take advantage of placenta-specific homing peptides, a drug delivery system would be required that utilises these peptides. Previous research in tumour drug delivery has investigated the use of nanoparticles decorated with a tissue-specific homing peptide as a targeted drug carrier system; these studies have shown that liposomes are ideal carriers to use with targeting peptides, being biocompatible, biodegradable and with lipids being easily modifiable to allow conjugation of peptides (Rezler et al., 2007, Lee et al., 2007, Wang et al., 2015).

A number of studies have highlighted the detrimental effects of different nanoparticle formulations in pregnant mice: silica nanoparticles, carbon nanotubes and titanium dioxide nanoparticles have all been examined, with these nanoparticles being chosen as they are already in common use in the fields of electronics, foods, cosmetics and drug delivery. Biodistribution in mice, in particular the mouse placenta, was assessed by the use of whole-body imaging, after injection with fluorescently labelled silica and titanium dioxide nanoparticles of different sizes or carbon nanotubes. Accumulation in the liver was seen for silica nanoparticles, carbon nanotubes and titanium dioxide nanoparticles, irrespective of size, and accumulation was also detected in the placental trophoblasts, fetal liver and fetal brain, following injections of silica nanoparticles of 70nm diameter and titanium dioxide nanoparticles of approximately 200nm diameter. These nanoparticles induced fetal resorption and fetal growth restriction; the complications were attributed to structural and functional changes observed in the placentas, which occurred following treatment with the silica nanoparticles of diameter 70nm (Yamashita et al., 2011).
Further studies have assessed the biocompatibility of gold nanoparticles, and the ability of these nanoparticles to cross the mouse placenta. Gold nanoparticles of 13nm diameter with different surface compositions were intravenously injected into pregnant mice at different gestations, from E5.5 to E15.5, and the distribution of these nanoparticles was assessed. Gold nanoparticles were observed in fetal tissues prior to E11.5, with amounts dropping significantly after E11.5, highlighting a correlation between gestational age and placental transit. No signs of fetal toxicity were found after administration and no significant differences in fetal weights were noted, as compared to a control group. PEG-coated gold nanoparticles accumulated in the placenta to significantly higher levels than citrate-capped nanoparticles and the PEG coated nanoparticles also exhibited an increased circulation time (Yang et al., 2012). Despite the lack of toxicity of the gold nanoparticles, the synthesis of these particles is more complex than that of other nanoparticles and controlling the size of gold nanoparticles is difficult, resulting in a broad size distribution, which would not be desirable in a delivery system (Sau et al., 2001).

Liposomes have been extensively characterised and are known to be non-toxic, biocompatible, and biodegradable, meaning that once the liposome has delivered its therapeutic cargo it can be metabolised and cleared by the body, whilst not causing any toxicity. This property makes liposomes more suitable for placental drug delivery than other nanoparticle preparations previously discussed (Malam et al., 2009). Liposomes encapsulating haemoglobin (diameter 250nm) have been tested using a pregnant rat model, with results showing that daily injections of the liposome formulation had no detrimental effects on the pregnancy, the organ function of the fetuses and mother, or on fetal maturation (Kaga et al., 2012).
From the previous research carried out, it can be seen that liposomes are an appropriate nanoparticle vehicle for drug delivery during pregnancy. Liposomes are vesicles that are composed of a phospholipid bilayer in which the hydrophobic tail regions interact, leaving the hydrophilic head groups on the outside, forming a conformation with the lowest possible entropy. It is then possible to manipulate this phospholipid bilayer in order to form a spherical liposome that contains a hydrophobic bilayer region and an aqueous core (Figure 1.2).

Liposomes have proven to be useful drug delivery systems, allowing drugs of differing solubilities to be encapsulated. Lipophilic drugs may be incorporated into the lipid bilayer of the liposome by introducing the drug in the initial stages of liposome synthesis. Hydrophilic drugs may be dissolved in the aqueous solution that is used to rehydrate the phospholipid bilayer, encapsulating the drug within the aqueous core of the liposome.

Liposomes have been utilised as drug delivery systems for a number of different therapies, especially recently, in effort to lower side effects associated with cancer therapy. Doxil®, the liposomal formulation of the chemotherapeutic doxorubicin, has been clinically approved and has been proven to reduce the side effects associated with systemic doxorubicin chemotherapy.

Throughout the development of Doxil®, a number of strategies were employed in order to ensure that drug delivery was successful and efficient. One of the major drawbacks to using liposomes as drug delivery systems were their rapid
clearance time from the body by the mononuclear phagocyte system (MPS), a part of the immune system that consists of phagocytic cells (van Etten et al., 1998). In order to overcome this, liposomes were coated in polyethylene glycol (PEG). This strategy proved to be successful, with PEGylated liposomes having a longer circulatory time than their non-PEGylated counterparts. Addition of PEG provides the outer surface with a hydrophilic character, thereby protecting the liposome from immune recognition, opsonisation and clearance (Hong et al., 1999, Zolnik et al., 2010).

Another issue to consider when using liposomes as drug delivery systems is the rate at which drugs are released. Liposomes that are made of only phospholipids have been shown to be highly permeable to water, and therefore drug release from the aqueous core is very fast. Incorporation of cholesterol into liposomes reduces their permeability, thereby prolonging drug retention and slow release (Malam et al., 2009).

Liposomes can also be tailored to release their drug payload at a specific pH range (Simoes et al., 2004). Inclusion of phosphatidylethanolamine (PE) into the liposome composition favours destabilisation under acidic conditions (Simoes et al., 2004); this may prove useful in the development of liposomes for use in drug delivery to the placenta, as the placental tissue in complicated pregnancies tends to be hypoxic and therefore of a lower pH than other maternal tissues (Hung et al., 2001, Kendall et al., 2011).

Previous studies have found that there is a correlation between the size of nanocarriers and their clearance profiles. Generally, liposomes of 300nm diameter and above are rapidly cleared by the body; it is believed that this occurs due to the activation of the complement system. There is a proportional relationship between liposome size and activation of the complement system, with smaller liposomes showing less complement activation (Harashima et al., 1994, Moghimi et al., 2001, Devine et al., 1994). Activation of complement causes the nanocarriers to be tagged with complement component proteins, therefore making them recognisable to macrophages; these macrophages then actively phagocytose the nanocarriers, in order to disable any threat that they may cause. For this reason it is important to use liposomes that are below 300nm for clinical applications, in order to prevent activation of the complement system and rapid clearance.
To summarise, it is evident that a number of factors must be taken into consideration in order to develop efficient nanocarriers for clinical use. Liposomes must include both PEG and cholesterol and they must also be below 300nm in diameter, in order to ensure that they have an appropriate stability and drug release profiles and that they circulate in the blood stream long enough to have a biological effect. The issue of off-target side effects caused by circulating liposomes being taken up by a number of tissues can be alleviated by decorating them with tissue-specific homing peptides. This would rise to a liposomal formulation that is selectively targets the maternal side of the placenta, potentially limiting accumulation of any encapsulated therapeutics in maternal and fetal tissues.

1.5.2 Liposomes with attached homing peptides as a targeted drug delivery system

To enable decoration of the liposomal surface with tissue-specific homing peptides, an appropriate reactive group must be incorporated during synthesis of the liposome; this can be achieved by the inclusion of PEG-maleimide (mPEG) into the lipid bilayer. Previous research has found that at least 2% of the unmodified PEG must be replaced with mPEG, allowing sufficient maleimide groups to be displayed on the outer surface of the liposome to allow peptide labelling (Nobs et al., 2004, Samad et al., 2007).

mPEG contains an electro-rich double bond that favours electrophillic addition reactions, in particular with thiol groups (Figure 1.3). Some peptides naturally contain a thiol, for example if they contain the amino acid cysteine. Cysteine may be added to either end of the peptide by a condensation reaction, provided that the end of the peptide is not an active part of the targeting moiety. It is also possible to attach a fluorophore, such as green fluorescent protein (GFP) or rhodamine, to the targeting peptide in order to track its binding.
1.5.3 Liposomes as a targeted drug delivery system – potential therapies

Liposomes are effective at encapsulating a broad range of drugs as they can accommodate molecules of both a hydrophilic and a hydrophobic nature; this is due to the liposome having an aqueous core in which hydrophilic drugs may dissolve and a bilayer in which lipophilic drugs may associate. As has been previously discussed, in some cases of FGR there is a down-regulation of expression of the growth factors IGF-I and IGF-II by the fetus, possibly as a physiological adaptation to lower its growth trajectory, in order to survive the
reduction in rate of nutrient transfer across the placenta (Brodsky and Christou, 2004). As down-regulation of circulating fetal IGF expression is a characteristic of FGR, and IGF-I and -II are known to enhance placental cell proliferation, survival and nutrient transport in human placental tissue in vitro (Forbes/Westwood review), it would seem logical to hypothesise that administering IGFs maternally, along with nutrients to support growth, would improve placental growth function, thereby indirectly having a positive effect on fetal growth and survival. However, systemic administration of IGF-I to pregnant rats resulted in accelerated growth of maternal organs with no effect on fetuses; although IGF-I was administered without the use of a targeting drug carrier (Robinson et al., 2000). Sferruzzi-Perri et al have also successfully demonstrated that treatment of pregnant guinea pigs with IGFs in early pregnancy increases fetal growth, nutrient transport to the fetus and the ability of the fetus to utilise maternal substrates near term (Sferruzzi-Perri et al., 2007). By encapsulating IGFs in a targeting liposome carrier it may be possible to deliver these growth factors directly to the placenta, thereby enhancing placental growth and optimising placental function. Encapsulation of IGFs in liposomes has been demonstrated previously, thereby indicating that liposomal delivery in pregnancy would be a possibility (Jeschke et al., 2005); a recent study has indeed shown this to be the case, with targeted delivery of IGF-II using a tumour homing peptide conjugated to a liposome was shown to increase fetal growth in a mouse model of FGR with a placental-specific \textit{IgfII} knock-out (King et al., 2016).

A characteristic of pre-eclampsia and some cases of FGR is reduced spiral artery remodelling, leading to impaired delivery of blood to the placenta. In order to improve uteroplacental perfusion it seems reasonable that a vasodilator would have beneficial effects. Administration of a vasodilator may encourage relaxation of the incompletely remodelled spiral arteries, and indeed all arteries in the uterine circulation, thereby increasing efficiency and volume of maternal blood flow.

Sildenafil citrate is a potent vasodilator and recent research has examined the effect of this drug on the human uterine vasculature and on pregnancy outcomes in FGR and pre-eclampsia. Sildenafil citrate causes vasodilation by the inhibition of phosphodiesterase type 5 (PDE5); this enzyme is responsible for the
breakdown of cGMP and so inhibition of PDE5 prevents the degradation of cGMP, leading to vasodilation (Francis and Corbin, 2005). Sildenafil citrate caused significant vasodilation of pre-constricted uterine arteries obtained from patients with FGR in vitro; arteries were dissected from uterine biopsies performed during caesarean; (Wareing et al., 2005a). Furthermore, correlations between maternal sildenafil citrate administration and increased fetal weights, as compared to an untreated group in severe-early onset FGR in humans have also been described, providing strong evidence that sildenafil citrate is a potential treatment for FGR (Maharaj et al., 2009, von Dadelszen et al., 2011).

Uteroplacental blood flow may also be enhanced via the use of nitric oxide (NO) donor drugs. NO donor drugs have commonly been used for treatment in cardiovascular disease, such as angina and hypertension, and examples include PYRO-NO, isosorbide mononitrate and glyceryl trinitrate (GTN). Clinical trials involving sublingual delivery of GTN to women with mild pre-eclampsia and threatened pre-term labour have previously been conducted. Results from these trials indicated that delivery of GTN reduced the resistance of the fetoplacental circulation, but effect on pregnancy outcome was not investigated. It has been suggested that this was due to GTN acting as a NO-donor, releasing NO into the placental vasculature, which compensated for any reduction of NO production by the pre-eclamptic placenta and correcting the impaired uteroplacental perfusion (Luzzi et al., 1999). Despite promising results from trials involving GTN and its precursor L-arginine, there was a significant risk of side-effects including severe headaches that caused cessation of treatment (Meher and Duley, 2007). The ability to deliver NO-donor drugs, such as GTN, selectively to the placenta may alleviate these off-target side-effects thereby providing the first treatment option for pre-eclampsia, and for FGR caused by abnormal uterine blood flow.

1.6 The Pregnant Mouse Model as a Tool for Testing Targeting and Drug Delivery

In order to develop mechanisms for tissue-specific targeting and assess drug delivery and release, an in vivo model must be used. The pregnant mouse shares a number of similarities with the human placenta and several well-
characterised mouse models of pre-eclampsia and FGR are available (Dilworth and Sibley, 2013). The anatomy and nomenclature of the mouse placenta is different to that of the human placenta, with the mouse labyrinth being functionally equivalent to the human chorionic vill, the junctional zone (also known as the spongiotrophoblast zone) of the mouse placenta being equivalent to the basal plate of the human placenta and the zona intima being equivalent to the terminal villi of the human placenta (Rossant and Cross, 2001, Georgiades et al., 2002). The main difference between the human and mouse placenta lies in the way in which the maternal and fetal blood supplies are kept separate. In the human placenta this is achieved by the trophoblast bilayer, consisting of the syncytiotrophoblast and the underlying cytotrophoblast that are in contact with the maternal blood. In the mouse placenta however, this trophoblast layer differs; rather than being a bilayer of trophoblasts in which the proliferative cytotrophoblasts fuse to form the outer syncytiotium, the mouse placenta contains a tri-layer of trophoblasts in which there is a mononuclear trophoblast layer in contact with the maternal blood supply, followed by two layers of syncytiotrophoblast. The murine syncytiotrophoblast differs from its human counterpart as there are two layers as opposed to one, and the syncytiotrophoblast layers are also not formed from fusion of an underlying cytotrophoblast, as in the human placenta (Georgiades et al., 2002, Carter, 2007, Rossant and Cross, 2001, Dilworth and Sibley, 2013). In this sense, the mouse placenta has a barrier capacity that is thicker than that of the human placenta. This difference may mean that the kinetics of transport of molecules across the mouse placenta may vary, with the possibility of transport being more complex in the mouse placenta with any diffusion distance being greater. Since the aim of the targeting liposome would be to reach the placenta and not cross it, this structural variation may not prove a hurdle to using the pregnant mouse model. A comparison of the murine and human placentas is shown in Figure 1.4 (Rossant and Cross, 2001).
Figure 1.4. Comparison of murine and human placenta from (Rossant and Cross, 2001)
1.7 Aims and Objectives of Current Research

It is the aim of the current research project to design and test a targeted drug delivery system for the administration of therapies to treat pre-eclampsia and FGR. Liposomes have been identified as useful drug carriers and the addition of a tissue-specific homing peptide makes them even more attractive as therapeutic options.

In previous research carried out by our laboratory, two novel, placental-specific homing peptides have been discovered through screening of peptides libraries using the phage display technique (Ruoslahti, 2002, Ruoslahti et al., 2010). These peptides, NKGLRNK and RSGVAKS, have been tested in a pregnant mouse model in order to validate placental homing and specificity of binding. Mice were intravenously injected with fluorescently tagged free peptide at different gestations, the mice were then sacrificed, their placentas fixed, frozen and cryosectioned, then peptide binding was assessed by fluorescence microscopy; maternal organs were also examined in order to assess extent of peptide binding and tissue specificity (Figure 1.5, Figure 1.6).
Figure 1.5. Mice were injected with peptide NKGLRNK at different gestations and then sacrificed, their placentas fixed, frozen and cryosectioned and then fluorescence microscopy was carried out to determine peptide binding. SA = spiral artery, Lab = labyrinth, JZ = junctional zone, Dec = decidua.
Figure 1.6. Mice were injected with peptide RSGVAKS at different gestations and then sacrificed, their placentas fixed, frozen and cryosectioned and fluorescence microscopy was carried out to determine peptide binding. SA = spiral artery, Lab = labyrinth, JZ = junctional zone, Dec = decidua.
These sequences selectively target the labyrinth and spiral arteries of the mouse placenta without significantly accumulating any of the major maternal organs. These results confirm that the two novel peptide sequences are active, placental-specific homing peptides. It remains to be shown whether this targeting specificity is retained when the same peptides are conjugated to a nanoparticle.

The two peptides that have been selected for use in this research project do not contain any cysteine amino acids; peptides with a cysteine attached to the N-terminal amino group must be used in order to permit coupling to the liposomal surface. The amino acid structure and R group details for each of the peptides are shown below (Figure 1.7, Table 0.1).

As previously described, it is possible to covalently couple cysteine residues to mPEG in the liposome formulation; thus liposomes containing mPEG, PEG and cholesterol will be synthesized. An extrusion technique will be used in order to
produce liposomes of a defined size of greater than 100nm, but less than 200nm; liposomes of this size have been shown to have enhanced in vivo circulation, limited effects on complement activation and have also been shown to be too large to easily cross the placental barrier (Moghimi et al., 2001, Bajoria and Contractor, 1997). Once the liposomes have been sized, the targeting peptide will be attached and the liposomes may be tested in vitro and in vivo.

Once these experiments have been performed, the ability of homing peptide-coated liposomes to selectively target the placenta will be known. It is important to ascertain whether both the liposome and the homing peptide reach the target tissue and the drug is released; it could be that the peptide reaches the target, as has been seen before, but that during this process it becomes detached from the liposome. It will therefore be beneficial to use differently coloured fluorophores, one on the peptide and one within the liposome, in order to demonstrate that both the peptide and the liposome have reached their target.

Once this drug delivery system has been refined, it will then be possible to load these liposomes with different therapeutics in order to try and alter different parameters of placental function, and determine the effect of fetal growth, using a pregnant mouse model. Such therapeutics could include vasodilators, for example sildenafil citrate and NO-donor drugs, in order to dilate the uterine vasculature or antioxidants to counteract the oxidative stress that characterises placental dysfunction; both treatments may need to be accompanied by an increase in maternal nutrient supply in order to account for the increased placental demand as function improves. Other therapeutics may also be introduced to try to alter the efficiency placental function, for example to induce increased expression or activity of nutrient transporters.

The specific aims of my research project are as follows:

- develop, synthesise and characterise a peptide-conjugated liposome
- assess binding ability of free peptide and peptide-conjugated liposomes using human placental explants, and study drug release using suitable encapsulated fluorophore
• assess binding ability and tissue distribution of free peptide and peptide-conjugated liposomes after tail vein intravenous injection into pregnant mice
• identify and encapsulate a suitable therapeutic, determine release profiles \textit{ex vivo}
• administer peptide-conjugated liposomes containing an encapsulated therapeutic to pregnant mice, in order to determine effects on placental size / weight, fetus size / weight and any side-effects in the mother

It is the ultimate aim of this research project to provide a viable treatment option for pregnancy-related complications. There have been major advancements made in the early detection of such complications by identifying and measuring markers in the blood of pregnant women and improvements in imaging/scanning technologies (Wortelboer et al., 2010). If these technologies are developed along with the targeting drug delivery system, it may be possible not only to provide treatment options for complications after their onset, but also to provide preventative treatment to those women identified to have the biomarkers that are predictive of disease.
Chapter 2 Methods
2.1 Materials

1,2-distearoyl-sn-glycer-3-phosphocholine (DSPC), 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-[methoxy(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG), 1,2-distearoyl-sn-glycer-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (ammonium salt) (DSPE-PEG-maleimide) and 1,2-distearoyl-sn-glycer-3-phosphoethanolamine-N-(7-nitro-2,1,3-benzoxadiazol-4-yl) (ammonium salt) (NBS-DSPE) were obtained from INstruchemie Netherlands. Cholesterol was obtained from Sigma-Aldrich UK. 2-[[4-[(nitrooxy)methyl]benzoyl]thio]-benzoic acid, methyl ester (SE175) was purchased from Cayman Chemical (>97% purity). Peptides were custom synthesised by Insight Biotechnology UK (>98% purity). All chemicals were used as received. All solvents used were of analytical grade. Slide-A-Lyzer Dialysis Cassettes, MWCO 3.5kDa, were obtained from Thermo Fisher Scientific UK. All plastics were purchased from Appleton Woods (Birmingham).

Phosphate buffered saline (PBS) was used at 1M and consisted of 137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4 and 1.8 mM KH2PO4 in 1 L distilled water, with pH adjusted to 7.4.

Tris-buffered saline (TBS) was used at 0.05M and consisted of 10 mM Tris and 150 mM NaCl in 1 L distilled water, with pH adjusted to 7.6.
2.2 Liposome preparation and characterisation

2.2.1 Synthesis of non-targeted liposomes

Liposomes were prepared by the lipid film rehydration method (Nallamothu et al., 2006). DPSC (32.5 µmol), cholesterol (15 µmol), DSPE-PEG (2.5 µmol) were weighed and dissolved in methanol:chloroform (9:1 ratio). Solvent was removed by rotary evaporation (40°C, 270 mbar) to produce a lipid film, which was incubated in a vacuum drier overnight (25°C, 0 mbar). The lipid film was rehydrated in 1 mL phosphate buffered saline (PBS) and the resultant solution placed in an oven at 62°C for 3 h to produce large multilamellar vesicles. The solution was then extruded through a 1mL thermobarrel Mini-Extruder using a 0.1µm, 19 mm polycarbonate membrane surrounded by 10 mm filter supports (Avanta Polar Lipids), 11 times in order to produce monodispersed unilamellar liposomes. Liposomes were then stored at 4°C until use.

