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**TITLE:** Circumventing Therapeutic Resistance and the Emergence of Disseminated Breast Cancer Cells Through Non-Invasive Optical Imaging

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Herein we explore a series of optically distinct near infrared emissive polymersomes (NIREPs; biodegradable polymer vesicles that manifest extraordinarily high irradiances and are ideally suited for in vivo optical imaging), each conjugated to different antibodies for the non-invasive molecular imaging of all breast cancer sites within a patient, including micrometastases. In Year 1, we have taken significant steps to optimize NIREP fabrication protocols to ensure immunoreactive antibodies can be reproducibly conjugated to the surface of NIREPs. We have designed and synthesized the most extensive family of PEO-b-PCL copolymers that vary over the largest range of number-average molecular weights (Mn: 3.6 - 57K), PEO weight fractions (fPEO: 0.08 - 0.33), and PEO chain lengths (0.75 – 5.8K) reported to date. With these, we have demonstrated using comparative morphology diagrams, that a multiplicity of PEO-b-PCL diblock copolymer compositions are able to give rise to nanoscale vesicles. The broad range of polymersome-forming PEO-b-PCL compositions suggest the ability to construct extensive families of nanoscale vesicles of varied bilayer thickness, providing the ability to tune the timescales of vesicle degradation in vivo. Lastly, we have established four pairs of parental and isogenic matched tamoxifen (tam)-resistant cell lines (MCF7, T47D, MDA-MB-361 and HCC1428), and characterized the changes in cell surface protein expression that occurs with onset of resistance. We have found that members of the HER receptor family (EGFR/HER1; HER2; HER3) are upregulated with the development of tam-resistance; furthermore, differences in receptor expression between ER+ cell lines have been characterized. These reagents are critical for evaluation of the selectivity of a panel of antibody-conjugated NIREPs.
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Introduction
Breast cancer (BC) is a molecularly heterogeneous disease, which can be categorized into subtypes based on the tumor biology.\(^1\)\(^2\) HER2 over-expressing cancers (HER2+) for example, account for 25–30% of diagnosed BCs and are associated with early metastasis and poor survival prognosis.\(^3\) Treatments for HER2+ BCs, such as Herceptin,\(^4\) directly target the HER2 cell membrane receptor.\(^5\) Such treatments are specific to HER2+ BCs, with no effect on other tumor subtypes e.g. estrogen receptor overexpressing cancers (ER+, luminal A subtype). Similarly, ER+ BC are treated with endocrine therapies targeting either ER directly e.g. tamoxifen,\(^6\) or production of the receptor ligand (estradiol) e.g. aromatase inhibitors.\(^7\)\(^8\) It is therefore crucial to molecularly profile a cancer to prescribe the correct program of treatment. Diagnosis and subsequent treatment of BC is often based on analysis of a single biopsy of a primary tumor, and the surrounding lymph nodes. However, this method is flawed in advanced stage cancers, as the molecular profile of the cancer may differ at metastatic sites and/or between metastases in different organs.\(^9\) Furthermore, most advanced stage breast cancers that initially respond to targeted therapy, whether HER2+ or ER+, become resistant to treatment, generally in less than 12 months, resulting in disease progression.\(^10\)\(^11\) The molecular heterogeneity of BC tumors, coupled with the diverse mechanisms and dynamic nature of therapy resistance,\(^12\)\(^13\)\(^14\)\(^15\)\(^16\)\(^17\)\(^18\) illustrate that this method of diagnosis is insufficient to predict a tumor’s response to treatment. This accounts for the mixed clinical responses to treatment observed in advanced stage BC patients, where some sites regress while others see progression. Developing effective treatment strategies for BC requires the ability to identify the molecular subtypes of each tumor site, and understand the mechanisms involved in the development of therapeutic resistance. However, biopsying each site multiple times over the course of treatment is impossible. We proposed that near-infrared emissive polymersomes (NIREPs), a brightly emissive nanostructure, can be targeted to signature cell surface proteins to enable quantitative, non-invasive optical imaging of molecularly heterogeneous sites of BC in vivo. In this DoD 2013-2014 annual report, we provide an update on our progress to develop antibody-conjugated NIREPs capable of distinguishing between phenotypically distinct BC cells.

