Exploiting the placenta for nanoparticle-mediated drug delivery during pregnancy

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ABSTRACT

A major challenge to treating diseases during pregnancy is that small molecule therapeutics are transported through the placenta and incur toxicities to the developing fetus. The placenta is responsible for providing nutrients, removing waste, and protecting the fetus from toxic substances. Thus, the placenta acts as a biological barrier between the mother and fetus that can be exploited for drug delivery. Nanoparticle technologies provide the opportunity for safe drug delivery during pregnancy by controlling how therapeutics interact with the placenta. In this Review, we present nanoparticle drug delivery technologies specifically designed to exploit the placenta as a biological barrier to treat maternal, placental, or fetal diseases exclusively, while minimizing off-target toxicities. Further, we discuss opportunities, challenges, and future directions for implementing drug delivery technologies during pregnancy.

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1. Introduction

Approximately 90% of pregnant women take over-the-counter or prescription medications during pregnancy, although many are not thoroughly tested in pregnant animal models or clinical trials [1,2]. The exclusion of pregnant women from clinical trials is due to the relatively small patient population size and ethical concerns regarding safety to the unborn fetus [3]. However, physiological changes during pregnancy, such as a 30–40% increase in cardiac output, alter drug pharmacokinetics [4]. Understanding drug pharmacokinetics during pregnancy, and how these therapeutics interact with the placenta to be transported to the fetal compartment, is critical to ensure therapeutic efficacy and safety for both the mother and fetus.

Prior to the 1960s, the placenta was thought to be an impenetrable barrier that protected the fetus from any toxins or xenobiotics present in the maternal circulation. However, this idea was challenged when thalidomide, a medication that was used to treat morning sickness during pregnancy, was found to cause birth defects [2]. This instigated a field of research geared towards understanding the transplacental transport of therapeutics during pregnancy. Once developed at 10–12 weeks of gestation, the placenta is solely responsible for providing nutrients, removing waste products, and protecting the fetus from foreign and toxic substances in maternal circulation [4,5]. In terms of drug delivery, the placenta serves as a highly organized and functional biological barrier between the mother and the fetus. As a biological barrier, the placenta can be exploited for targeted drug delivery by surpassing, targeting, or inhibiting the passage of therapeutics to treat diseases and disorders during pregnancy [1,6]. In this Review, we present recent studies that use drug delivery technologies, and in particular nanoparticles (NPs), to treat fetal, placental, or maternal diseases exclusively, by controlling drug interactions with the placenta.

NPs have been extensively studied to treat cancer and other diseases in adults, and several technologies are in clinical trials [7–10]. Therapeutic molecules, such as small molecules, nucleic acids, and antibodies, are encapsulated within or conjugated to the surface of NPs, which yields several benefits over the unbound molecules [8,11]. First, NPs improve the stability of poorly soluble molecules and protect the encapsulated molecules from degradation from serum endonucleases and immune recognition [10,12]. Further, they enable tissue specificity by adding surface modifications including targeting ligands, or stealthing agents, or by changing the NP composition itself [13]. Lastly, NPs enable cell uptake and endosomal escape for intracellular drug delivery, and they offer controlled degradation and drug release over time or in response to internal or external stimuli [10,12]. Thus, NPs can greatly improve the therapeutic efficacy and decrease off-target effects compared to unbound molecules, supporting the clinical translation of these technologies. The first NP technology to be introduced into the clinic in 1995 was Doxil, a NP formulation comprised of the chemotherapeutic doxorubicin encapsulated within liposomes that is used to treat solid tumors with reduced cardiotoxicity compared to free doxorubicin [14]. More recently in 2018, the first lipid NP platform for nucleic acid delivery, ONPATTRO, was approved by the U.S. Food and Drug Administration (FDA) to treat hereditary transthyretin-mediated amyloidosis, paving the way for the clinical translation of nucleic acid-NP conjugates [15]. While drug delivery platforms have been extensively studied to treat adult diseases and are now used clinically, their use for treating diseases and disorders during pregnancy is in early stages.

This article focuses on NP drug delivery technologies and their abilities to control interactions with the placenta to enable maternal, placental, or fetal delivery exclusively following intravenous administration. NPs designed to avoid crossing the placenta would be applicable to treat a range of maternal conditions including pre-existing conditions, cancers, and pregnancy complications without harm to the developing fetus. Alternatively, targeting NPs to the placenta can treat pregnancy disorders that originate in the placenta, such as pre-eclampsia or fetal growth restriction, which are life threatening to the mother and fetus.

Lastly, the development of NPs that can actively cross the placenta can be used to non-invasively treat fetal diseases *in utero*, which can greatly improve therapeutic success compared to treatment post-birth. In this Review, we discuss drug delivery technologies, with a focus on NPs, that have been developed specifically for use during pregnancy to treat both pre-existing conditions and pregnancy-related diseases of the mother, placenta, or fetus following intravenous administration to the mother. Here, our goal is to shed light on how NP drug delivery technologies can be specifically designed and engineered for use during pregnancy while ensuring both maternal and fetal safety.

2. The placenta as a biological barrier

The placenta rapidly develops starting in early pregnancy into a highly organized and functional organ to support fetal growth [4]. The main functions of the placenta are to provide the fetus with nutrients from the mother and excrete waste back into the maternal circulation [4,16]. Further, the placenta serves to protect the fetus from infection, immune attack from the maternal immune system, and harmful environmental toxins present in the maternal circulation [5,16]. Thus, the placenta is a biological barrier between the fetus and the mother that facilitates maternal-fetal transport and can be exploited to control drug delivery exclusively for maternal, placental, or fetal therapy. Below, we describe the physiology of the placenta in the context of developing drug delivery technologies that yield specific interactions with the placenta. For a more thorough description of placenta development and physiology, we refer readers to previously published literature [4,16,17].

Trophoblasts are the main placenta cells that control the formation and function of the placenta (Fig. 1A). Following blastocyst implantation into the uterine wall, trophoblasts surrounding the blastocyst proliferate and differentiate into an inner layer of cytotrophoblasts and outer layer of syncytiotrophoblasts [4,18]. During placental development, cytotrophoblasts fuse to form syncytiotrophoblasts that invade the uterine wall and induce remodeling of the maternal spiral arteries (Fig. 1A) [4,16,19]. As syncytiotrophoblasts and cytotrophoblasts proliferate, the branched chorionic villi are formed, which are comprised of fetal veins and arteries and are the main functional units of transport in the placenta [4]. The villi are surrounded by an internal layer of cytotrophoblasts and an external layer of syncytiotrophoblasts separating the villi from the intervillous space (Fig. 1B). Fetal capillaries extending from the villi towards the fetus carry oxygenated blood and nutrients to the umbilical vein, and fetal arteries carry nutrient-depleted blood back to the villi for excretion through the maternal circulation [4,20]. The intervillous space is filled with extravillous trophoblasts (EVTs) that extend from the villi to the uterine basal plate to invade and remodel the decidua and arteries, establishing blood flow to the placenta and fetus [4,19].

As the placenta develops throughout pregnancy to support the growing fetus, the chorionic villi extend additional and more complex branches to support increased blood and nutrient supply to the fetus. Further, the syncytiotrophoblast population grows as cytotrophoblast layer that lines the chorionic villi becomes thinner and less continuous in later stages of pregnancy [16,21]. The maternal portion of the placenta also undergoes changes as the pregnancy progresses. Early in pregnancy, EVTs plug the spiral arteries to restrict blood flow to the placenta, but these arteries become remodeled later in pregnancy to accommodate the higher required blood volume [19]. These physiological changes in the placenta at different stages of gestation alter blood flow and circulation, and must be considered in the development of drug delivery technologies for maternal, placental, or fetal diseases. Below, we describe some of the transport mechanisms that drive delivery through the placenta or out of the placenta that can be exploited for drug delivery during pregnancy.
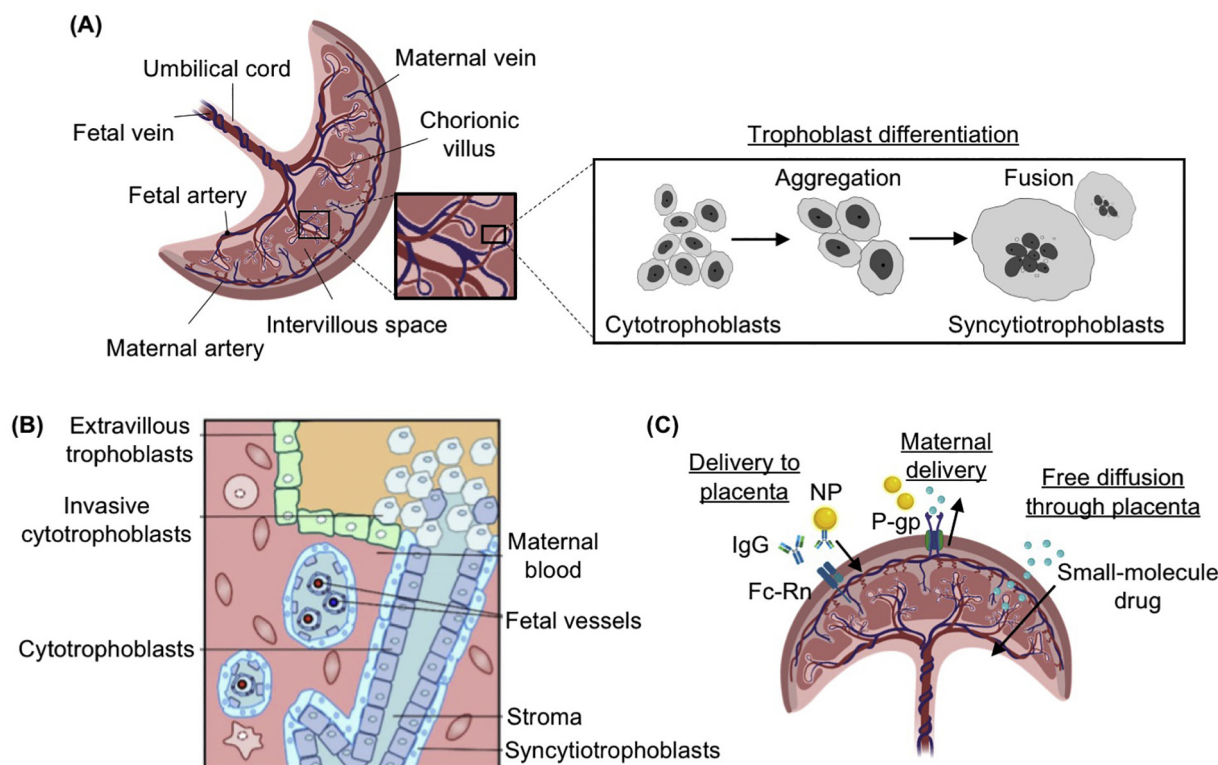
Fig. 1. (A) Schematic showing the overall physiology of the placenta and trophoblast proliferation and differentiation. As the placenta develops, cytotrophoblasts fuse to form multi-nucleated syncytiotrophoblasts. Trophoblast differentiation figure adapted with permission from Reference [47]. (B) Cross-section of a chorionic villi, which is the main functional unit for placental transport, showing an inner layer of cytotrophoblasts and and an outer layer of syncytiotrophoblasts. Adapted with permission from Reference [18]. (C) Active and passive transport mechanisms in the placenta that can be exploited for drug delivery.