2.2.2 Synthesis of control PBS-encapsulated homing peptide-conjugated liposomes

Liposomes were prepared as described in 2.2.1. 10% of the DSPC was replaced with DSPC-PEG to allow peptide conjugation. The lipid film was prepared and rehydrated with PBS followed by extrusion as described above. Peptide (0.27 µmol; sequence: CNKGLRKN or CRSGVAKS) was added to 1 mL of extruded liposome suspension immediately after extrusion in order to present any maleimide cross-reaction and allowed to covalently couple overnight at room temperature. Unreacted peptide was removed by dialysis (Slide-A-Lyzer Dialysis Cassettes, MWCO 3.5 kDa) against PBS. Liposomes were then stored at 4°C until use.

2.2.3 Synthesis of SE175 encapsulated homing peptide-conjugated liposomes

Liposomes were prepared as described in 2.2.2 with SE175 being added at the point of lipid rehydration. In short, after the lipid film was formed and dehydrated
overnight, 1 mL of 320 µM SE175 was added, the solution placed in an oven at 62°C for 3 h to produce large multilamellar vesicles. The solution was then extruded as previously described and peptide (0.27 µmol; sequence: CNKGLRKN or CRSGVAKS) was added to 1 mL of extruded liposome suspension immediately after extrusion in order to present any maleimide cross-reaction and allowed to covalently couple overnight at room temperature. Unreacted peptide and SE175 was removed by dialysis (Slide-A-Lyzer Dialysis Cassettes, MWCO 3.5 kDa) against PBS. Liposomes were then stored at 4°C until use.

SE175 encapsulation efficiency was unable to be measured and was presumed at a maximum of 320 µM or less.

2.2.4 Liposome characterisation

The size (hydrodynamic diameter) distribution (SD) and polydispersity index (PDI) were measured by dynamic light scattering (DLS) and the zeta potential (ZP) measured by laser Doppler micro-electrophoresis (LDE) (25°C; scattering angle of 173° (Zetasizer Nano ZS, Model ZEN3600, fitted with a 632 nm laser, Malvern Instruments Ltd., UK)). To measure SD and PDI, 300 µL diluted liposome suspension (50 µL suspension in 950 µL PBS) was loaded into a disposable semi-micro cell (Model ZEN0040, Malvern Instruments Ltd., UK), the measurement taken in triplicate and the Mark Houwink parameter applied. The ZP was measured by loading 500 µL of liposome suspension, prepared as described above, into a disposable folded capillary cell (Model DTS1070, Malvern Instruments Ltd., UK) and the Smoluchowski model applied to the measurement. The pH of all suspensions was maintained at 7.4. Measurements were repeated three times per formulation for a minimum of three independent samples where possible. Size distribution and zeta potential were determined for all liposomes. 300µL liposome suspension was added to a disposable low volume sizing cuvette (Malvern, UK) and the Z-average size calculated from size distributions measured by dynamic light scattering on the The Zetasizer Nano
ZS (Malvern, UK). Zeta potential was also measured using a folded capillary zeta cell (Malvern, UK).

2.2.5 Liposome stability

To assess liposome stability, flocculation and degradation at 4°C, the SD and PDI was measured at 24 h, 7 days, 14 days, 21 days and 28 days.
2.3 Tissue culture and microscopy

2.3.1 Collection of first trimester and term human placenta

First trimester human placental tissue was acquired from medical or surgical terminations of pregnancy at gestations of 6 - 15 weeks under local ethical approval (ethics number 13/NW/0205). Term human placental tissue was acquired from elective Caesarean sections or vaginal deliveries at gestations of 35 - 42 weeks under local ethical committee approval (ethics numbers; September 2012 – August 2013 08/H1010/55, August 2013 – April 2016 08/H1010/55(+5), April 2016 onwards 15/NW/0829).

2.3.2 Tissue culture

2.3.2.1 Culture of free peptide with human placental tissue

Biopsies of villous tissue were removed from term or first trimester human placenta within 30 minutes of delivery and washed 3 times with sterile PBS to remove excess blood. Under sterile conditions, 1mm² explants of villous tissue were dissected and added to 24-well plates, pre-coated with agarose gel (1% (w/v)), with 900μL culture medium (DMEM/Ham’s F12 medium containing penicillin (100 IU/mL), streptomycin (100 µg/mL), glutamine (2 nM) and 10% (v/v) fetal bovine serum), warmed to 37°C. 100μL rhodamine labelled peptide (CNKGLRNK or CRSGVAKS, 0.27µmol) was added to each explant and cultured (37°C, 95% air, 5% carbon dioxide) for 30 mins 1 h, or 3 h. Untreated explants were used as a control.

2.3.2.2 Culture of homing peptide-conjugated liposomes with human placental tissue

Homing peptide-conjugated liposomes were synthesised as described in 2.2.2 using fluorescently labelled NBD-DSPE (2.7 µmol) and rhodamine labelled peptide (CNKGLRNK or CRSGVAKS, 0.27µmol). Human term explants were cultured as described in 2.3.2.1 for 0 h, 3 h, 6 h, 24 h or 48 h. Non-targeted
liposomes, as described in 2.2.1, containing fluorescently labelled NBD-DSPE (2.7 µmol) and untreated explants were used as controls.

2.3.2.3 Culture of DAPI encapsulated homing peptide-conjugated liposomes with human placental tissue

Homing peptide-conjugated liposomes were synthesised as described in 2.2.2 using fluorescently labelled NBD-DSPE (2.7 µmol), rhodamine labelled peptide (CNKGLRNK or CRSGVAKS, 0.27µmol) and rehydrated with DAPI (50 µL 1mM in 0.95 mL PBS). Human term explants were cultured as described in 2.3.2.1 for 0 h, 3 h, 6 h, 24 h or 48 h. Non-targeted liposomes, synthesised as described in 2.2.1, with fluorescently labelled NBD-DSPE (2.7 µmol) and untreated explants were used as controls.

2.3.2.4 Culture of free SE175 with human placental tissue

Human term explants were cultured as described in 2.3.2.1 for 24 h, 48 h or 72 h with free SE175 (320 µM, in PBS (1:10 DMSO)). Tissue was fixed in neutral buffered formalin (4% (v/v), pH 7.4; overnight), then paraffin-embedded and stored at room temperature.

2.3.3 Fluorescence microscopy

Tissue was fixed in neutral buffered formalin (4% (v/v), pH 7.4; overnight), embedded in OCT (Sakura) and stored at -80°C. Tissue was sectioned (10 µm), post-fixed to slides with paraformaldehyde (4% (w/v) in PBS, 15 min), washed in PBS (2 x 5 min) and mounted in Vectashield fortified with DAPI, or unfortified Vectashield in the case of tissue from 2.3.2.3. Tissue was then imaged on the Zeiss Axio Observer fluorescence microscope. Images were taken using the same exposure time to allow for comparisons to be made across samples.
2.4 Immunohistochemistry

Sections of paraffin-embedded tissue (5 µm) were deparaffinised in Histoclear and alcohol and then rehydrated in dH₂O. Heat-induced antigen retrieval was performed by microwaving the slides in antigen retrieval buffer (sodium citrate buffer (0.01 M), containing 0.05% (v/v) Tween 20 (pH 6.0), 20 mins), followed by cooling for 10 mins. After cooling, tissue sections were incubated with hydrogen peroxidase (3% (w/v), 10 mins) to block endogenous peroxide activity, washed 3 times in tris-buffered saline (TBS) and incubated with bovine serum albumin (BSA; 5% (w/v) in TBS, 30 mins) to block non-specific binding. Tissue sections were then incubated with primary antibodies (Table 2.1) or control IgG (matched concentration; Sigma Aldrich) overnight at 4°C. Tissue sections were washed 3 times (TBS; 5 mins) and incubated at room temperature with secondary antibodies for 30 mins. Tissue sections were then washed 3 times (TBS; 5 mins) and incubated at room temperature with avidin peroxidase (5 µg/mL in TBS, 30 mins; Sigma Aldrich). Tissue sections were then washed with dH₂O, incubated for 2-10 mins with diaminobenzidine (0.05% (w/v); Sigma Aldrich) and urea hydrogen peroxide (0.015% (v/v); Sigma Aldrich) and washed again with dH₂O. Tissue sections were then counterstained with filtered Harris’s haematoxylin, dehydrated in alcohol and histoclear and mounted in DPX mountant (Sigma Aldrich). All sections were stained in the same staining run to allow for direct comparison of staining intensity between groups.

Three random images of each immunostained tissue section were captured on the Olympus B641 Light Microscope using Image-Pro Plus 7 software. Images were taken at the same exposure to allow comparisons to be made between groups.
<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host Species</th>
<th>Target Species</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67 Clone MIB-1 #M7240</td>
<td>Mouse</td>
<td>Human</td>
<td>Dako</td>
<td>1:500</td>
</tr>
<tr>
<td>M30 (CytoDEATH) #12-140-322-001</td>
<td>Mouse</td>
<td>Human</td>
<td>Roche</td>
<td>1:100</td>
</tr>
<tr>
<td>COX-1 aa 274 – 288 #160109</td>
<td>Rabbit</td>
<td>Mouse</td>
<td>Cayman</td>
<td>1:250</td>
</tr>
<tr>
<td>COX-2 aa 584 – 598 #160126 (batch 0463352-2)</td>
<td>Rabbit</td>
<td>Mouse</td>
<td>Cayman</td>
<td>1:1000</td>
</tr>
<tr>
<td>HNE #HNE11-S</td>
<td>Rabbit</td>
<td>Mouse</td>
<td>Alpha Diagnostics</td>
<td>1:500</td>
</tr>
<tr>
<td>IgG #I8765</td>
<td>Mouse</td>
<td>Human</td>
<td>Sigma Aldrich</td>
<td>Matched</td>
</tr>
<tr>
<td>IgG #I8140</td>
<td>Rabbit</td>
<td>Mouse</td>
<td>Sigma Aldrich</td>
<td>Matched</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Host Species</th>
<th>Target Species</th>
<th>Conjugate</th>
<th>Supplier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Goat</td>
<td>Human</td>
<td>Biotinylated</td>
<td>Dako</td>
<td>1:200</td>
</tr>
<tr>
<td>Swine</td>
<td>Mouse</td>
<td>Biotinylated</td>
<td>Dako</td>
<td>1:200</td>
</tr>
</tbody>
</table>

Table 2.1. Antibodies and dilutions used for immunohistochemistry
2.5 Animal procedures

2.5.1 Animal housing and husbandry

All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act of 1986. Work was carried out under a Home Office project licence awarded to Professor Colin Sibley (PPL number 40/3385) or a Home Office project licence awarded to Dr Mark Dilworth (PPL number 70/8504). Mice were housed with suitable nesting material and were provided with standard pellet mouse chow (Beekay Rat and Mouse Diet; Bantin & Kingman; Hull, UK), water ad libitum and caged under a 12 hour light/dark cycle at 21-23°C with 65% humidity. For timed matings, males were mated with 8-16 week old virgin females. Identification of a vaginal plug was considered the beginning of pregnancy, embryonic day 0.5 (E0.5). Mice were weighed at E2.5, E6.5 and E10.5 to track weight gain as an indicator of pregnancy prior to commencing dosing regimens. Dams were euthanased at E18.5 by cervical dislocation unless otherwise stated. A surgical laparotomy was performed on the dam to expose the uterine horn and fetuses were identified by horn position. Fetal and placental tissues were removed and fetal tail tips collected for retrospective gender genotyping.

2.5.2 Validation of liposome targeting

PBS-encapsulated targeted liposomes or non-targeted control liposomes were synthesised as described in 2.2.2 using fluorescently labelled NBD-DSPE (2.7 µmol) and rhodamine labelled peptide (CNKGLRNK or CRSGVAKS, 0.27µmol). Liposomes were administered via tail vein injection to time-mated, pregnant mice (C57/BK6 or eNOS<sup>-/-</sup>) at E13.5 or E15.5 and allowed to circulate for 24, 48 or 72 hours. To remove unbound liposomes and peptide mice were terminally cardiac perfused. Mice were placed under general anaesthetic (isoflurane, O<sub>2</sub> mixture; adjusted to maintain unconsciousness), the abdomen opened and the ribcage incised at both sides to reveal the heart. The superior vena cava was cut and a steady flow of PBS was perfused into left ventricle until the maternal kidney and liver were pale in colour (3-5 mins), the right ventricle was then perfused with a...
steady flow of PBS until the maternal lungs were pale in colour (1-3 mins). Organs were collected, fixed and visualised as described in 2.3.3.

2.5.3 Liposome treatment study

Time-mated, pregnant mice (C57/BK6 or eNOS<sup>−−</sup>) were intravenously injected with 100 µL of SE175-encapsulated targeted liposomes (as synthesised in 2.2.3, CNKGLRNK peptide, approximately 0.44 mg/kg), PBS-encapsulated targeted liposomes (as synthesised in 2.2.2, CNKGLRNK peptide), free SE175 (320 µM, approximately 0.44 mg/kg) or vehicle (PBS) on E11.5, E13.5, E15.5 and E17.5. Mice were sacrificed by cervical dislocation at E18.5 and the following measurements taken: litter size, number of resorptions, fetal weight, placental weight, maternal kidney weight, maternal spleen weight and maternal heart weight. Organs were fixed in neutral buffered formalin (4% (v/v), pH 7.4; overnight), then paraffin-embedded and immunostained as described in 2.4.

The numbers of mice recommended to achieve a statistically significant result was pre-determined by a power calculation using the GPower 3.1 software. Sample size was determined by using mean and standard deviations from previous studies. An increase in fetal weight of 10% in the SE175-encapsulated targeted liposomes was assumed and power of 0.95 and α error (false positive) probability of 0.05 was used.

This gave a total sample size recommendation of 28 with an actual power of 0.9545; this equated to 7 mice per treatment group (Figure 2.1).
Figure 2.1. G Power sample size calculation based on a power of 0.95 and α error probability of 0.05

2.5.4 Gender genotyping

Sex-specific genes were used to assess the gender of fetuses, using genomic DNA extracted from fetal tail tip tissue as previously described (Kunieda et al., 1992). Fetal tail tips were incubated with 150 µL DirectPCR lysis buffer (Bioquote Ltd, UK) and 1µL proteinase K (Quiagen stock; 20 mg/mL) overnight at 55°C in a hybridisation oven (Hybaid, VWR, UK). Samples were then vortexed and proteinase K inactivated by incubation on a hot block at 85°C for 30 min. Samples were centrifuged at 13,000 rpm for 1 min and then diluted by the addition of 1 mL milliQ H₂O. Extracted genomic DNA was stored at -20°C for no longer than 4 weeks before polymerase chain reaction (PCR).
Primers for the male SRY gene (Sry2/JG and Sry4/JG) and for the DXNds3 locus of the X chromosome (NDS3 and NDS4) were used. Full primer sequences are shown below:

Sry2  5' TCTTAAACTCTGAAGAAGAGAC 3'
Sry4  5' GTCTTGCGTGTATGTGATGG 3'
NDS3  5' GAGTGCCCTCATCTATACTTACAG 3'
NDS4  5' TCTAGTTGATTGATTGATGTC 3'

Genomic DNA was amplified using a Techne Prime thermal cycler (Bibby Scientific, UK) and the Expand High Fidelity PCR system. Concentrations of primers and reagents can be found in Table 2.2. PCR conditions were as follows:

| step 1: | 94°C for 5mins |
| step 2: | 94°C for 1min |
| step 3: | 55°C for 1min |
| step 4: | 72°C for 1min |
| step 5: | repeat steps 2-4 for 35 cycles |
| step 6: | 72°C for 10mins |

18µL PCR product was then loaded onto a 1.5% agarose gel. 5µL Hyperladder IV molecular weight marker (Bioline, UK) was added to lane 1. The gel was run at 35 min at 120 volts then visualised using the Fel UV Transilluminator.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>25 µL MyTaq™ Red Mix (Bioline, UK)</td>
<td>-</td>
</tr>
<tr>
<td>5 µL PCR H₂O (Sigma-Aldrich, UK)</td>
<td>-</td>
</tr>
<tr>
<td>4 µL SRY2 primer</td>
<td>500 nM</td>
</tr>
<tr>
<td>4 µL SRY4 primer</td>
<td>500 nM</td>
</tr>
<tr>
<td>4 µL NDS3 primer</td>
<td>500 nM</td>
</tr>
<tr>
<td>4 µL NDS4 primer</td>
<td>500 nM</td>
</tr>
<tr>
<td>4µL genomic DNA</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.2. Reagents for a 1 X PCR reaction using the Expand High Fidelity PCR system
2.6 Blood vessel dissection and normalization for wire myography

2.6.1 Mouse uterine arteries

Pregnant female mice (eNOS<sup>−/−</sup> and C57BL/6J) were mated and at E18.5, dams were euthanized by cervical dislocation. A surgical laparotomy was performed on the dam to expose the uterine horn. The entire horn was removed and placed into ice-cold PBS. The horn was pinned out as previously described (Kusinski et al., 2012) and main loop uterine artery carefully dissected using a dissection microscope (model S6E, Leica, UK). The dissected artery was trimmed into approximately 40 µm length sections and kept in cold physiological salt solution (PSS, Table 2.3) prior to mounting on the wire myograph.

Dissected arterial sections were mounted by inserting two 40 µm steel wires into the lumen, which were secured in the jaws of a Multi Myograph System 610M (Danish Myo Technology A/S, Denmark) as previously described (Wareing et al., 2002). Arterial section lengths were then measured using a calibrated eyepiece graticule. Arterial sections were equilibrated to physiological conditions as previously described in 6 mL PSS, warmed to 37°C and gassed with 20 % O<sub>2</sub> / 5 % CO<sub>2</sub> / balanced with N<sub>2</sub> (BOC Gases, Worsley, UK). 20 % O<sub>2</sub> was chosen to mimic in vivo oxygen conditions. Arterial sections were classically normalised to ensure that arterial sections of different diameters had the same resting tension as previously described (Kusinski et al., 2009); briefly arterial sections were normalised to 0.9 of \(L_{13.3}\) kPa, where \(L\) is the internal diameter of the vessel section, using Laplace’s relationship. Arterial sections were then left to equilibrate for 20 minutes.

2.6.2 Human chorionic plate arteries

Term human placentas were acquired as described in 2.3.1. Placental biopsies of chorionic plate were taken and placed into ice-cold PSS. Small chorionic plate arterial branches originating from the main umbilical arteries were identified and carefully dissected and trimmed into approximately 40 µm length sections. Vessels were then mounted and normalised as described in 2.6.1.
2.7 Wire myography

After dissection and normalisation, arterial sections were exposed to a high potassium salt solution (KPSS, Table 2.3) for 5 minutes to determine maximal smooth muscle contraction, before being washed twice with PSS to return arterial sections back to baseline. Arterial sections were left to re-equilibrate for 20 minutes before being exposed to the α-1 agonist phenylephrine (PE, $10^{-5}$ mol/L) for 10 minutes to determine maximal contraction. After 10 minutes, arterial sections were exposed to acetylcholine (ACh, $10^{-5}$ mol/L) to determine maximal relaxation, before being washed twice with PSS and left to equilibrate for 20 minutes. PE exposure was then repeated and relaxation to SE175 determined following the application of incremental doses ($10^{-9}$ – $10^{-5}$, 2 minute intervals). Equivalent concentrations of DMSO (vehicle control) and no treatment (time control) were also used. In the case of wire myography using SE175-encapsulated targeted liposomes, the extent of relaxation to a single dose of SE175 was determined (100 µL liposome suspension).

Relaxation data were expressed as percentage relaxation of maximum pre-constriction to PE. Data were expressed as mean ± SEM unless otherwise stated. Myodata software (version 2.02; Myonic Software, National Instruments Corporation, USA) was used to visualise and interpret data.

<table>
<thead>
<tr>
<th>Physiological salt solution (PSS)</th>
<th>High potassium salt solution (KPSS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>119 mM / L NaCl</td>
<td>12.45 mM / L NaCl</td>
</tr>
<tr>
<td>4.69 mM / L KCl</td>
<td>120 mM / L KCl</td>
</tr>
<tr>
<td>2.4 mM / L MgSO$_4$</td>
<td>2.4 mM / L MgSO$_4$</td>
</tr>
<tr>
<td>25 mM / L NaHCO$_3$</td>
<td>25 mM / L NaHCO$_3$</td>
</tr>
<tr>
<td>1.18 mM / L KH$_2$PO$_4$</td>
<td>1.18 mM / L KH$_2$PO$_4$</td>
</tr>
<tr>
<td>6.05 mM / L Glucose</td>
<td>6.05 mM / L Glucose</td>
</tr>
<tr>
<td>0.034 mM / L EDTA</td>
<td>0.034 mM / L EDTA</td>
</tr>
<tr>
<td>1.6 mM / L CaCl$_2$</td>
<td>1.6 mM / L CaCl$_2$</td>
</tr>
<tr>
<td>pH 7.4 HCl</td>
<td>pH 7.4 HCl</td>
</tr>
<tr>
<td>95 % O2 / 5 % CO2 10 min</td>
<td>95 % O2 / 5 % CO2 10 min</td>
</tr>
</tbody>
</table>

Table 2.3. Composition of physiological salt solutions used for wire myography
2.8 Data analysis

All data were analysed using GraphPad Prism software (version 7). Myography data were analysed using two-way analysis of variance (Soloviev et al.) with Bonferroni post hoc test for comparisons between groups. All data were tested for normality using the D'Agostino-Pearson omnibus test. Data that passed this test were analysed using one-way ANOVA with Dunnett post hoc test for comparisons between groups; any data that did not pass the test for normality was analysed using a Kruskal-Wallis with Dunn’s post hoc test. Frequency distribution curves were generated by performing non-linear regression on fetal / placental weight histograms. Unless otherwise stated, data are shown as mean ± SEM and significance was taken as $P < 0.05$. 
Chapter 3
3.1 Introduction

More than 10% of pregnancies are complicated by conditions such as fetal growth restriction (FGR) and pre-eclampsia, resulting in maternal and fetal morbidity and mortality (MacKay et al., 2001, Bernstein et al., 2000). Current management strategies for such complications are limited to increased monitoring and induction of pre-term labour if the condition worsens (Alberry and Soothill, 2007). Pre-term delivery itself is associated with increased morbidity and mortality, which is exacerbated in those fetuses with growth restriction (Garite et al., 2004). There is also evidence that being born small or prematurely can lead to developmental programming, leading to an increased risk of adulthood diseases such as diabetes, cardiovascular disease, obesity and type 2 diabetes (Gluckman et al., 2008). Despite the short and long term health implications, development of therapeutics for use in pregnancy is hindered by the risk of systemic side-effects.