Body
Aim 1 (Years 1–3): Profile a panel of molecularly heterogeneous human breast cancer cell lines using cocktails of antibody-conjugated NIREPs.

Therien Group: In our prior work, we demonstrated that NIREPs featuring surfaces functionalized with the humanized anti-HER2 monoclonal antibody trastuzumab (Herceptin), were able to discriminate between HER2+ and HER2– cell lines in \textit{in vitro} experiments (Figure 1d). The intent of this project is to extend these studies by developing a series of other antibody-conjugated NIREPs, each targeting a specific breast cancer-relevant cell surface antigen. Each unique NIREP would contain a porphyrin oligomer fluorophore to enable wavelength-based detection of cell receptor type (Figure 1a–c).

In a separate DOD project (PI: Armstrong) it was found that NIREPs bearing antibodies targeted to EPCAM saw batch-to-batch variability in flow cytometry experiments for binding to EPCAM over-expressing cells. Investigation of new batches of anti-HER2-NIREPs found similar difficulties in reproducibility (Figure 2). In some batches, high uptake of up to 85,000 NIREPs per HER2+ SKBR3 cell were observed; in others, uptake levels matched that of control NIREPs conjugated to an isotype-matched IgG antibody. This prompted us to reinvestigate our protocols in detail. Our previously used bicinchoninic assay (BCA) used to determine the degree of antibody functionalization on the surface on the NIREP was unable to distinguish between covalently bound-antibody and surface associated antibody. Therefore, the use of this assay to determine the efficiency of different antibody-coupling chemistries in some cases may have yielded inaccurate data.

We have therefore invested time optimizing our coupling protocols and characterization methods as described below.
Figure 1 Schematic depiction of an antibody-conjugated NIREP: a) Polymersomes consist of a bilayer of amphiphiles which self-assemble in aqueous conditions. The resulting vesicle has the hydrophilic head groups pointing into the interior of the vesicle, and towards bulk solvent, with the hydrophobic chains sequestered within the membrane; b) The lipophilic membrane can be loaded with near-IR fluorophores (PZn₃ compounds, e.g. PZn₃: λₑₓ = 786 nm; λₑ𝑚 = 809 nm). Number of porphyrins N and solubilizing groups R can be varied to alter emission wavelength; c) Antibody-conjugated NIREPs enable tumor specific cell targeting. (d) Anti-HER2 NIREPs can discriminate between HER2+ cells (HCC1569, BT474 and SKBR3 cells) and HER2 negative cells (MCF7). Left column: nuclei stained with Hoechst 33258 (λₑᵐ = 461 nm). Center column: PZn₃ anti-HER2-NIREPs (λₑᵐ = 809 nm) located on the membrane surface and in the cell cytoplasm. Right column: Overlay of the micrographs from the blue and red channels.

Figure 2 Flow sorting of HER2+ SKBR3 cells using anti-HER2-NIREPs; detection using the Cy7 channel (>790 nm near infrared wavelength). a) Batch 1: Large uptake of anti-HER2-NIREPs into SKBR3 cells gives a large increase in fluorescence compared to unstained cells; b) Batch 2: Negligible uptake of anti-HER2-NIREPs into SKBR3 cells, with very similar levels of fluorescence to cells incubated with control IgG-conjugated NIREPs.

Conjugation chemistries

We have reexplored two orthogonal conjugation methods for the coupling of antibodies to the surface of NIREPs. The first method utilizes a fluoronitrobenzoic acid (FNB) functionality which is introduced onto the block-copolymer end hydroxyl group in a single, high yielding step (Scheme 1).
Scheme 1 Functionalization of PDB-b-PEO diblock copolymer OB18 with FNB. Conjugation of a bioligand via a lysine residue yields a chromophoric ortho-nitroaniline linker highlighted in yellow.