2.1. Drug transport in the placenta

Early in pregnancy, when the placenta is not fully developed (prior to 10–12 weeks of gestation), nutrient transfer is histotrophic [4,22]. After this phase, the functional placenta is responsible for maternal-fetal transport, which can be through diffusion or active transport mechanisms (Fig. 1B) [23]. In this Review, we describe active transport as a mechanism by which natural or synthetic substances engage with transporter proteins on cells to be carried through the placenta. This form of active transport is critical for drug delivery technologies that are large compared to small molecule therapeutics, and thus are not freely diffused through the placenta. Small molecule therapeutics, such as some chemotherapies, may interact with the placenta by passive and active mechanisms, depending on the age of gestation and the physicochemical properties of the molecules (Fig. 1C).

Though advances have been made to establish active transport mechanisms for the development of drug delivery technologies during pregnancy, there is still much to explore (Fig. 1C) [24]. Active transport of immunoglobulins (IgG) provides a substantial foundation for placental and fetal-targeted drug delivery technologies. IgG antibodies represent a key class of molecules that can cross the placenta regardless of their large size (150 kDa). Importantly, active transport of IgG is dependent on gestational age (transport begins mid-gestation) and the subtype of IgG (IgG 1 is transported more than other IgG subtypes) [22,25]. Other antibody structures, including IgM and IgA, are unable to cross the placenta and remain in maternal circulation. IgG antibodies cross the placenta by engaging neonatal major histocompatibility complex-related (MHC) Fc receptors (FcRn) that are expressed on the apical membrane of the syncytiotrophoblasts [24]. Following uptake into the syncytiotrophoblast layer, it is hypothesized that receptors on the endothelium of placental vasculature mediate

receptor-mediated transport across the placenta, although this is still under investigation [24,26].

I think FcRn mediates the transport of IgG through the placenta via transcytosis rather than active transport. This may be splitting hairs a bit tho. In addition to active transport through FcRn, clathrin and caveolin proteins have also been shown to mediate active placental transport, although this transport mechanism requires further investigation [27]. Towards this goal, Rattanapinyopituk et al. intravenously injected pregnant mice with gold NPs to study clathrin and caveolin-mediated placental transport [28]. The results showed that administration of gold NPs increased clathrin expression in syncytiotrophoblasts and fetal endothelial cells, and increased caveolin expression in the fetal endothelium [28]. These results demonstrate that clathrin- and caveolin-mediated cellular uptake may be an additional active transport mechanism that can be exploited for placental drug delivery [27,28]. Drug delivery technologies can use IgG- or clathrin/caveolin-mediated active transport by conjugating antibodies or other targeting molecules to NPs to drive active transport across the placenta, and we present several examples of active placental targeting in this Review [29].

Other types of active transport mechanisms present in the placenta also hold potential to be exploited for drug delivery during pregnancy. However, these mechanisms haven't been leveraged for drug delivery to the same extent as those described above. For example, organic anion-transporting polypeptides (OATPs) are a family of transporter proteins expressed by the placenta that mediate maternal-fetal transport of hormones, waste, and metabolites [30]. Likewise, other types of transport such as amino acid, glucose, and transferrin transporters mediate transport of specific substrates for maternal-fetal transfer across the placenta [31,32]. Of note, the transferrin receptor plays a critical role in iron transport to the placenta *via* receptor-mediated endocytosis [31]. These active transport mechanisms that mediate transfer of specific substrates across the placenta have not been extensively

studied for drug delivery. However, the known mechanisms of placenta transport can be exploited with the development of drug delivery technologies to enable highly specific receptor-mediated transport into the fetal compartment. Moving forward, we anticipate that these active transport mechanisms will be further evaluated to mediate placental and fetal drug delivery.

In opposition to the goal of crossing the placenta, researchers have also explored active transport mechanisms to enable efflux out of the placenta following uptake, which is applicable for achieving maternal delivery while minimizing fetal exposure. For example, ATP-binding cassette (ABC) transporters expressed on trophoblasts are responsible for drug efflux to protect the fetus from exposure [33]. The most widely studied ABC transporter in the placenta is P-glycoprotein (P-gp), which plays a critical role in the efflux of molecules out of trophoblasts [22,33–35]. Importantly, inhibiting P-gp expression in animal models increases drug accumulation in the fetus, leading to detrimental effects on fetal health [33,36]. Thus, P-gp-mediated efflux is critical to protect the fetus from xenobiotics, but it can also be exploited for maternal drug delivery during pregnancy. For example, coating NPs with P-gp targeting molecules holds potential for the therapeutics to be continuously effluxed from the placenta to treat maternal diseases while avoiding fetal toxicities.

Here, we provide an introduction to these transport mechanisms to demonstrate the vast potential to develop drug delivery technologies for active placental transport. For a more thorough discussion of other types of trans-placental transport including passive transport and other types of active transport not described here (metabolism, hydrolysis, oxidation, and others), we refer readers to published literature focused solely on this topic [4,22]. Below, we describe experimental models used to study placenta transport, and we provide examples of innovative drug delivery technologies geared at exploiting these transport mechanisms to enable controlled drug delivery during pregnancy.

3. Experimental models to study drug delivery during pregnancy

There are multiple experimental techniques established for studying transport through the placenta, including *in vitro, ex vivo,* and *in vivo* models, that can be used and adapted for studying drug delivery technologies (Fig. 2) [37]. Below, we briefly introduce these experimental models, and we discuss the advantages and limitations of each. In addition to discussing traditional experimental techniques, we will also introduce several recent advances that provide new opportunities to study the placenta in the laboratory.

3.1. In vitro experimental techniques to study the placenta

In vitro assays that use static culture environments to study placenta transport enable high-throughput analysis of drug delivery technologies or therapeutics simultaneously. Two commonly used *in vitro* experimental techniques are 2D cell culture, in which placental cells are treated with therapeutics directly to study uptake and the therapeutic effects, and transwell assays (Fig. 2A). These techniques use either cell lines, such as the human placenta choriocarcinoma cell line BeWo, or primary placenta cells collected from animal models or human tissue [38–41]. In transwell assays, cell culture inserts are coated with either (i) a trophoblast monolayer on the apical (maternal) side of the membrane [42], or (ii) a co-culture of a trophoblast monolayer on the apical side and a human placental endothelial cell (HPEC) monolayer on the basolateral side [38]. Following the formation of cell monolayers, therapeutics are added and transport through the cell layers is evaluated [40].
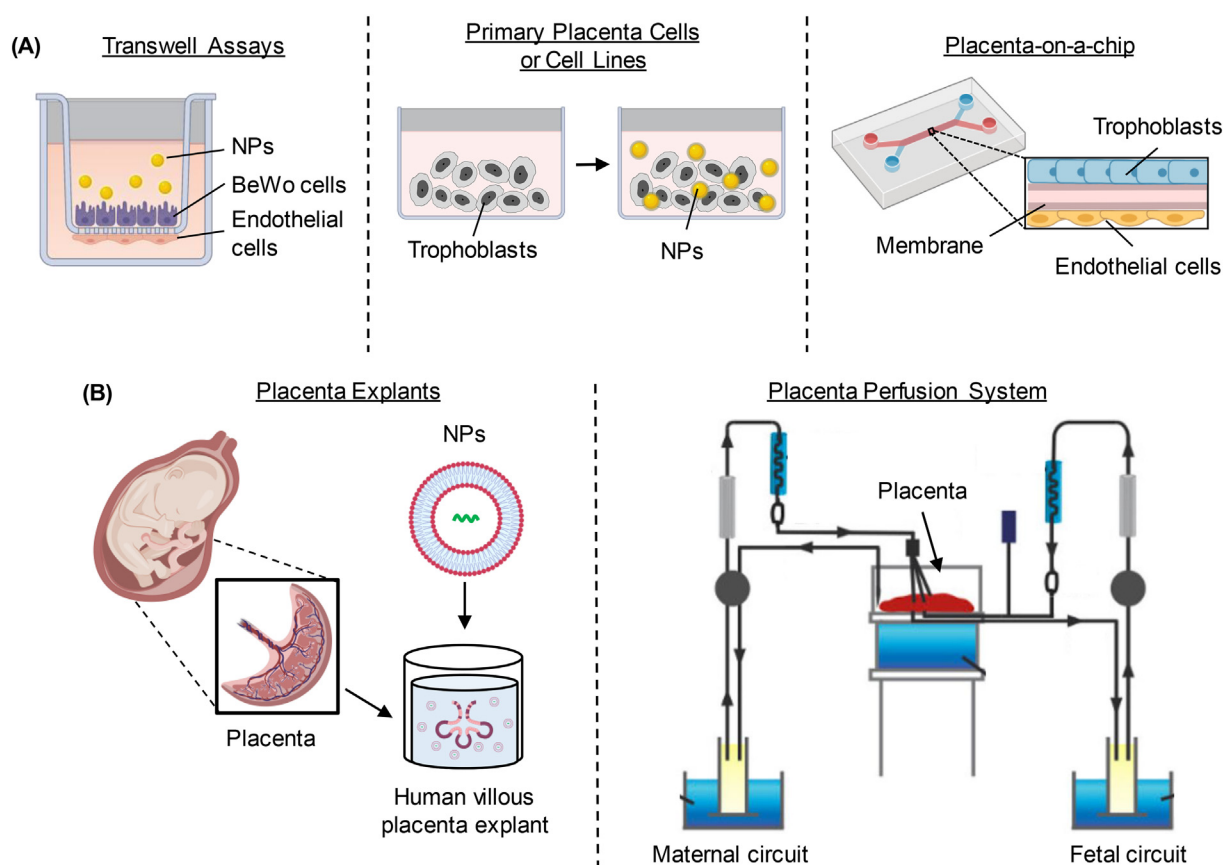

Fig. 2. (A) *In vitro* and (B) *ex vivo* experimental models to study drug transport through the placenta. *In vitro* models utilize cell lines or primary cells to study how therapeutics are taken up and transported through cell monolayers. *Ex vivo* experiments utilize human or animal placenta tissue to study placental uptake and transport. Figures adapted with permission from References [38,48](A), and [49,6](B).