Peptide-mediated targeting of liposomes to specific vascular beds is a strategy that has been explored to allow selective delivery of therapeutics, in an attempt to alleviate off-target, systemic side-effects (Ruoslhti et al., 2010). Much of the current research in this area has focussed on targeted delivery of chemotherapeutics to tumours, and have achieved this by utilising targeting specific receptors that are overexpressed on cancer cells (Accardo and Morelli, 2015, Gray et al., 2013). The villous placenta and surrounding uterine vasculature overexpress a number of receptors, similar to those expressed by solid tumours and their associated vasculature. Indeed, the placenta exhibits similar behaviours to that of solid tumours, including the abilities to be highly proliferative, to be invasive and migratory and evade the immune system (Ferretti et al., 2007). These commonalities led to the hypothesis that existing tumour-homing sequences could be utilised to facilitate targeted drug delivery to the placenta, in the hope of alleviating maternal and fetal side-effects associated with systemic drug delivery.

It is has been demonstrated that impaired uteroplacental blood flow contributes to fetal growth restriction in both human and mouse pregnancies (Swanson and David, 2015, Krishna and Bhalerao, 2011), suggesting that improving uteroplacental blood flow may provide a means to improve fetal growth in utero.
Attempts have been made to enhance uteroplacental blood flow with the use of vasodilators, with a clinical trial showing that administration of sildenafil citrate to pregnant women presenting with FGR improved uteroplacental perfusion as identified by Doppler ultrasound (Dastjerdi et al., 2012). Sildenafil citrate has also been assessed in a mouse model of FGR, and while these studies were able to demonstrate that sildenafil citrate significantly increased fetal weight, they also showed that maternal administration of sildenafil citrate caused impaired vasorelaxation of fetal abdominal aortas, lead to female offspring exhibiting impaired glucose tolerance, and caused the offspring to have elevated blood pressure as adults (Dilworth et al., 2013, Renshall et al., 2014a, Renshall et al., 2014b). These studies demonstrate the potential side-effects of systemic maternal delivery of therapeutics in pregnancy and lend support to the potential of targeted drug delivery to the placenta.

Previous research from our group has demonstrated that the tumour homing peptide iRGD can be used to facilitate selective liposomal delivery of insulin-like growth factor 2 to the mouse placenta, leading to an improvement in fetal weight distribution in a model of FGR (King et al., 2016). Importantly, this study demonstrated that peptide-mediated targeting of liposomes to placenta did not cause any detectable detrimental effects in the mother or the fetus, providing proof-of-principle for targeted drug delivery to the placenta.

In a related study, we have identified novel, non-tumour targeting peptide sequences that selectively bind to the villous placenta and uterine spiral arteries blood vessels. The initial aims of this particular study were to 1) conjugate these newly identified peptide sequences to liposomes, validating the characteristics and stability of this formulation, and 2) to determine the extent of binding and uptake of these peptide-conjugated liposomes in vitro and in vivo.

These aims will address the following questions; a) do peptide-conjugated liposomes decorated with novel peptide sequences exhibit suitable size and stability profiles for use as a drug delivery system? If so b) are the binding, uptake and drug release profiles in human placenta appropriate and c) does intravenous delivery of the peptide-conjugated liposomes lead to selective targeting of mouse placenta?
3.2 Results

3.2.1 Characterisation of the size, zeta potential and polydispersity index of homing peptide-conjugated liposomes

Homing peptide-conjugated liposomes, prepared by lipid film hydration, were characterised using the Zetasizer Nano ZS to determine size distribution and zeta potential. Homing peptide (CRSGVAKS and CNKGLRNK)-conjugated liposomes had mean diameters of 136 ± 1.2 nm and 151 ± 1.0 nm, respectively. Undecorated liposomes, lacking a homing peptide, had a mean diameter of 171 ± 1.1 nm. Polydispersity index (PDI) is a measure of particle size heterogeneity, with values closer to 0 indicating a narrow size distribution and values closer to 1 indicating wider distribution. Conjugation of peptides to liposomes reduced the PDI, indicating a narrower particle size distribution and therefore a more uniform, monodisperse liposome suspension (Figure 3.1, Table 3.1).

The zeta potential of undecorated liposomes was -13.7 ± 0.29 mV. RSG-conjugated and NKG-conjugated liposomes had zeta potentials of +0.7 ± 0.47 mV and -0.5 ± 0.46 mV respectively. This indicates that conjugation of peptides to liposomes brought the zeta potential closer to neutral.
Figure 3.1 Size distribution of undecorated liposomes (green), RSG-conjugated liposomes (blue) and NKG-conjugated liposomes (red). Representative plot from n = 3 liposome preparations.

<table>
<thead>
<tr>
<th></th>
<th>Mean diameter / nm</th>
<th>Polydispersity index (PDI)</th>
<th>Mean zeta potential / mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Undecorated liposome</td>
<td>170.9 ± 1.1</td>
<td>0.181</td>
<td>-13.7 ± 0.29</td>
</tr>
<tr>
<td>RSG-conjugated</td>
<td>135.5 ± 1.2</td>
<td>0.118</td>
<td>0.7 ± 0.47</td>
</tr>
<tr>
<td>NKG-conjugated</td>
<td>150.7 ± 1.0</td>
<td>0.109</td>
<td>-0.5 ± 0.46</td>
</tr>
</tbody>
</table>

Table 3.1 Mean diameter and zeta potential measurements of undecorated liposomes, RSG-conjugated liposomes and NKG-conjugated liposomes, where means are calculated from triplicate measurements of three separate sample preparations ± standard error.
3.2.2 Assessment of the stability of homing peptide-conjugated liposomes

Homing peptide-conjugated liposomes remained stable at 4°C for 28 days. The average particle size and PDI values remained consistent, with mean PDI values of between 0.081 and 0.154 (Table 3.2). RSG-conjugated liposomes showed a lower mean particle size after 28 days; the presence of an additional peak between 10 nm and 100 nm may account for this, indicative of liposome degradation.

Undecorated liposomes were stable for up to 21 days, with the average size remaining relatively consistent and PDI values remaining between 0.141 and 0.181. Additional peaks were present in the size distribution of undecorated liposomes at 28 days (Figure 3.2). There were both peaks of a smaller diameter and of a larger diameter and the PDI increased to 0.848. This suggested that both aggregation and degradation were occurring.
Table 3.2 Size and PDI measurements of undecorated liposomes, RSG-conjugated liposomes and NKG-conjugated liposomes over a 28 day period. Size and PDI measurements are means are calculated from triplicate measurements of three separate sample preparations ± standard error.

<table>
<thead>
<tr>
<th></th>
<th>Initial</th>
<th>24 h</th>
<th>7 day</th>
<th>14 day</th>
<th>21 day</th>
<th>28 day</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Size / nm</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Undecorated liposomes</td>
<td>170.9 ± 1.1</td>
<td>172.6 ± 1.7</td>
<td>171.5 ± 1.1</td>
<td>169.8 ± 0.9</td>
<td>146.1 ± 0.5</td>
<td>458.1 ± 25.2</td>
</tr>
<tr>
<td>RSG-conjugated liposomes</td>
<td>135.5 ± 1.2</td>
<td>131.3 ± 2.4</td>
<td>129.0 ± 0.5</td>
<td>131.1 ± 0.7</td>
<td>129.6 ± 1.3</td>
<td>112.0 ± 0.9</td>
</tr>
<tr>
<td>NKG-conjugated liposomes</td>
<td>150.7 ± 1.0</td>
<td>146.7 ± 0.6</td>
<td>143.6 ± 0.5</td>
<td>146.2 ± 1.5</td>
<td>154.2 ± 1.5</td>
<td>154.4 ± 0.7</td>
</tr>
</tbody>
</table>

| **PDI**                |         |       |       |        |        |        |
| Undecorated liposomes  | 0.181   | 0.171  | 0.153  | 0.141  | 0.171  | 0.848  |
| RSG-conjugated liposomes | 0.109   | 0.107  | 0.094  | 0.081  | 0.086  | 0.141  |
| NKG-conjugated liposomes | 0.118   | 0.135  | 0.154  | 0.123  | 0.140  | 0.107  |
Figure 3.2 Size distribution curves of liposome diameters (representative plots from n = 3 liposome preparations) across 28 days for RSG-conjugated liposomes (A), NKG-conjugated liposomes (B) and undecorated liposomes (C).
3.2.3 Validation of placental homing peptide binding to human first trimester and term placental explants

Placental-specific homing peptides required the addition of an additional cysteine to facilitate liposome conjugation; the modified sequence required validation of binding to ensure the additional cysteine did not interrupt the targeting capabilities. Rhodamine-labelled, placental-specific homing peptides, CRSGVAKS (CRSG) and CNKGLRNK (CNKG) accumulated in the syncytiotrophoblast of human first trimester (Figure 3.3) and term (Figure 3.4) placental explants. Accumulation was observed at 30 minutes and was still present at 3 h. Limited accumulation of peptide in the villous stroma (VS) was observed across the time course. Untreated explants did not emit any fluorescent signal in the rhodamine channel.

Figure 3.3. First trimester placental explants incubated with rhodamine labelled (red) CRSKVAKS (0.27 µmol), CNKGLRNK (0.27 µmol) or left untreated to determine background fluorescence. Blue, DAPI (nuclei). ST, syncytiotrophoblast; VS, villous stroma. Scale bars, 50 µm. n = 3 (3 images taken per sample, representative images shown).
3.2.4 Binding of homing peptide-conjugated liposomes to human term placental explants

Rhodamine labelled, homing peptide-conjugated liposomes prepared using NBD-labelled lipids (green) were incubated with term placental explants to determine the time course of binding; term placenta was used since clinical therapy is more likely to be administered at the end of pregnancy. Homing peptide-conjugated liposomes were observed in the ST of human term placental explants at 1 h, 3 h and 6 h; limited accumulation was seen in the VS at these time points (Figure 3.5). Accumulation was observed in both the ST and VS at 24 h and 48 h. Control undecorated liposomes were observed in the VS at 1 h and 3 h; a markedly weaker signal were observed at 24 h and 48 h. This may indicate that the lipids were degraded by the placenta and hence why the fluorescent signal was lost.
Figure 3.5. Term placental explants incubated with rhodamine-labelled (red) CRSGVAKS-conjugated, CNKGLRNK-conjugated or control undecorated liposomes with NBD lipid (green). Blue, DAPI (nuclei). ST, syncytiotrophoblast; VS, villous stroma. Scale bars, 50 µm. n = 5 (3 images taken per sample, representative images shown).
3.2.5 Monitoring the release of a fluorescent payload from homing peptide-conjugated liposomes

Rhodamine-labelled, homing peptide-conjugated liposomes, prepared using NBD-labelled lipids and encapsulated DAPI were incubated with human term placental explants to assess the time course of cargo release from liposomes (Figure 3.6).

CRSGVAKS peptide-conjugated liposomes were present in the ST after 1 h; limited DAPI-positive nuclei were observed at this time point. Accumulation was seen in the ST after 3 h and the number of DAPI-positive nuclei was slightly increased. The number of DAPI-positive nuclei was slightly increased in the ST after 6 h. After 24 h, CRSGVAKS peptide-conjugated liposomes were observed in the ST and at a low level in the CTB; DAPI-positive nuclei were present in the ST and CTB. After 48 h, peptide-conjugated liposomes were present at a lower level in the ST, CTB and throughout the stroma; DAPI-positive nuclei were observed throughout the ST, CTB and were increased in the stroma.

CNKGLRNK peptide-conjugated liposomes were present in the ST after 1 h; limited DAPI-positive nuclei were observed at this time point. Accumulation was seen in the ST after 3 h and the number of DAPI-positive nuclei was slightly increased. The number of DAPI-positive nuclei was markedly increased in the ST and underlying CTB after 6 h. After 24 h, peptide-conjugated liposomes were observed in the ST and at a low level in the CTB and stroma; DAPI-positive nuclei were present in the ST, CTB and stroma. After 48 h, peptide-conjugated liposomes were present at a lower level in the ST, CTB and stroma; DAPI-positive nuclei were observed throughout the ST, CTB and were increased in the stroma. The localisation of CNKGLRNK was similar to that of CRSGVAKS but the uptake of DAPI was faster in CNKGLRNK peptide-conjugated liposomes.

Explants treated with control liposomes, lacking a homing peptide, showed a slower time course of uptake, with limited DAPI-positive nuclei being observed until 6 h. DAPI-positive nuclei were restricted to the ST and CTB until 24 h; after 48 h DAPI-positive nuclei were observed in a low amount in the stroma (Figure 3.7).
Explants treated with control liposomes, which lacked a homing peptide, exhibited a significantly reduced number of DAPI-positive nuclei compared to explants treated with NKG-conjugated liposomes at all time points; the number of DAPI-positive nuclei in explants treated with RSG-conjugated liposomes were not significantly different from explants treated with control explants at 1 h and 24 h (Figure 3.8). These data suggest that CNKGLRNK may be a more effective homing peptide for mediating internalisation of a liposome cargo.
Figure 3.6. Term placental explants incubated with DAPI-encapsulated (blue), rhodamine labelled (red) CRSGVAKS-conjugated, CNKGLRNK-conjugated liposomes with NBD lipid (green). ST, syncytiotrophoblast; VS, villous stroma. Scale bars, 50 µm. n = 5 (3 images taken per sample, representative images shown).
Figure 3.7. Term placental explants incubated with DAPI-encapsulated (blue) undecorated liposomes with NBD lipid (green). ST, syncytiotrophoblast; VS, villous stroma. Scale bars, 50 µm. n = 5 (3 images taken per sample, representative images shown).
3.2.6 Tissue distribution of homing peptide-conjugated liposomes in pregnant mice

Rhodamine-labelled, homing peptide-conjugated liposomes with NBD-labelled lipids were intravenously injected into pregnant mice at E18.5 to show distribution at full term (Figure 3.9) and at E15.5 to show distribution at an earlier gestation, representing a clinically relevant gestation where FGR could be detected (Figure 3.10). Cardiac perfusion was performed prior to tissue collection to ensure that any unbound or freely circulating peptide-conjugated liposomes were removed.

Peptide-conjugated liposomes accumulated in the labyrinth and within the maternal spiral arteries of placentas from E18.5 and E15.5 mice after 6 h (Figure 3.9, Figure 3.10; A and B) and were still present after 24 h (Figure 3.9, Figure 3.10; C and G). After 48 h, peptide-conjugated liposomes were not observed within the placentas of E18.5 mice (Figure 3.9; D and H); accumulation was still observed in the labyrinth of placentas from E15.5 mice (Figure 3.10; D and H).

Figure 3.8. Number of DAPI-positive nuclei in term human placental explants from undecorated, RSG-conjugated and NKG-conjugated liposomes. n = 5 (3 images taken and counted per sample, total n = 15 images). Data are shown as mean ± SEM. Statistical analyses, compared to undecorated liposomes at each individual time point, were performed using two-way ANOVA with Dunnett’s post hoc test. * P<0.05, **** P<0.0001.
No accumulation was observed in the junctional zone of placentas at any time or gestation.

Peptide-conjugated liposomes were not observed in the maternal brain, heart, lungs, liver or in the fetus (Figure 3.11, Figure 3.12, Figure 3.13, Figure 3.14); some accumulation in the liver was observed in E15.5 mice after 24 h but this was diminished by 48 h (Figure 3.13, Figure 3.14; P – R). Peptide-conjugated liposomes were observed in the kidney and spleen after 6 h, with accumulation being markedly reduced after 24 h and mostly diminished after 48 h. Control undecorated liposomes were observed transiently in the labyrinth of the placenta after 6 h; no liposomes were observed after 24 h and 48 h (Figure 3.9, Figure 3.10; I – L).
Figure 3.11. Organs from pregnant C57 mice at E18.5 after tail vein injection of rhodamine labelled (red), homing peptide (CRSGVAKS)-conjugated liposomes with NBD lipids (green). Blue, DAPI (nuclei). SA, spiral artery; LB, labyrinth. Scale bars = 50 µm. n = 3 organs from n = 3 mice (representative images shown).
Figure 3.12. Organs from pregnant C57 mice at E18.5 after tail vein injection of rhodamine labelled (red), homing peptide (CNKGLRNK)-conjugated liposomes with NBD lipids (green). Blue, DAPI (nuclei). SA, spiral artery; LB, labyrinth. Scale bars = 50 µm. n = 3 organs from n = 3 mice (representative images shown).
Figure 3.13. Organs from pregnant C57 mice at E15.5 after tail vein injection of rhodamine labelled (red), homing peptide (CRSGVAKS)-conjugated liposomes with NBD lipids (green). Blue, DAPI (nuclei). SA, spiral artery; LB, labyrinth. Scale bars = 50 µm. n = 3 organs from n = 3 mice (representative images shown).
Figure 3.14. Organs from pregnant C57 mice at E15.5 after tail vein injection of rhodamine labelled (red), homing peptide (CNKGLRNK)-conjugated liposomes with NBD lipids (green). Blue, DAPI (nuclei). SA, spiral artery; LB, labyrinth. Scale bars = 50 µm. n = 3 organs from n = 3 mice (representative images shown).
3.2.7 Binding of free placental specific homing peptides with isolated mouse uterine artery

Prior to \textit{in vivo} treatment studies it was necessary to validate peptide binding to mouse uterine arteries. Uterine arteries from pregnant C57 mice (E18.5) were isolated and cultured with free homing peptide. The rhodamine-labelled, placental specific homing peptide, CNKGLRNK (NKG) accumulated in the endothelium of isolated mouse uterine arteries (Figure 3.15). Accumulation was observed by 1 h and was still present at 3 h.

![Figure 3.15. Isolated uterine artery incubated with rhodamine-labelled (red) CNKGLRNK (0.27 µmol). Blue, DAPI (nuclei). LU, lumen; ED, endothelium. Scale bars, 50 µm. \( n = 2 \) (3 images taken per sample, representative images shown).]
3.3 Discussion

This study utilised a novel placental-specific targeting peptide to determine whether conjugation to a biocompatible liposome could facilitate targeted drug delivery to the placenta. The placental-specific homing peptide sequences CNKGLRNK and CRSGVAKS were conjugated to liposomes and the characteristics and stability assessed. Homing peptide-conjugated liposomes were of similar size and zeta potential to undecorated liposomes, showed a narrower particle size distribution and also showed greater stability across 28 days. When incubated with human placental explants, homing peptide-conjugated liposomes bound to the outer syncytiotrophoblast layer of human term placental explants and facilitated the internalisation of a mock drug cargo over 48 hours, a physiologically relevant time-course for uptake. Tissue distribution and the time course of tissue targeting was assessed in vivo by tail vein injection of homing-peptide conjugated liposomes in pregnant mice; peptide-conjugated liposomes selectively bound to the mouse placental labyrinth and spiral arteries, being cleared from placenta within 48 hours. These results collectively demonstrate that peptide-conjugated liposomes bearing a novel placental-specific peptide are suitable for targeted delivery of a drug cargo.

3.3.1 Characterisation and stability of homing peptide-conjugated liposomes

The first aim of this study was to conjugate newly identified placental-specific homing peptides to liposomes and assess the characteristics and stability in phosphate buffered saline at 4°C in order to assess appropriateness for manufacture and use in a clinical setting. Liposome mean diameters and zeta potentials were determined immediately after preparation. Homing peptide (CRSGVAKS and CNKGLRNK)-conjugated liposomes had diameters that were very similar, approximately 140 nm (Figure 3.1). This diameter is within the size range that has previously been reported to be suitable for in vivo use, with nanoparticles above 200 nm being more susceptible to immune detection and clearance, and nanoparticles below 100 nm having potential to cross the placenta (Moghimi et al., 2001, Keelan, 2011).
Homing peptide-conjugated liposomes had greater stability over 28 days, exhibiting less aggregation of particles compared to liposomes lacking a homing peptide (Figure 3.2). Previous studies have shown peptide-conjugated liposomes to be stable for periods of 28 days at 4°C, indicating that liposomes prepared in this study are of similar stability (Mourtas et al., 2011, Nahar et al., 2014). Peptide-conjugation brought the zeta potential closer to neutral; the greater the charge of the particle the more likely they are to repel each other and therefore the less likely they are to aggregate. This enhanced stability as the result of peptide-conjugation despite a neutral zeta potential is previously unreported, but it can be postulated that addition of a peptide to the surface of the liposomes provides steric hindrance, meaning particles are not able to aggregate. This finding is clinically relevant, as enhanced stability of any liposome preparation would be a desirable characteristic.

### 3.3.2 Binding and uptake of homing peptide-conjugated liposomes in human placenta

In order to facilitate conjugation of placental-specific homing peptides to the surface of liposomes, 10% of PEG was replaced with PEG-maleimide; this allowed for conjugation of homing peptides to the outside of the liposome by a well characterised thiol-maleimide Michael addition reaction, as has been previously reported (Chen et al., 2011b). Placental-specific homing peptides were initially identified from a phage display with peptide sequences RSGVAKS and NKGLRKN; in order to enable conjugation with maleimide groups on the surface of liposomes, a free cysteine was required. Homing peptides were therefore synthesized with the sequences CRSGVAKS and CNKGLRKN. This modified sequence was validated in human first trimester and term placental explants (Figure 3.3, Figure 3.4); addition of a cysteine did not interfere with targeting capabilities of either peptide sequence, with accumulation being observed in the outer syncytiotrophoblast layer of placenta.