The advantage of this method is twofold. Firstly, FNB is reactive to primary amines, which in the case of antibodies, is readily available via the protein N-terminus or surface lysine residues. The abundance of these residues in antibodies means prior chemical modification of antibodies is unnecessary, minimizing the possibility of antibody-deactivation through inadvertent modification of the complementarity determining regions (CDR). Secondly, conjugation of primary amines to the para-position of FNB yields a chromophoric ortho-nitroaniline, which absorbs strongly at 428 nm. This provides an internal ‘reporter’ that enables us to quantify covalently bound antibody using absorption at this wavelength. Our group has previously used FNB-based chemistry to successfully functionalize NIREPs with the cell-penetrating Tat peptide for tracking dendritic cells in vivo, so we are confident we can adapt this protocol for the attachment of antibodies.

The second method we are exploring uses a sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-SMCC) linker, which we can use to couple antibodies to the NIREP surface via a sulfhydryl group (Scheme 2).

Scheme 2 Synthesis of SMCC-functionalized PEO-b-PBD co-polymer OB18. SMCC-OB18 reacts with a thiol group introduced onto an antibody using 2-iminothiolane (Traut’s reagent).

Sulfhydryl functionality can be introduced onto the surface of antibodies by modification of native lysines with 2-iminothiolane (Traut’s reagent), without destruction of antibody immunoreactivity. Many examples of sulfo-SMCC being used as a protein-drug conjugate linker exist in the literature, most notably in the case of the 2013 FDA-approved chemotherapy drug ado-trastuzumab emtansine (Kadcyla®), where Herceptin is ligated through sulfo-SMCC to the cytotoxic agent mertansine (DM1). We will evaluate the conjugation efficiency of antibodies to sulfo-SMCC functionalized NIREPs using size exclusion chromatography and SDS-PAGE.

NIREP construction

Using our newly validated functionalized OB18 polymer, we can construct NIREPs using our well-established methodology (Figure 3).
Figure 3 Construction of antibody-conjugated NIREPs via FNB- and sulfo-SMCC routes.

FNB/amine functionalized PEO(3600)-b-PBD(6800) diblock copolymer (OB18) was mixed with PEO(1300)-b-PBD(2500) diblock copolymer (OB2) in a 5:95 molar ratio. The mixed diblock copolymer and porphyrin fluorophore (PZnN) were dissolved in dichloromethane in a 40:1 polymer:fluorophore molar ratio. The solution was plated onto a roughened Teflon film and dried under vacuum overnight. Polymersomes were formed upon the addition of aqueous buffer (for FNB: 0.1 M sodium borate buffer, pH 8.5; for amine-OB18: 0.1 M PBS buffer, pH 7.5) and sonication for 1 h. A narrow size distribution of nano-size polymersomes was achieved with serial extrusion using a Liposofast Basic handheld extruder equipped with 400-, 200- and 100 nm polycarbonate membranes (Avestin Inc., Ottawa, Ontario). The resulting polymersomes are characterized by dynamic light scattering (DLS) and confocal microscopy.

To avoid maleimide deactivation by hydrolysis under aqueous conditions, SMCC-activated NIREPs are assembled using the precursor amine-OB18. The amine-NIREP can subsequently be treated with sulfo-SMCC, rapidly purified on a desalting column to remove excess linker, and then incubated with thiolated antibody. The presence of the cyclohexane ring in sulfo-SMCC confers significantly greater stability under aqueous conditions than our previously used \(m\)-maleimidobenzoyl-N-hydrosuccinimide ester (MBS) linker,\(^{23}\) which will improve conjugation efficiencies of the antibody to the NIREP surface.

