Published in final edited form as:

J Drug Target. 2014 December ; 22(10): 935–947. doi:10.3109/1061186X.2014.950666.

A Polypeptide Drug Carrier for Maternal Delivery and Prevention of Fetal Exposure

Eric M. George, Ph.D.^a, Huiling Liu, M.D., Ph.D.^b, Grant G. Robinson, M.S.^b, and Gene L. Bidwell III, Ph.D.^{b,c}

^aDepartment of Physiology and Biophysics, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, USA

^bDepartment of Neurology, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, USA

Abstract

Background—Pregnant females are largely overlooked in drug development due to concerns for fetal health. Additionally, pregnancy is often an exclusion criterion in clinical trials, so the safety of many drugs during pregnancy is unknown.

Purpose—The goal of this study was to evaluate Elastin-like Polypeptide (ELP), a synthetic protein derived from human elastin, for maternally sequestered drug delivery. ELP is a versatile drug carrier with a long plasma half life, low immunogenicity, and the ability to be fused to nearly any small molecule or protein-based therapeutic.

Methods—We determined the pharmacokinetics, biodistribution, and fetal exposure to the ELP drug carrier using quantitative fluorescence techniques in a rat pregnancy model.

Results—After either bolus IV administration or continuous infusion over five days, ELPs accumulated strongly in the kidneys, liver, and placenta, but importantly, little to no ELPs were detectable in the fetus. Within the placenta, ELPs were localized to the chorionic plate and broadly distributed within the labyrinth, but were excluded from the fetal portion of the chorionic villi.

Conclusion—These data indicate that ELP does not cross the placenta, and they suggest that this adaptable drug delivery system is a promising platform for prevention of fetal drug exposure.

Keywords

Elastin-like Polypeptide; pregnancy; drug delivery; placental transport; preeclampsia; fetal exclusion

^cCorresponding Author: Gene L. Bidwell, III, Assistant Professor of Neurology, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216, Phone: (1) 601-984-6787, Fax: (1) 601-984-6626, gbidwell@umc.edu.

Declaration of Interest.

Direct funding for this work was provided by American Heart Association grant 13SDG16490006, NIH NHLBI grant R01HL121527, and startup funds from the University of Mississippi Medical Center to G.L.B. Partial salary support for E.G. was provided by NIH grant R00HL116774. *Ex vivo* specimen imaging was partially supported by the Animal Imaging Core Facility of the University of Mississippi Medical Center. G.L.B. is owner of Leflore Technologies, LLC, a private company working to develop ELP-based therapies for various diseases.

Introduction

Pregnant females have been a vastly overlooked patient population in drug development. Due to concerns of risk to the developing fetus, pregnancy is very often an exclusion criterion for clinical trials. As a result, the safety of drugs during pregnancy is often unknown. Other agents have exhibited toxicity in preclinical animal models, and have therefore been ruled out as therapeutics for the management of pregnancy-related disorders. Perhaps the most notable of these in the cardiovascular field are endothelin type A (ET-A) receptor antagonists. Though they show promising therapeutic effects in preclinical models of preeclampsia, ET-A receptor antagonists cause serious and even fatal effects on offspring in rodents (Alexander et al., 2001; Taniguchi and Muramatsu, 2003). Furthermore, there are disorders of pregnancy, including preeclampsia, for which there are no efficacious therapeutic interventions. Antihypertensives such as labetalol or nifedipine are sometimes prescribed with marginal success at controlling the hypertension, but there is little evidence that they affect pregnancy prolongation or the incidence of low birth weight (Fenakel et al., 1991; Sibai et al., 1992). Furthermore, these agents cross the placenta and pose some risk to the fetus (Bartels et al., 2007). Drug development for disorders of pregnancy has been nearly stagnant for decades due to concerns for fetal health and risk aversion from drug developers.

Small molecule drugs often cross the placenta, and can thus pose a threat to fetal health and development. Proteins, on the other hand, are generally too large to passively cross the placental barrier. Exceptions include immunoglobulins, which bind to the neonatal Fc receptor inside the endosomes of syncytiotrophoblasts following pinocytosis from maternal plasma and are subsequently transcytosed and released into the fetal plasma (Firan et al., 2001; Leach et al., 1996; Story et al., 1994), and transferrin, which is bound by transferrin receptors on trophoblasts and actively transported (Galbraith et al., 1980; McArdle and Morgan, 1982). This suggests that protein-fusion, using proteins that are not substrates for active placental transport, could be a viable strategy to prevent fetal drug exposure. However, little research has been performed in this area. Other high molecular weight drug carriers such as nanoparticles have been examined for placental transport and fetal uptake with mixed results. Yang et al. studied the fetal exposure to maternally administered gold nanoparticles (13 nm) in a mouse pregnancy model. They found that these particles readily transferred to the developing fetus during early gestation (prior to GD11.5), but were mostly restricted when given in late pregnancy (Yang et al., 2012). Consistent with fetal exclusion in late pregnancy, gold nanoparticles ranging from 10 to 30 nm and capped with polyethylene glycol were not transferred to the fetal side in an *ex vivo* perfusion model of human placentas harvested after birth (Myllynen et al., 2008). Yamashita et al. injected 70 nm silica nanoparticles and 35 nm titanium dioxide nanoparticles intravenously into pregnant mice, and they observed these particles in the fetal liver and the fetal brain. Nanoparticle injection lead to placental structural abnormalities and reduced fetal and uterine sizes relative to control mice (Yamashita et al., 2011). Quantum dots (QD) also cross the placental barrier, though the efficiency of transfer decreases as the QD size increases (Chu et al., 2010). Fetal transfer can be reduced but not totally eliminated by capping the QDs with silica or polyethylene glycol shells. A separate study using QDs coated with mercaptopropionic acid or polyethylene glycol reported no placental transfer or

teratogenicity when injected on GD6, GD13, or GD18, but the QDs did induce intraperitoneal necrosis, slightly decreased birth weight, and rarely, spots of placental and embryonic necrosis (Zalgeviien et al., 2012). Using a different type of nanoparticle, Menjoge et al. found that PAMAM dendrimers with molecular weights of approximately 16 kDa exhibited very little transfer to the fetal side (fetal levels < 1% of maternal levels) in an *ex vivo* human placental transfer model (Menjoge et al., 2011). These results indicate that many nanoparticles are efficiently excluded from trans-placental transfer, especially when administered in late pregnancy. But some types of nanoparticles, including some particles with diameters as large as 70 nm, are susceptible to penetration into fetal tissue. These results demonstrate that prediction of which types of macromolecules will penetrate the placenta is not always possible *a priori*. The goal of this study was to determine whether a protein-based macromolecular drug carrier called Elastin-like Polypeptide (ELP) could be used for maternal drug delivery and prevention of fetal drug exposure.