Placental uptake of homing peptide-conjugated liposomes was then investigated; initial binding to the syncytiotrophoblast of term human placenta was observed, with internalisation into villous stroma noted by 48 hours (Figure 3.5). Delivery of a mock drug cargo in human placental explants, investigated
using a fluorescent payload, demonstrated that explants treated with CNKGLRNK-conjugated liposomes showed greater uptake of liposomal cargo at all time points compared with CRSGVAKS-conjugated and liposomes displaying no homing peptide. These results suggested that the CNKGLRNK homing peptide sequence was more effective at mediating liposome uptake and internalisation of a liposome cargo.

Previous studies have shown that uptake of peptide-conjugated liposomes is mediated by clathrin-dependant endocytosis (Bareford and Swaan, 2007); targeted liposomes interact with receptors on the cell membrane, initiating clathrin-dependant internalisation of targeted liposomes and their cargo (Kou et al., 2013). The mechanism of uptake of peptide-conjugated liposomes in placenta is previously unreported. A previous study has examined placental uptake mechanism of liposome-encapsulated doxorubicin, demonstrating that endocytosis by the syncytiotrophoblast was responsible for liposome uptake (Soininen et al., 2015); these liposomes were lacking a targeting peptide so it is possible that the placenta may process peptide-conjugated liposomes by a different mechanism. It has been reported that ligand-modified liposomes have increased cellular uptake compared to those lacking a targeting ligand (Gao et al., 2013). A study by Chen et al demonstrated that conjugation of a cyclic RGD-bearing tumour homing peptide to liposome surface enhanced cellular uptake compared to non-targeted liposomes (Chen et al., 2012); results from this study supported previous findings, with peptide-conjugated liposomes showing increased and faster cellular uptake than those lacking a homing peptide. The iRGD peptide is known to bind to the alpha V integrin and has been shown to be taken up into cells by a C-end Rule (CendR) mechanism, an endosomal pathway (Teesalu et al., 2013). It has also been reported that liposomes conjugated to the cell penetrating peptide TAT are taken up into cells by an endocytosis mediated mechanism (Fretz et al., 2004). It is not known what receptors the homing peptides CRSGVAKS and CNKGLRNK bind to, but it may be speculated that the receptor target for CNKGLRNK is more highly expressed on human placental explants, thereby leading to a faster time course of binding and internalisation by endocytosis or a faster mechanism.
3.3.3 Tissue distribution and clearance of homing peptide-conjugated liposomes in pregnant mice

It is well documented that liposomes are rapidly cleared after intravenous injection by the mononuclear phagocyte system (MPS) and previous studies have concentrated on minimising immune detection and clearance by the MPS (Ishida et al., 2002a). One strategy that has proven successful is the incorporation of polyethylene glycol (PEG) into liposomes, with PEGylation becoming a commonly used strategy (Hong et al., 1999). PEGylation provides a ‘stealth’ coating by suppressing uptake of liposomes by the MPS in order to evade immune detection, preventing rapid clearance and thereby extending blood circulation of liposomes in vivo (Immordino et al., 2006). For the purposes of this study, PEG was incorporated into liposomes to ensure an adequate circulation time. Previous studies have also suggested that incorporation of cholesterol into liposomes improves stability by reducing their permeability and therefore breakdown both in vitro and in vivo (Bozzuto and Molinari, 2015), importantly a balance between stability and enabling drug release when taken up by cells and tissue must be reached. For the purposes of this study, cholesterol was incorporated into liposomes at a molar ratio of 70-30%, consisting of 70% DSPC and 30% cholesterol, a ratio that has previously been shown to be optimal to provide a stable liposome formulation for in vivo targeting (Briuglia et al., 2015).

This study found that after intravenous injection, peptide-conjugated liposomes accumulated in the labyrinth and within the maternal spiral arteries of placentas at E18.5 of gestation and after 6 h, with liposomes still being present after 24 h. After 48 h, peptide-conjugated liposomes were not observed within the placentas of E18.5 mice, but liposomes did persist in the labyrinth of placentas from E15.5 mice at this time point. It has previously been reported that uteroplacental perfusion increases across gestation (Osol and Mandala, 2009); this increased blood flow through the placenta may account for the accelerated time course of liposome clearance from the placenta at a later gestation. Liposomes with no conjugated homing peptide did not show significant accumulation in the placenta at any time point, demonstrating that homing peptides are responsible for the selective accumulation in the placenta.
Liposomes with conjugated CNKGLRNK peptide appeared to preferentially target the maternally facing uterine spiral arteries that are responsible for delivering blood to the placenta; this result is of physiological importance as CNKGLRNK-conjugated liposomes may provide enhanced delivery of compounds such as vasodilators to these maternal arteries.

Maternal organs and fetuses were visualised to determine whether there was any off-target accumulation of peptide-conjugated liposomes. Peptide-conjugated liposomes were not observed in the maternal brain, heart, lungs, liver or in the fetus, confirming selectivity of targeting and suggesting that the receptors for both peptides must be highly expressed in placenta, but not expressed, or expressed at a level low enough as to mediate significant uptake, in other maternal organs. A number of previous studies have shown that nanoparticles are able to cross the placenta, being found in organs of the fetus; polystyrene beads of diameters from 20 to 500 nanometers have been shown to be taken up by placenta, with diameters of 20 and 40 nanometers inducing trophoblast apoptosis (Huang et al., 2015). Iron oxide nanoparticles have also been shown to freely cross the placenta, accumulating in the fetal liver and causing fetal toxicity (Di Bona et al., 2014). The lack of detectable peptide-conjugated liposomes in the fetus indicated that placental transfer of liposomes did not occur, an important factor for clinical translation of this drug delivery system.

Peptide-conjugated liposomes were observed in the maternal spleen, liver and kidneys 6 hours after intravenous injection; accumulation was markedly reduced by 24 and 48 hours. As has been discussed previously, the MPS is responsible for the processing and clearance of liposomes from the circulation. The spleen is the largest unit of the MPS, containing a large number of macrophages that have been demonstrated to be involved in the clearance of liposomes (Takahashi et al., 2011) and the kidneys have been shown to be responsible for the renal clearance of liposomes (Krishna et al., 1999), explaining why transient accumulation may be seen in these organs.
CNKGLRNK-conjugated liposomes demonstrated lesser accumulation in maternal spleen and kidneys than the CRSGVAKS-decorated liposomes, indicating that it may be a more favourable peptide to facilitate targeted drug delivery to the placenta. This observation was also confirmed by the lack of accumulation of CNKGLRNK-conjugated liposomes in the maternal liver; CRSGVAKS-conjugated liposomes showed accumulation in the maternal liver after 24 hours in E15.5 mice. Limitations in the comparison of accumulation of peptide-conjugated liposomes must be noted, as all assessments of fluorescent images were qualitative; however it is promising that CNKGLRNK appeared to exhibit a more clinically relevant profile of uptake and clearance, both in placenta and maternal organs.

Free CNKGLRNK homing peptide demonstrated more favourable tissue targeting for the delivery of vasodilators when injected in vivo, showing preferential targeting to the uterine spiral arteries. CNKGLRNK proved to be superior in limiting accumulation in maternal clearance organs. It can be postulated that the receptor target for this homing peptide is more selectively expressed than that of CRSGVAKS, meaning that it more efficiently targets the placenta. It is also possible that CNKGLRNK acts as an additional ‘stealth’ layer on the outside of the liposome that the CRSGVAKS peptide does not, preventing recognition of the liposome by the maternal immune system and therefore fewer CNKGLRNK-conjugated liposomes are identified and taken up by the maternal clearance organs.

Since CNKGLRNK peptide was found to effectively target maternal spiral arteries in vivo, uterine arteries from the main loop of the uterine horn of a pregnant mouse were incubated with fluorophone-labelled free peptide to determine whether binding could be observed. It was found that CNKGLRNK homing peptide bound to the endothelium of mouse uterine arteries, further lending support for the use of CNKGLRNK for the potential delivery of vasodilators in vivo.

3.4 Summary
The collective data from this chapter provide evidence to support the hypothesis that novel placental-specific homing peptide may be used to facilitate liposomal
targeting and drug delivery to the placenta. Homing peptides were conjugated to the outside of liposomes, with the resultant liposome formulation showing suitable size, zeta potential and enhanced 28 day stability compared to non-conjugated liposomes. These homing peptide-conjugated liposomes were validated \textit{in vitro} using a human placental explant model, with the data indicating that peptide-conjugated liposomes target the outer syncytiotrophoblast layer of placenta and show internalisation and release of a mock drug cargo over a 48 hour time course. When tested \textit{in vivo}, homing peptide-conjugated liposomes exhibited targeting to the vessels that supply blood to the placenta, along with the exchange region of mouse placenta. CNKGLRNK peptide-conjugated liposomes exhibited greater specificity, showing less maternal organ accumulation. This peptide also showed a strong affinity toward maternal uterine blood vessels, a finding further validated by the \textit{ex vivo} culture of uterine artery vessels with CNKGLRNK. These data demonstrate that CNKGLRNK homing peptide-conjugated liposomes may provide a suitable delivery system for the delivery of vasodilators to the uteroplacental vasculature.
Chapter 4
4.1 Introduction

As has been described in the previous chapter, peptide-conjugated liposomes have been validated as a strategy to allow the selective delivery of a drug cargo to the uteroplacental unit. The homing peptide CNKGLRNK demonstrated more favourable characteristics than CRSGVAKS, exhibiting reduced maternal organ localisation and enhanced binding to the mouse uterine vasculature. Previous strategies by our group to target therapeutics to the placenta have used iRGD-conjugated liposomes with encapsulated insulin-like growth factor 2 (IGF2) in an attempt to rescue fetal growth (King et al., 2016). Since CNKGLRNK showed more selective binding to the mouse uterine arteries, it would be logical to develop a therapeutic strategy to exploit this selective targeting.

It is well documented that the maternal uterine arteries undergo extensive remodeling in the first trimester of gestation in order to facilitate a high flow, low resistance blood flow through the placenta (Osol and Mandala, 2009). When this remodeling is incomplete or impaired, the maternal spiral arteries do not permit the adequate perfusion of the placenta, and as such, impaired spiral artery remodeling and impaired uteroplacental blood flow has been identified as causative in some cases of FGR (Kaufmann et al., 2003, Krishna and Bhalerao, 2011). These cases are accompanied by abnormal umbilical artery Doppler velocity waveforms, with one study indicating a 50% reduction in uterine artery blood flow compared with non-FGR pregnancies (Ferrazzi et al., 2011).

The homing peptide CNKGLRNK is able to selectively target the maternal uterine vasculature that is responsible for ensuring adequate placental perfusion. Since impaired uteroplacental perfusion is observed in some cases of FGR, it stands to reason that reducing the resistance of these vessels by inducing vasodilation may improve uteroplacental perfusion and improve fetal growth. The use of vasodilators to treat FGR in both animal models and humans is well documented, but has had limited success due to a number of factors. One such study assessed the use of transdermal glyceryl trinitrate (GTN) in a rat model FGR, induced by lipopolysaccharide (LPS) injection; the results demonstrated that GTN was able to improve uteroplacental perfusion and pregnancy outcome by preventing LPS-induced FGR (Cotechini et al., 2014). Another study assessed the effects of transdermal GTN on Doppler velocity waveforms in women with
severe placental insufficiency (Trapani et al., 2011); whilst this study was able to show that GTN could improve uterine artery blood flow, it is also well documented that continued use of GTN is associated with the development of tolerance, and increased incidence of severe headaches, making it unsuitable for use in pregnancy (Bagdy et al., 2010, Munzel et al., 2005). Sildenafil citrate (SC) has also been explored as a possible vasodilator to improve uteroplacental perfusion in pregnancy. SC has been shown to improve both fetal and placental weight and normalized umbilical artery blood flow in a mouse model of FGR (Dilworth et al., 2013), and more recently in an ovine model of FGR (Oyston et al., 2016). It is known that SC is able to freely cross the placenta; a study to examine the long term adulthood health of fetuses from SC-treated mice demonstrated evidence of negative developmental programming, with offspring exhibiting altered blood vessel function, as well as altered metabolic and cardiovascular functions (Renshall et al., 2014b).

Considering this evidence, it is important to select a vasodilator for encapsulation into targeted liposomes that is locally acting, so as to limit off target effects and potential placental transfer. Nitric oxide (NO) donor molecules are compounds that are able to release NO in order to induce vasodilation. NO is not able to diffuse over large distances since it is a highly reactive free radical with a short half-life, and rapidly induces vasodilation through the production of cGMP which in turn activates myosin phosphatase, causing myosin dephosphorylation which leads to vasorelaxation (Zhao et al., 2015); for these reasons NO donor molecules represent attractive compounds for the targeted delivery to the uteroplacental vasculature.

A class of NO donor molecule that exhibits suitable characteristics for in vivo use are the NONOates. NONOates are nitric oxide donor molecules that are able to self-liberate NO, requiring no enzymatic conversion (Miller and Megson, 2007). In order to assess the stability and half-life of different NONOates, a literature review was undertaken, the results of which are summarized in Table 4.1. This literature search demonstrated that the molecule SE175 was the most suitable NO donor, being stable in PBS and having the ability to self-liberate NO.
SE175, shown in Figure 4.1., is a nitroxyacylated thiosalicylate that has previously been shown to induce vasodilation of isolated rat aorta, (Endres et al., 1999). SE175 is composed of a NO-donating nitrate group (Figure 4.1, C) attached to a NO-liberating thiosalicylate (Figure 4.1, D); this class of compound was created in an effort to achieve spontaneous release of NO from nitrate groups, based on the evidence that a free thiol (SH) is able to liberate nitric oxide from nitrate groups (Lundberg et al., 2008). Esterase breakdown of SE175 allows the thiosalicylate group to self-liberate NO from the nitrate group, thus SE175 should not cause the same tolerance as observed following repeated administration of traditional nitrates.

SE175 has not previously been explored for use as a vasodilator in the uteroplacental vasculature. The aims of this study were 1) to determine whether SE175 is able to induce vasodilation of the human and mouse uterine vasculature, 2) to encapsulate SE175 in peptide-conjugated liposomes, validating the characteristics and stability of this formulation and 3) to assess the effects of SE175 on human trophoblast cell turnover.

These aims will address the following questions; a) is SE175 able to induce vasodilation of human and mouse uteroplacental vessels? If so, b) can SE175 be encapsulated in homing-peptide conjugated liposomes whilst retaining suitable size and stability profiles and c) does SE175 impair human placental function by altering basal levels of apoptosis or proliferation?
<table>
<thead>
<tr>
<th>Compound</th>
<th>Half-life pH 7.4, 37°C</th>
<th>Half-life pH 7.4, 22-25°C</th>
<th>Stable Stock Solution</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEA NONOate</td>
<td>2 mins</td>
<td>16 mins</td>
<td>10 mM NaOH, pH 12</td>
<td>-</td>
</tr>
<tr>
<td>DETA NONOate</td>
<td>20 hrs</td>
<td>56 hrs</td>
<td>10 mM NaOH, pH 12</td>
<td>-</td>
</tr>
<tr>
<td>DPTA NONOate</td>
<td>3 hrs</td>
<td>5 hrs</td>
<td>10 mM NaOH, pH 12</td>
<td>-</td>
</tr>
<tr>
<td>β-gal NONOate</td>
<td>6 mins (pH 5)</td>
<td>-</td>
<td>10 mM NaOH, pH 12</td>
<td>Activation by beta-galactosidase to release NO</td>
</tr>
<tr>
<td>MAHMA NONOate</td>
<td>1 min</td>
<td>3 mins</td>
<td>10 mM NaOH, pH 12</td>
<td>-</td>
</tr>
<tr>
<td>PAPA NONOate</td>
<td>15 mins</td>
<td>77 mins</td>
<td>10 mM NaOH, pH 12</td>
<td>-</td>
</tr>
<tr>
<td>SPERMINE NONOate</td>
<td>39 mins</td>
<td>230 mins</td>
<td>10 mM NaOH, pH 12</td>
<td>-</td>
</tr>
<tr>
<td>SE175</td>
<td>Stable</td>
<td>Stable</td>
<td>1:9 in DMSO:PBS, 100µg/ml</td>
<td>Able to self-liberate NO from nitrate</td>
</tr>
</tbody>
</table>

Table 4.1. Summary of potential nitric oxide donor compounds for encapsulation in homing peptide-conjugated liposomes
Figure 4.1. Structure and breakdown mechanism of SE175. [A] SE175. Esterase breakdown of [B] leads to the production of [C] Linker molecule with a nitric-oxide donor moiety and [D] Thiosalicylate, from which the free thiol (SH) is able to liberate nitric-oxide from the nitrate group of [C]. Molecules drawn using eMolecules® Building Blocks.
4.2 Results

4.2.1 Validation of SE175 activity in isolated mouse uterine arteries from C57BL/6 mice with free SE175

SE175 has previously been shown to induce vasodilation of isolated rat aorta (Endres et al., 1999) but there are no data to demonstrate the action of SE175 on isolated mouse uterine arteries. Prior to in vivo treatment studies, it was necessary to validate the vasodilatory capabilities of SE175 in mouse uterine arteries. Isolated mouse uterine arteries were mounted onto a wire myograph, pre-constricted with phenylephrine and the relaxation to increasing doses of SE175 recorded.

SE175 dose-response curves were constructed by expressing relaxation as a percentage of maximal constriction to phenylephrine (Figure 4.2; A). SE175 was prepared in a solution containing DMSO; it has previously been reported that DMSO induces vasodilation in isolated rat aorta (Kaneda et al., 2016). To ensure that any relaxation observed was as a result of SE175 and not DMSO, dose-response curves for equivalent DMSO concentrations were also constructed (Figure 4.2; B). Untreated mouse uterine arteries were used as a time control to assess the longevity of phenylephrine constriction, to ensure that any relaxation observed was not due to the arteries naturally relaxing over time (Figure 4.2; C).

A dose-dependent relaxation with free SE175 solution was observed in pre-constricted mouse uterine arteries. Vessels relaxed to 21% of the pre-constricted maximum at $10^{-5} \text{ M}$. This was a significantly greater relaxation response than to that observed with DMSO and time controls at concentrations of $10^{-7} \text{ M}$, $10^{-6} \text{ M}$ and $10^{-5} \text{ M}$ (Figure 4.2; D). Uterine arteries treated with DMSO remained constricted, as did arteries that were left untreated, indicating that relaxation was induced by SE175 and was not as a result of DMSO or due to a natural relaxation over time.
Figure 4.2. Dose response curves from C57 mouse uterine arteries. [A] Dose response to SE175; [B] Dose response to equivalent DMSO; [C] Untreated time control; [D] Comparison of responses. Key: SE175 (black circle, n=6), DMSO (blue square, n=6), time control (red triangle, n=5). Data shown are mean ± SEM; error bars not visible if smaller than symbol. SE175 dose response was compared to DMSO (blue *) and time (red *) controls using two-way ANOVA with Bonferroni’s post hoc test. **P<0.01, ****P<0.0001.
4.2.2 Validation of SE175 activity in term human chorionic plate arteries

It was necessary to confirm the vasodilatory activity of SE175 in term human chorionic plate arteries, to ensure that effects were consistent between species and that the treatment could be translated into humans. Ideally, human myometrial arteries would have been used for these experiments due to their maternal origin; however, due to time constraints placental arteries were used for this bioassay. Human term placentas were collected, the chorionic plate arteries dissected and mounted onto a wire myograph. Vessels were pre-constricted with phenylephrine and vessel relaxation to increasing doses of SE175 recorded. DMSO and time controls were used as previously described in 4.2.1.

A dose dependent relaxation with free SE175 solution was observed when added to pre-constricted human chorionic plate arteries (Figure 4.3; A). Vessels relaxed to 23% of the pre-constricted maximum at $10^{-5}$ M. This relaxation was significantly greater than that observed in the DMSO and time controls at concentrations of $10^{-9}$ M, $10^{-7}$ M, $10^{-6}$ M and $10^{-5}$ M (Figure 4.3; D). Chorionic plate arteries treated with DMSO remained constricted (Figure 4.3; B), as did untreated arteries (Figure 4.3; C), indicating that relaxation was induced by SE175, and was not as a result of DMSO or due to a natural relaxation over time.
Figure 4.3. Dose response curves of human chorionic plate arteries. [A] Dose response to SE175; [B] Dose response to DMSO; [C] Untreated time control; [D] Comparison of responses. Key: SE175 (black circle, n=6), DMSO (blue square, n=6), time control (red triangle, n=6). Data shown are mean ± SEM; error bars not visible if smaller than symbol. SE175 dose response was compared to DMSO (blue *) and time (red *) using two-way ANOVA with Bonferroni’s post hoc test. **P<0.01, ****P<0.0001.
4.2.3 Validation of SE175 activity when delivered to isolated mouse uterine arteries from C57BL/6 mice using CNKGLRNK peptide-conjugated liposomes

Prior to undertaking in vivo treatment studies with SE175 encapsulated in peptide-conjugated liposomes, it was necessary to ensure that SE175 was able to induce vasodilation in vitro over a suitable time frame. Mouse uterine arteries were pre-constricted with phenylephrine, SE175-encapsulated CNKGLRNK peptide-conjugated liposomes introduced to the myography bath in a single bolus and vessel relaxation recorded at 10 minute intervals for 1 hour. DMSO and time controls were used as previously described in 4.2.1. SE175 concentration was assumed to be at a maximum 320 µM or lower.