While these assays provide a relatively simple and high-throughput means of assessing placental transport using relevant cell types, there are several aspects that limit their applicability to human placental transport. First, the separation of the cell layers by the transwell membrane is not present in placental tissue. Towards the goal of developing physiologically relevant *in vitro* techniques, Muoth et al. developed an advanced *in vitro* model that uses engineered 3D microtissues [43]. This platform is comprised of placental fibroblasts encompassed by a trophoblast layer and provides direct cell-cell contact to more closely mimic cellular and tissue behavior *in vivo* [43]. The authors showed increased trophoblast differentiation and reduced toxicity in response to NP treatment using their 3D microtissues compared to 2D culture, providing a new high-throughput *in vitro* technique to study drug interactions with the placenta [43].

In addition to cell-to-cell interactions, another limitation is that the static *in vitro* environment is not physiologically comparable to the dynamic *in vivo* environment [44]. Recently, placenta-on-a-chip technologies have emerged to more closely replicate fluid flow within the placenta (Fig. 2B). These technologies utilize micro-engineered devices with both maternal and fetal compartments to more closely replicate the complex *in vivo* dynamics (Fig. 2A) [23]. Such devices have been used to study bacterial infections [45], caffeine [46], glucose [47], and NP transport [48], demonstrating the vast applicability of these technologies. In an elegant placenta-on-a-chip study, Blundell et al. developed a multilayered system with trophoblast and fetal endothelial cells under fluid flow that enabled the formation of syncytialized epithelium and microvilli representative of the human placenta [47]. They showed that their device closely mimics glucose transport compared to an *ex vivo* placenta perfusion model, demonstrating that sophisticated microfluidic designs have immense potential to study placental transport *in vitro* [47].

3.2. Ex vivo models of placenta transport

Although not as high-throughput as the *in vitro* assays described above, *ex vivo* experimental models offer the opportunity to study native human or animal placenta tissue in the laboratory (Fig. 2B) [41]. Below, we discuss two of the most commonly used *ex vivo* techniques for studying placental transport: tissue explant culture and placenta perfusion models.

For tissue explant culture experiments, placenta tissue is collected from the placenta immediately following cesarean or vaginal delivery. Explant culture has been used for drug delivery [49], diagnostics [50], and for harvesting extracellular vesicles [51], and the precise experimental setup depends on the desired goal. Broadly, these experiments involve extraction of placenta villous explants that are typically collected from first or third trimester placentas. The extracts are cultured in well plates or transwell inserts with or without matrix support, such as collagen [52]. In this case, the matrix support allows for the trophoblasts to invade through the matrix and transwell insert into the bottom of the well in order to evaluate trophoblast invasion [52]. Explants can be cultured for up to 11 days and are continuously monitored to evaluate tissue integrity, as any damage during culture can negatively impact tissue function and experimental results [16,41]. For drug delivery experiments, the explants in culture are treated with the test therapeutics and are then analyzed for therapeutic efficacy, toxicity, or uptake, among any other experimental output of interest (Fig. 2B). A critical benefit of explant experiments is that multiple explants can be collected and cultured from a single placenta, which is particularly useful to study tissue function in response to various drugs or drug delivery platforms simultaneously [49]. In addition to drug delivery, placenta explants can also be used to study physiological differences between tissue collected from abnormal and healthy pregnancies for diagnostics and to identify disease biomarkers [41,50]. In a less common but highly sophisticated application, placenta explants are used to study placental secretion of extracellular vesicles [51,53–55]. These extracellular

vesicles carry fetal molecular cargo, such as nucleic acids, and can be used as a diagnostic tool to detect pregnancy complications [55]. Thus, explants are an invaluable tool for secretion of extracellular vesicles that can then be used for diagnostic purposes, and they hold potential as a drug delivery technology, the latter of which we discuss in the "Future Directions" section of this Review.

The benefit of the explant models described above is that they use human tissue to study the placenta on a relatively small experimental scale. However, explant culture does not directly recapitulate how the placenta functions in the dynamic *in utero* environment during pregnancy. The most physiologically relevant *ex vivo* experiment is the placenta perfusion model (Fig. 2B). This model uses whole placentas or a dissected intact cotyledon to study how natural or synthetic molecules are transported through the placenta. *Ex vivo* perfusion setups can be open circulation for studies utilizing steady state concentrations, or closed circulation to evaluate the transport of drugs from the maternal to fetal compartment, the latter of which we discuss below [20,39]. To study drug transport, placenta tissue is perfused from a "maternal" compartment containing the therapeutic of interest. The output into the "fetal" compartment and the placenta tissue itself are evaluated for drug content and tissue damage [6,20,39,56]. To ensure the integrity of the perfusion system, multiple quality assurance checkpoints need to be assessed throughout the experiments including leaks, changes in pH, glucose consumption, flow, and pressure throughout these experiments [20,27,39,57]. While *ex vivo* perfusion experiments provide a high level of physiological relevance, they are low-throughput as it can be challenging to acquire placentas, and only one experiment can be completed with each placenta. Further, the complexity of perfusion models results in a success rate of the experimental setup of only 15–20% [6]. Further, repeatability and validation is challenging as researchers utilize different experimental protocols across laboratories [6]. However, *ex vivo* perfusion is the only experimental model that utilizes the bulk human placenta, making it highly relevant for studying transport and function.

3.3. In vivo animal models of pregnancy to study the placenta

A critical step towards the translation of therapeutics and drug delivery technologies into the clinic requires the use of animal models to evaluate placenta transport and therapeutic efficacy in living systems. Mouse models are most commonly used in initial pre-clinical studies of pregnancy and placental transport. The short gestational period of mice (19–21 days) and their ability to carry >10 fetuses simultaneously makes mice ideal for high-throughput initial *in vivo* studies of placental transport and toxicity. Mouse placentas share a key structural similarity to the human placenta in that both are hemochorial, which means that the trophoblast layer is in direct contact with maternal blood [58,59]. However, several key differences exist between the mouse and human placenta, and reproduction entirely, that must be considered when using mice as a model organism. For example, unlike humans, mice have an inverted yolk sac, or choriovitelline, placenta [58]. When investigating therapeutics or drug delivery technologies in mice, this key difference may lead to higher levels of toxicity to placenta development compared to what would be experienced in humans [58]. Further, therapeutics may be transported through the yolk sac placenta differently than the human placenta, and may be even more protective against some extraneous substances [58]. Another important difference between the mouse and human placenta is the inter-placental anatomy. The mouse placenta has junctional and labyrinth zones that are responsible for endocrine function and maternofetal exchange, respectively [60]. Comparatively, the human placenta contains two trophoblast layers in early pregnancy that evolves into one functional zone, containing layered trophoblasts with villi extending into the maternal blood space [23,60].

While their large litter size and short gestation makes mice an attractive animal model for initial pre-clinical investigations, it is also

critical to consider these key differences that may impact how therapeutics are transported through the placenta into the fetal compartment. Other rodent models, such as rats and guinea pigs, are also used in studies of placental transport. Similar to mice, rat and guinea pig placentas are hemachorial [61] but have physiological similarities to humans that mice do not. Namely, placentation in rats involves deep invasion of trophoblasts, making the spiral artery remodeling more consistent with humans. However, rat placentas are similar to mouse placentas in that they contain junctional and labyrinth zones, rather than the trophoblast layers seen in human placentas [61]. Alternatively, guinea pig gestation is longer than mice or rats and can be split into three trimesters to be more comparable to human gestation [62]. However, two critical differences between the guinea pig and human placentas is that the guinea pig experiences labyrinthine placentation and a lobulated placenta structure [62]. The three rodent models described here, including mice, rats, and guinea pigs, each hold important similarities and differences in gestation and placenta development compared to humans. Thus, the choice of rodent models in early studies of drug delivery should be carefully chosen based on the most important features to test based on the application.

The use of larger animal models such as sheep or non-human primates that more closely resemble the human placenta and gestation are necessary as drug delivery technologies approach clinical translation. Two widely used large animal models for pregnancy-related studies are sheep and nonhuman primates. Sheep offer long gestations of ~152 days, making them amenable to longer term studies and advanced procedures that require testing in animals before being implemented in humans [63]. The cotyledon and vascular structure in sheep placentas are similar to the humans, making it useful to study placental development and transport [63]. However, the sheep placenta is epitheliochorial, contains binucleate cells that invade the epithelium, and is characterized by superficial implantation, all of which are stark differences from the human placenta [63]. Nonhuman primates are the most physiologically relevant models for pregnancy compared to humans [63,64]. They offer long gestations, similar hormonal control throughout pregnancy, similar uterine physiology and villous placentation, among other similarities [63]. The main difference between nonhuman primates and humans is that the primate trophoblast invasion is not as deep as what is seen in humans [63]. However, due to ethical considerations, cost, and long gestational periods, nonhuman primates are not always feasible for use in initial investigations of drug delivery technologies during pregnancy. Therefore, this Review focuses on pre-clinical studies in mice and rats due to their widespread use in initial investigations, but we refer readers to articles that focus on placenta anatomy and physiology for more thorough discussions of placentation and placenta structure across species [58,60]. Below, we discuss how the experimental techniques and placenta models described above are used to study and design drug delivery technologies for use during pregnancy.

4. Need for drug delivery technologies to treat maternal, placental, or fetal disorders during pregnancy

The need to develop drug delivery technologies specifically for use during pregnancy stems from critical physiological changes, such as a 30–40% increase in cardiac output, that occur during pregnancy [4]. Drug pharmacokinetics, including blood clearance and biodistribution, are different in pregnant *versus* non-pregnant women due to the increased blood flow and volume necessary to support fetal growth [4]. The majority of therapeutics used clinically are not targeted to specific tissues, and depending on their physicochemical characteristics, they can accumulate in both maternal and fetal tissues resulting in off-target toxicities [65]. The overarching goal of drug delivery technologies, such as NPs, is for selective accumulation of the therapeutics within maternal tissues, the placenta, or fetal tissues, while minimizing exposure to non-targeted tissues.