ELP is a bioengineered protein with many attractive properties that make it an ideal drug carrier (Bidwell and Raucher, 2010). ELP is made of repeated units of a VPGxG pentapeptide motif, where x can be any amino acid except proline (Dan W. Urry et al., 1991). Because it is genetically encoded, the sequence of ELP is easily manipulated to change the repeat length and resulting molecule size, and the sequence can be modified to incorporate targeting peptides, reactive sites for drug attachment, or therapeutic peptides and proteins (Bidwell and Raucher, 2009; Raucher et al., 2009). ELP has previously been used for delivery of small molecule chemotherapeutics (Bidwell et al., 2007; Dreher et al., 2003; MacKay et al., 2009; Moktan et al., 2012, 2010), peptide-based therapeutics (Bidwell and Raucher, 2010, 2005; Bidwell et al., 2013, 2012, 2010; Massodi et al., 2010, 2009b, 2005), and even protein cargo (Shamji et al., 2008a, 2008b, 2007). A second advantage of ELP derives from the fact that it exhibits reversible temperature-induced aggregation. ELP is soluble below a characteristic and tunable transition temperature, but forms aggregates when the solution is raised above the transition temperature (Dan W. Urry et al., 1991; Urry et al., 1974). The aggregation is fully reversible when the solution is cooled below the polypeptide's transition temperature. This property is leveraged in two ways. First, purification of ELP after recombinant expression is achieved by simple centrifugation at a temperature above the transition temperature, thus bypassing the need for complicated chromatographic purification protocols (Bidwell and Raucher, 2005; Meyer and Chilkoti, 1999). Second, the heat-induced aggregation allows for thermal targeting of ELP to a desired site *in vivo* by applying focused, externally generated hyperthermia at that site (Bidwell et al., 2013, 2012; Liu et al., 2006; Meyer et al., 2001). A third advantage of ELP for drug delivery is that it is non-immunogenic (D. W. Urry et al., 1991). The VPGxG repeat in ELP was originally derived from human elastin, and it does not induce an immune response *in vivo* (D. W. Urry et al., 1991). Two versions of ELP were tested in this study, one with 160 VPGxG repeats, a molecular weight of about 61 kDa, and a transition temperature of 65 °C, and a second with the same ELP modified at the N-terminus by the addition of a cell penetrating peptide (CPP) called SynB1 (Bidwell et al., 2010; Rousselle et al., 2001). We have shown previously that the addition of a CPP to ELP enhances its cellular uptake *in vitro* (Bidwell and Raucher, 2005; Bidwell et al., 2009, 2007; Massodi et al., 2010, 2005) and influences its biodistribution *in vivo* (Bidwell et al., 2013, 2012; Moktan et al.,

2012). To date, most of the preclinical development of ELP has been for drug delivery applications in cancer models (Bidwell and Raucher, 2010, 2006; Bidwell et al., 2013, 2012, 2007; Dreher et al., 2003; Liu et al., 2012, 2010; MacKay et al., 2009; Massodi et al., 2009a, 2009b, 2010; Meyer et al., 2001; Moktan and Raucher, 2012; Moktan et al., 2012). This study defines the pharmacokinetics, biodistribution, placental uptake, and fetal uptake of ELP and SynB1-ELP in a preclinical pregnancy model and lays the foundation for the use of ELP to safely deliver therapeutics in pregnant patients.

Materials and Methods

Purification of Elastin-like Polypeptides

The coding sequence for ELP with the sequence (VPGxG)_n where x is V, G, or A in a 1:7:8 ratio, respectively and n = 160 was cloned into the pET 25b expression vector as described previously (Bidwell and Raucher, 2005). Similarly, the coding sequence for SynB1-ELP (containing the same ELP moiety modified N-terminally with the SynB1 CPP) was cloned into pET 25b. Both polypeptides were expressed recombinantly in *E. coli* BLR(DE3) and purified by inverse transition cycling as described previously (Bidwell and Raucher, 2005).

Fluorescent Labeling of ELP

ELP or SynB1-ELP were covalently labeled at an engineered cysteine residue with rhodamine-5-maleimide or AlexaFluor 633 maleimide (Life Technologies) as described in (Bidwell and Raucher, 2005).

Animal Use

All animal use was approved by the Institutional Animal Care and Use Committee at the University Of Mississippi Medical Center and was carried out in accordance with the National Institutes for Health Guide for the Care and Use of Laboratory Animals. Timed pregnant Sprague Dawley rats (Charles River) were received at gestational day 11 (GD11) and acclimated to the animal housing facility.

For acute experiments, rats at GD14 were anesthetized with isoflurane and carotid catheters were placed for blood sampling. Rhodamine or AlexaFluor 633 labeled ELP or SynB1-ELP or saline control was injected by IV bolus at a dose of 100 mg/kg via the femoral vein. The injection syringe was weighed before and after injection to determine the exact dosage delivered. Blood was sampled intermittently for four hours, then the major organs, placentas, and pups were removed for fluorescence analysis. From each animal, two intact feto-amnio-placental units were rapidly frozen in liquid nitrogen for subsequent quantitative fluorescence and histological analysis.

For chronic experiments, rats at GD14 were anesthetized with isoflurane, a dose of carprofen (5 mg/kg) was administered SC, and minipumps were placed in the peritoneal cavity via a small abdominal incision. The pumps delivered 30 mg/kg/day of saline or rhodamine labeled ELP or SynB1-ELP. The incision was closed with 4-0 gut suture followed by skin closure with 4-0 prolene suture. Blood was sampled at 1h, 2h, 4h, and 8h following pump implantation and twice daily thereafter by tail prick until GD19. Bacon

flavored carprofen tablets (2 mg carprofen / tablet, BioServe) were fed daily for 2 days after pump implantation. On GD19, rats were again anesthetized with isoflurane, blood was drawn from the abdominal aorta, and organs, placentas, and pups were harvested for fluorescence analysis. Two feto-amnio-placental units from each animal were rapidly frozen for quantitative fluorescence and histological analysis.

Pharmacokinetic Analysis

Fluorescence intensity of plasma from both acute and chronic biodistribution experiments was determined using a Nanoquant plate and a fluorescence plate reader (Tecan). 2 μ L of undiluted plasma was measured at 543 nm excitation and 575 nm emission wavelengths and a PMT gain of 90. In addition to plasma measurements, the fluorescence of standards made from the injected proteins was determined at the same fluorometer settings. Generation of standard curves from the same batch of protein that was injected allows for correction for any variation in labeling efficiency between proteins. Plasma fluorescence was corrected for autofluorescence using plasma from saline injected animals, and the corrected fluorescence values were fit to the standard curves to determine plasma ELP concentration (μ g/mL). For acute experiments, plasma concentration versus time was fit to a two-compartment pharmacokinetic model, and pharmacokinetic parameters were calculated as described in (Bidwell et al., 2012).

For chronic IP infusion experiments, once steady state plasma levels were achieved (24 h and later time points), plasma data were fit linearly to determine the average plasma ELP level. All fitting was performed using GraphPad Prism.

In Vitro Dye Release

Rhodamine-labeled ELP or SynB1-ELP was incubated at a concentration of 100 μ M in serum from pregnant rats for 0 – 24 h at 37 °C. An aliquot of each sample was set aside for measurement of total fluorescence, then the protein fraction was precipitated by addition of 1:1 volume of 10 % trichloroacetic acid (TCA) and separated by centrifugation for five minutes at 13,000 $\times$ g. The fluorescence of the samples before and after TCA precipitation was measured with a fluorescence plate reader and a Nanoquant plate (Tecan) using 543 nm excitation and 575 nm emission and a gain value of 75. The fluorescence of the TCA supernatant was corrected for dilution and divided by the total sample fluorescence before precipitation to determine the % free dye. Data represent the mean $\pm$ s.d. of two independent experiments.