A time-dependent relaxation was observed when SE175-encapsulated CNKGLRNK peptide-conjugated liposomes were added to pre-constricted mouse uterine arteries (Figure 4.4; A). As observed in 4.2.1, free SE175 caused vessels to relax to 21% of the pre-constricted maximum after 8 minutes; after 10 minutes SE175-encapsulated liposomes had relaxed vessels to 72% of the pre-constricted maximum, indicating that free SE175 acted faster than liposome encapsulated SE175. A gradual relaxation was observed over 1 hour, with relaxation reaching 18% of the pre-constricted maximum.

Relaxation of vessels by SE175 was significantly greater than the DMSO and time controls from 10 minutes onwards (Figure 4.4; D). Mouse uterine arteries treated with DMSO remained constricted (Figure 4.4; B), as did arteries that were left untreated (Figure 4.4; C), indicating that relaxation was induced by SE175 and was not as a result of DMSO or due to a natural relaxation over time.
Figure 4.4. Time response curves of C57 mouse uterine arteries. [A] Time response to SE175-encapsulated CNKGLRNK peptide-conjugated liposomes; [B] Time response to DMSO; [C] Untreated time control; [D] Comparison of responses. Key: SE175-encapsulated (black circle, n=6), DMSO (blue square, n=6), time control (red triangle, n=6). Data shown are mean ± SEM; error bars not visible if smaller than symbol. SE175 time response was compared to DMSO (blue *) and time (red *) controls using two-way ANOVA with Bonferroni's post hoc test. **P<0.01, ***P<0.001, ****P<0.0001.
4.2.4 Validation of SE175 activity when delivered to term human chorionic plate arteries using CNKGLRNK peptide-conjugated liposomes

Term human chorionic plate arteries were pre-constricted with phenylephrine, SE175-encapsulated CNKGLRNK peptide-conjugated liposomes were introduced to the myography bath in a single bolus and vessel relaxation recorded at 10 minute intervals for 1 hour. DMSO and time controls were used as described in 4.2.1.

A time-dependent relaxation was observed when SE175-encapsulated CNKGLRNK peptide-conjugated liposomes were added to pre-constricted human chorionic plate arteries (Figure 4.5; A). As observed in 4.2.2, addition of free SE175 caused vessels to relax to 23% of the pre-constricted maximum after 8 minutes; after 10 minutes SE175-encapsulated liposomes had relaxed vessels to 84% of the pre-constricted maximum, indicating that as observed in mouse uterine arteries, free SE175 was able to act faster than liposome encapsulated SE175. A gradual relaxation was observed over 1 hour, with relaxation reaching 18% of the pre-constricted maximum.

Relaxation of vessels to SE175 was significantly greater than the DMSO and time controls from 10 minutes onwards (Figure 4.5; D). Mouse uterine arteries treated with DMSO remained constricted (Figure 4.5; B), as did arteries that were left untreated (Figure 4.5; C), indicating that relaxation by SE175 was not as a result of DMSO or due to a natural relaxation over time.
Figure 4.5. Time response curves of human chorionic plate arteries. [A] Time response to SE175-encapsulated CNKGLRNK peptide-conjugated liposomes; [B] Time response to DMSO; [C] Untreated time control; [D] Comparison of responses. Key: SE175-encapsulated (black circle, n=6), DMSO (blue square, n=6), time control (red triangle, n=6). Data shown are mean ± SEM; error bars not visible if smaller than symbol. SE175 time response was compared to DMSO (blue *) and time (red *) controls using two-way ANOVA with Bonferroni’s post hoc test. **P<0.01, ****P<0.0001.
4.2.5 Validation of SE175 activity in isolated mouse uterine arteries from eNOS<sup>−/−</sup> mice

The endothelial nitric oxide synthase knock out mouse (eNOS<sup>−/−</sup>), which will be discussed in more detail in the next chapter, was selected as an appropriate mouse model of FGR for <i>in vivo</i> treatment studies. Prior to these studies, it was necessary to determine whether SE175 could induce vasodilation of uterine arteries from the eNOS<sup>−/−</sup> mouse. eNOS<sup>−/−</sup> uterine arteries were pre-constricted with phenylephrine and relaxation to increasing doses of free SE175 determined.

A dose-dependent relaxation to free SE175 was previously observed in C57 mouse uterine arteries (Figure 4.2). In pre-constricted eNOS<sup>−/−</sup> uterine arteries, no relaxation was observed with increasing doses of SE175; a slight relaxation was observed at 10<sup>−5</sup> M (Figure 4.6). In C57 uterine arteries, relaxation at 10<sup>−5</sup> M was 21% of the pre-constricted maximum; in uterine arteries from eNOS<sup>−/−</sup> mice relaxation at 10<sup>−5</sup> M was 83%.

eNOS<sup>−/−</sup> uterine arteries were left for 60 minutes after the 10<sup>−5</sup> M addition of SE175 and relaxation measured every 10 minutes. A time-dependent vasodilatation was observed in eNOS<sup>−/−</sup> uterine arteries, with relaxation reaching 32% of the pre-constricted maximum after 1 hour (Figure 4.7; A). Uterine arteries treated with DMSO remained constricted (Figure 4.7; B), as did arteries that were left untreated (Figure 4.7; C), indicating that relaxation was induced by SE175 and was not as a result of DMSO or due to a natural relaxation over time. Relaxation of vessels to SE175 was significantly greater than the DMSO and time controls from 10 minutes onwards (Figure 4.7; D).
Figure 4.6. Dose response curves of eNOS-/- mouse uterine arteries. [A] Dose response to free SE175; [B] Dose response to DMSO; [C] Untreated time control; [D] Comparison of responses. Key: free SE175 (black circle, n=4), DMSO (blue square, n=4), time control (red triangle, n=4). Data shown are mean ± SEM; error bars not visible if smaller than symbol.
Figure 4.7. Time response curves of eNOS−/− mouse uterine arteries. [A] Time response to free SE175; [B] Time response to DMSO; [C] Untreated time control; [D] Comparison of responses. Key: Free SE175 (black circle, n=4), DMSO (blue square, n=4), time control (red triangle, n=4). Data shown are mean ± SEM; error bars not visible if smaller than symbol. SE175 time response was compared to DMSO (blue *) and time (red *) controls using two-way ANOVA with Bonferroni’s post hoc test. ***P<0.001, ****P<0.0001.
4.2.6 Characterisation of the size, zeta potential and polydispersity index of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes

SE175-encapsulated CNKGLRNK peptide-conjugated liposomes, prepared by lipid film hydration, were characterised using the Zetasizer Nano ZS to determine size distribution and zeta potential; these results were compared to PBS-encapsulated, CNKGLRNK peptide-conjugated liposomes in order to determine whether encapsulation of SE175 altered liposome characteristics.

SE175-encapsulated CNKGLRNK peptide-conjugated liposomes had a mean diameter of 164.2 ± 1.1 nm, a polydispersity index of 0.049 and a zeta potential of -0.9 ± 0.57 mV. PBS-encapsulated CNKGLRNK peptide-conjugated liposomes had a mean diameter of 159.8 ± 0.8 nm, a polydispersity index of 0.065 and a zeta potential of -0.6 ± 0.36 mV; these results indicate that encapsulation of SE175 did not alter the mean particle size, particle size heterogeneity or zeta potential of liposomes (Figure 4.8, Table 4.2).

The stability of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes was determined over 28 days with liposomes remaining stable, showing no signs of aggregation or degradation (Figure 4.9).

![Figure 4.8. Size distribution of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (red) and PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (blue). Representative plot from n = 3 liposome preparations.](image-url)
Table 4.2. Mean diameter and zeta potential measurements of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes and PBS-encapsulated CNKGLRNK peptide-conjugated liposomes, where means are calculated from triplicate measurements of three separate sample preparations ± standard error.

<table>
<thead>
<tr>
<th></th>
<th>Mean diameter / nm</th>
<th>Polydispersity index (PDI)</th>
<th>Mean zeta potential / mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>SE175-encapsulated</td>
<td>164.2 ± 1.1</td>
<td>0.049</td>
<td>-0.9 ± 0.57</td>
</tr>
<tr>
<td>liposome</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS-encapsulated</td>
<td>159.8 ± 0.8</td>
<td>0.065</td>
<td>-0.6 ± 0.36</td>
</tr>
<tr>
<td>liposome</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 4.9. Size distribution curves of liposome diameters (representative plots from n = 3 liposome preparations) across 28 days for SE175-encapsulated CNKGLRNK peptide-conjugated liposomes.
4.2.7 Assessment of the effects of SE175 on human trophoblast cell turnover

Prior to embarking on in vivo treatment studies it was necessary to ensure that SE175 did not cause any adverse effects on placental cell growth or function. This information is also important for determining the suitability of SE175 for use in human clinical trials. In order to examine this, human term placental explants were cultured with SE175 for 24, 48 or 72 hours; explants were then immunostained for Ki67, a marker of proliferation, and M30, a marker of apoptosis. Untreated explants were used as a control to compare staining.

Qualitative assessment of immunostaining showed that exposure of human placental explants with SE175 did not alter the proportion of Ki67-positive cells at 24, 48 or 72 hours (Figure 4.10). This indicated that SE175 did not negatively affect the basal rate of proliferation in term placental explants. There was also no observable difference in the proportion of M30 positive cells 24, 48 or 72 hours, indicating that SE175 did not induce apoptosis in human placental explants (Figure 4.11). Ki67 immunopositive cells were sometimes observed in the placental arteries after SE175 treatment, as seen in Figure 4.10 A.
Figure 4.10. Ki67 immunostaining of SE175 cultured (320 μM) or untreated term human placental explants. [A - C] SE175 cultured; [D – E] Control untreated. Scale bars, 50 μm. n = 3 (3 images taken per sample, representative images shown).
Figure 4.11. M30 immunostaining of SE175 cultured (320 µM) or untreated term human placental explants. [A - C] SE175 cultured; [D – E] Control untreated. Scale bars, 50 µm. n = 3 (3 images taken per sample, representative images shown).
4.3 Discussion

This study assessed the suitability of a novel vasodilator compound, the nitroxyacylated thiosalicylate SE175, for use as a vasodilator of uteroplacental vessels. SE175 was added to pre-constricted mouse uterine arteries and human chorionic plate arteries and a dose-dependent vasodilatation was observed in both C57BL6/J mouse uterine arteries and human chorionic plate arteries. In uterine arteries obtained from pregnant eNOS\(^{-/-}\) mice, SE175 took longer to induce vasorelaxation, and acted in a time-dependent rather than a dose-dependent manner; although not previously reported in eNOS\(^{-/-}\) mice, since SE175 requires transformation by an esterase it may be that eNOS\(^{-/-}\) have impaired esterase function. When encapsulated in CNKGLRNK homing peptide-conjugated liposomes, SE175 did not alter liposome characteristics or stability. When incubated with term human placental villous explants, SE175 did not alter basal rates of apoptosis or proliferation. These results collectively identify SE175 as a suitable vasodilator for encapsulation in targeted liposomes for the induction of vasodilation of the uteroplacental vasculature.

4.3.1 Confirmation of the bioactivity of free and liposomal SE175 in human chorionic plate arteries

The first aim of this study was to determine whether free and liposome-encapsulated SE175 were able to induce vasodilation of pre-constricted human chorionic plate arteries. Previous studies have assessed the differential relaxation of placental chorionic plate arteries and maternal myometrial arteries; these vessels show a similar responsiveness, with the main observable difference being that chorionic plate arteries are less sensitive to endothelial-dependent vasodilators (Wareing et al., 2005b, Sweeney et al., 2008, Dordea et al., 2013).

The nitric oxide donor SE175 was able to induce vasorelaxation of pre-constricted human chorionic plate arteries. When free SE175 was used, dose-dependent relaxation was observed. This finding is the same as has been observed with the endothelium-independent nitric oxide donor sodium nitroprusside (SNP), which also induced a dose-dependent relaxation of pre-constricted chorionic plate arteries (Hayward et al., 2013).
Liposome-encapsulated SE175 was also able to induce vasorelaxation, but over a slower time-course than free SE175; free SE175 induced relaxation to 23% of the pre-constricted maximum after 10 minutes, whereas liposome-encapsulated SE175 took 1 hour to induce relaxation to 18% of the pre-constricted maximum. These data confirm that SE175 remains functional after encapsulation and is being released from the liposomes, since it is able to exhibit a bioactive effect. The exact concentration of SE175 between free and encapsulated may be altered due to encapsulation efficiency. These results are similar to those observed in previous studies; Jain et al. showed that the liposome encapsulated vasodilator iloprost was slower acting than when added in free form to mouse pulmonary arteries, but was also able to enhance vasorelaxation of these vessels compared to those treated with free drug (Jain et al., 2014). Whilst we didn’t observe a statistically significant enhancement of relaxation with liposomal encapsulation over the time frame of our in vitro experiments, we may observe a similar effect in vivo. The slower action of liposome-encapsulated SE175 may prove clinically beneficial, since a sustained relaxation would be preferred to a rapid but transient response. It has also been reported that pulmonary-specific peptide targeted liposomes containing fasudil were able to induce site-specific pulmonary vasodilation with no systemic effects on arterial pressure (Nahar et al., 2014); this study supports the use of site-specific targeting of vasodilators for localised action and limited off-target side-effects.

**4.3.2 Confirmation of the bioactivity of free and liposomal SE175 activity in C57BL6/J and eNOS−/− mouse uterine arteries**

After confirming SE175 bioactivity in human vessels, the efficacy of SE175 was also examined in mouse uterine arteries, both in wild-type C57BL6/J mice and eNOS−/− mice.

The nitric oxide donor SE175 was able to induce vasorelaxation of pre-constricted C57BL6/J uterine arteries. When free SE175 was used, a dose-response relaxation was observed; dose-dependent relaxation of wild-type mouse uterine arteries with the nitric oxide donor SNP has previously been described (Kusinski et al., 2009), indicating that SE175 is able to induce
vasodilation by a similar method. Liposome-encapsulated SE175 acted in the same manner on mouse uterine arteries as was previously described in human chorionic plate arteries, causing a time-dependent relaxation that was slower acting than free SE175. These data again demonstrate that SE175 was released from liposomes, as indicated by the biological response observed over a 1 hour time period. The exact concentration of SE175 between free and encapsulated may be altered due to encapsulation efficiency.

When examining the activity of free SE175 in eNOS\(^{-/-}\) uterine arteries, no dose-dependent relationship was observed; no relaxation occurred until the final concentration of \(10^{-5}\) M SE175 was added to the bath, after which a non-significant vasorelaxation to 83% of the pre-constricted maximum was observed. This result was in stark contrast to the relationship observed in C57 vessels, in which relaxation to 21% of the pre-constricted maximum was observed after addition of \(10^{-5}\) M SE175. However the SE175-treated eNOS\(^{-/-}\) uterine arteries appeared to relax over time, so a time-response experiment was undertaken; these data demonstrated that SE175 induced vasorelaxation in eNOS\(^{-/-}\) uterine arteries in a time-dependent manner, with relaxation reaching 32% of the pre-constricted maximum after 1 hour. Time-dependent relaxation of eNOS\(^{-/-}\) uterine arteries is previously unreported. It has previously been described that eNOS\(^{-/-}\) uterine arteries have impaired uterine artery function in vitro when compared to wild-type C57 vessels (Kusinski et al., 2012), with endothelium dependent-relaxation to ACh being significantly reduced. These responses are similar to those of human maternal myometrial arteries obtained from women with FGR, in which a reduced endothelium-dependent relaxation to bradykinin is observed (Wareing et al., 2005a). Thus eNOS\(^{-/-}\) mouse uterine arteries represent a valid model of human FGR for testing the efficacy of different vasodilators.

### 4.3.3 Characterisation and stability of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes

The second aim of this study was to determine whether encapsulation of SE175 in CNKGLRNK homing peptide-conjugated liposomes altered size, zeta potential or stability in phosphate buffered saline at 4°C. Liposome mean diameters and zeta potentials were determined immediately after preparation.
SE175-encapsulated, CNKGLRNK peptide-conjugated liposomes had a mean diameter of approximately 160 nm, which was almost identical to PBS-encapsulated liposomes. The polydispersity index of both SE175 and PBS-encapsulated liposomes was below 0.1, indicating that encapsulation of SE175 did not alter particle size heterogeneity. Both SE175 and PBS-encapsulated liposomes had a neutral zeta potential, again indicating that SE175 encapsulation did not alter the characteristics of the liposome preparation.

The stability of SE175-encapsulated, CNKGLRNK peptide-conjugated liposomes was investigated over 28 days with liposomes remaining stable, showing no signs of aggregation or degradation. These results collectively demonstrate that SE175 can be encapsulated in peptide-conjugated liposomes without altering characteristics or stability. These data are supported by previous studies that demonstrate that encapsulation of the vasodilator fasudil in liposomes did not alter characteristics or stability when stored at 4°C (Ishida et al., 2002b). This stability is clinically relevant as it means that SE175-encapsulated, CNKGLRNK peptide-conjugated liposomes may be suitability stored and transported for therapeutic use.

4.3.4 Assessment of cell turnover in human placental explants treated with SE175

In order to identify any potential toxicological effects of SE175 toward human placental tissue, placental cell turnover was assessed to examine whether SE175 altered basal rates of apoptosis and proliferation. Human term placental explants were cultured with free SE175 and immunostained for Ki67, a marker of proliferation, and M30 a marker of apoptosis.

It has previously been demonstrated that NO plays an important role in placental vascular function, having roles in promoting angiogenesis and regulating vascular tone (Krause et al., 2011). The role of NO in regulating cell turnover has also been examined, indicating that NO has a protective effect in human extravillious trophoblast cells, protecting them from apoptosis through the induction of cyclic GMP production and the inhibition of tumour necrosis factor
alpha signaling or secretion (Dash et al., 2003). Nitric oxide administration has also been shown to improve outcome in ischaemia-reperfusion injury in rat liver (Kurabayashi et al., 2005); uteroplacental ischaemia-reperfusion injury has been indicated in pathological pregnancies, and as such, nitric oxide donors may alleviate damage caused by such injury (Hung et al., 2001).

Culture of placental explants with SE175 did not alter proliferation suggesting a lack adverse effects. SE175 also did not alter the extent of M30 immunostaining, indicating that the basal level of apoptosis in these tissues was unchanged and that SE175 did not cause damage to placental explants and was well tolerated for the 72 hour culture. These results collectively demonstrate the safety of SE175 in human placental tissue. This information, plus further safety studies, will be essential if SE175 were to be taken in to clinical trials; in order to demonstrate further safety of SE175, studies to assess nutrient transport, hCG production and lactate dehydrogenase release in placental tissue could be undertaken.

4.4 Summary

The collective data from this chapter lend evidence to support the hypothesis that the novel nitric oxide donor SE175 may be encapsulated in peptide-conjugated liposomes to induce vasodilation of the uteroplacental vasculature. Both free and liposome-encapsulated SE175 were able to induce vasodilation of human chorionic plate arteries and mouse uterine arteries, with eNOS-/- vessels exhibiting slower relaxation than C57BL6/J vessels. SE175 was encapsulated in CNKGLRNK peptide-conjugated liposomes, with these preparations showing no difference in characteristics compared to PBS-encapsulated liposomes. SE175 did not adversely affect proliferation of human placental tissue and also did not induce apoptosis. The same concentration (320 µM) was used across all studies, as this was the concentration at which all vessels exhibited maximal relaxation. At this concentration, SE175 was bioactive, both free and when delivered inside liposomes; future in vivo studies will use this concentration as it demonstrates an optimal dose. In summary, these results indicate potential for in vivo success of SE175.
Chapter 5
5.1 Introduction

CNKGLRNK homing peptide-conjugated liposomes have been validated as nanocarriers that are able to deliver a drug cargo to the placenta. The novel vasodilator SE175, a nitroxyacylated thiosalicylate, was selected after a literature review of NO donor molecules; its bioactivity was demonstrated on human chorionic plate arteries and isolated mouse uterine arteries ex vivo. SE175 was successfully encapsulated into liposomes, which had favourable stability and release profiles, causing vasodilation of human and mouse vessels over a suitable time course. The effect of SE175 on placental function was assessed in human placental explants; the compound was well tolerated and had no effect on trophoblast turnover. In order to assess whether SE175-encapsulated CNKGLRNK homing peptide-conjugated liposomes can improve fetal growth or placental function, the formulation needed to be validated in vivo, using a mouse model of pregnancy.

SE175-encapsulated CNKGLRNK homing peptide-conjugated liposomes have not previously been studied in mice and therefore the safety and efficacy of this preparation was first validated in a mouse model of uncomplicated pregnancy, the wild-type control mouse C57BL6/J. In order to assess effects on fetal growth in a clinically relevant manner, it was important to determine the effect of SE175-encapsulated peptide-conjugated liposomes in a mouse model of FGR; these data would then provide evidence as to whether this treatment is not only safe, but also of potential therapeutic use in the treatment of FGR.

Previous studies have identified a number of mouse models of FGR, each with differing phenotypes and underlying pathology. The placental-specific Igf-II knock out mouse (P0) exhibits FGR, with evidence indicating that a decrease in nutrient transport is responsible for the onset of growth restriction (Constancia et al., 2002). P0 mice have no abnormalities in uteroplacental vascular function (Kusinski et al., 2011). As our approach is to use a vasodilator to treat FGR caused by dysregulated vascular adaptations to pregnancy, the P0 mouse model may not prove useful. A model of FGR that exhibits reduced uteroplacental perfusion is the endothelial nitric oxide synthase (eNOS-/-) knockout mouse.
Hence, we also examined the effects of SE175-encapsulated peptide-conjugated liposomes on fetal growth in these mice.