There are multiple opportunities to use NPs to study placental development and to safely treat disorders and diseases during pregnancy. NPs enable active targeting of cells to promote uptake, require lower dosages, and provide enhanced cellular uptake than naked molecules [8,66]. NPs can be used to study placental development throughout gestation by comparing placental uptake and transport of different NP platforms in animal models and *ex vivo* in human tissue [24,44,67]. In an elegant example of using nanoparticles to study placental development, Yang et al. showed that gold nanoparticles administered intravenously had higher fetal accumulation when injected early in mouse gestation (<E11.5) compared to later in gestation (E > 11.5) [67]. This indicates that the placenta has the ability to protect the fetus from foreign substances as it develops during pregnancy [67]. Repeating this study with other types of NPs could provide valuable information comparing several nanomaterials throughout gestation that could subsequently be used to develop drug delivery platforms to treat diseases at different stages of pregnancy. The discussion below describes how the benefits afforded by NPs can enable the exploitation of the placenta as a biological barrier to safely treat maternal, fetal, and placental diseases during pregnancy.
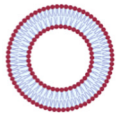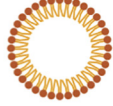
The NP design features that dictate tissue specificity for drug delivery both in pregnant and non-pregnant models include the type of biomaterial, size, surface charge and modifications, and surface ionization [16,24,44,67–70]. In general, smaller, lipophilic materials cross the placenta more readily than larger NPs with a cutoff of ~25 nm in diameter, although this also depends on the age of gestation, transport mechanism, and material composition, among other properties [24,69]. Therefore, larger platforms may be more amenable to treating maternal or placental diseases while avoiding placental transport to the fetal compartment [69]. Surface charge is another property that dictates placental transport, as cationic NPs cross the placenta more than anionic NPs, which is likely due to higher trophoblast uptake through the negatively charged cell membrane [69]. However, positively charged NPs are also rapidly cleared from the bloodstream and induce higher toxicity than negatively charged NPs due to increased tissue and cell uptake. Thus, surface charge must be carefully controlled to balance between tissue toxicity and placental uptake and transport. In the following sections, we present specific examples of how these NP characteristics, and others, enable control over delivery to maternal, placental, or fetal tissues. Further, we present specific examples of drug delivery technologies that have recently been developed for use during pregnancy by exploiting the placenta.
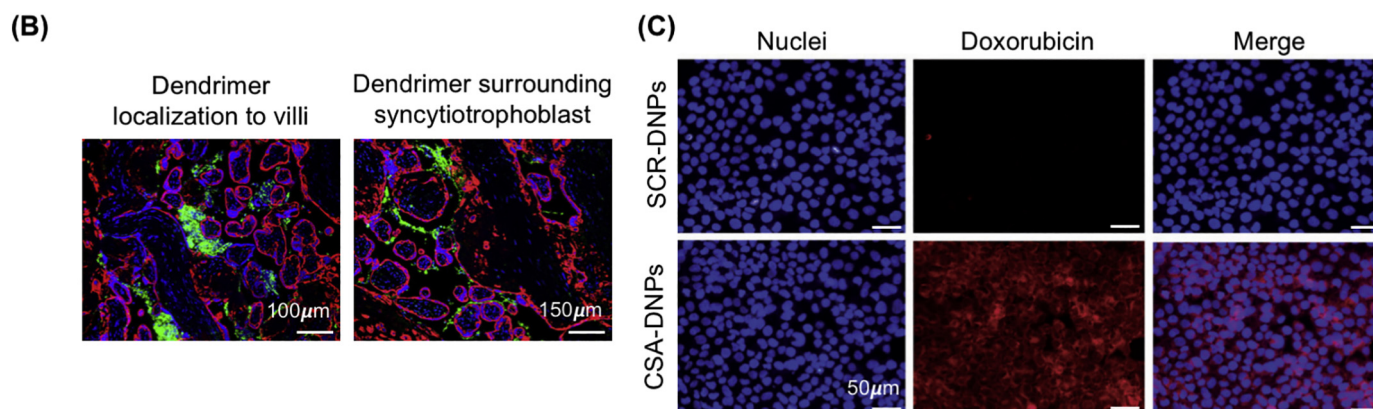
5. Drug delivery technologies to treat maternal conditions during pregnancy

The overarching goal of drug delivery to treat maternal diseases is to avoid crossing the placenta to minimize exposure and toxicity to the developing fetus. Many therapeutics currently used to treat maternal pregnancy complications and pre-existing conditions hold the risk of crossing the placenta and interfering with normal fetal and/or placental development. The extent of placental transport depends on the properties of the therapeutic, such as size and charge, and the gestational age of the pregnancy [24]. NPs engineered to minimize placental crossing can enable safe and effective maternal therapy while avoiding fetal exposure and toxicity. One of the first demonstrations of NP-mediated maternal therapy used liposomes with encapsulated valproic acid (VPA), which is an antiepileptic drug that induces fetal malformations when freely administered. Encapsulating VPA within liposomes decreased placental transfer to the fetal compartment compared to free VPA in an *ex vivo* human placenta perfusion model, demonstrating that NPs can safely deliver therapeutics that are otherwise toxic to the fetus [71].

Since this early study, researchers have investigated other types of NPs for maternal drug delivery including gold, silver, and polymeric NPs (Fig. 3A) [72–75]. Of note, dendrimers, which are branched, tree-like polymers, hold great promise for drug delivery [75–78]. The transplacental transport of poly(amidoamine) (PAMAM) dendrimers has

(A) Nanoparticle Technologies for Maternal Drug Delivery

NP	NP and Experimental Design	Experimental Results and Conclusions
Liposomes	• Size: 233 nm • ∝-tocopherol modification • *Ex vivo* perfusion model	• First demonstration of reduced placental transport compared to free drug [71]
	• Size: 200 nm • Oxytocin antibodies • Mouse models and explants	• Increased uterus localization and decreased contractions compared to nontargeted NPs for prolonged pregnancy [84]
Polymers PAMAM	• Size: 5.6 nm • Charge: neutral • *Ex vivo* perfusion model	• 18-fold lower fetal accumulation compared to maternal circulation • Dendrimers mostly seen in intervillous spaces and outer rim of branches [75]
PLGA	• Sizes: 82 – 110 nm • Net charge: -30 – -20 mV • CSA targeting peptides • Choriocarcinoma mouse model	• NPs targeted choriocarcinoma cells and CSA targeting decreased primary and secondary tumor formation [93]
Micelles	• Sizes: 33 – 174 nm • Net charge: -44.5 – -3.2 mV • PEG-PLA, clonazepam NPs • *In vitro* transwells	• Micelles reduced transport through BeWo cell layers 26% compared to free drug [74]
Gold	• Sizes: 10 – 30 nm • PEG surface coating • *Ex vivo* perfusion model	• PEGylation of small gold NPs inhibits their ability to cross the placenta [72]
	• Sizes: 1.4, 18, and 80 nm • Charge: anionic • sulfonized triphenylphosphine (S-TPP) modification • *In vivo* rat model	• 80 nm anionic gold NPs were unable to cross the placenta and accumulate in fetal tissues • Strict size dependency for S-TPP-modified gold NPs [68]
Silver	• Sizes: 2 – 15 nm • Net charge: -32.5 – -10.7 mV • Sodium carboxylate (COON) or PEG modifications • *Ex vivo* perfusion model	• Transport dependent on size and modification • Aggregated NPs remained in maternal circulation • PEG modification decreases transport of small NPs (<30 nm) compared to COON [73]

(B)

Dendrimer localization to villi

Dendrimer surrounding syncytiotrophoblast

100μm   150μm

(C)

	Nuclei	Doxorubicin	Merge
SCR-DNPs			
CSA-DNPs	50μm		

Fig. 3. (A) NP drug delivery technologies that have been studied for maternal drug delivery during pregnancy. (B) Sub-placental distribution of fluorophore-labeled dendrimers (green) in placental stem villi and surrounding syncytiotrophoblasts (red). Nuclei appear blue. Figure adapted with permission from Reference [75]. (C) PLGA-NPs modified with chondroitin sulfate A binding peptides actively target choriocarcinoma cells (nuclei are blue), leading to intracellular delivery of doxorubicin (red). NPs coated with scramble control peptide do not bind cells (top). Figure adapted with permission from Reference [93]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

been evaluated in *ex vivo* human placenta perfusion models (Fig. 3B) [75,79]. Fluorescently tagged PAMAM dendrimers were administered to the maternal inlet of the perfusion model and dendrimer accumulation in the fetal outlet was measured after 5.5 h. The fetal perfusate contained 18 times lower amounts of PAMAM dendrimer compared to the maternal perfusate, indicating it was not crossing through the placenta. Comparatively, antipyrine, which is freely diffused through the placenta, was rapidly transported into the fetal compartment within 15 min [75]. These results demonstrate that PAMAM dendrimers can limit the transfer of drugs through the placenta, setting the foundation for the evaluation of other types of branched polymers for maternal drug delivery.

In addition to the material composition, size and surface modifications also play a critical role in avoiding placental transport. The most direct comparisons between NP size and surface modifications have been conducted using gold NPs. Semmler-Behnke et al. provide a direct size comparison between 1.4 nm, 18 nm, and 80 nm anionic gold NPs that were modified with sulfonized triphenylphosphine [68]. Following intravenous injection of each NP in pregnant rats, they found that 1.4 nm and 18 nm NPs, but not 80 nm NPs, crossed the placenta and accumulated in fetal tissues. However, the overall amount of gold NPs found for both smaller sizes was a small fraction of the injected dose [68]. This demonstrates a strict size dependency of anionic gold NPs, and shows that >80 nm sizes may be viable candidates for drug delivery to maternal tissues. Similarly, Myllynen et al. presented a direct comparison of 10–30 nm gold NPs coated with PEG using the *ex vivo* placenta perfusion model [72]. They demonstrated that PEG-coated NPs were unable to cross the human placenta over the 6 h perfusion [72]. This demonstrates that the addition of stealthing agents, such as PEG, to NPs inhibits their ability to cross the placenta. Thus, smaller NPs may be coated with PEG to overcome the size restriction for maternal therapy. Although PEG surface modification decreases placental transport, excess PEG can also inhibit uptake into maternal tissues and cells, making it necessary to balance the use of PEG surface modifications, placental transport, and maternal cell uptake [80].

The major challenge to treating maternal conditions during pregnancy is the potential risks to the fetus and placenta. Thus, there is vast opportunity to develop and study NPs for drug delivery to treat maternal conditions during pregnancy including pregnancy-related conditions, cancer, and pre-existing conditions, among others. Below, we present recent studies that utilize maternally-targeted drug delivery technologies to treat some of these conditions during pregnancy while minimizing fetal exposure.

5.1. NPs for preterm birth, pre-existing conditions, and cancer

Approximately 12% of pregnancies result in preterm birth, or birth prior to 37 weeks of gestation [81]. Depending on the gestational age at birth, preterm birth can cause short- and long-term neurological and physiological impairments in the fetus, and increased risk of infection to both the fetus and the mother, the severity of which ranges from mild to life threatening [82]. Although the underlying causes of preterm birth varies, the immediate precursor to preterm birth is premature uterine contractions. Uterine contractions can be slowed with the treatment of tocolytics, such as magnesium sulfate, to prolong the pregnancy [83]. However, often tocolytics prolong pregnancy by only a few days. To modulate uterine contractions to prolong pregnancy, Paul et al. developed liposomes coated with antibodies against the oxytocin receptor to bind uterine tissue expressing oxytocin [84]. Following intravenous administration in mice, uterine accumulation of the targeted liposomes was increased 7-fold compared to nontargeted liposomes. Further, liposomes were used to encapsulate the medications (nifedipine, salbutamol, and rolipram) to evaluate their ability to inhibit contractions. The targeted liposomes were administered to human uterine tissue *in vitro* and resulted in a total suspension of myometrial contractions [84]. Lastly, inhibition of preterm birth rates was determined using a

mouse model of LPS-inducted preterm birth. Intravenous injection of LPS (to trigger preterm birth) and the targeted liposomes resulted in a preterm birth rate of 18%. Comparatively, mice treated with LPS and free drug, or LPS alone experienced preterm birth rates of 31% and 67%, respectively. Importantly, the targeted liposomes did not accumulate within fetal tissues, demonstrating that oxytocin-targeted liposomes enable active targeting of therapeutics to maternal uterine tissue while avoiding fetal exposure and toxicities [84].