Antibody modification
Chemical modification of an antibody has the potential to adversely affect its immunoreactivity. Trastuzumab contains between 80–95 lysine residues which potentially can act as a chemical handle either for direct attachment to FNB-NIREPs, or for conversion to a SMCC-reactive thiol group. We will explore the effects of antibody functionalization in the following experiments:

1. **Thiolation.** We will assay the number of thiol groups introduced by 2-iminothiolane onto the surface of the antibody using Elmann’s reagent. Reaction conditions (temperature, reaction time and reagent concentrations) will be altered accordingly to minimize the number of thiol groups introduced.

2. **Coupling reactions.** Trastuzumab will be conjugated to triethylene glycol (TEG) models as shown in Scheme 3. The immunoreactivity of the resulting conjugates will be evaluated using flow cytometry with HER2+ BC cells, and ELISA. If appropriate, different coupling conditions (temperature, buffer, reaction times, concentrations) will be explored.

![Scheme 3](image)

**Scheme 3** Reaction of antibodies with functionalized triethylene glycol (TEG) models to evaluate the effect of covalent modification on immunoreactivity.

**Aim 1a (Year 1): Cell Lines**

*Spector Group: Our hypothesis* is that a cocktail of optically distinct near infrared NIREPs (soft matter, biodegradable, NIR emissive nanoscale vesicles that manifest extraordinarily high irradiances and are ideally suited for *in vivo* optical imaging), each conjugated to different antibodies that target one of multiple therapeutically relevant cell surface proteins expressed on breast cancer cells, can provide non-invasive molecular images of the active BC sites within a patient, including micrometastases. A NIREP-based imaging approach could then be used to select appropriate combinations of targeted therapies taking into account the molecular heterogeneity that exists in a patient.

Furthermore, NIREP-based *in vivo* optical imaging will enable spatial and temporal characterization of the effects of targeted therapies on the molecular profile of tumors, some of which have been shown to contribute to the development of therapeutic auto-resistance. To test our hypothesis, particularly the second aspect having to do with characterizing the molecular changes associated with therapeutic resistance to targeted therapies, we must first develop isogenic matched HER2+ human breast cancer cell lines, parental and cell counterparts that have become resistant to targeted therapies used in breast cancer treatment. As provided in the original grant application, we have developed lapatinib resistant HER2+ human breast cancer cell lines and characterized

![Figure 4](image)

**Figure 4** Increased HER receptor expression in response to tam-treated MDA-MB-361 cells and tam-resistant 361 cells. Steady-state levels of the indicated proteins in control 361 cells treated with vehicle (0.01% DMSO) alone (lane 1); parental 361 cells treated with 1 µM tamoxifen for 72 hrs (lane 2); and tamoxifen-resistant 361 cells cultured in the continuous presence of 1 µM tamoxifen. Actin steady-state protein levels served as controls for equal loading of protein.
differences between the lapatinib-sensitive parental and resistant cell counterparts.\textsuperscript{12,15} For example, lapatinib-resistant SKBR3 cells (rSKBR3) preferentially express the stem cell antigen CD146 compared with isogenic matched parental cells. CD146 is one of the targets for our antibody-conjugated NIREP based imaging approach. During the past year we sought to expand our repertoire of isogenic matched parental and targeted therapy resistant breast cancer cell lines to include ER+ tumors. We have established several tamoxifen (tam) resistant ER+ human breast cancer cell lines by continuously exposing parental cells to clinically relevant concentrations of tamoxifen. Establishing stable tam-resistant cell lines turned out to be a challenging and protracted process that took months. We now have four pairs of parental and matched tam-resistant cell lines (MCF7; T47D; MDA-MB-361; HCC1428).

We have found that protein expression in members of the HER receptor family (EGFR/HER1; HER2; HER3) is up-regulated with the development of tam-resistance (Figure 4). Even acute treatment of parental MDA-MB-361 cells led to an increase in HER receptor expression. We can now use antibody-conjugated NIREPs targeting EGFR, HER2, and HER3 to distinguish parental from tam-resistant cell counterparts. In contrast, we see a different effect of tam-resistance on HER receptor expression in ER+ MCF7 and T47D human breast cancer cell lines (Figure 5). In MCF7 cells, expression of EGFR is increased while expression of HER2 and HER3 decreased in tam-resistant cells compared with parental cell counterparts. Similarly, HER2 and HER3 expression are decreased with the development of tam-resistance in T47D. We expect to characterize these changes using specific antibodies to HER receptors conjugated to NIREPs.