The endothelial nitric oxide synthase (eNOS) enzyme converts L-arginine to nitric oxide (NO), causing vasodilation by inducing the relaxation of smooth muscle cells (Moncada, 1997, Denninger and Marletta, 1999). eNOS⁻/⁻ mice (Jackson Laboratories; strain B6.129P2-\textit{Nos3}^{tm1Unc/J}), are homozygous for the for the nitric oxide synthase 3 (eNOS) knockout allele. The lack of a functional eNOS enzyme leads to pregnant eNOS⁻/⁻ dams exhibiting dysregulated vascular adaptations to pregnancy, and fetuses that are growth restricted (Kusinski et al., 2012, Pallares and Gonzalez-Bulnes, 2010). Offspring from eNOS⁻/⁻ mice are on average 10% lighter than C57 wild-type mice, with placentas showing no histological abnormalities (Hefler et al., 2001a). It has been demonstrated that eNOS⁻/⁻ mice have significantly reduced cardiac output throughout pregnancy (Kulandavelu et al., 2006); these mice also exhibit raised blood pressure before and throughout gestation (Hefler et al., 2001b). Placental nutrient transport has also been shown to be reduced in eNOS⁻/⁻ mice, along with placentas exhibiting oxidative stress and uteroplacental hypoxia (Kusinski et al., 2012). With these characteristics in mind, the eNOS⁻/⁻ mouse represents a suitable model of FGR to study the effects of a targeted vasodilator.

Along with a NO-donating nitrate group, SE175 also contains a thiosalicylate group, which is related to aspirin. As such, SE175 has the potential to act as a non-selective cyclooxygenase (COX) inhibitor. There is evidence that increased oxidative stress in mouse placenta leads to the induction of both cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2), leading to increased prostaglandin synthesis and apoptosis (Burdon et al., 2007). COX-1 and COX-2 are upregulated in pregnancy complications such as pre-eclampsia (Wetzka et al., 1997) and in uterine artery ischaemia-reperfusion injury (Yamazaki et al., 2006). It has also been shown that placentas from FGR fetuses exhibit excess oxidative stress, which in turn leads to reduced protein translation of signaling proteins in the AKT and mTOR pathways, which are important in mediating nutrient transport and angiogenesis (Yung et al., 2008, Scifres and Nelson, 2009).
It has been demonstrated that fetal sex plays a role in determining growth rate and chances of survival in response to an adverse maternal environment (Clifton, 2010). Maternal asthma during pregnancy has been shown to exhibit sexually dimorphic effects on fetal growth and placental function, suggesting that female fetuses are able to respond to an adverse maternal environment by adjusting placental function and reducing growth trajectory in an effort to compensate for maternal disease, whereas male fetuses fail to alter placental function, resulting in no change in fetal growth but an increased risk of morbidity and mortality (Clifton, 2005, Murphy et al., 2003). With sexual dimorphism being an important factor in response to maternal environment, determining gender-specific responses to treatments was investigated.

The specific aims of the study were 1) to determine whether SE175-encapsulated CNKGLRNK peptide-conjugated liposomes have any adverse effects in wild-type C57L6/J mice, 2) to examine the effects of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes in the eNOS\(^{-/-}\) mouse, a model of FGR, 3) to assess whether any effects of treatment are gender-specific and 4) to determine whether SE175-encapsulated CNKGLRNK peptide-conjugated liposomes alter the basal level of placental oxidative stress or COX expression.

These aims address the following questions; a) is delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes safe and well tolerated in wild-type mice? If so, b) can treatment with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes alter fetal or placental growth in a model of FGR? If there are differences in fetal or placental growth, c) does fetal gender have any effect on response to treatment? Finally, d) does treatment with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes alter placental oxidative stress or COX expression in the mouse placenta?
5.2 Results

5.2.1 Effects of treatment with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes in pregnant C57BL/6J mice

C57BL/6J mice were time-mated, with the confirmation of a copulation plug being considered as embryonic (E) day 0.5. Mice were injected with four doses of PBS to control for the stress of restraint and intravenous injections, PBS-encapsulated CNKGLRNK peptide-conjugated liposomes, SE175-encapsulated CNKGLRNK peptide-conjugated liposomes or free SE175. Mice were dosed at E11.5, E13.5, E15.5 and E17.5; mice were sacrificed at E18.5 and fetuses and placentas collected.

5.2.1.1 Effect of SE175-encapsulated CNKGLRNK peptide-conjugated liposome treatment on fetal weight

Delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes did not alter fetal weight compared to vehicle control in C57BL/6J mice (Figure 5.1). Delivery of free SE175 significantly reduced fetal weight compared to vehicle control, indicating that targeted delivery of SE175 may be safer than systemic delivery of free SE175. Mean litter weights were not significantly altered compared to vehicle control in any of the treatment groups (Figure 5.2).

Construction of a fetal weight distribution curve showed that delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes narrowed fetal weight distribution; delivery of PBS-encapsulated CNKGLRNK peptide-conjugated liposomes had the same effect (Figure 5.3). Delivery of free SE175 shifted the fetal weight distribution curve to the left, indicating that more fetuses lay below the fifth and tenth weight centiles. This observation was validated by calculating the percentage of fetuses falling below the fifth and tenth weight centiles; delivery of free SE175 increased the percentage of fetuses in both weight centiles (Table 5.1).
Figure 5.1. Individual fetal weights. C57BL/6J mice (N = dams, n = fetuses) were intravenously injected with 100 µL of PBS (N = 17, n = 115; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, n = 58; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11, n = 72; downward facing black triangle) or free SE175 (N = 10, n = 56; open black square). Horizontal red dashed line represents vehicle control mean fetal weight. Data points represent individual fetuses; data shown are mean ± SEM. Means were compared using one-way ANOVA with Dunnett’s post hoc test. **P<0.001.
Figure 5.2. Fetal weight litter means. C57BL/6J mice (N = dams) were intravenously injected with 100 µL of PBS (N = 17; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11; downward facing black triangle) or free SE175 (N = 10; open black square). Horizontal red dashed line represents vehicle control mean litter weight. Data points represent litter averages; data shown are mean ± SEM. Means were compared using one-way ANOVA with Dunnett's post hoc test.
Figure 5.3. Individual fetal weight distributions. C57BL/6J mice (N = dams, n = fetuses) were intravenously injected with 100 µL of PBS (N = 17, n = 115; blue line), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, n = 58; red line), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11, n = 72; green line) or free SE175 (N = 10, n = 56; fuchsia line). Data were plotted as population distribution curves. Vertical red dashed line represents the fifth weight centile of control PBS treated mice, vertical black dashed line represents the tenth weight centile of control PBS treated mice.

<table>
<thead>
<tr>
<th></th>
<th>Percent of mice falling below 5th centile of weight</th>
<th>Percent of mice falling below 10th centile of weight</th>
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</thead>
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<td>Vehicle control</td>
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<td>10 %</td>
</tr>
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<tr>
<td>peptide-targeted liposomes</td>
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<td></td>
</tr>
<tr>
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<td>5 %</td>
</tr>
<tr>
<td>peptide-targeted liposomes</td>
<td></td>
<td></td>
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<tr>
<td>Free SE175</td>
<td>7 %</td>
<td>13 %</td>
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Table 5.1. Percentage of individual C57BL/6J fetuses below the fifth and tenth weight centiles of control PBS treated mice.
5.2.1.2 Effect of SE175-encapsulated CNKGLRNK peptide-conjugated liposome treatment on litter size and resorptions

Delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes did not significantly alter litter size compared to vehicle control in C57BL/6J mice (Figure 5.4). Delivery of PBS-encapsulated liposomes and free SE175 also did not significantly affect litter size.

To ensure that any differences in litter size were not due to the loss of fetuses during gestation, the percentage of resorptions per litter was investigated. No treatment significantly affected the percentage of resorptions per litter (Figure 5.5).

![Figure 5.4](image)

Figure 5.4. Number of pups per litter. C57BL/6J mice (N = dams) were intravenously injected with 100 µL of PBS (N = 17; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11; downward facing black triangle) or free SE175 (N = 10; open black square). Data points represent individual litter sizes; data shown are mean ± SEM. Means were compared using one-way ANOVA with Dunnett’s post hoc test.
Figure 5.5. Percentage of resorptions per litter. C57BL/6J mice (N = dams) were intravenously injected with 100 µL of PBS (N = 17; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11; downward facing black triangle) or free SE175 (N = 10; open black square). Data points represent individual litter percentage resorptions; red lines represent median. Data were compared using Kruskal-Wallis with Dunn’s post hoc test.
5.2.1.3 Effect of SE175-encapsulated CNKGLRNK peptide-conjugated liposome treatment on placental weight and efficiency

Delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes did not significantly alter individual placental weights compared to vehicle control in C57BL/6J mice; no significant differences in placental weights were observed with any treatment groups (Figure 5.5). No treatment group significantly altered mean litter placental weight compared to vehicle control (Figure 5.7).

Placental efficiency is commonly described in terms of fetal-placental (F:P) weight ratio, indicating how many grams of fetus are produced per gram of placenta (Wilson and Ford, 2001). F:P weight ratio was not significantly altered in any treatment group compared to vehicle control, indicating that treatments did not significantly alter placental growth or function (Figure 5.8).
Figure 5.6. Individual placental weights. C57BL/6J mice (N = dams, n = fetuses) were intravenously injected with 100 µL of PBS (N = 17, n = 115; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, n = 58; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11, n = 72; downward facing black triangle) or free SE175 (N = 10, n = 56; open black square). Horizontal red dashed line represents vehicle control mean placental weight. Data points represent individual placental weights; data shown are mean ± SEM. Means were compared using one-way ANOVA with Dunnett’s post hoc test.
Figure 5.7. Placental weight litter means. C57BL/6J mice (N = dams) were intravenously injected with 100 µL of PBS (N = 17; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11; downward facing black triangle) or free SE175 (N = 10; open black square). Horizontal red dashed line represents vehicle control mean placental weight. Data points represent average litter placenta weights; data shown are mean ± SEM. Means were compared using one-way ANOVA with Dunnett’s post hoc test.
Figure 5.8. C57BL/6J mice (N = dams, n = fetuses) were intravenously injected with 100 µL of PBS (N = 17, n = 115; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, n = 58; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11, n = 72; downward facing black triangle) or free SE175 (N = 10, n = 56; open black square). Horizontal red dashed line represents vehicle control mean F:P weight ratio. Data points represent individual F:P weight ratio; data shown are mean ± SEM. Means were compared using one-way ANOVA with Dunnett’s post hoc test.
5.2.1.4 Effect of gender on fetal weight, placental weight and placental efficiency after SE175-encapsulated CNKGLRNK peptide-conjugated liposome treatment

Sexual dimorphism has been reported in previous studies related to placental and fetal development, with results indicating that sex-dependent adaptations in the placenta appear to protect female fetuses from adverse outcomes (Tarrade et al., 2013, Rosenfeld, 2015). Given previous evidence of sexual dimorphism in placental adaptations, fetuses were gender-typed and differences in fetal weight, placental weight and placental efficiency examined between male and female offspring in each treatment group.

No significant differences in fetal weight were observed between male and female fetuses within each treatment group (Figure 5.9). Placental weights were examined within each treatment group, with placentas from female fetuses were significantly smaller in the SE175-encapsulated CNKGLRNK peptide-conjugated liposome treatment group, being on average 11.3% smaller, compared to those from male fetuses (Figure 5.10). Although no significant differences were observed between placentas from male and female fetuses in any other treatment group, it was seen that placentas from female fetuses were on average 9.5% smaller than those from male fetuses across all treatment groups.

F:P weight ratios were also assessed within each treatment group, with F:P weight ratios being significantly greater in female fetuses compared to male fetuses in the SE175-encapsulated CNJGLRNK peptide-conjugated liposome treatment group (Figure 5.11). Although no significant differences were observed between F:P weight ratios in any other treatment group, F:P weight ratios from female fetuses were on average 8.2% higher than those from male fetuses across all treatment groups.
Figure 5.9. Individual male and female fetal weights. C57BL/6J mice (N = dams, f = female fetuses, m = male fetuses) were intravenously injected with 100 µL of PBS (N = 4, m = 11; closed blue square, f = 16; closed orange square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, m = 28; upward facing blue triangle, f = 29; upward facing orange triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11, m = 38; downward facing blue triangle, f = 33; downward facing orange triangle) or free SE175 (N = 10, m = 29; open blue square, f = 28; open orange square). Data points represent individual fetuses; red line represents median. Data were compared using Kruskal-Wallis with Dunn’s post hoc test.
Figure 5.10. Individual male and female placental weights. C57BL/6J mice (N = dams, f = placentas from female fetuses, m = placentas from male fetuses) were intravenously injected with 100 µL of PBS (N = 4, m = 11; closed blue square, f = 16; closed orange square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, m = 28; upward facing blue triangle, f = 29; upward facing orange triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11, m = 38; downward facing blue triangle, f = 33; downward facing orange triangle) or free SE175 (N = 10, m = 29; open blue square, f = 28; open orange square). Data points represent individual placentas; red lines represent median. Data were compared using Kruskal-Wallis with Dunn’s post hoc test. **P<0.01.
Figure 5.11. Individual male and female F:P weight ratios. C57BL/6J mice (N = dams, f = F:P weight ratios from female fetuses, m = F:P weight ratios from male fetuses) were intravenously injected with 100 µL of PBS (N = 4, m = 11; closed blue square, f = 16; closed orange square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, m = 28; upward facing blue triangle, f = 29; upward facing orange triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11, m = 38; downward facing blue triangle, f = 33; downward facing orange triangle) or free SE175 (N = 10, m = 29; open blue square, f = 28; open orange square). Data points represent individual F:P weight ratios; red lines represent median. Data were compared using Kruskal-Wallis with Dunn’s post hoc test. *P<0.005.
5.2.1.5 Assessment of the effect of SE175-encapsulated CNKGLRNK peptide-conjugated liposome treatment on murine placenta oxidative stress and cyclooxygenase expression

Placentas from treated C57BL/6J mice were immunostained for hydroxynonenal (Casey et al.), a product of lipid peroxidation and therefore a marker of oxidative stress. HNE immunoreactivity was observed throughout the decidua, junctional zone and labyrinth of vehicle-treated mouse placetas (Figure 5.12; A - C). No treatment altered HNE immunoreactivity compared to vehicle control, in any regions of the placenta (Figure 5.12; D - L). Control immunoglobulin G (IgG) showed no immunoreactivity (Figure 5.12; M – O).

To allow for quantitative analysis of immunostaining, three treated mice per group were selected at random and three placentas per mouse immunostained in the same staining run to allow for direct comparison. Nine images were taken per placenta, equating to twenty-seven images per mouse (taken as n = 1). Images were then analysed using TissueGnostics HistoQuest® Analysis Software. Haematoxylin (nuclear stain) total area and diaminobenzidine (DAB; HNE stain) total area were determined and a ratio of DAB area to haematoxylin area calculated to control for tissue area.

Delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes did not alter the ratio of DAB to haematoxylin compared to vehicle control, indicating that targeted delivery of SE175 did not increase lipid peroxidation in C57BL/6J mice (Figure 5.13). No difference in the ratio of DAB to haematoxylin were observed in any other treatment groups indicating that all treatments were well tolerated and did not induce lipid peroxidation, a marker of oxidative stress (Figure 5.13).
Figure 5.12. HNE immunostaining of mouse placenta. Blue = haematoxylin nuclear stain; Brown = DAB; HNE stain. [A – C] Vehicle control treated; [D – F] PBS-encapsulated CNKGLRNK peptide-conjugated liposome treated; [G – I] SE175-encapsulated CNKGLRNK peptide-conjugated liposome treated; [J – L] Free SE175 treated; [M – O] IgG control. LB = labyrinth; DEC = decidua; JZ = junctional zone. Scale bars = 50µm. n = 3 placentas from n = 3 mice (representative images shown).
Figure 5.13. Placentas (n = 3 placentas from n = 3 mice) from C57BL/6J treated with PBS (closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (downward facing black triangle) or free SE175 (open black square) were immunostained for HNE and the total areas of haematoxylin staining and DAB staining determined using HistoQuest. Data points represent individual DAB:Haematoxylin ratios.
Cyclooxygenase is responsible for the formation of prostaglandins and there is evidence that oxidative stress is linked to cyclooxygenase induction, causing the production of prostaglandins that may contribute to inflammation and generalised placental dysfunction (Burdon et al., 2007). SE175 contains a thiosalicylate group which may inhibit cyclooxygenase activity. Placentas from treated C57BL/6J mice were immunostained for cyclooxygenase 1 (COX-1) and cyclooxygenase 2 (COX-2) to determine whether targeted delivery of SE175 had any effect on cyclooxygenase protein expression in C57BL/6J mice.

COX-1 and COX-2 immunoreactivity was observed throughout the decidua, junctional zone and labyrinth of vehicle-treated murine placenta (A - C). No treatment altered immunoreactivity of COX-1 or COX-2 compared to vehicle control in any regions of the placenta (D - L). Control immunoglobulin G (IgG) showed no immunoreactivity (M – O).

Haematoxylin (nuclear stain) total area and diaminobenzidine (DAB; COX-1 or COX-2 stain) total area were determined and a ratio of DAB area to haematoxylin area calculated to control for tissue area using TissueGnostics HistoQuest® Analysis Software. No treatment altered the ratio of DAB to haematoxylin for COX-1 (Figure 5.15) or COX-2 (Figure 5.17) compared to vehicle control.
Figure 5.15. Placentas (n = 3 placentas from n = 3 mice) from C57BL/6J treated with PBS (closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (downward facing black triangle) or free SE175 (open black square) were immunostained for COX-1 and the total areas of haematoxylin staining and DAB staining determined using HistoQuest. Data points represent individual DAB:Haematoxylin ratios.
Figure 5.16. COX-2 immunostaining of mouse placenta. Blue = haematoxylin nuclear stain; Brown = DAB; HNE stain. [A – C] Vehicle control treated; [D – F] PBS-encapsulated CNKGLRNK peptide-conjugated liposome treated; [G – I] SE175-encapsulated CNKGLRNK peptide-conjugated liposome treated; [J – L] Free SE175 treated; [M – O] IgG control. LB = labyrinth; DEC = decidua; JZ = junctional zone. Scale bars = 50 µM. n = 3 placentas from n = 3 mice (representative images shown).
Figure 5.17. Placentas (n = 3 placentas from n = 3 mice) from C57BL/6J treated with PBS (closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (downward facing black triangle) or free SE175 (open black square) were immunostained for COX-2 and the total areas of haematoxylin staining and DAB staining determined using HistoQuest. Data points represent individual DAB:Haematoxylin ratios.
5.2.2 Effects of treatment with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes in pregnant endothelial nitric oxide synthase knock-out mice

The endothelial nitric oxide synthase knock-out (eNOS\(^{-/-}\)) mouse was used to assess the effect of targeted delivery of SE175 in a model of fetal growth restriction (FGR). This is a clinically relevant experiment as it is anticipated that any therapeutic intervention would be administered to women with established FGR.

eNOS\(^{-/-}\) mice were time-mated, with the confirmation of a copulation plug being considered as embryonic (E) day 0.5. Mice were injected with four doses of PBS to control for the stress of restraint and intravenous injections, PBS-encapsulated CNKGLRNK peptide-conjugated liposomes, SE175-encapsulated CNKGLRNK peptide-conjugated liposomes or free SE175. Mice were dosed at E11.5, E13.5, E15.5 and E17.5; mice were sacrificed at E18.5 and fetuses and placentas collected.

5.2.2.1 Effect of SE175-encapsulated CNKGLRNK peptide-conjugated liposome treatment on fetal weight in eNOS\(^{-/-}\) mice

Delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes significantly increased fetal weight compared to vehicle control in eNOS\(^{-/-}\) mice (Figure 5.18). No other treatment significantly altered fetal weight. Fetuses from eNOS\(^{-/-}\) mice had a mean fetal weight 13 % smaller than that of wild type C57BL/6J mice; delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes increased mean fetal weight by 4 % compared to vehicle control in eNOS\(^{-/-}\) mice. Plotting individual fetal weights demonstrated that fewer fetuses of lower weight were observed, whereas the number of fetuses that were of appropriate weight was not changed. This suggests that targeted delivery of SE175 may increase the growth of the smallest fetuses, but not affect the growth of appropriate weight fetuses.

The litter mean fetal weight of vehicle control eNOS\(^{-/-}\) mice was significantly decreased compared to vehicle control C57BL/6J, being 14% smaller. Delivery
of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes did not significantly alter litter mean fetal weight compared to vehicle control, further suggesting that the treatment affects only a proportion of the individual fetuses rather than the whole litters (Figure 5.19). No other treatment significantly altered litter mean fetal weight.

Construction of a fetal weight distribution curve showed that delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes increased the weights of the smallest fetuses to above the tenth centile of eNOS−/− vehicle control fetal weights; delivery of PBS-encapsulated CNKGLRNK peptide-conjugated liposomes and free SE175 had no effect (Figure 5.20). This observation was confirmed by calculation of percentage of fetuses below the 5th and 10th centiles of weight, with delivery of SE175-encapsulated CNKGLRNK peptide-targeted liposomes resulting in no fetuses falling below the fifth or tenth centiles of weight (Table 5.2).

These results collectively demonstrate that treatment of eNOS−/− mice, a model of fetal growth restriction, with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes improved the weight of those fetuses below the tenth centile of weight, the clinical definition of fetal growth restriction.
Figure 5.18. Individual fetal weights. eNOS<sup>−/−</sup> mice (N = dams, n = fetuses) were intravenously injected with 100 µL of PBS (N = 14, n = 113; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, n = 67; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 10, n = 68; downward facing black triangle) or free SE175 (N = 8, n = 57; open black square). C57BL/6J vehicle control data are shown are comparison (N = 17, n = 115; closed black circle). Horizontal red dashed line represents eNOS<sup>−/−</sup> vehicle control mean fetal weight. Data points represent individual fetuses; data shown are mean ± SEM. Means were compared using one-way ANOVA with Dunnett’s post hoc test. *P<0.05, ****P<0.0001.
Figure 5.19. Fetal weight litter means. eNOS<sup>-/-</sup> mice (N = dams) were intravenously injected with 100 µL of PBS (N = 17; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11; downward facing black triangle) or free SE175 (N = 10; open black square). C57BL/6J vehicle control data are shown as comparison (N = 17; closed black circle). Horizontal red dashed line represents eNOS<sup>-/-</sup> vehicle control mean fetal weight. Data points represent litter averages; data shown are mean ± SEM. Means were compared using one-way ANOVA with Dunnett’s *post hoc* test. ****P<0.0001.
**Table 5.2.** Percentage of individual eNOS\(^{-/-}\) fetuses below the fifth and tenth weight centiles of control PBS treated mice.