In Vivo Protein Stability

Plasma from each sample from the pharmacokinetic experiment (5 μ L) was electrophoresed on a 10% SDS-PAGE gel under non-reducing conditions, and 10 μ g of the injected protein was used as a loading control. Gels were imaged with a Chemi-doc imager with transillumination and an ethidium bromide emission filter to directly visualize the rhodamine label. One gel was run for each animal in the study, and all gels were imaged with 1.0 s exposure time. Total lane intensity and intensity of all bands < 50 kDa were determined using Image J. The percentage of the total lane intensity < 50 kDa was

calculated and corrected for the % < 50 kDa of the loading control. Data represent the average $\pm$ s.d. of 4 rats per group. One representative gel from each group is shown.

Ex vivo Fluorescence Imaging

Tissue levels of fluorescent proteins were determined by *ex vivo* whole organ fluorescence imaging using an IVIS Spectrum (Perkin Elmer). 8 placentas and their associated pups from each experimental rat were dissected from the amniotic sac and imaged in the IVIS with 535 nm excitation and 580 nm emission, field-of-view B, f stop = 2, and auto exposure. Heart, liver, kidney, spleen, lungs, and brain were imaged similarly. Standards were prepared from the injected proteins and plated 100 μ L per well in a 96-well plate. The plate of standards was imaged using identical parameters. Mean fluorescence radiant efficiency of each organ or standard was determined using a closely drawn region of interest using Living Image 4.3 software (Perkin Elmer). The mean fluorescence radiant efficiency was averaged for all placentas and pups from each rat, levels were corrected for autofluorescence determined from tissues of the saline injected animals, and fluorescence data were fit to the standard curve of the injected protein. Mean standardized fluorescence intensity was calculated for all rats in each group (n = 8 placentas and pups per rat and 4 rats per group). To generate images for publication, one representative image of placentas and pups from each group was opened using Living Image's "load as group" tool, and all images were displayed on the same scale to allow direct comparison of the fluorescence intensity among groups.

Quantitative Fluorescence Histology Analysis

Intact, frozen feto-amnio-placental units were sectioned using a cryomicrotome and placed on microscope slides (20 μ m sections). Slides were dried and scanned using a slide scanner with a 543 nm excitation laser (ScanArray Express, Perkin Elmer, laser power 70%, PMT gain 85% for sections from the acute experiment and laser power 80%, PMT gain 95% for sections from the chronic experiment, scan resolution 50 μ m). Standards of the injected protein were prepared by dilution into OCT cryo-embedding medium, drawn into a 1 cc syringe, frozen, and sectioned at 20 μ m thickness to create disks of each standard cut at the same thickness as the tissue. Standards were scanned with identical scan settings. The mean fluorescence intensity of standards, placenta, and pups was determined using ImageJ software. The mean tissue fluorescence intensity was corrected for autofluorescence (determined from scans of tissues from saline injected animals) and for exact injection dosage in each animal, then fit to the standard curve to determine the amount of ELP in each tissue (μ g ELP / g tissue). Placenta and pup levels were averaged for all animals (n = 10 – 20 sections / tissue and 4 rats per group), and the data represent the mean $\pm$ s.e.

Histological Staining and Microscopy

For whole-slide imaging of intact feto-amnio-placental units in rats injected with AlexaFluor 633-labeled ELPs, frozen tissue sections (20 μ m) were fixed for 10 min with 4% paraformaldehyde, rinsed, and stained with rhodamine-phalloidin (5 U/mL in PBS + 1% bovine serum albumin (BSA), Life Technologies) for 20 min at room temperature. Slides were washed three times, dried, and scanned using a fluorescence slide scanner. A 543 nm excitation laser and a 633 nm excitation laser were used for detection of rhodamine and

AlexaFluor 633, respectively, at a 10 μm resolution scan setting. Image brightness and contrast were adjusted manually using Image J to optimize the placental fluorescence and to detect the presence of any fetal fluorescence.

For microscopy, frozen tissue sections (20 μm) of feto-amnio-placental units from the acute and chronic injection of rhodamine-labeled ELP and SynB1-ELP were mounted on glass slides and fixed in -20°C acetone for 10 min. Slides were briefly washed with PBS and blocked in 1% BSA in PBST solution for 1 hour, followed by incubation with 1:200 dilution of the primary mouse monoclonal antibody (anti-Pan cytokeratin C-11, Sigma-Aldrich) in 1% BSA/PBST at 4°C overnight. After 3 washes in PBST (5 min each), the slides were incubated with 1:100 Alexa Fluor 488-conjugated rabbit, anti-mouse secondary antibody at room temperature for 1 hour. Slides were then washed and counterstained with 4', 6-diamidino-2-phenylindole (DAPI) and covered with coverslips. The sections were examined using a fluorescent microscope interfaced with Meta-morph 6.3 software. Images were pseudocolored using Image J, and brightness and contrast were adjusted to optimize image clarity.

Statistical Analysis

Ex vivo whole-organ fluorescence data were analyzed using a two-way ANOVA with treatment parameters for protein (ELP or SynB1-ELP) and tissue, and post-hoc multiple comparisons were made by Bonferroni analysis. Differences between ELP and SynB1-ELP in the quantitative placental and pup histology assay were assessed using t-tests. A p value of < 0.05 was considered statistically significant. Statistics were calculated using GraphPad Prism.

Results

Acute Pharmacokinetics of ELPs in Pregnant Rats

We determined the plasma clearance pharmacokinetics of ELP and SynB1-ELP in pregnant Sprague Dawley rats on GD14. As shown in Figure 1, after IV bolus administration, both ELP and SynB1-ELP showed a similar clearance profile with a rapid distribution phase occurring in the first 60 min followed by a slower elimination phase. ELP had a slightly higher initial plasma concentration of 3,168 $\mu\text{g/mL}$ than did SynB1-ELP (2,376 $\mu\text{g/mL}$), but SynB1-ELP cleared with a longer terminal half-life of 190.2 min relative to ELP (77.5 min). The data fit well to a two-compartment pharmacokinetic model, and the results of the fitting are shown in Table 1. SynB1-ELP had a faster plasma to tissue rate and a slower elimination rate than ELP, suggesting that the CPP promoted extravasation of the polypeptide and deposition within tissues. The faster plasma to tissue rate and the slower elimination rate contributed to the reduced apparent C_0 and longer terminal half-life of SynB1-ELP relative to ELP.

Protein Stability and Dye Release

When using fluorescently labeled proteins, it is imperative that the label be stably bound in order to get accurate pharmacokinetic and biodistribution data. To determine the stability of the rhodamine label attached to the proteins via maleimide chemistry, we incubated the

labeled protein in plasma from pregnant rats for various times at 37 °C. After incubation, all proteins were precipitated using a 1:1 mixture with 10% trichloroacetic acid, and the fluorescence of the remaining supernatant was measured and compared to the pre-precipitation fluorescence. As shown in Figure 2A, almost no label separated from the protein when incubated in rat plasma. Even after 24 h incubation, less than 2% of the dye was released from the protein. This demonstrates that the chemistry used to label these proteins is sufficient to produce a stable bond and confirms that our measurements are indeed of the labeled protein and not of released dye.