Treating pre-existing conditions, or conditions that began before pregnancy, is another abundant opportunity for drug delivery technologies. Medications used to treat pre-existing conditions, including both physical and mental disorders, may be stopped or changed, or the dose is reduced during pregnancy either due to a lack of studies during pregnancy, or known fetal toxicities [85]. Of note, benzodiazepines, which include a variety of anxiety and panic disorder medications, may cause preterm birth and low birth weights if used during pregnancy [86]. Thus, the use of benzodiazepines is not recommended during pregnancy. Towards the goal of enabling safe benzodiazepine delivery during pregnancy, Sezgin-Bayindir et al. used micelles to encapsulate clonazepam [74]. These micelle-like NPs were comprised of polystyrene-poly(acrylic acid), PEG-poly(lactic acid), and PEG-lipid conjugates [74]. Placental transport of clonazepam-loaded NPs was evaluated *in vitro* using transwell assays to evaluate transport through BeWo cell layers. Free clonazepam or NP-encapsulated clonazepam was placed onto the apical side of the membrane, and drug content in the basolateral side of the transwell insert was quantified. After a treatment time of 6 h, 48% and 22.2% of the clonazepam was in basolateral side of the membrane after treatment with free drug or NPs, respectively. This reduction in drug content demonstrates that these NPs inhibit transport through BeWo cell monolayers [74]. Future work should evaluate this platform in *ex vivo* human tissue and animal models, although these initial results warrant further investigation of micelle-like NPs for maternal drug delivery.

A final application that can benefit greatly from NP drug delivery technologies during pregnancy is cancer. Cancer is diagnosed in 1:1000 pregnancies, and this statistic is predicted to rise as the average maternal age at conception increases [87]. However, most cancer therapeutics are not studied in pregnancy models or in clinical trials due to ethical concerns and the high cost for a restricted patient population [88]. Depending on the age of gestation, chemotherapies bind to or cross the placenta and interfere with normal fetal and placental development [89]. The precise impact that chemotherapy has on the pregnancy is dependent on the gestational age, as multiple chemotherapies have been shown to exhibit high fetal toxicity in the first trimester. The detrimental impact that chemotherapy has on early fetal development is demonstrated by one study that showed that the chemotherapy doxorubicin can be used to eliminate ectopic pregnancies [90]. Thus, patients diagnosed with cancer early in pregnancy decide to terminate the pregnancy or delay treatment until later in pregnancy or after birth [88,91]. However, taxane chemotherapies are still known to cross the placenta and enter the fetal compartment in late pregnancy, as taxanes administered in the second and third trimesters are present in infant meconium at birth [92]. Thus, using NPs to deliver chemotherapies during pregnancy can avoid fetal exposure and target the therapeutics to maternal tissues, as described below, although this application is in early stages.

The potential of NPs to treat cancer during pregnancy was recently demonstrated by Zhang et al., who used polymeric NPs comprised of poly(lactic-co-glycolic acid) (PLGA), soybean lecithin, and lipid-PEG conjugates to deliver doxorubicin to treat choriocarcinoma (Fig. 3C) [93]. Choriocarcinoma is a type of gestational trophoblastic disease resulting from hyperproliferation of trophoblast cells that requires aggressive chemotherapy regimens [94]. To target diseased trophoblasts, NPs were coated with peptides specific to chondroitin sulfate A (CSA), which is exclusively expressed on trophoblasts [93]. Targeted NPs were taken up by choriocarcinoma cells and resulted in a decrease in cell viability *in vitro* compared to unbound doxorubicin or NPs coated

with a control peptide. Following intravenous administration in mice bearing choriocarcinoma tumors, CSA-coated NPs inhibited both primary tumor growth and the formation of metastatic lesions [93]. Future work should evaluate these NPs in pregnant mouse models to evaluate placental transport, maternal biodistribution, and fetal accumulation. Although CSA is a promising target for choriocarcinoma, targeting ligands for treating other types of maternal cancers during pregnancy must be carefully chosen. Many of the overexpressed receptors on cancer cells, including EGFR, are also expressed on trophoblast cells and play a critical role in placenta development [95]. Thus, it is critical to use targeting ligands specific to receptors overexpressed in cancer while avoiding trophoblasts to treat other types of maternal cancers.

Here, we focused on maternal diseases and disorders during pregnancy that have been explored for NP-mediated drug delivery thus far. However, the development of drug delivery technologies that avoid the placenta to treat maternal conditions safely during pregnancy has just begun. There is vast potential to use existing technologies, and develop new ones, to treat other types of maternal conditions such as gestational diabetes, opioid addiction, and other pre-existing conditions, by hindering the ability for therapeutics to cross the placenta [95].

6. Targeting the placenta to treat pregnancy-related conditions

Pregnancy-related conditions, such as pre-eclampsia and fetal growth restriction, originate from abnormal placental development and function and can affect both the mother and the fetus. Pre-eclampsia is the most common pregnancy-related disorder, affecting 3–5% of pregnancies, and is the focus of the discussion below [96]. Pre-eclampsia is characterized by abnormally high blood pressure and increased protein content, and severe disease can lead to seizures and maternal and fetal morbidity [96]. One cause of the severe hypertension is believed to be a result of high levels of circulating soluble fms-like tyrosine kinase-1 (sFlt-1) leading to decreased angiogenesis in the placenta and maternal tissues compared to normal pregnancies [96]. Due to the lack of therapeutics for treating pregnancy-related conditions, there is an unmet need and opportunity to develop new therapies to treat pre-eclampsia, and other placental disorders, during pregnancy.

The only therapy currently used to treat pre-eclampsia is low-dose aspirin to lower hypertension, and there are currently no therapies that treat the underlying placenta abnormalities [97,98]. Pre-clinical studies have evaluated existing therapeutics to improve upon placental function and fetal growth [96,99,100]. Pravastatin, which is typically used to treat high cholesterol in non-pregnant adults, was evaluated as a therapy for pre-eclampsia by inducing expression of placental growth factor (PGF), which counteracts the high levels of sFlt-1 [96]. Pravastatin treatment increased PGF levels leading to significantly decreased sFlt-1 expression and reduced hypertension in a pre-eclampsia mouse model [96]. Recently, pravastatin was evaluated in clinical trials to treat early-onset pre-eclampsia (presented between week 24–31 of pregnancy) [99]. However, treatment did not yield significant changes in disease progression or time until delivery compared to placebo, although the medication had no adverse effects to the infants at the doses studied [99]. These preclinical and clinical studies demonstrate that statins may be a worthwhile therapy for pre-eclampsia if administered after week 24, although future work should investigate early administration prior to the onset of pre-eclampsia in high risk populations to assess therapeutic efficacy [99].

The preclinical study described above, and others, demonstrate the potential to treat the underlying mechanisms behind abnormal placenta function [96,99,100]. However, there are currently no therapeutic options that have yielded strong clinical efficacy. Further, pravastatin and sildenafil (another therapeutic evaluated in clinical trials), have been shown to cause birth defects, inhibited placental growth, and abnormal vascular function in ex vivo and in vivo experiments [101–103]. These adverse effects limit the dosages that can be used during pregnancy, particularly in early pregnancy when the fetus is most vulnerable to

these therapeutics [101–103]. Thus, treating placenta disorders are an excellent application for drug delivery technologies that are specifically designed to accumulate in the placenta with minimal fetal exposure.
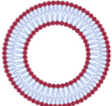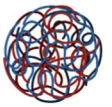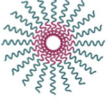
6.1. Drug delivery technologies for placenta-specific therapy

Preferential placenta accumulation of NP drug delivery technologies can be achieved by controlling the physicochemical properties of the NPs or by coating the NPs with targeting ligands. NP material composition, size, and surface charge can be optimized for placenta delivery, although a critical consideration is to maximize placenta accumulation while minimizing transport to the fetal compartment. Several types of NPs have been studied for placental drug delivery including liposomes [104], polymers [105,106], and gold NPs (Fig. 4A) [39,44]. The size and surface-modification dependency of NPs on placental accumulation has been most thoroughly examined using gold NPs. For example, 3–4 nm gold NPs penetrate further into human placenta microtissues compared to 13–14 nm gold NPs [44]. Further, gold NPs modified with sodium carboxylate yielded increased trophoblast uptake compared to PEG modifications [39,44]. However, in an ex vivo placenta perfusion model, only PEGylated NPs (3–4 nm), and not carboxylated NPs, were able to cross the placenta and enter fetal circulation after 6 h, demonstrating that PEG may not be a suitable surface modification for placenta-specific drug delivery [39].

A critical consideration for placenta-targeted NPs is the gestational age of the pregnancy, as placenta physiology and transport changes throughout gestation. The impact that gestational age has on placenta accumulation was directly investigated by Ho et al., who utilized fluorescently-labeled poly(glycidyl methacrylate) (PGMA) NPs surface-modified with PEI in pregnant rats (Fig. 4B) [105]. Following intravenous injection in early gestation (E10), PEI-coated PGMA NPs accumulated in the decidua and were taken up by trophoblast giant cells [105]. Comparatively, injection in late gestation at E20 resulted in NP accumulation within the chorionic plate. Further, higher levels of cationic PEI-coated NPs were present compared to anionic uncoated NPs, and no NPs were found within the fetuses [105]. Together, this study demonstrates that modifying NPs with PEI may be a viable platform for placenta-specific delivery depending on the gestational age. Moving forward, it would be interesting to evaluate how PEI surface modifications influences the ability for other types of NPs to preferentially accumulate within the placenta.