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<th>Percent of mice falling below 10(^{th}) centile of weight</th>
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<td>Vehicle control</td>
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<td>10 %</td>
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<td>PBS-encapsulated CNKGLRNK peptide-targeted liposomes</td>
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<td>0 %</td>
</tr>
<tr>
<td>Free SE175</td>
<td>7 %</td>
<td>10 %</td>
</tr>
</tbody>
</table>

Figure 5.20. Individual fetal weight distributions. eNOS\(^{-/-}\) mice (N = dams, n = fetuses) were intravenously injected with 100 µL of PBS (N = 17, n = 115; blue line), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, n = 58; red line), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11, n = 72; green line) or free SE175 (N = 10, n = 56; fuchsia line). C57BL/6J vehicle control data are shown are comparison (N = 17, n = 115; black line). Data were plotted as population distribution curves. Vertical red dashed line represents the fifth weight centile of control PBS treated mice, vertical black dashed line represents the tenth weight centile of control PBS treated mice.
5.2.2.2 Effect of SE175-encapsulated CNKGLRNK peptide-conjugated liposome treatment on litter size and resorptions

Delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes did not significantly alter litter size compared to vehicle control in eNOS\(^-\) mice (Figure 5.21). Delivery of PBS-encapsulated liposomes and free SE175 also did not significantly affect litter size.

To ensure that any differences in litter size were not due to the loss of fetuses during gestation, the percentage of resorptions per litter was investigated. No treatment significantly affected the percentage of resorptions per litter, indicating that all treatments were well tolerated (Figure 5.22).

![Figure 5.21. Number of pups per litter. eNOS\(^-\) mice (N = dams) were intravenously injected with 100 µL of PBS (N = 14; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 10; downward facing black triangle) or free SE175 (N = 8; open black square). C57BL/6J vehicle control data are shown are comparison (N = 17; closed black circle). Data points represent individual litter sizes; data shown are mean ± SEM. Means were compared\(^{3}\) using one-way ANOVA with Dunnett's post hoc test.](image-url)
Figure 5.22. Percentage of resorptions per litter. eNOS^{+} mice (N = dams) were intravenously injected with 100 µL of PBS (N = 14; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 10; downward facing black triangle) or free SE175 (N = 8; open black square). C57BL/6J vehicle control data are shown for comparison (N = 17; closed black circle). Data points represent individual litter percentage resorptions; red lines represent median. Data were compared using Kruskal-Wallis with Dunn's post hoc test.
5.2.2.3 Effect of SE175-encapsulated CNKGLRNK peptide-conjugated liposome treatment on placental weight and efficiency

Delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes significantly decreased individual placental weight compared to vehicle control in eNOS+/− mice; a significant decrease in placental weight was also observed in free SE175 treated mice (Figure 5.23). PBS-encapsulated CNKGLRNK peptide-conjugated liposomes did not affect placental weight.

eNOS+/− placentas are larger than that of wild type C57BL/6J mice, being on average 6 % heavier; treatment with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes reduced placental weight by 9 % compared to eNOS+/− vehicle control, bringing mean weight to 4 % below that of wild type C57BL/6J mice. Placental weight litter mean was also decreased compared to vehicle control in eNOS+/− mice (Figure 5.24).

Placental efficiency, described in terms of the F:P weight ratio, is significantly reduced in vehicle control eNOS+/− mice compared to vehicle control C57BL/6J mice, being on average 18 % less efficient (Figure 5.25). Delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes significantly increased F:P weight ratio compared to vehicle control in eNOS+/− mice, improving efficiency by 15 %. Delivery of free SE175 also improved F:P weight ratio compared to vehicle control, but this improvement in efficiency did not translate into an improvement in fetal weight.

These results collectively demonstrate that treatment of eNOS+/− mice with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes significantly reduced placental weight, leading to an improvement in placental efficiency and an increase in fetal weight.
Figure 5.23. Individual placental weights. eNOS<sup>−/−</sup> mice (N = dams, n = fetuses) were intravenously injected with 100 µL of PBS (N = 14, n = 113; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, n = 67; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 10, n = 68; downward facing black triangle) or free SE175 (N = 8, n = 57; open black square). C57BL/6J vehicle control data are shown as comparison (N = 17, n = 115; closed black circle). Horizontal red dashed line represents eNOS<sup>−/−</sup> vehicle control mean placental weight. Data points represent individual placentas; data shown are mean ± SEM. Means were compared using one-way ANOVA with Dunnett’s post hoc test. **P<0.006, ****P<0.0001.
Figure 5.24. Placental weight litter means. eNOS\textsuperscript{−/−} mice (N = dams) were intravenously injected with 100 µL of PBS (N = 17; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 11; downward facing black triangle) or free SE175 (N = 10; open black square). C57BL/6J vehicle control data are shown as comparison (N = 17; closed black circle). Horizontal red dashed line represents eNOS\textsuperscript{−/−} vehicle control mean placental weight. Data points represent litter averages; data shown are mean ± SEM. Means were compared using one-way ANOVA with Dunnett's post hoc test. **P<0.005.
Figure 5.25. Individual F:P weight ratio. eNOS<sup>−/−</sup> mice (N = dams, n = fetuses) were intravenously injected with 100 µL of PBS (N = 14, n = 113; closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, n = 67; upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 10, n = 68; downward facing black triangle) or free SE175 (N = 8, n = 57; open black square). C57BL/6J vehicle control data are shown as comparison (N = 17, n = 115; closed black circle). Horizontal red dashed line represents eNOS<sup>−/−</sup> vehicle control mean F:P. Data points represent individual F:P; data shown are mean ± SEM. Means were compared using one-way ANOVA with Dunnett’s post hoc test. ****P<0.0001.
5.2.2.4 Effect of gender on fetal weight, placental weight and placental efficiency after SE175-encapsulated CNKGLRNK peptide-conjugated liposome treatment

Given previous evidence of sexual dimorphism in placental adaptations, fetuses were gender-typed and differences in fetal weight, placental weight and placental efficiency examined between male and female offspring in each treatment group.

No significant differences in fetal weight were observed between male and female fetuses within each treatment group (Figure 5.26). Placental weights were examined within each treatment group; placentas from female fetuses were significantly smaller in vehicle-treated mice, being on average 9.9% smaller than those from male fetuses (Figure 5.27). Although no significant differences were observed between placentas from male and female fetuses in any other treatment group, placentas from female fetuses were on average 7.5% smaller than those from male fetuses across all treatment groups.

F:P weight ratios were also assessed within each treatment group, with no significant differences between F:P weigh ratios in any treatment group between male and female fetuses (Figure 5.28). F:P weight ratios from female fetuses were on average 7.0% higher than those of male fetuses across all treatment groups, with the exception of those from mice treated with free SE175, in which female F:P was 1.5% greater compared to male F:P.
Figure 5.26. Individual male and female fetal weights. eNOS<sup>−/−</sup> mice (N = dams, f = female fetuses, m = male fetuses) were intravenously injected with 100 µL of PBS (N = 9, m = 33; closed blue square, f = 30; closed orange square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, m = 36; upward facing blue triangle, f = 31; upward facing orange triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 10, m = 34; downward facing blue triangle, f = 33; downward facing orange triangle) or free SE175 (N = 8, m = 23; open blue square, f = 34; open orange square). Data points represent individual fetuses; red lines represent median. Data were compared using Kruskal-Wallis with Dunn's post hoc test.
Figure 5.27. Individual male and female placental weights. eNOS$^{-/-}$ mice (N = dams, f = female fetuses, m = male fetuses) were intravenously injected with 100 µL of PBS (N = 9, m = 33; closed blue square, f = 30; closed orange square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, m = 36; upward facing blue triangle, f = 31; upward facing orange triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 10, m = 34; downward facing blue triangle, f = 33; downward facing orange triangle) or free SE175 (N = 8, m = 23; open blue square, f = 34; open orange square). Data points represent individual placentas; red lines represent median. Data were compared using Kruskal-Wallis with Dunn’s post hoc test. **P<0.01.
Figure 5.28. Individual male and female F:P weight ratios. eNOS<sup>-/-</sup> mice (N = dams, f = female fetuses, m = male fetuses) were intravenously injected with 100 µL of PBS (N = 9, m = 33; closed blue square, f = 30; closed orange square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 9, m = 36; upward facing blue triangle, f = 31; upward facing orange triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (N = 10, m = 34; downward facing blue triangle, f = 33; downward facing orange triangle) or free SE175 (N = 8, m = 23; open blue square, f = 34; open orange square). Data points represent individual F:P weight ratios; red lines represents median. Data were compared using Kruskal-Wallis with Dunn’s post hoc test.
5.2.2.5 Assessment of the effect of SE175-encapsulated CNKGLRNK peptide-conjugated liposome treatment on mouse placental oxidative stress and cyclooxygenase expression

Placentas from treated eNOS\(^{-/-}\) mice were immunostained for HNE as a marker of oxidative stress; C57 vehicle control treated placentas were also immunostained for comparison. HNE immunoreactivity was observed throughout the decidua, junctional zone and labyrinth of vehicle treated murine placenta (A - C). HNE immunoreactivity was higher in placentas from eNOS\(^{-/-}\) vehicle control mice (A - C) compared to C57 vehicle control mice (D - F). Placentas treated with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes exhibited significantly reduced immunoreactivity of HNE compared to eNOS\(^{-/-}\) vehicle control. (); no other treatment group exhibited altered HNE immunoreactivity. Control immunoglobulin G (IgG) showed no immunoreactivity (P – R).

Quantitative analysis of immunostaining was performed using TissueGnostics HistoQuest® Analysis Software. Haematoxylin (nuclear stain) total area and diaminobenzidine (DAB; HNE stain) total area were determined and a ratio of DAB area to haematoxylin area calculated to control for tissue area. Delivery of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes showed a trend to reduce the ratio of DAB to haematoxylin compared to that observed in the eNOS\(^{-/-}\) vehicle control, indicating that targeted delivery of SE175 reduced lipid peroxidation in eNOS\(^{-/-}\) mice (Figure 5.30). No difference in the ratio of DAB to haematoxylin was observed in any other treatment groups indicating that all treatments did not induce lipid peroxidation (Figure 5.30).

Placentas from treated eNOS\(^{-/-}\) mice were immunostained for COX-1 and COX-2 to determine whether targeted delivery of SE175 had any effect on cyclooxygenase expression; C57 vehicle control-treated placentas were also immunostained for comparison. COX-1 and COX-2 immunoreactivity was observed throughout the decidua, junctional zone and labyrinth of eNOS\(^{-/-}\) placentas. eNOS\(^{-/-}\) vehicle control placentas (D - F) expressed more COX-1 and COX-2 than C57 vehicle control...
placentas (A - C); treatment with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes appeared to reduce the expression of both COX-1 and COX-2 compared to eNOS+/− vehicle control.

Haematoxylin (nuclear stain) total area and diaminobenzidine (DAB; COX-1 or COX-2 stain) total area were determined and a ratio of DAB area to haematoxylin area calculated to control for tissue area using TissueGnostics HistoQuest® Analysis Software.

No treatment altered the ratio of DAB to haematoxylin for COX-1 (Figure 5.32) or COX-2 (Figure 5.34) compared to vehicle control, indicating that treatment with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes did not reduce COX expression.
Figure 5.29. HNE immunostaining of mouse placenta. Blue = haematoxylin nuclear stain; Brown = DAB; HNE stain. [A – C] C57 vehicle control treated; [D – F] eNOS$^{-/-}$ vehicle control treated; [G – I] eNOS$^{-/-}$ PBS-encapsulated CNKGLRNK peptide-conjugated liposome treated; [J – L] eNOS$^{-/-}$ SE175-encapsulated CNKGLRNK peptide-conjugated liposome treated; [M – O] eNOS$^{-/-}$ free SE175 treated; [P – R] eNOS$^{-/-}$ IgG control. LB = labyrinth; DEC = decidua; JZ = junctional zone. Scale bars = 50 µM. n = 3 placentas from n = 3 mice (representative images shown).
Figure 5.30. HNE immunostaining. Placentas (n = 3 placentas from n = 3 mice) from eNOS\(^{-/-}\) mice treated with PBS (closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (downward facing black triangle) or free SE175 (open black square) were immunostained for HNE and the total areas of haematoxylin staining and DAB staining determined using HistoQuest. C57BL/6J vehicle control data are shown are comparison (closed black circle). Data points represent individual DAB:Haematoxylin ratios.
Figure 5.31. COX-1 immunostaining of mouse placenta. Blue = haematoxylin nuclear stain; Brown = DAB; HNE stain. [A – C] C57 vehicle control treated; [D – F] eNOS⁻/⁻ vehicle control treated; [G – I] eNOS⁻/⁻ PBS-encapsulated CNKGLRNK peptide-conjugated liposome treated; [J – L] eNOS⁻/⁻ SE175-encapsulated CNKGLRNK peptide-conjugated liposome treated; [M – O] eNOS⁻/⁻ free SE175 treated; [P – R] eNOS⁻/⁻ IgG control. LB = labyrinth; DEC = decidua; JZ = junctional zone. Scale bars = 50 µM. n = 3 placentas from n = 3 mice (representative images shown).
Figure 5.32. COX-1 immunostaining. Placentas (n = 3 placentas from n = 3 mice) from eNOS-/- mice treated with PBS (closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (downward facing black triangle) or free SE175 (open black square) were immunostained for COX-1 and the total areas of haematoxylin staining and DAB staining determined using HistoQuest. C57BL/6J vehicle control data are shown as comparison (closed black circle). Data points represent individual DAB:Haematoxylin ratios.
Figure 5.33. COX-2 immunostaining of mouse placenta. Blue = haematoxylin nuclear stain; Brown = DAB; HNE stain. [A – C] C57 vehicle control treated; [D – F] eNOS\textsuperscript{−/−} vehicle control treated; [G – I] eNOS\textsuperscript{−/−} PBS-encapsulated CNKGLRNK peptide-conjugated liposome treated; [J – L] eNOS\textsuperscript{−/−} SE175-encapsulated CNKGLRNK peptide-conjugated liposome treated; [M – O] eNOS\textsuperscript{−/−} free SE175 treated; [P – R] eNOS\textsuperscript{−/−} IgG control. LB = labyrinth; DEC = decidua; JZ = junctional zone. Scale bars = 50 µM. n = 3 placentas from n = 3 mice (representative images shown).
Figure 5.34. COX-2 immunostaining. Placentas (n = 3 placentas from n = 3 mice) from eNOS<sup>-/-</sup> mice treated with PBS (closed black square), PBS-encapsulated CNKGLRNK peptide-conjugated liposomes (upward facing black triangle), SE175-encapsulated CNKGLRNK peptide-conjugated liposomes (downward facing black triangle) or free SE175 (open black square) were immunostained for COX-2 and the total areas of haematoxylin staining and DAB staining determined using HistoQuest. C57BL/6J vehicle control data are shown as comparison (closed black circle). Data points represent individual DAB:Haematoxylin ratios.
5.3 Discussion

This study utilised novel CNKGLRNK homing peptide-conjugated liposomes to facilitate the targeted delivery of the vasodilator SE175 to the uteroplacental vasculature in a mouse model of pregnancy, to determine the effects on pregnancy end-points in mice with uncomplicated and FGR complicated pregnancies. Treatment with SE175-encapsulated targeted liposomes did not affect fetal or placental growth in wild-type C57 mice; however delivery of free SE175 in C57 mice significantly reduced fetal weight compared to vehicle control, indicating that targeted drug delivery may improve safety. No treatment, altered placental growth or efficiency in C57 mice and no treatment significantly altered litter size or resorptions. Although not statistically significant, it was observed across all groups that female fetuses tended to have lower mean placental weights and therefore improved placental efficiency, compared to male fetuses. Placental oxidative stress and COX expression was not altered by any treatment in C57 mice.

When administered to pregnant eNOS<sup>−/−</sup> mice, a well characterised model of FGR, SE175-encapsulated targeted liposomes significantly improved fetal growth compared to vehicle-treated controls; no other treatment significantly altered fetal growth. Moreover, SE175-encapsulated targeted liposomes increased the weight of those fetuses that fell below the tenth centile, the clinical definition of FGR. No treatment significantly altered litter size or the number of resorptions, indicating that all treatments were well tolerated. Both free and targeted delivery of SE175 significantly reduced mean placental weight, compared to vehicle control in eNOS<sup>−/−</sup> mice; eNOS<sup>−/−</sup> placentas were found to be significantly heavier than that of wild-type C57 mice. Both free and targeted delivery of SE175 significantly improved placental efficiency compared to vehicle control in eNOS<sup>−/−</sup> mice, but only targeted delivery had a beneficial effect on fetal weight. As was noted in C57 mice, female fetuses had smaller placentas and therefore improved placental efficiency compared to male fetuses in all groups, except for those treated with free SE175. SE175-encapsulated targeted liposomes significantly decreased HNE, a marker of oxidative stress, in eNOS<sup>−/−</sup> placentas compared to vehicle control. Although not significant, SE175-
encapsulated targeted liposomes also decreased the mean level of COX-1 and COX-2 immunoreactivity in eNOS\(^{-/-}\) placentas. These results collectively suggest that targeted delivery of the novel vasodilator SE175 to the uteroplacental vasculature is well-tolerated and is able to enhance fetal growth, placental efficiency and reduce placental oxidative stress in a mouse model of FGR.

5.3.1 Examination of effects of SE175-encapsulated targeted liposomes on fetal weight, litter size and resorptions

This study aimed to determine whether treatment with SE175-encapsulated peptide-conjugated liposomes enhanced fetal weight in C57 wild-type control mice and in eNOS\(^{-/-}\) mice as a model of FGR.

Treatment with SE175-encapsulated peptide-conjugated liposomes did not alter fetal growth in C57 mice, but did significantly improve fetal growth in eNOS\(^{-/-}\) mice. Selective action of a vasodilator on growth restricted fetuses is a phenomenon that has been reported before. In the P0 mouse, there are both wild-type and P0 fetuses, with only P0 fetuses being growth restricted. Treatment of P0 mothers with the vasodilator sildenafil citrate significantly improved the growth of P0, but not of wild-type fetuses (Dilworth et al., 2013). It may therefore be postulated that fetuses from C57 mice do not show an improvement in fetal growth in response to treatment with a vasodilator as these fetuses are already appropriately grown and excess fetal growth may therefore be of detriment; however, in growth restricted eNOS\(^{-/-}\) mice, fetuses have not yet reached their genetic growth potential, and as such, therapeutic intervention is able to improve fetal growth to no apparent detriment.

SE175-encapsulated peptide-conjugated liposomes did not alter fetal weight distribution or the percentage of pups falling below the tenth centile in C57 mice. In eNOS\(^{-/-}\) mice, SE175-encapsulated peptide-conjugated liposomes shifted fetal weight distribution to the right, indicating that a greater proportion of fetuses were heavier. When the number of fetuses falling below the tenth and fifth centiles was examined, it was noted there were no fetuses fell below these centiles in SE175-encapsulated peptide-conjugated liposome treated mice; these data
demonstrate that it is the smallest fetuses whose growth is rescued, supporting the hypothesis that the treatment only improves the growth of fetuses that have not yet reached their growth potential. Previous studies have also demonstrated enhancement of growth of the smallest fetuses in a litter following therapeutic intervention. Treatment of pregnant eNOS\textsuperscript{-/-} mice with the IGF-II analog Leu\textsuperscript{27} has been shown to halve the number of fetuses falling below the fifth centile of weight, despite having no significant effect on mean fetal weight (Charnock et al., 2016). Another study examined targeted delivery of IGF-II to the placentas of P0 mice and found that the growth of the smallest fetuses was enhanced following treatment (King et al., 2016).

Across all treatment groups in C57 and eNOS\textsuperscript{-/-} mice, no differences in litter size or the percentage of resorptions per litter were observed; these results demonstrate that treatment was well tolerated and confirms that the smallest fetuses indeed had their growth enhanced, as opposed to being lost, with treatment nor interfering with normal fetal and placental development and function. In addition, no changes in maternal behavior were noted and no fetal abnormalities were apparent.

5.3.2 Examination of effects of SE175-encapsulated targeted liposomes on placental weight and efficiency

SE175-encapsulated peptide-conjugated liposomes did not alter placental weight in C57 mice. In eNOS\textsuperscript{-/-} mice, both targeted and free delivery of SE175 decreased placental weight compared to eNOS\textsuperscript{-/-} vehicle control. This study demonstrated that eNOS\textsuperscript{-/-} placentas were significantly heavier than C57; this finding is in contrast to what has previously been reported, with placental weights being reported as being unaltered (Kusinski et al., 2012). eNOS\textsuperscript{-/-} mice are known to have elevated blood pressure and impaired vascular reactivity and so it may be postulated that placentas from these mice are heavier in an attempt to adapt to the lack of uteroplacental blood flow. It has been reported that placental weight and placental blood flow are directly correlated with fetal growth (Fowden et al., 2006); since in eNOS\textsuperscript{-/-} mice placental blood flow is reduced, it stands to reason that the overgrowth of placentas from eNOS\textsuperscript{-/-} mice may be in an attempt to increase placental size and therefore surface area for nutrient transport to
maintain a normal fetal growth trajectory. Since both targeted and systemic delivery of SE175 reduced this placental overgrowth, it may be that there is an improvement in uteroplacental perfusion in both of these treatment groups, resulting in no requirement to adapt to poor uteroplacental perfusion and therefore no need to increase the size of the placenta.