In addition to measuring dye release *in vitro*, we also examined the degradation of the protein in plasma samples *in vivo*. Plasma from the pharmacokinetic experiment above was analyzed by SDS-PAGE using direct fluorescence imaging to detect the labeled protein. As shown in Figure 2B and D, the total protein intensity decreased over time as the protein was cleared from circulation. However, very little protein degradation was apparent in either the ELP or the SynB1-ELP plasma samples. The amount of degradation was determined by measuring the total band intensity of the entire lane versus the total intensity of all bands < 50 kDa. Plotting the total lane intensity (Figure 2 C and E) revealed a clearance curve that closely overlaid the clearance seen with direct plasma fluorescence measurement in the pharmacokinetic experiment (Figure 1). Analysis of the percentage of the band intensities at <50 kDa molecular weight revealed that very little degraded protein was present (right axis in Figure 2C and E). Even at the 4 hour time point, less than 20% of the total signal was present in these degraded bands. This analysis revealed that these proteins were quite stable in circulation. This analysis must be considered with two caveats. First, since these proteins are labeled with fluorophors only at their termini, only fragments containing the labeled terminus will be visible. Second, because small fragments will likely be cleared by renal filtration, they may be under-represented in this PAGE analysis.

Acute Biodistribution of ELPs in Pregnant Rats

Four hours after the IV bolus dose, tissues were removed to determine the biodistribution of the polypeptides and their placental deposition and fetal uptake. We first examined the tissues by *ex vivo* whole organ fluorescence imaging. As shown in Figure 3A, both ELP and SynB1-ELP accumulated to high levels in the placenta, but no polypeptide was detectable in the pups. Quantitation of the whole organ fluorescence relative to standards of the injected proteins revealed that the highest polypeptide levels were found in the kidney at this time point after bolus dosing, and high levels were also seen in the liver (Figure 3B). The placenta also accumulated high levels of the polypeptides, but the pups had barely detectable (ELP) or completely undetectable (SynB1-ELP) polypeptide levels relative to autofluorescence. The addition of the SynB1 peptide to ELP increased its uptake in most organs. The kidneys were the most affected, with SynB1-ELP levels reaching more than 4 times the ELP levels ($p < 0.0001$). SynB1-ELP also accumulated to significantly higher levels in the liver and heart ($p = 0.014$ and $p = 0.0017$, respectively). The mean placental levels of SynB1-ELP were almost two-fold higher than the ELP levels, but the difference did not reach statistical significance ($p = 0.076$). The only exception to the rule of organ levels increasing with the addition of the CPP was in the spleen, where SynB1-ELP levels were significantly lower than ELP levels ($p = 0.00013$).

The *ex vivo* whole organ analysis gives a good snapshot of the polypeptide's biodistribution and an estimate of the actual tissue polypeptide levels. However, due to differences in organ size and therefore variability in the transmission of light through the tissue, combined with the difficulty of creating appropriate standards to correctly assess the absorbance and scattering of light, this technique has a limited ability to assess absolute tissue polypeptide levels. Therefore, we also carried out quantitative fluorescence analysis of placental and pup polypeptide levels using cryosections of intact feto-amnio-placental units. By sectioning tissue and polypeptide standards to the same thickness, this technique allows for accurate quantitation of tissue polypeptide levels. As shown in Figure 4A, this analysis confirmed that both polypeptides accumulated strongly in the placenta, but no polypeptide was detectable over autofluorescence in the pups. The images in Figure 4A, all collected at the same scan settings, also indicate that SynB1-ELP accumulated at higher levels in the placenta than did ELP. The quantitative analysis revealed that ELP placental levels were approximately 50 µg/g of tissue (Figure 4B). The placental level was increased over two-fold by the addition of the SynB1 CPP ($p < 0.0001$). The quantitative fluorescence analysis also confirmed the fetal exclusion of both ELP and SynB1-ELP. Neither peptide was detectable in the pups using this method (Figure 4B). To further confirm fetal exclusion, we injected rats on gestational day 14 with ELP and SynB1-ELP labeled with the near-infrared dye AlexaFluor 633. In the near-infrared region, the tissue autofluorescence is greatly decreased. Four hours after injection, feto-amnio-placental units were frozen and sectioned as above, slides were stained with rhodamine-phalloidin to mark actin and make both the placenta and the pups visible, and scanned using a fluorescence slide scanner. As shown in Figure 4C, this method confirmed that both ELP and SynB1-ELP accumulated highly in the placenta, but were undetectable in the pups. (Note that the images in Figure 4C were individually exposed to maximize the placental fluorescence and detect any fetal fluorescence. Therefore, the image intensities do not accurately reflect the differences in placental levels between the two polypeptides.)

We also examined the placental tissue microscopically with a cytokeratin counterstain to detect trophoblast cells. Low magnification revealed that both ELP and SynB1-ELP accumulated highly at the chorionic plate (Figure 5A and B, solid arrows) and distributed diffusely within the labyrinth zone. Higher magnification revealed that both polypeptides accumulated in the cytoplasm of trophoblast cells. However, the interior of chorionic villi, which contain fetal blood and are detected by voids in the cytokeratin staining, contained no ELP or SynB1-ELP (open arrows in Figure 5A and B). These results confirm at the cellular level the observations from the whole-organ and cryosection imaging that the ELP-based drug carrier is capable of entering cytotrophoblasts in the placenta but is excluded from transport into fetal circulation.

Biodistribution of ELPs in Pregnant Rats after Continuous Administration

Ex vivo whole organ and quantitative histological fluorescence analysis revealed that ELP and SynB1-ELP accumulate highly in the placenta but are excluded from the fetus four hours after bolus administration on GD14. We also examined whether the fetal exclusion held after five days of continuous infusion of the polypeptides. ELP or SynB1-ELP was administered continuously from GD14 to GD19 using an IP minipump. As shown in Figure

6A, this technique lead to a steady state plasma level of the polypeptides beginning 24 h after pump implantation. At the dose used (30 mg/kg/day), the plasma levels were maintained at 33.94 $\mu\text{g/mL}$ for ELP and 69.15 $\mu\text{g/mL}$ for SynB1-ELP. These plasma levels are a reflection of many *in vivo* kinetic processes, including the rate of transport from the peritoneal fluid to the blood, the rate of extravasation from the blood to the tissues, and the plasma clearance rate. Since SynB1-ELP has a longer terminal plasma half-life than ELP, this likely explains why there is a higher steady-state level of SynB1-ELP in the plasma than ELP. These plasma concentration data also provide useful information for the formulation of dosages for future studies, in which ELP will be fused with therapeutic agents.

We again performed *ex vivo* whole organ fluorescence analysis of the placentas, pups, and organs on GD19 following five days of continuous polypeptide infusion. Relative to the acute experiment, the placental levels of the polypeptides were lower, which resulted from the difference in dose (100 mg/kg in the bolus dosing versus 30 mg/kg/day in the chronic infusion). However, similar to the acute data, the polypeptides accumulated at high levels in the placenta but were undetectable over autofluorescence in the pups (Figure 6B). The kidneys still accumulated the most polypeptide, followed by the liver and the placenta (Figure 6C). Also, after chronic infusion, the effect of the CPP on the polypeptide biodistribution was much less pronounced. Only the kidneys contained significantly more SynB1-ELP than ELP (kidney levels of SynB1-ELP were increased four-fold relative ELP kidney levels, $p = 0.01$). This indicates that the increases seen in the tissues immediately after infusion were the result of faster tissue deposition kinetics for SynB1-ELP relative to ELP, and after chronic administration, the tissue levels of the two polypeptides eventually became equivalent (with the exception of the kidneys).