For a more active approach for targeting the placenta, NPs can be surface-modified with targeting ligands such as peptides, antibodies, or aptamers that bind placenta-specific cell surface receptors [107]. Tumor-homing peptides, including CGKRK and iRGD, have emerged as viable targets for placenta-specific delivery [108,109]. For example, iron oxide nanoworms coated with either of these tumor-homing peptides selectively accumulated within mouse placentas following intravenous administration, whereas nanoworms coated with nontargeted peptides did not [108]. The same study evaluated sub-placental localization of CGKRK or iRGD-targeted liposomes. Liposomes modified with CGKRK peptides accumulated in the labyrinth zone of mouse placentas following intravenous administration. Comparatively, liposomes coated with iRGD peptides were present in the labyrinth and spiral arteries. Importantly, liposomes coated with control peptides were also present within the decidua and labyrinth, although at lower levels than the targeted NPs, demonstrating that tumor-homing peptides increase placental selectivity [108]. This technology was evaluated in an insulin-like growth factor 1 (IGF-1) knockout mouse model of fetal growth restriction, in which untreated pups are born at 69% of the wild-type pups at birth [108]. IGF-1 plays a critical role in placental growth and function, and low levels of IGF-1 induces fetal growth restriction [106]. iRGD-coated liposomes with encapsulated insulin-like growth factor 2 (IGF-2) were delivered intravenously to pregnant mice, which resulted in pups born at 83% of the wild-type birth weight. Alternatively, pregnant mice treated with free IGF-2 did not experience an increase in pup

(A)

<u>Nanoparticle Technologies for Targeting the Placenta</u>

| Nanoparticle	NP and Experimental Design	Experimental Results and Conclusions
<u>Liposomes</u>	• Sizes: 142 – 156 nm • Charge: cationic, anionic, neutral • Coated with tumor homing peptides CGKRK or iRGD • Mouse model of fetal growth restriction	• CGKRK-NPs present in labyrinth zone • iRGD-NPs present in labyrinth and spiral arteries • iRGD-targeted NPs delivered IGF2 to improve fetal birth weights [108]
<u>Polymers</u>		
PGMA	• Sizes: 135.6 nm, 186.2 nm • Net charge: -26.2 mV, 25.8 mV • PEI modification • Pregnant rat model	• Accumulation in decidua and trophoblast giant cells at E10, or chorionic plate at E20. • PEI modification increases placenta accumulation compared to uncoated NPs [105]
DMAEMA	• Diblock copolymer complexed with hIGF-1 plasmid DNA • Mouse model of fetal growth restriction with direct injection in placenta [106] • *Ex vivo* perfusion model [110]	• NPs induce hIGF1 expression and alleviate IUGR following direct placental injection [106] • NPs taken up by syncytiotrophoblast cells with minimal transport to fetal compartment [110]
PAMAM	• Sizes: 70 – 80 nm • Net charge: -22 – 15 mV • Complexed with sFlt-1 siRNA • Rat model of pre-eclampsia	• Dendrimers inhibited sFlt-1 secretion and alleviated symptoms of pre-eclampsia [79]
PLA	• Sizes: ~100 nm • Net charge: 3 – 6 mV • PEG-PLA, DOTAP, CSA binding peptides • Mouse model of pre-eclampsia	• CSA-targeting increased placental accumulation and lowered sFlt-1 mRNA and protein levels compared to non-targeted NPs • No toxic effects to fetuses or dams [112]
<u>Gold</u>	• Sizes: 3 – 4 nm, 13 – 14 nm • Net charge: -37 – -16.5 mV • Modifications: sodium carboxylate, PEG • Human placenta micro-tissues	• 3-4 nm NPs and COON modification yields higher uptake than PEG modification and larger NPs • PEG-modified NPs minimally surpass trophoblast layer [44]

(B)
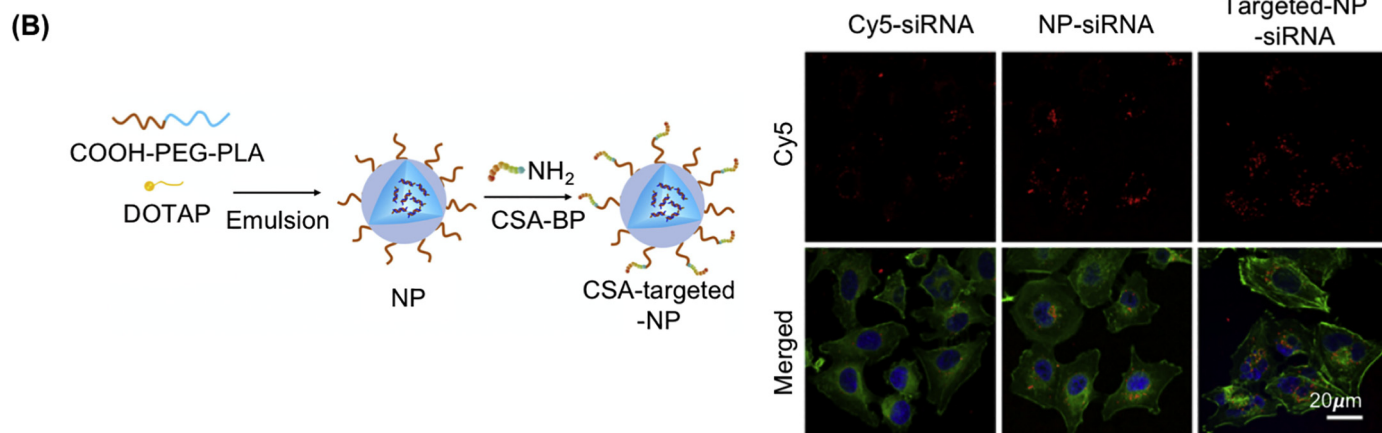
Fig. 4. (A) NP drug delivery technologies that have been evaluated to treat placental disorders during pregnancy. (B) (left) Schematic showing double emulsion synthesis of NPs comprised of PLA, COOH-PEG-PLA, and DOTAP. CSA binding peptides are conjugated to NPs using an NH$_2$ linker, and sFlt1 siRNA is encapsulated within NPs. (right) CSA-targeted NPs yield increased uptake in trophoblast cells compared to nontargeted NPs, as demonstrated by intracellular siRNA signal (red). Cytoskeleton is green and nuclei are blue. Adapted with permission from Reference [112]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

weight at birth [108]. Together, these results demonstrate that tumor homing peptides may enable successful treatment of placental insufficiencies by enhancing accumulation within the placenta.

A driving factor of placental insufficiencies, including pre-eclampsia and fetal growth restriction, is abnormal cell signaling. NPs enable intracellular delivery of gene therapies, such as nucleic acids, to regulate cell signaling in the placenta and promote its normal function. In an important demonstration of placental gene therapy, Ellah et al. developed a DMAEMA diblock copolymer NP complexed with a plasmid encoding human hIGF-1 [106]. These complexes were directly injected into placentas in a mouse model of fetal growth restriction. Following injection, the NPs induced hIGF1 expression in trophoblasts and replenished fetal birth weights to similar weights as healthy pups [106]. More recently, this research group examined uptake of these NPs into syncytiotrophoblasts using a combination of *in vitro* and *ex vivo* explants and perfusion experiments [110]. Using the placental perfusion model, they show that the NPs are taken up by syncytiotrophoblast cells, with no detectable NPs in the fetal outlet. Further, *in vitro* treatment of human tissue explants and BeWo cells resulted in enhanced hIGF1 expression and protection against oxidative stress, respectively, compared to untreated controls [110]. While these studies demonstrate the potential for gene therapies for treating placental insufficiencies, future work should evaluate the ability for diblock copolymer NP complexes to target the placenta following intravenous administration.

In addition to using plasmids for gene therapy, there is also ample opportunity for transient gene regulation using siRNA or mRNA to treat placental disorders during pregnancy. The use of siRNA to treat pre-eclampsia was recently demonstrated by Turanov et al. by inhibiting the secretion of sFlt-1 from trophoblasts [79,111]. This study identified an siRNA sequence that inhibited sFlt-1 mRNA levels in the placenta by 40% following intravenous injection in mice at 20 mg/kg. Further, siRNA injection at 10 mg/kg into a baboon model of pre-eclampsia resulted in a 50% silencing of sFlt1 mRNA, resulting in improvements in the clinical signs of preeclampsia [111]. Both animal models demonstrate the opportunity for siRNA-mediated knockdown of sFlt-1 to treat the underlying cause of pre-eclampsia. Towards the goal of lowering treatment dosages and maximizing placenta-specific delivery, researchers have recently begun developing NPs for siRNA delivery to the placenta [79,112]. For example, sFlt-1 siRNA encapsulated within PAMAM dendrimers inhibited sFlt-1 secretion following intravenous injection in pregnant rats [79], which improved dam hypertension and prolonged pregnancy with doses as low as 0.3 mg/kg [79,111]. More recently, Li et al. developed a NP drug delivery technology that combined both active placenta-targeting and siRNA delivery to treat pre-eclampsia. NPs comprised of PEG-poly(D,L-lactide) (PEG-PLA) and the cationic lipid DOTAP coated with CSA binding peptides were intravenously injected into pregnant mice (Fig. 4C). The targeted NPs yielded significantly higher accumulation in the placenta compared to non-targeted NPs. This increased placental targeting resulted in lower sFlt-1 mRNA and protein levels in the serum of pregnant mice [112]. Importantly, the targeted NPs showed no toxic effects to injected mice or fetuses, and pup birth weights were comparable those treated with untargeted NPs [112]. These studies demonstrate the potential for siRNA to treat diseases that originate in the placenta, such as pre-eclampsia. Further, they set the foundation for the development of new technologies to explore transient gene regulation to treat placenta disorders while ensuring fetal safety.

7. Drug delivery for treating fetal diseases

In stark opposition to treating maternal and placental diseases during pregnancy, which aim to avoid fetal exposure, the goal of fetal therapy is to access the fetal compartment to treat fetal diseases. Many fetal diseases can be diagnosed early in gestation using advanced imaging and other diagnostic techniques, but there are few options to treat these diseases *in utero*. Currently, fetal surgery *in utero* can correct structural defects for diseases including spina bifida, congenital cystic adenomatoid malformations, and sacrococcygeal teratoma resections, among others [3,113]. Fetal surgery can yield substantial improvements in quality of life and survival for the affected fetus by correcting the defect early in the onset of irreversible pathology. However, these procedures are highly invasive and present risks to the mother and fetus including infection and preterm birth [114]. The development of drug delivery technologies to facilitate delivery through the placenta for fetal diseases is in early stages. Below, we present initial studies of fetal drug delivery *in utero* either through direct fetal injection or systemic injection to the mother. Further, we present initial studies of NP drug delivery technologies designed to cross the placenta and enter fetal circulation.

Thus far, fetal gene therapy has mostly been explored through direct injection to the fetuses during surgery in mice. In these experiments, a midline laparotomy is conducted on the pregnant mouse to expose the fetuses and inject the therapeutic through the vitelline vein or directly into the amniotic fluid [81,115,116]. Intra-amniotic injections are useful for treating lung diseases, as amniotic fluid is ingested by the fetuses. In an example of this, Alapati et al. used adenovirus to deliver CRISPR-Cas9 gene editing material to mediate gene editing in fetal lungs to treat interstitial lung diseases [113]. Intra-amniotic injection of this system inactivated the human SFTPC^{I73T} mutation, which resulted in substantial improvements in both lung morphology and pup survival [113]. This demonstrates that intra-amniotic fluid injections of gene therapies may be a viable approach to treat lethal monogenic lung diseases in fetuses before birth [113].