Fetal placental weight ratio is used as an indicator of placental efficiency. SE175-encapsulated peptide-conjugated liposomes did not alter F:P weight ratio in C57 mice; all other treatment groups did also not have an effect of F:P weight ratio. In eNOS−/− mice, F:P weight ratio was improved in mice treated with both targeted and free SE175. This improvement in placental efficiency only translated to an improvement in fetal growth in those mice treated with targeted SE175, indicating that delivery of SE175-encapsulated peptide-conjugated liposomes is more beneficial at improving pregnancy outcome than delivery of free SE175. The beneficial effects of targeted drug delivery versus systemic drug delivery have been reported in previous studies. Targeted delivery of IGF-II to the placenta was found to improve fetal growth in a model of FGR, whereas free IGF-II did not affect fetal growth (King et al., 2016). It has also been reported that targeted drug delivery can aid the activity and specificity of anti-cancer therapeutics, with doxorubicin-encapsulated iRDG-conjugated liposomes demonstrating enhanced tumour activity and improved survival of mice, compared to systemic delivery of doxorubicin (Holig et al., 2004).

5.3.3 Examination of the effect of gender on fetal weight, placental weight and placental efficiency

Sexual dimorphism has been reported in previous studies related to placental and fetal development. It has been documented that gender-dependent adaptations in the placenta protect female fetuses from adverse outcomes, with male fetuses being more prone to adverse pregnancy outcomes (Tarrade et al., 2013, Rosenfeld, 2015). It has been shown that the maternal environment can have gender-specific programming effects; a low protein maternal diet throughout pregnancy programmed male fetuses, but not female fetuses, to exhibit increased arterial pressure and impaired \textit{ex vivo} relaxation to
acetylcholine (Roghair et al., 2009). It has also been demonstrated that a high fat maternal diet leads to only male fetuses exhibiting dyslipidaemia (Tarrade et al., 2013), again demonstrating the gender-specific responses to external stimuli. Given previous evidence of sexual dimorphism, gender-specific differences to each treatment group were examined.

In both C57 and eNOS−/− mice and within all treatment groups it was observed that female fetuses had lighter placentas than male fetuses, being on average 9.5% lighter in C57 mice and 7.5% lighter in eNOS−/− mice. Previous studies in wild type mice have shown that placentas from female fetuses are not significantly lighter than placentas from male fetuses in early gestation, but are significantly lighter at E17.5 (Ishikawa et al., 2006) and at E18.5 (Blakley, 1978). It is promising to note that across all treatment groups, this relationship remained constant, indicating that all treatments were well tolerated by both male and female fetuses.

F:P weight ratio was also altered in C57 and eNOS−/− mice and within all treatment groups, with the exception of eNOS−/− mice treated with free SE175. Female placentas had a greater F:P weight ratio, indicating that female placentas are more efficient than male placentas, producing more grams of fetus per gram of placenta. Female placentas were on average 8.2% more efficient in C57 mice and 7.0% more efficient in eNOS−/− mice. Female placentas from eNOS−/− mice treated with free SE175 were only 1.5% more efficient than male placentas, indicating that treatment with free SE175 may have disrupted the gender-specific growth trajectory of the placenta. These data provide further evidence to support the use of SE175-encapsulated CNKGLRNK peptide-conjugated liposome as opposed to free SE175.

5.3.4 Assessment of oxidative stress and cyclooxygenase expression in mouse placenta

Increased oxidative stress in the placenta has previously been reported in pregnancies complicated by FGR (Biri et al., 2007). Previous studies have demonstrated the link between oxidative stress and hypoxia, showing that oxidative stress contributes to the increased production of sFlt-1 as a result of
placental hypoxia in trophoblast cells, with evidence that increased levels of sFlt-1 may contribute to maternal endothelial dysfunction (Li et al., 2005). It has also been shown that eNOS−/− mice exhibit reduced uterine blood flow, leading to reduced placental oxygenation and therefore oxidative stress in the placenta (Kulandavelu et al., 2012). eNOS−/− mice have also been shown to have increased markers of hypoxia, indicating that oxidative stress may be a factor in the reduction in fetal growth (Kusinski et al., 2012).

Taking into account this previous evidence of the importance of oxidative stress in regulating fetal growth and pregnancy complications, placentas from all treatment groups were immunostained for the lipid peroxidation marker HNE, a marker of oxidative stress. There is also evidence that HNE is cytotoxic; an investigation of the role of HNE after an oxidative insult in neurons has shown that HNE induced neuronal apoptosis (Kruman et al., 1997). It has also been reported that elevated HNE levels are associated with the induction of pro-inflammatory mediators, such as tumour necrosis factor alpha, and vascular dysfunction in the uteroplacental vasculature, which may contribute to FGR and pre-eclampsia (Chapple et al., 2013, Chamy et al., 2006, Negre-Salvayre et al., 2010).

No treatment significantly altered placental HNE immunoreactivity compared to vehicle control in C57 mice. When the placentas from eNOS−/− were examined, it was found that treatment with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes significantly reduced HNE immunoreactivity compared to eNOS−/− vehicle control, indicating that targeted delivery of SE175 was able to reduce lipid peroxidation in the placenta, a marker of oxidative stress. Although not a statistically significant result, eNOS−/− placentas appeared to display more HNE immunoreactivity than those from C57 mice; these data agree with previously reported data that eNOS−/− placentas exhibit more oxidative stress (Kusinski et al., 2012) and suggest that targeted delivery of SE175 is able to ameliorate this oxidative stress. It must be noted these data were from n = 3 mice per treatment group and so further investigation would need to be undertaken to verify this result.

SE175 contains a thiosalicylate group that may act as a COX inhibitor after esterase breakdown in vivo. Oxidative stress have been shown to induce COX-
1 and COX-2 in the mouse placenta, with COX enzymes being linked to the production of prostaglandins (PGs) (Burdon et al., 2007, Malek et al., 2001). Although the differential roles for COX-1 and COX-2 are not completely understood in placenta, it has been demonstrated that an overexpression of COX-2 in mouse placenta leads to increased circulating PGs, reduced fetal growth and maternal hypertension; selective inhibition of COX-2 by celecoxib was shown to improve fetal growth and alleviate maternal hypertension (Sones et al., 2016).

Across all treatment groups in C57 mice, COX immunoreactivity was not altered, indicating that while no treatment reduced COX enzyme expression, it also did not induce COX expression, again lending support to the safety of treatment. In eNOS−/− placentas, although not significant, mean levels of COX-1 and COX-2 immunoreactivity was observed to be greater compared to those seen in C57 placentas; treatment with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes reduced the mean level of immunoreactivity for both COX-1 and COX-2, indicating that the thiosalicylate group of SE175 may have a role in reducing COX expression in placentas from mice with FGR.

5.4 Summary

The collective results from this chapter demonstrate that SE175-encapsulated CNKGLRNK homing peptide-conjugated liposomes may be used to rescue growth of the smallest fetuses in a mouse model of FGR, while also alleviating oxidative stress. Targeted delivery of SE175 did not have any adverse effects in wild-type control mice and also did cause negative effects on litter size or pregnancy losses in both wild-type mice and in a mice exhibiting FGR; these results indicate that treatment was well tolerated and safe in these models. Treatment with both free and targeted SE175 reduced adaptive placental overgrowth in eNOS−/− mice, leading to an improvement in placental efficiency; despite this increase in efficiency, only targeted delivery of SE175 resulted in an improvement in fetal weight, demonstrating the potential benefits of targeted delivery of a vasodilator to the uteroplacental vasculature. The data presented here supports further pre-clinical testing of targeted liposomes for the treatment of FGR, eventually leading to clinical trials.
Chapter 6
6.1. General Discussion

There were three main findings of this programme of research. Firstly, that the novel and placental-specific homing peptide sequences CNKGLRNK and CRSGVAKS can be conjugated to biocompatible liposomes, with the resultant preparation selectively binding to the outer syncytiotrophoblast layer of human placenta \textit{ex vivo} and to the labyrinth exchange regions and maternal arteries \textit{in vivo} in the mouse placenta. The homing peptide CNKGLRNK exhibited greater selectivity of binding to the maternal uterine vasculature, indicating that it may be more suitable for delivery of vasodilator compounds to this vascular bed. Secondly, that a novel vasodilator, the NO donor molecule SE175, is able to induce vasodilation of human chorionic plate arteries and mouse uterine arteries \textit{ex vivo}. SE175 did not cause any adverse effects in human placental explants, indicating tolerance and safety, and SE175 was successfully encapsulated in peptide-conjugated liposomes. Thirdly, treatment of a mouse model of FGR with a dysregulated vascular phenotype with SE175-encapsulated CNKGLRNK homing peptide-conjugated liposomes was able to rescue fetal growth of the smallest fetuses, whilst also improving placental efficiency and reducing markers of placental oxidative stress.

FGR affects 5 - 10% of pregnancies in the UK, and is a significant cause of stillbirth, neonatal morbidity and neonatal mortality (MacKay et al., 2001, Bernstein et al., 2000). Therapeutic interventions for FGR are currently lacking, with intervention being limited to pre-term delivery, which in turn is associated with increased risk of neonatal complications (Alberry and Soothill, 2007). Current management strategies for such complications are limited to increased monitoring and induction of pre-term labour if the condition worsens (Alberry and Soothill, 2007). FGR has also been shown to cause developmental programming, with growth restricted fetuses having an increased risk of disease in adulthood (Galjaard et al., 2013). With short and long term health implications in mind, it is of importance to investigate therapeutic strategies for the treatment of FGR in utero.

A major barrier in the development of therapeutic interventions for the treatment of FGR has been the risk of off-target systemic side-effects in both the mother and the fetus. A strategy that has proven useful in limiting off-target side-effects
in cancer treatment has been the development of targeted drug delivery systems. Previous research from our group has shown the potential of peptide-conjugated liposomes as a means of directly enhancing placental function which influences fetal growth without systemic side-effects, with targeted placental delivery of IGF-II proving more effective in improving fetal growth than systemically administered free IGF-II (King et al., 2016). Although this study demonstrated the potential of targeted liposomes for therapeutic intervention, it utilised peptide sequences that have also been demonstrated to selectively bind to tumour vasculature. With this in mind, this thesis aimed to validate newly identified, placental-specific homing peptides for the targeted delivery of a drug cargo to the placenta; binding of these peptides to cancer cells has not been validated.

Placental-specific homing peptides were identified by phage display, a technique that allows peptides that bind to molecular markers that are unique to specific organs to be determined (Pasqualini and Ruoslahti, 1996). Although this technique allows homing peptides to be identified, it does not identify the molecular marker or receptor that the peptide is binding to on the organ of interest. In the case of the two homing peptides used in this study, a receptor target it not known for either and so future work could focus on identification of these. Despite not knowing the identity of the receptors for the homing peptides, repeated administration of CNKGLRNK peptide-conjugated liposomes did not cause any adverse maternal, placental or fetal effects, indicating that binding of the receptor for this peptide does not cause any adverse downstream effects associated with maintenance of pregnancy.

This study aimed to induce vasodilation of the uteroplacental vasculature, as evidence in humans indicates that impaired blood flow through the uteroplacental unit can contribute to FGR (Alberry and Soothill, 2007). Previous studies have assessed the systemic delivery of vasodilators in an effort to improve fetal growth in FGR fetuses. Systemic delivery of sildenafil citrate was shown to improve fetal weight, but was also found to impair fetal aortic vascular function and caused impaired glucose tolerance in female offspring (Dilworth et al., 2013, Renshall et al., 2014b). A previous study has assessed targeted delivery of growth factors as a means of improving fetal growth (King et al., 2016); with systemic side-effects in mind,
this thesis aimed to determine whether targeted delivery of the vasodilator NO donor molecule SE175, which exhibits a rapid half-life \textit{in vivo} and a short diffusion distance once released, may also have positive effects on fetal growth in a mouse model of FGR. SE175 was shown to not exhibit any cytotoxicity when cultured with human placenta explants; it did not induce apoptosis, and was also well tolerated when delivered in systemically via tail vein injection to pregnant mice. SE175 has not currently been approved for clinical use and therefore future work would need to complete full toxicity studies in order to determine if SE175 is safe for use in humans.

Much previous work in targeted nanoparticles has concentrated on the targeted delivery of chemotherapeutics to tumours, in an attempt to alleviate off-target side-effects in non-cancerous tissues. To this end, biocompatibility and enhanced safety of such nanoparticles has been of lesser importance, with any reduction in systemic side-effects being beneficial. Furthermore, as the primary aim is to kill off the tumour cells, cytotoxic metallic nanoparticles are often used as delivery vehicles. The safety of nanoparticles and their effects on the placenta during pregnancy is a field that is lacking; a limited number of studies have examined nanoparticle biodistribution and fetoplacental toxicity in pregnancy, determining the effects of size, surface charge and composition on pregnancy outcome, and focussing on placental uptake, fetal accumulation and fetal toxicity (reviewed by (Muoth et al., 2016). These limited studies have demonstrated that most of the nanoparticles assessed, for example quantum dots, silica, gold and iron oxide nanoparticles, would not be suitable for use in pregnancy, exhibited placental transfer and fetal accumulation. This review did identify some nanoparticle formulations that are not able cross the placenta; cadmium oxide nanoparticles showed no placental transfer, but still caused fetal-toxicity (Blum et al., 2012). Therefore, liposomes represent an attractive option for nanoparticle mediated delivery of a drug cargo to the placenta. The peptide-conjugated liposomes used in this study did not show any signs of placental transfer, exhibiting no accumulation in any part of the fetus. Repeated administration did not lead to any apparent fetal toxicity, with fetuses showing no obvious signs of deformities. The safety of these peptide-conjugated liposomes in respect to fetal toxicity is an area that requires further investigation; studies to examine long-
term health implications on fetal survival and development would represent logical future work.

Maternal toxicity of peptide-conjugated nanoparticles is also an area that would require further investigation. In this study, maternal side-effects were examined in brief; mice were examined for obvious signs of distress, such as facial grimacing and irregular behaviour patterns (e.g. over-grooming). Treated dams did not display any signs of distress, indicating that treatment was well tolerated. Gross morphological analysis of maternal organs by weight and visual inspection, indicated no apparent abnormalities.

Previous studies have shown that solid lipid nanoparticles induced haematological changes, increasing red blood cell number and haemolysis ratio when administered intraperitoneally to mice (Silva et al., 2014). It has also been shown that polyurethane nanoparticles elicited inflammatory responses in mice, so any nanoparticle formulation being considered for clinical use would need to undergo full biocompatibility evaluation using relevant human in vitro models (Silva et al., 2016). Even more importantly, some nanoparticle formulations have been shown to activate the human complement cascade, leading to production of anaphylatoxins and chemoattractants that can cause pseudoallergic responses in sensitive individuals (Moghimi and Hunter, 2011). The addition of a targeting moiety to nanoparticles may alter the extent of immune cell or complement activation, since the surface chemistry and architecture of nanoparticles are important determinants of biological responses. To date, the effect of the addition of targeting moieties on biological responses has not been investigated.

The biocompatibility of targeted nanoparticles in pregnancy requires unique consideration since immune function in pregnancy is altered. The immune system adapts, becoming more immunotolerant in order to support the pregnancy, thus the responses to nanoparticle challenge may be significantly different and are therefore critical to understand (Ozen et al., 2015). Regulation of the complement cascade is essential for a successful pregnancy and dysregulation of complement has been observed in pregnancy complications such as pre-eclampsia, with selected complement components showing promise.
as biomarkers for the condition (Regal et al., 2015). Furthermore, enhanced complement activity in pregnancy disease states could cause unexpected responses to nanoparticles, which also warrant investigation. Studies to assess nanoparticle biocompatibility in pregnancy have to date focused on nanoparticulate air pollutants and their associated fetal toxicity (Hougaard et al., 2015, Stapleton, 2016). Assessment of the biological response to targeted and non-targeted nanoparticles during pregnancy has not been investigated and will be an essential step of the translational pathway, for clinical development of peptide-conjugated nanoparticles.

Another necessary area of investigation when developing targeted nanoparticles is to understand their in vivo biodistribution, with targeting approaches showing promise in improving the accumulation of nanotherapeutics at their intended sites. This study utilised fluorescent labelling of both lipids within liposomes and conjugated peptides; this approach was able to demonstrate the gross accumulation of the peptide-conjugated nanoparticles in placenta and clearance organs, but it likely did not give an accurate representation of whole body biodistribution, how this changes over time or the absorption, distribution, metabolism and excretion of nanoparticles. Since sequential measurements cannot be taken from the same animal with the methodologies used in this study, more work would be required using a different method to further understand how nanoparticles are processed in real time.

Recent advances have moved toward real-time imaging of nanoparticles in order to determine biodistribution in a non-invasive, real-time and whole body manner. Efforts have been made to develop nanoparticles that are capable of incorporating a positron-emitting nuclide for use with positron emission tomography (PET). Molecular imaging by PET is increasingly being exploited as a tool to understand drug delivery systems and their interactions in vivo (Bartneck et al., 2015).

At present, imaging of nanoparticles by PET is an area that is very much contained within the field of oncology, with emphasis being placed on determination of tumour size, location and its response to treatment (Willmann et al., 2008, Jacobson and Chen, 2013, Janib et al., 2010). Studies have
assessed the use of targeted nanoparticles as theranostic imaging agents, for example to determine tumour presence and size, to determine chemokine receptor expression in inflammatory atherosclerosis, to assess the initiation of inflammatory response in the murine fetal brain and as a guide for photothermal therapy (Hansen et al., 2015, Chen et al., 2014, Luehmann et al., 2016, Girardi et al., 2015). There have been some efforts to use PET to elucidate the comparative biodistribution of targeted nanoparticles as compared to their non-targeted counterparts in the field of oncology; the few studies to date have shown that PET can be used to compare targeted and non-targeted nanoparticle biodistribution (Hong et al., 2012, Yang et al., 2011). PET imaging has also been used in limited studies of placental growth and function, including imaging of placental trophoblastic tumours and determination of placental amino acid transport (Cerci et al., 2015, Berglund et al., 1990). However, the use of PET imaging to assess placental-specific targeting has not been investigated, but could easily be exploited to provide a greater understanding of targeted and non-targeted drug delivery to this unique organ, generating high resolution placental images.

In thinking about future clinical translation of peptide-conjugated liposomes as a potential treatment for FGR, a few factors must be considered. Scale up of the preparation is possible, with equipment such as the Maximator® high pressure extruder being able to synthesise liposomes on a large, reproducible scale. Stability of the preparation has been assessed, however further work would have to ensure that all preparations were tested to the International Conference on Harmonisation (ICH) guidelines. The route of administered to pregnant women is essential to consider in regards to clinical translation; the preparation demonstrated in this study must be intravenously administered and as such it is likely that any treatments would need to take place in clinical by IV infusion every few days. In order to move toward clinical translation, first in human trials would need to be carried-out, however it is likely that any first in human trials would take place in women having terminations, followed by in women who present with severe FGR in which there is a dismal prognosis.

Novel therapeutics for use in pregnancy is an area in which it has proven difficult to move from bench to clinical, with clinical translation being a sticking point. Recently, a novel gene therapy using adenoviral delivery of VEGF, which has
been shown to increase uterine blood flow and fetal growth, has made it from guinea pigs to sheep and now is recruiting for clinical trial (Sheppard et al., 2016, Mehta et al., 2012, Carr et al., 2015, Mehta et al., 2015), so this shows promise for clinical translation of this work.

6.2. Conclusions

- Placental-specific homing peptides CNKGLRNK and CRSGVAKS can be conjugated to liposomes to facilitate targeted delivery of an encapsulated cargo to the uteroplacental unit.

- The novel vasodilator SE175 is able to cause vasorelaxation of human chorionic plate arteries and mouse uterine arteries \textit{ex vivo} and can be encapsulated in peptide-conjugated liposomes.

- Treatment of wild-type C57 mice with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes did not alter fetal or placental weight, nor placental efficiency; no adverse effects were observed indicating safety of this therapeutic strategy.

- Treatment of the eNOS$^{-/-}$ mouse model of FGR with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes significantly improved fetal weight and prevented any fetuses from being below the tenth centile of weight, the clinical definition of FGR.

- Treatment of the eNOS$^{-/-}$ mouse model of FGR with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes and free SE175 significantly improved placental efficiency; however, only targeted delivery of SE175 was able to translate to an improvement in fetal growth, providing evidence to support the use of SE175-encapsulated CNKGLRNK peptide-conjugated liposomes for the treatment of FGR.

6.3. Short and long term plans for future Work

- Determine the receptor targets of the placental specific homing peptides used in this study.

- Undertake a full toxicity study of SE175 in mice to determine suitability for clinical use.
- Assess potential maternal toxicity and the safety profile of peptide-conjugated liposomes, including the potential for immune cell and complement activation, both \textit{in vitro} using biological samples from pregnant women and \textit{in vivo} in pregnant mice.
- Further investigate the effects of treatment with SE175-encapsulated CNKGLRNK peptide-conjugated liposomes on neonatal outcome by off-spring follow up.
- Examine real-time biodistribution and clearance of targeted and non-targeted nanoparticles using PET.
- Determine if the homing-peptides used in this study can selectively bind to the uterine arteries of non-pregnant mice; if so, examine the possibility of using this targeted drug delivery strategy to deliver compounds the non-pregnant uterus, e.g. for improvement of implantation, for the treatment of endometriosis.
- Investigate the use of other classes of compounds for the treatment of other pregnancy complications, e.g. targeted delivery of compounds for the prevention of pre-term labour.
Chapter 7 References


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