We also examined the feto-amnio-placental units from the chronically infused rats by quantitative fluorescence histology. As shown in Figure 7A, both ELP and SynB1-ELP were present at high levels in the placenta and in the chorionic membranes. Because the laser power and PMT gain were set higher in this experiment relative to the acute experiment, the fetal autofluorescence is more evident. But even with this higher PMT gain, the polypeptides were not readily detectable in the fetus. Quantitative analysis revealed that placental polypeptide levels were about 17 – 22 $\mu\text{g/g}$ of tissue for both ELP and SynB1-ELP (Figure 7B). Pup levels were barely detectable over autofluorescence for ELP (about 3.5 $\mu\text{g/g}$, and all detection came from the fetal liver which is highly autofluorescent) and completely undetectable for SynB1-ELP. This finding is significant because CPPs have been shown to mediate uptake across plasma membranes in many systems (Heitz et al., 2009), and SynB1 has even been shown to deliver some small molecule cargo types across the blood brain barrier (Rousselle et al., 2001). However, even after five days of continuous exposure, the SynB1-ELP polypeptide did not penetrate the placental barrier.

Microscopic analysis of the placentas revealed that both polypeptides accumulated in the cytoplasm of trophoblasts. However, similar to the acute exposure results, both ELP and SynB1-ELP were excluded from the fetal side of the chorionic villi (Figure 7C). These results indicate that ELP can be used to deliver therapeutic agents to the placenta, and that the carrier can even penetrate into placental trophoblasts to deliver therapeutics to

intracellular targets. However, therapeutics fused to ELP or SynB1-ELP will not cross the placental barrier and reach the fetal circulation.

Discussion

One of the most vexing problems in the development of therapeutic agents for the pregnant patient is ensuring restricted fetal exposure to the agent, or in determining that the agent has no adverse effects on fetal development. This is especially true in obstetrical complications, one of the most common of which is hypertension, which affects approximately 10% of all pregnancies ("Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy," 2000). Concerns for fetal outcome have significantly hampered development of therapeutics for the most common complications of pregnancy, including preeclampsia (Podymow and August, 2008). A number of commonly utilized antihypertensive agents have not been fully studied in pregnant populations. Others, such as ACE-I inhibitors, cause adverse fetal outcomes and death (Lambot et al., 2001). While in most cases severe hypertension can be ameliorated to acceptable levels to allow for extension of the pregnancy to near term, early induction of labor is often the outcome, making preeclampsia a leading cause of premature birth and maternal/fetal perinatal morbidity and mortality (Roberts et al., 2003). Several potential therapies, such as endothelin-1 type A receptor antagonists, have been suggested from preclinical studies, but the known teratogenic effects have prohibited their application in the clinical population (George and Granger, 2011a). The dearth of new therapeutic options for the treatment of preeclampsia that are safe for the developing fetus is a significant inadequacy in obstetrical therapeutics.

Here, we report a novel peptide polymer-based delivery system for the safe, maternally sequestered delivery of therapeutics during pregnancy. Acute administration of ELP with or without the cell penetrating peptide SynB1 demonstrated similar plasma kinetics, though the SynB1-tagged polypeptide exhibited both a faster extravasation into the tissues in the distribution phase as well as a slower elimination rate in the elimination phase. These data can also be contrasted with a previous study in which the pharmacokinetics of similar ELP and SynB1-ELP polypeptides were determined in non-pregnant female Sprague Dawley rats of about the same age (Bidwell et al., 2013). In that study, we determined terminal half-lives of 243 and 278 minutes for ELP and SynB1-ELP, respectively. The biodistribution in the non-pregnant rats was similar, with strong accumulation in the kidneys and liver. The significantly longer half-lives in non-pregnant rats are consistent with the expected increase in drug elimination rate during pregnancy. Future work will test the effects of modifying the ELP size on the pharmacokinetics, placental deposition, and fetal transfer in order to optimize delivery to the target maternal organs while still preventing fetal exposure.

Most significantly, when the acute biodistribution of the two peptides was examined, it became readily clear that ELP with or without the SynB1 CPP was unable to significantly cross the maternal/fetal interface and was restricted to the maternal compartment. When the placenta was examined histologically, both ELP and SynB1-ELP were found ubiquitously in the placental tissue, but were excluded from the fetal-derived placental villi, showing that the interface between the two circulations was the effective barrier of the molecules (Figure

5). Highest concentrations of both polypeptides were observed in the maternal kidney, liver, and placenta, with SynB1 increasing tissue levels in most of these organs. It is significant for potential therapeutics for preeclampsia that both the kidneys and placenta exhibit significant accumulation of the carriers. The placenta itself is the source of many of the pathogenic factors known to cause the maternal symptoms of preeclampsia, and the changes in blood pressure seen in the disorder are linked to decreased renal function and glomerular endotheliosis (Jeyabalan and Conrad, 2007; Moran et al., 2003). These organs, therefore, are the most likely candidates for intervention in the preeclampsia patient. The fetal exclusion of ELP even held after longer chronic exposures. When ELP or SynB1-ELP were administered chronically from GD14-19 (to mimic treatment throughout the final trimester of pregnancy as would be appropriate for preeclampsia therapy), we saw distributions quite similar to the acute experiments. The pattern of tissue biodistribution was similar to that seen in the acute experiments, with accumulation in the kidney, liver, and placenta but undetectable or barely detectable in the pups (Figure 6C). These data indicated that even in the setting of prolonged administration of ELP or SynB1-ELP as would be done for preeclampsia therapy, significant fetal exposure is unlikely to occur.

Our current focus is to utilize the ELP drug delivery system for delivery of therapeutics to treat preeclampsia. Preeclampsia is marked by new-onset hypertension, proteinuria, and maternal vascular dysfunction. If left unchecked, eclampsia can result, leading to maternal grand mal seizures, and in extreme cases, death. While the maternal hypertension is a significant health concern, it is not entirely clear that antihypertensive therapy *per se* is desirable. Preeclampsia is now thought to result from hypoperfusion and chronic ischemia of the placenta, which in turn secretes pathogenic factors into the maternal blood stream such as the VEGF antagonist sFlt-1 and inflammatory cytokines (George and Granger, 2011b). Lowered maternal pressure conceivably could lower placental perfusion even further and significantly worsen the effects of the disease. One potential therapeutic approach, then, is to directly target these placental-derived factors to prevent worsening of the maternal syndrome. However, ensuring fetal safety of new therapeutics remains a primary concern. The ELP-based delivery system described here could provide a safe carrier with which to administer new therapeutics, (either peptide or small molecule-based) to the preeclamptic patient with significantly reduced risk of causing fetal harm. Our current work is focused on development of ELP-fused protein and peptide-based antagonists of s-Flt function or inflammatory cytokine production. In addition to the management of preeclampsia, a vast array of contraindicated therapies for a spectrum of disorders could also be safely delivered in the pregnant patient. The data here support the utility of ELP conjugation as a method of maternally sequestering these potentially harmful agents. Future work testing ELP transport in human placental tissue *ex vivo* and assessing efficacy of ELP-conjugated therapeutics in preclinical models of pregnancy disorders such as preeclampsia should prove enlightening.