Another method of direct fetal injections in mice is through the vitelline vein, which directly feeds into the liver sinusoids [117]. This technique was used to deliver CRISPR/Cas9 or base editors complexed in viral vectors to edit metabolic genes in fetal mouse livers [117]. Following injection, these complexes successfully edited Pcsk9 in wild-type mice and disrupted the Hpd gene in a murine model of hereditary tyrosinemia type 1 (HT1) [117]. Editing the Hpd gene resulted in pup survival rates of 89% three months after birth, compared to 0% survival of fetuses treated with control base editors [117]. This study demonstrates the opportunity to treat congenital genetic disorders *in utero*. However, the clinical translation of viral vectors is precluded by immunogenicity, carcinogenesis, and broad tropism [12]. Recently, researchers have begun developing non-viral delivery vehicles for nucleic acid delivery to fetuses. Towards this goal, Ricciardi et al. used PLGA NPs to deliver peptide nucleic acids and donor DNA to treat β-thalassemia by correcting a mutation in the β-globin gene [118]. Mouse fetuses injected with the PLGA NPs at a gestational age of 15.5 days (E15.5) experienced increased hemoglobin levels, reduced reticulocytes, and improved survival compared to untreated controls [118]. These results reveal the potential for non-viral delivery vectors for *in utero* gene editing.

The examples presented above demonstrate techniques to treat mouse fetuses directly *via* surgery. Importantly, there are several key considerations for translating this approach to humans. Vitelline vein and intra-amniotic injections in mouse fetuses are highly invasive and require a laparotomy to enable direct access to each individual fetus [116]. Translation to large animal models or humans can utilize ultrasound-guided approaches similar to an amniocentesis or cord blood transfusion, which are performed regularly by physicians. However, these procedures still hold risk to the developing fetus and mother including infection, maternal morbidity, preterm birth, and fetal demise [3]. Towards the goal of noninvasive fetal delivery, researchers have begun developing NPs that can cross the placenta and enter fetal circulation following intravenous injection to pregnant mice, as described below.

7.1. Drug delivery technologies for placental transport to treat fetal diseases

Transplacental gene delivery was first presented in 1995 with the goal of enabling noninvasive fetal drug delivery to treat fetal diseases

in utero [119,120]. In this technique, nucleic acids are administered intravenously to pregnant mice, cross the placenta, and enter fetal tissues [119,120]. This first demonstration of transplacental gene delivery utilized plasmid DNA:lipopolyamine complexes to introduce exogenous genes into mouse fetuses [120]. Since then, researchers have used this technique to study fetal immune responses to plasmid DNA for vaccination [121], to deliver T7 bacteriophages [122], and to study delivery technologies [123–125]. Most recently, Nakumura et al. used a commercially available nonliposomal transfection reagent complexed with a plasmid vector of Cas9 and gRNA targeted to eGFP cDNA [126]. These complexes were intravenously injected in pregnant mice and resulted in activation of eGFP in fetal hearts, demonstrating successful gene editing and heart-specific delivery [126].

The use of commercially available cationic lipids has also been explored for fetal delivery through the placenta [119,124]. The strong positive charge of cationic lipids enables cellular uptake through negatively charged cell membranes for intracellular gene delivery, and positively charged NPs cross the placenta more readily than anionic NPs [8,95]. However, the clinical translation of cationic lipid carriers is challenged due to widespread uptake in both diseased and healthy cells, leading to high toxicity to healthy tissues, and immunogenicity [8,127–129]. To overcome these challenges of cationic lipids, immunoliposomes were used to deliver luciferase plasmid DNA to fetal brains [123]. In this study, the NPs were coated with 8D3 antibodies to target the transferrin receptor that is expressed in the human and mouse placenta and brain. Following intravenous injection in pregnant mice, the targeted immunoliposomes traversed the placenta and induced luciferase expression in the fetal brain, demonstrating that targeting NPs to transferrin can surpass the placenta and is a viable target for fetal brain conditions [123]. Further, this targeted platform forms the basis for understanding how drug delivery technologies can be designed for fetal therapy through the placenta.

The size, surface charge, and material composition of NPs impacts their ability to cross the placenta into the fetal compartment. Several types of NP materials have been evaluated for this application including (but not limited to) gold [68,130], silver [73,131], pullulan acetate [132], polymers [74,105,133], polystyrene [134], quantum dots [135], and liposomes (Fig. 5A) [104]. In general, smaller NPs are able to cross the placenta and enter fetal circulation more readily compared to larger NPs. One study used negatively charged gold NPs to demonstrate how size impacts NP accumulation in fetal fluids and tissues [68]. Gold NPs that were 1.4 nm, 18 nm, or 80 nm in diameter were intravenously injected in pregnant rats at E18. The 1.4 nm gold NPs accumulated within the amniotic fluid two orders of magnitude higher than the larger NPs, and the fetuses themselves contained small amounts of the 1.4 nm and 18 nm NPs [68]. This size dependency is directly related to the biomaternal composition of the NPs. For example, 20, 40, 100, 200, and 500 nm carboxylated polystyrene NPs were intravenously injected into pregnant mice at E17, and all sizes crossed the placenta and accumulated within fetal organs [134]. In this study, the authors found no linear correlation between NP size and uptake in fetuses [134]. These studies indicate that the ability for NPs to cross the placenta depends on their size only in a material- and modification-dependent, manner.

Surface modifications that impart a net positive charge on NPs yield increased placental transport compared to negatively charged NPs [105]. Yang et al. directly compared how various surface modifications and the gestational age impact maternal-fetal-placental biodistribution of gold NPs [67]. In this study, 13 nm gold NPs were modified with ferritin, poly(ethylene) glycol, or citrate and intravenously injected into pregnant mice at E5.5 – E15.5 (Fig. 5B) [67]. When injected in early gestation (<E11.5), all of the NPs accumulated in fetal tissues. However, injection later in pregnancy (>E11.5), which is when the placenta is developed and controls fetal transport, resulted in decreased accumulation in fetal tissues. However, ferritin and PEG-modifications yielded higher fetal accumulation compared to citrate-capped NPs overall (Fig. 5B). The zeta potential of citrate-capped NPs was −17.0 mV,

compared to −1.6 mV and 6.0 mV for the ferritin and PEG-modified NPs, respectively [67]. This indicates that surface charge and modification is a critical factor that dictates placental transport to the fetal compartment, and strong negative charges can hinder delivery through the placenta.

The importance of surface charge for fetal delivery is supported by a study by Sezgin-Bayindir et al. who evaluated lipofectamine, a cationic transfection reagent, as a surface modification for fetal delivery. In this study, polymeric micelle-like NPs with encapsulated clonazepam were coated with lipofectamine, and placental transport was evaluated using *in vitro* transwell models [74]. Transwell inserts with either BeWo or B.end3 monolayers were treated with free clonazepam or clonazepam encapsulated within lipofectamine-modified micelles. Coating the micelles with lipofectamine resulted in a 1.4-fold increase in transport of clonazepam compared to free drug, which may be attributed to cationic charges afforded by the lipofectamine [74]. This study demonstrates that cationic lipids may improve transport across the placenta, although future studies are needed to evaluate these lipofectamine-coated micelles *in vivo.* This is important because the clinical translation of transfection reagents is challenged by limited *in vivo* efficacy and high toxicities, which may be detrimental to a developing fetus [136].
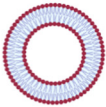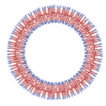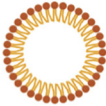
Many of the studies presented above demonstrate the impact that NP design has on placental transport and fetal accumulation. The development of drug delivery technologies is in early stages, and there is much opportunity to develop NPs that can cross the placenta to treat fetal diseases. By combining knowledge gained from direct fetal injections to treat diseases, and NP-mediated placental transport, researchers can now begin to develop drug delivery technologies for noninvasive fetal therapy to treat specific diseases.

8. Challenges and future directions

In this Review, we presented NP drug delivery technologies that exploit the placenta as a biological barrier for treating maternal, placental, or fetal diseases and disorders during pregnancy. Moving forward, the development of these technologies can build off of the extensive research that has evaluated environmental exposures of NPs during pregnancy [73,131,137–139]. For example, exposure to single-walled carbon nanotubes (SWCNT), which are used in industrial settings, have been evaluated for toxicity to embryos and fetuses during pregnancy using animal models [137,138]. Pregnant mice were intravenously injected with SWCNTs early in pregnancy (postcoital day 5.5) and reproductive tissues, including uteri, placentas, and fetuses, were extracted after 10 days. NPs administered at as low as 100 ng/mouse caused fetal malformations and loss. However, surface modifications on SWNTs had a substantial impact on fetal toxicity, as 56% and 23.7% of pregnant mice injected with oxidized SWCNTs or pristine SWCNTs, respectively, experienced total fetal loss or malformed fetuses [138]. Alternatively, coating SWCNTs with PEG drastically decreased their toxicity, with teratogenic effects found in only 1 out of 10 fetuses at the highest administered dose (30 μg/mouse) [137].

The opportunity for surface modifications to reduce placental and fetal toxicity was also demonstrated by Yamashita et al. who showed that 70 nm silica NPs and 35 nm titanium dioxide NPs injected intravenously into pregnant mice accumulated in the placentas, fetal livers, and fetal brains, demonstrating that these materials cross the placenta into the fetal compartment [140]. However, this resulted in high levels of fetal toxicity including restricted uterus and fetal growth due to placenta abnormalities [140]. However, coating silica NPs with carboxyl and amine groups alleviated these placenta and fetal toxicities, demonstrating the importance of surface coatings on placental and fetal toxicity [140]. Another technology that holds potential for future studies evaluating surface chemistry are quantum dots. Quantum dots are often used for diagnostics, and have been evaluated for use during pregnancy in animal models [135]. Zalgeviciene et al. evaluated the placental transport and resultant placental and fetal toxicity of quantum dots in

(A)

Nanoparticle Technologies for Fetal Diseases

Nanoparticle	NP and Experimental Design	Experimental Results and Conclusions
Liposomes	• Sizes: 75-100 nm • PEG modification with 8D3 targeting antibodies • Pregnant mouse model	• Plasmid DNA encapsulated in liposomes targeted with 8D3 antibodies was delivered to fetal brains • Transferrin-targeted NPs can enable noninvasive therapy to treat fetal brain diseases *in utero* [123]
Polymers — Polystyrene	• Sizes: 20 - 500 nm • Carboxylate modification • Pregnant mouse model	• All NP sizes crossed the placenta and accumulated within fetal tissues • No linear correlation between NP size and fetal accumulation [134]
Pullulan Acetate	• Sizes: 200 – 300 nm • Fluorophore-labelled NPs • *In vitro* transwell assays	• NPs were taken up by BeWo cells through endocytosis and pinocytosis with no toxicity to cell layers • NPs accumulated in the BeWo cell layer and in the fetal compartment [132]
Micelles	• Sizes: 33 – 174 nm • Net charge: -44.50 – -3.23 mV • PEG-PLA with clonazepam and lipofectamine modification • *In vitro* transwell assays	• Coating micelles with lipofectamine increased transport of clonazepam through monolayers 1.4-fold compared to free drug [74]
Gold	• Size: 13 nm • Net charge: -17 – -1.6 mV • ferritin, PEG, or citrate modifications • Pregnant mouse model	• All NPs accumulated in fetal tissues in early gestation (<E11.5) • Ferritin and PEG-modified NPs had higher accumulation in extraembryonic tissues and fetuses than citrate-modified NPs [67]
Silver	• Sizes: 2 – 15 nm • Net charge: -32.5 – -10 mV • COON or PEG modifications • *Ex vivo* perfusion model	• Slightly increased placental transport to fetal compartment of PEG-NPs compared to COON-NPs overall [73]