Conclusion

We have tested the protein based drug carrier ELP to determine its distribution during pregnancy in a rat model. We found that ELP can be used to target drugs to the maternal kidneys, liver, or placenta, but that little to no ELP crosses the placenta and reaches the developing fetus. ELP is a versatile drug carrier that is being developed for delivering drugs

for many diseases, from cancer to cardiovascular disease. This work demonstrates that ELP can be used as a carrier to prevent placental transfer of drugs. Therefore, ELP fusion could transform drugs that are currently unsafe, or whose safety is unknown, into a formulation that is safe for use during pregnancy. Additionally, ELP fusion could allow for the development of new therapeutics for disorders of pregnancy for which we currently have no therapies.

References

- Alexander BT, Rinewalt AN, Cockrell KL, Massey MB, Bennett WA, Granger JP. Endothelin type a receptor blockade attenuates the hypertension in response to chronic reductions in uterine perfusion pressure. *Hypertension*. 2001; 37:485–489. [PubMed: 11230323]
- Bartels PA, Hanff LM, Mathot RA, Steegers EA, Vulto AG, Visser W. Nicardipine in pre-eclamptic patients: placental transfer and disposition in breast milk. *BJOG*. 2007; 114:230–3. doi:10.1111/j.1471-0528.2006.01200.x. [PubMed: 17166219]
- Bidwell GL, Davis AN, Raucher D. Targeting a c-Myc inhibitory polypeptide to specific intracellular compartments using cell penetrating peptides. *J Control Release*. 2009; 135:2–10. doi:10.1016/j.jconrel.2008.11.015. [PubMed: 19095020]
- Bidwell GL, Fokt I, Priebe W, Raucher D. Development of elastin-like polypeptide for thermally targeted delivery of doxorubicin. *Biochem Pharmacol*. 2007; 73:620–31. [PubMed: 17161827]
- Bidwell GL, Perkins E, Hughes J, Khan M, James JR, Raucher D. Thermally targeted delivery of a c-Myc inhibitory polypeptide inhibits tumor progression and extends survival in a rat glioma model. *PLoS One*. 2013; 8:e55104. doi:10.1371/journal.pone.0055104. [PubMed: 23372821]
- Bidwell GL, Perkins E, Raucher D. A thermally targeted c-Myc inhibitory polypeptide inhibits breast tumor growth. *Cancer Lett*. 2012; 319:136–43. doi:10.1016/j.canlet.2011.12.042. [PubMed: 22261328]
- Bidwell GL, Raucher D. Application of thermally responsive polypeptides directed against c-Myc transcriptional function for cancer therapy. *Mol Cancer Ther*. 2005; 4:1076–85. doi: 10.1158/1535-7163.MCT-04-0253. [PubMed: 16020665]
- Bidwell GL, Raucher D. Enhancing the antiproliferative effect of topoisomerase II inhibitors using a polypeptide inhibitor of c-Myc. *Biochem Pharmacol*. 2006; 71:248–56. doi:10.1016/j.bcp.2005.10.041. [PubMed: 16316634]
- Bidwell GL, Raucher D. Therapeutic peptides for cancer therapy. Part I - peptide inhibitors of signal transduction cascades. *Expert Opin Drug Deliv*. 2009; 6:1033–47. [PubMed: 19637980]
- Bidwell GL, Raucher D. Cell penetrating elastin-like polypeptides for therapeutic peptide delivery. *Adv Drug Deliv Rev*. 2010; 62:1486–96. doi:10.1016/j.addr.2010.05.003. [PubMed: 20478348]
- Bidwell GL, Whittom AA, Thomas E, Lyons D, Hebert MD, Raucher D. A thermally targeted peptide inhibitor of symmetrical dimethylation inhibits cancer-cell proliferation. *Peptides*. 2010; 31:834–41. doi:10.1016/j.peptides.2010.02.007. [PubMed: 20167239]
- Chu M, Wu Q, Yang H, Yuan R, Hou S, Yang Y, Zou Y, Xu S, Xu K, Ji A, Sheng L. Transfer of quantum dots from pregnant mice to pups across the placental barrier. *Small Wein. Bergstr. Ger*. 2010; 6:670–678. doi:10.1002/smll.200902049.
- Dreher MR, Raucher D, Balu N, Michael Colvin O, Ludeman SM, Chilkoti A. Evaluation of an elastin-like polypeptide-doxorubicin conjugate for cancer therapy. *J Control Release*. 2003; 91:31–43. [PubMed: 12932635]
- Fenakel K, Fenakel G, Appelman Z, Lurie S, Katz Z, Shoham Z. Nifedipine in the treatment of severe preeclampsia. *Obstet Gynecol*. 1991; 77:331–7. [PubMed: 1992393]
- Firan M, Bawdon R, Radu C, Ober RJ, Eaken D, Antohe F, Ghetie V, Ward ES. The MHC class I-related receptor, FcRn, plays an essential role in the maternofetal transfer of gamma-globulin in humans. *Int. Immunol*. 2001; 13:993–1002. [PubMed: 11470769]
- Galbraith GM, Galbraith RM, Faulk WP. Immunological studies of transferrin and transferrin receptors of human placental trophoblast. *Placenta*. 1980; 1:33–46. [PubMed: 6255455]

- George EM, Granger JP. Endothelin: key mediator of hypertension in preeclampsia. *Am. J. Hypertens.* 2011a; 24:964–969. doi:10.1038/ajh.2011.99. [PubMed: 21677700]
- George EM, Granger JP. Mechanisms and potential therapies for preeclampsia. *Curr. Hypertens. Rep.* 2011b; 13:269–275. doi:10.1007/s11906-011-0204-0. [PubMed: 21465139]
- Heitz F, Morris MC, Divita G. Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Br J Pharmacol.* 2009; 157:195–206. [PubMed: 19309362]
- Jeyabalan A, Conrad KP. Renal function during normal pregnancy and preeclampsia. *Front. Biosci. J. Virtual Libr.* 2007; 12:2425–2437.
- Lambot MA, Vermeylen D, Noël JC. Angiotensin-II-receptor inhibitors in pregnancy. *Lancet.* 2001; 357:1619–1620. [PubMed: 11386314]
- Leach JL, Sedmak DD, Osborne JM, Rahill B, Lairmore MD, Anderson CL. Isolation from human placenta of the IgG transporter, FcRn, and localization to the syncytiotrophoblast: implications for maternal-fetal antibody transport. *J. Immunol. Baltim. Md 1950.* 1996; 157:3317–3322.
- Liu W, Dreher MR, Furgeson DY, Peixoto KV, Yuan H, Zalutsky MR, Chilkoti A. Tumor accumulation, degradation and pharmacokinetics of elastin-like polypeptides in nude mice. *J Control Release.* 2006; 116:170–178. [PubMed: 16919353]
- Liu W, MacKay JA, Dreher MR, Chen M, McDaniel JR, Simnick AJ, Callahan DJ, Zalutsky MR, Chilkoti A. Injectable intratumoral depot of thermally responsive polypeptide-radionuclide conjugates delays tumor progression in a mouse model. *J Control Release.* 2010; 144:2–9. doi:10.1016/j.jconrel.2010.01.032. [PubMed: 20117157]
- Liu W, McDaniel J, Li X, Asai D, Quiroz FG, Schaal J, Park JS, Zalutsky M, Chilkoti A. Brachytherapy using injectable seeds that are self-assembled from genetically encoded polypeptides in situ. *Cancer Res.* 2012; 72:5956–65. doi:10.1158/0008-5472.CAN-12-2127. [PubMed: 23155121]
- MacKay JA, Chen M, McDaniel JR, Liu W, Simnick AJ, Chilkoti A. Self-assembling chimeric polypeptide-doxorubicin conjugate nanoparticles that abolish tumours after a single injection. *Nat Mater.* 2009; 8:993–9. doi:10.1038/nmat2569. [PubMed: 19898461]
- Massodi I, Bidwell GL, Davis A, Tausend A, Credit K, Flessner M, Raucher D. Inhibition of ovarian cancer cell metastasis by a fusion polypeptide Tat-ELP. *Clin Exp Metastasis.* 2009a; 26:251–60. doi:10.1007/s10585-009-9237-z. [PubMed: 19169893]
- Massodi I, Bidwell GL, Raucher D. Evaluation of cell penetrating peptides fused to elastin-like polypeptide for drug delivery. *J Control Release.* 2005; 108:396–408. doi:10.1016/j.jconrel.2005.08.007. [PubMed: 16157413]
- Massodi I, Moktan S, Rawat A, Bidwell GL, Raucher D. Inhibition of ovarian cancer cell proliferation by a cell cycle inhibitory peptide fused to a thermally responsive polypeptide carrier. *Int J Cancer.* 2010; 126:533–44. doi:10.1002/ijc.24725. [PubMed: 19588502]
- Massodi I, Thomas E, Raucher D. Application of thermally responsive elastin-like polypeptide fused to a lactoferrin-derived peptide for treatment of pancreatic cancer. *Molecules.* 2009b; 14:1999–2015. [PubMed: 19513001]
- McArdle HJ, Morgan EH. Transferrin and iron movements in the rat conceptus during gestation. *J. Reprod. Fertil.* 1982; 66:529–536. [PubMed: 7175808]
- Menjoge AR, Rinderknecht AL, Navath RS, Faridnia M, Kim CJ, Romero R, Miller RK, Kannan RM. Transfer of PAMAM dendrimers across human placenta: prospects of its use as drug carrier during pregnancy. *J. Control. Release Off. J. Control. Release Soc.* 2011; 150:326–338. doi:10.1016/j.jconrel.2010.11.023.
- Meyer DE, Chilkoti A. Purification of Recombinant Proteins by Fusion with Thermally Responsive Polypeptides. *Nat. Biotechnol.* 1999; 17:1112–1115. [PubMed: 10545920]
- Meyer DE, Kong GA, Dewhirst MW, Zalutsky MR, Chilkoti A. Targeting a Genetically Engineered Elastin-like Polypeptide to Solid Tumors by Local Hyperthermia. *Cancer Res.* 2001; 61:1548–1554. [PubMed: 11245464]
- Moktan S, Perkins E, Kratz F, Raucher D. Thermal targeting of an acid-sensitive doxorubicin conjugate of elastin-like polypeptide enhances the therapeutic efficacy compared with the parent compound in vivo. *Mol Cancer Ther.* 2012; 11:1547–56. doi:10.1158/1535-7163.MCT-11-0998. [PubMed: 22532601]

- Moktan S, Raucher D. Anticancer activity of proapoptotic peptides is highly improved by thermal targeting using elastin-like polypeptides. *Int J Pept Res Ther.* 2012; 18:227–237. doi:10.1007/s10989-012-9295-y. [PubMed: 23105921]
- Moktan S, Ryppa C, Kratz F, Raucher D. A thermally responsive biopolymer conjugated to an acid-sensitive derivative of paclitaxel stabilizes microtubules, arrests cell cycle, and induces apoptosis. *Invest New Drugs.* 2010 doi:10.1007/s10637-010-9560-x.
- Moran P, Baylis PH, Lindheimer MD, Davison JM. Glomerular ultrafiltration in normal and preeclamptic pregnancy. *J. Am. Soc. Nephrol. JASN.* 2003; 14:648–652.
- Myllynen PK, Loughran MJ, Howard CV, Sormunen R, Walsh AA, Vähäkangas KH. Kinetics of gold nanoparticles in the human placenta. *Reprod. Toxicol. Elmsford N.* 2008; 26:130–137. doi: 10.1016/j.reprotox.2008.06.008.
- Podmow T, August P. Update on the use of antihypertensive drugs in pregnancy. *Hypertension.* 2008; 51:960–969. doi:10.1161/HYPERTENSIONAHA.106.075895. [PubMed: 18259046]
- Raucher D, Moktan S, Massodi I, Bidwell GL. Therapeutic peptides for cancer therapy. Part II - cell cycle inhibitory peptides and apoptosis-inducing peptides. *Expert Opin Drug Deliv.* 2009; 6:1049–64. doi:10.1517/17425240903158909. [PubMed: 19743895]
- Report of the National High Blood Pressure Education Program Working Group on High Blood Pressure in Pregnancy. *Am. J. Obstet. Gynecol.* 2000; 183:S1–S22.
- Roberts JM, Pearson G, Cutler J, Lindheimer M, NHLBI Working Group on Research on Hypertension During Pregnancy. Summary of the NHLBI Working Group on Research on Hypertension During Pregnancy. *Hypertension.* 2003; 41:437–445. doi:10.1161/01.HYP.0000054981.03589.E9. [PubMed: 12623940]
- Rousselle C, Smirnova M, Clair P, Lefauconnier JM, Chavanieu A, Calas B, Scherrmann JM, Temsamani J. Enhanced delivery of doxorubicin into the brain via a peptide-vector-mediated strategy: saturation kinetics and specificity. *J Pharmacol Exp Ther.* 2001; 296:124–31. [PubMed: 11123372]
- Shamji MF, Betre H, Kraus VB, Chen J, Chilkoti A, Pichika R, Masuda K, Setton LA. Development and characterization of a fusion protein between thermally responsive elastin-like polypeptide and interleukin-1 receptor antagonist: sustained release of a local antiinflammatory therapeutic. *Arthritis Rheum.* 2007; 56:3650–61. doi:10.1002/art.22952. [PubMed: 17968946]
- Shamji MF, Chen J, Friedman AH, Richardson WJ, Chilkoti A, Setton LA. Synthesis and characterization of a thermally-responsive tumor necrosis factor antagonist. *J Control Release.* 2008a; 129:179–86. doi:10.1016/j.jconrel.2008.04.021. [PubMed: 18547669]
- Shamji MF, Jing L, Chen J, Hwang P, Ghodsizadeh O, Friedman AH, Richardson WJ, Setton LA. Treatment of neuroinflammation by soluble tumor necrosis factor receptor Type II fused to a thermally responsive carrier. *J Neurosurg Spine.* 2008b; 9:221–8. doi:10.3171/SPI/2008/9/8/221. [PubMed: 18764758]
- Sibai BM, Barton JR, Akl S, Sarinoglu C, Mercer BM. A randomized prospective comparison of nifedipine and bed rest versus bed rest alone in the management of preeclampsia remote from term. *Am J Obstet Gynecol.* 1992; 167:879–84. [PubMed: 1415419]
- Story CM, Mikulska JE, Simister NE. A major histocompatibility complex class I-like Fc receptor cloned from human placenta: possible role in transfer of immunoglobulin G from mother to fetus. *J. Exp. Med.* 1994; 180:2377–2381. [PubMed: 7964511]
- Taniguchi T, Muramatsu I. Pharmacological knockout of endothelin ET(A) receptors. *Life Sci.* 2003; 74:405–9. [PubMed: 14607269]
- Urry DW, Long MM, Cox BA, Ohnishi T, Mitchell LW, Jacobs M. The synthetic polypentapeptide of elastin coacervates and forms filamentous aggregates. *Biochim Biophys Acta.* 1974; 371:597–602. [PubMed: 4474024]
- Urry DW, Luan C-H, Parker TM, Gowda DC, Prasad KU, Reid MC, Safavy A. Temperature of Polypeptide Inverse Temperature Transition Depends on Mean Residue Hydrophobicity. *J Am Chem Soc.* 1991; 113:4346–4348.
- Urry DW, Parker TM, Reid MC, Gowda DC. Biocompatibility of the bioelastic materials, poly(GVGVP) and its gamma-irradiation cross-linked matrix - summary of generic biological test results. *Bioact Compat Polym.* 1991; 6:263–282.