(B)
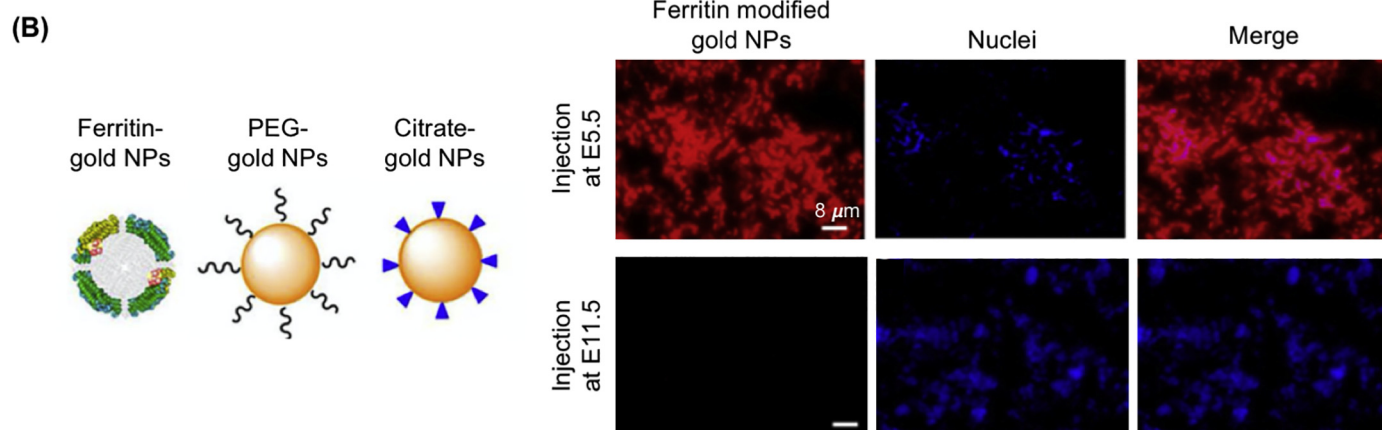
Fig. 5. (A) NP drug delivery technologies that have been studied for placental transport to treat fetal diseases. (B) Ferritin, PEG, and citrate-modified gold NPs (left) were directly compared for fetal biodistribution. Fetal accumulation of NPs was highly dependent on surface modification and gestational age. (right) Ferritin-modified NPs (red) accumulated in fetal tissues following injection at E5.5, but had minimal accumulation following injection at E11.5. Figure adapted with permission from Reference [67]. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

rats [141]. In this study, pregnant rats were injected on E13 and fetal and placental health was evaluated by histology on E20. Their results demonstrated that quantum dot toxicity is highly dose-dependent, as rats treated with 5 mg/kg or 20 mg/kg resulted in 97% or 43% fetal survival, respectively [141]. Further, there was apparent placental damage and reduced fetal growth following treatment with quantum dots [141]. This demonstrates that maternal exposure to quantum dots during pregnancy should be avoided. However, there is much opportunity in the future to study how quantum dots can be modified for reduced fetal toxicity. Since quantum dots are able to cross the placenta, improving their safety profiles can potentially enable their use to treat placental and fetal diseases, similar to the carboxyl and amine functionalization described above. Together, these studies focused on NP toxicity demonstrate the opportunity to study how surface modifications can enable the use of otherwise toxic NPs that can cross the placenta for fetal drug delivery.

In addition to synthetic NPs, which are the focus of this Review, there is vast potential to explore biological materials for drug delivery. Recently, it was discovered that extracellular vesicles (EVs) are secreted from the fetus and placenta during pregnancy and facilitate fetal-maternal communication [55,142,143]. These EVs contain fetal or placental-derived cargo, such as microRNAs, proteins, and immuno-modulatory molecules, that regulate the local immune system to support the pregnancy [142]. Further, EVs collected from maternal blood contain markers for pregnancy complications and can be used as diagnostic platforms to detect pregnancy abnormalities [55,143–148]. For example, micro- and nano-sized EVs collected from pregnant women with severe pre-eclampsia contain higher levels of Flt-1 compared to EVs collected from healthy pregnancies [51]. Similarly, the amount of EVs present in maternal blood can be diagnostic, as elevated levels of EVs are strongly correlated with the severity of hypertensive disorders [144]. Thus, EVs hold potential as diagnostic markers for pre-eclampsia and other pregnancy-related conditions, several of which are still being explored [55,143–147]. EVs are yet to be explored as drug delivery vehicles for use during pregnancy, but they have been extensively studied in non-pregnant disease models [149,150], setting the stage for their use during pregnancy. The innate ability of EVs to facilitate fetal-maternal communication and transport through the placenta, combined with existing studies that utilize exosomes for drug delivery in non-pregnant mouse models, makes EVs promising tools that should be explored for placental or fetal therapy in the future.

Elastin-like polypeptides (ELPs) represent an additional class of drug delivery technologies that have been developed for use during pregnancy to treat maternal diseases. Their large size inhibits their ability to passively cross the placenta, and they are specifically designed to avoid active transport mechanisms [151]. ELPs are engineered peptides containing any amino acid sequence that can be carefully selected to control the size of the overall construct. Further, the amino acid sequence can be adapted to incorporate therapeutics, thereby acting as a non-immunogenic drug delivery platform [151]. Due to these advantages for drug delivery, George et al. evaluated ELPs for maternal drug delivery during pregnancy. Pregnant rats were injected intravenously on E14, and their biodistribution was evaluated after 4 h. The ELPs accumulated in maternal tissues and the placenta, but minimal polypeptide, if any, was detectable in the pups [151]. To demonstrate therapeutic relevance of this technology, ELPs were used to treat pre-eclampsia in a rat model. One molecular mechanism believed to be a precursor to pre-eclampsia is the presence of high levels of soluble fms-like tyrosine kinase-1 that sequesters VEGF, leading to endothelial dysfunction and severe hypertension. To interrupt this process, ELPs were fused to vascular endothelial growth factor (VEGF) to form VEGF-ELP complexes [152]. Following intravenous injection into pregnant rats, VEGF-ELPs decreased free plasma soluble fms-like tyrosine kinase-1 levels, resulting in reduced maternal hypertension [152]. Although promising for pre-eclampsia treatment, future work should further evaluate allowable dosages, as these complexes were shown to have dose-dependent

adverse effects in initial studies [152]. Regardless, these studies demonstrate that ELPs are promising technologies to treat maternal diseases during pregnancy with minimal fetal exposure, and they offer the necessary flexibility to treat a range of maternal diseases by adjusting the peptide size and carefully choosing the fused protein [151,152].

As new drug delivery technologies emerge for use during pregnancy, it is also important to consider the ethical concerns towards the goal of clinical translation in humans. Treating diseases during pregnancy involves two patients: the mother and the fetus, both of which are impacted by the disease and the treatment [3]. This ethical dilemma requires balance between the therapeutic need for one patient with the risk imposed on the other, and it is one reason for the exclusion of pregnant women in clinical trials [1]. These concerns have been thoroughly discussed in the field of maternal-fetal surgery and are applicable to drug delivery as well. In the field of maternal-fetal surgery, the decision to conduct surgery can be summarized by three main criteria for the procedure in that: (i) pre-clinical animal studies indicate that the treatment is lifesaving or prevent irreversible damage to the fetus, (ii) the intervention minimizes risk of mortality and morbidity to the fetus compared to alternatives, and (iii) pre-clinical animal studies and theoretical risks indicate low risk to the pregnant woman, the current pregnancy, and future pregnancies [153,154]. For a more thorough discussion regarding the ethical concerns of materno-fetal surgery and drug delivery, we refer readers to articles focused on this topic [153,154]. As future innovations in developing drug delivery technologies to treat maternal, fetal, or placental diseases during pregnancy arise, researchers should carefully balance the clinical need for the therapy and the risks faced by the current and future health of the mother and child.

In addition to ethical considerations, the development of drug delivery technologies is faced by several experimental challenges. Studying drug delivery *in vivo* is difficult due to the underlying differences between animal and human reproduction and placenta physiology, as briefly described in the "Experimental Models of the Placenta" section of this Review. Mice are most often used to evaluate delivery in initial pre-clinical studies, but key differences exist including multiple fetuses per pregnancy, short gestations of 19–21 days, and physiological differences in the placenta [59]. Larger animal models, such as sheep, carry one or two fetuses per pregnancy with a longer gestation time of approximately 150 days, but the sheep placenta lacks trophoblast invasion into the uterus compared to human placentation [63]. Other animal models that are used to study pregnancy include guinea pigs, rats, and non-human primates, and we refer readers to a more thorough review of these models and how they compare to human gestation [63]. Due to these key differences, researchers must choose the *in vivo* model appropriate for their experimental goals while considering their limitations [63]. *Ex vivo* models are excellent alternatives to animal models in that they use human tissue to model tissue behavior. The *ex vivo* placenta perfusion experiments in particular provide the opportunity to study the bulk human placenta in the laboratory. Although powerful, it is difficult to standardize the experimental conditions and checkpoints across laboratories, making it challenging to directly compare results and replicate experiments [20]. Towards the goal of standardizing the placenta perfusion model, Conings et al. presented key parameters and checkpoints that should be followed in these experiments, and these criteria should be met to identify issues and compare results across laboratories [20]. This is particularly important as new NP technologies arise for use during pregnancy, as the placenta perfusion model is an invaluable tool to study now NP design influences placental transport.

In this article, we describe how drug delivery technologies can be used during pregnancy to exploit the placenta for maternal, placental, or fetal therapy, exclusively. By controlling the engineered properties of NPs including their material composition, surface modifications, surface charge, and size, these technologies can be specifically designed to avoid, cross, or target the placenta to maximize drug delivery to the

desired tissues. Thus, drug delivery technologies offer several benefits that can ensure effective and safe therapy during pregnancy including: (i) noninvasive fetal therapy through the placenta to treat fetal genetic diseases, (ii) targeting the placenta to treat diseases that originate from abnormal placenta development, and (iii) enabling the treatment of a wide range of maternal diseases by avoiding fetal exposure. The studies presented here demonstrate the opportunity afforded by NP drug delivery technologies for targeting maternal, fetal, or placental tissues, forming the foundation for future innovations that can broadly impact the field of drug delivery during pregnancy.

Declaration of Competing Interest

The authors declare no competing interests.

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