- Yamashita K, Yoshioka Y, Higashisaka K, Mimura K, Morishita Y, Nozaki M, Yoshida T, Ogura T, Nabeshi H, Nagano K, Abe Y, Kamada H, Monobe Y, Imazawa T, Aoshima H, Shishido K, Kawai Y, Mayumi T, Tsunoda S-I, Itoh N, Yoshikawa T, Yanagihara I, Saito S, Tsutsumi Y. Silica and titanium dioxide nanoparticles cause pregnancy complications in mice. *Nat. Nanotechnol.* 2011; 6:321–328. doi:10.1038/nnano.2011.41. [PubMed: 21460826]
- Yang H, Sun C, Fan Z, Tian X, Yan L, Du L, Liu Y, Chen C, Liang X, Anderson GJ, Keelan JA, Zhao Y, Nie G. Effects of gestational age and surface modification on materno-fetal transfer of nanoparticles in murine pregnancy. *Sci. Rep.* 2012; 2:847. doi:10.1038/srep00847. [PubMed: 23150793]
- Zalgevi ien V, Kulvietis V, Bulotien D, Didžiapetrien J, Rotomskis R. The effect of nanoparticles in rats during critical periods of pregnancy. *Med. Kaunas Lith.* 2012; 48:256–264.

Figure 1. Plasma Clearance of ELPs in Pregnant Rats. Rhodamine-labeled ELP or SynB1-ELP was injected IV (100 mg/kg) in pregnant rats on GD14. Plasma was sampled over time, and fluorescence measurements were fit to standard curves of the injected proteins to calculate plasma levels. Data represent the mean $\pm$ s.d. of four rats per group. Lines represent the fit to a two-compartment pharmacokinetic model.

Figure 2.

Dye release and protein stability in plasma. A. Rhodamine-labeled ELP and SynB1-ELP were incubated in plasma from pregnant rats for the indicated time at 37 °C. Percentage of dye release is shown for an average of two experiments, bars indicate s.d. *In vivo* protein stability was determined by SDS-PAGE analysis of plasma samples from the pharmacokinetic experiment (B – E). A representative gel from one animal in each group (B, ELP; D, SynB1-ELP) is shown. The numbers indicate time points, and the final lane was loaded with 10 µg of the injected protein as a loading control. The total band intensity and %

<50kDa (C, ELP; E, SynB1-ELP) are shown for an average of four animals per group, bars indicate s.d.

Figure 3.

Biodistribution, placental deposition, and fetal exposure to ELPs. **A.** Four hours after IV administration of rhodamine-labeled ELP or SynB1-ELP, polypeptide levels were determined in the placentas, pups, and major organs by *ex vivo* whole organ fluorescence imaging. A representative image of eight placentas and corresponding pups from one animal from each group is shown. **B.** Fluorescence intensities were quantified, corrected for autofluorescence, and fit to standards of the injected proteins. Data represent the mean $\pm$ s.e. of eight placentas and eight pups per rat and four rats per group. * Statistically significant as determined by a two-way ANOVA with post-hoc Bonferroni multiple comparisons ($p < 0.05$). ** Levels were not detectable over autofluorescence.

Figure 4. Quantitative Fluorescence Histology of Feto-amnio-placental Units. Frozen feto-amnio-placental units were cut to 20 µm sections, and slides were scanned with a fluorescence slide scanner. **A.** Representative images from each animal were collected with identical scan settings. **B.** Data from all slide scans were quantified relative to fluorescence standards made from the injected protein cut to the same thickness. * Statistically significant as determined by a t-test ($p < 0.05$). ** Levels were not detectable over autofluorescence. **C.** A similar analysis was conducted in rats injected with AlexaFluor 633 – labeled polypeptides. Shown are representative images individually exposed to maximize placental levels in order to search for any fetal fluorescence.

Figure 5. Intra-placental Distribution of ELPs. Slides from Figure 4A were immunostained with a cytokeratin antibody to mark trophoblast cells (green), and fluorescence of the rhodamine-labeled ELP (A) and SynB1-ELP (B) was detected (red). The 40× magnification shows polypeptide accumulation at the chorionic plate (solid arrows) and in the labyrinth. The 100× magnification shows polypeptide in the cytoplasm of trophoblast cells but excluded from the fetal chorionic villi (open arrows).

Figure 6.

Plasma Levels and Biodistribution of ELPs after Chronic Infusion. **A.** Rhodamine-labeled ELP or SynB1-ELP was administered chronically by IP minipump from GD14 – GD19. Plasma was sampled throughout the experiment, and polypeptide levels were determined relative to standards of the injected protein. Data represent the mean $\pm$ s.d. of four rats per group. **B.** *Ex vivo* fluorescence imaging of eight pups and corresponding placentas from one rat from each group is shown. **C.** Fluorescence intensities were quantified, corrected for autofluorescence, and fit to standards of the injected proteins. Data represent the mean $\pm$ s.e. of eight placentas and eight pups per rat and four rats per group. * Statistically significant as determined by a two-way ANOVA with post-hoc Bonferroni multiple comparisons ($p < 0.05$). ** Levels were not detectable over autofluorescence.

Figure 7. Quantitative Fluorescence Histology and Intra-placental Distribution of ELPs after Chronic Infusion. **A.** Intact feto-amnio-placental units were sectioned and analyzed by quantitative fluorescence histology as described above. A representative section of one slide from each group is shown. **B.** Data from all slide scans were quantified relative to fluorescence standards made from the injected protein cut to the same thickness. ** Levels were not detectable over autofluorescence. **C and D.** Intra-placental distribution of ELP (red, C) and SynB1-ELP (red, D) was determined following immunostaining for cytokeratin (green).

Table 1

			ELP	SynB1-ELP
Initial Concentration	C_o	(ug/mL)	3,168	2,376
Central Compartment Volume of Distribution	V_c	(mL)	7.7	13.0
Plasma Clearance	Cl	(mL·min ⁻¹)	0.11	0.08
Area Under Curve	AUC	(μg·min·mL ⁻¹)	215,254.3	401,975.3
Tissue to Plasma rate constant	k_{tp}	(min ⁻¹)	0.023	0.023
Plasma to Tissue rate constant	k_{pt}	(min ⁻¹)	0.009	0.012
Elimination rate constant	k_{el}	(min ⁻¹)	0.015	0.006
Distribution Half Life	$t_{1/2, dist}$	(min)	18.3	18.8
Terminal Half Life	$t_{1/2, term}$	(min)	77.5	190.2