**Key Research Accomplishments**

- Synthesis of nanosized bioresorbable NIREPs for \textit{in vivo} applications

Nanoparticles formed from diblock copolymers of FDA-approved PEO and PCL have generated considerable interest as \textit{in vivo} drug delivery vehicles. We designed and synthesized the most extensive family of PEO-b-PCL copolymers that vary over the largest range of number-average molecular weights ($M_n$: 3.6 - 57K), PEO weight fractions ($f_{\text{PEO}}$: 0.08 - 0.33), and PEO chain lengths (0.75 – 5.8K) reported to date.\textsuperscript{24} These polymers were synthesized in order to establish the full range of aqueous phase behaviours of these diblock copolymers and to specifically identify formulations that were able to generate bilayered polymersomes. Cryogenic transmission electron microscopy (cryo-TEM) was utilized in order to visualize the morphology of these structures upon aqueous self-assembly of dry polymer films. Nanoscale polymersomes were formed from PEO-b-PCL copolymers over a wide range of PEO weight fractions ($f_{\text{PEO}}$: 0.14 – 0.27) and PEO molecular weights (0.75 – 3.8K) after extrusion of aqueous suspensions. Comparative morphology diagrams, which describe the nature of self-assembled structures as a function of diblock copolymer molecular weight and PEO weight fraction, show that in contrast to micron-scale polymersomes, which form only from a limited range of PEO-b-PCL diblock copolymer compositions, a multiplicity of PEO-b-PCL diblock copolymer compositions are able to give rise to nanoscale vesicles. These data underscore that PEO-b-PCL compositions that spontaneously form micron-sized polymersomes, as well as those that have previously been reported to form polymersomes via a co-solvent fabrication system, provide only
limited insights into the distribution of PEO-b-PCL diblocks that give rise to nanoscale vesicles. The broad range of polymersome-forming PEO-b-PCL compositions suggest the ability to construct extensive families of nanoscale vesicles of varied bilayer thickness, providing the ability to tune the timescales of vesicle degradation and encapsulant release based on the intended *in vivo* application. Furthermore, these bioresorbable polymersome-forming PEO-b-PCL compositions provide important building blocks necessary for the construction of NIREPs suitable for the non-invasive optical imaging of molecularly heterogeneous sites of BC *in vivo*.

- **Development of stable tamoxifen (tam) resistant ER+ human breast cancer cell lines for the characterization of targeted therapy resistance using NIREPs.**

We have established a panel of parental and isogenic matched human breast cancer cell lines that developed resistance to different targeted therapies used in the treatment of HER2+ and ER+ breast cancers. These reagents are critical to successful achievement of our goals. They provide a molecularly diverse set of tumor cell lines to evaluate the selectivity of a panel of antibody-conjugated NIREPs. They also provide insight into the molecular changes that are associated with the development of therapeutic resistance to HER2 and ER-targeted therapies, which we propose can be temporally and spatially detected during the course of therapy using antibody-conjugated NIREPs. When the antibody-conjugated NIREPs are generated as described above, we will evaluate their selectivity against these panels of parental and resistant cell lines. In year 2, we will establish luciferase-tagged breast cancer cell lines from the panels described above in preparation for the *in vivo* tumor xenograft studies (Aim 2) proposed for years 2 and 3.

**Reportable Outcomes**

**Publication:**


**Degrees:**


**Cell Lines:**

Parental and matched tam-resistant cell lines MCF7, T47D, MDA-MB-361, HCC1428.

**Conclusion**

While experimental setbacks we have experienced in the first year of our Department of Defense funding have delayed us in achieving the expected outcomes outlined in our Statement of Work, significant steps have been taken to optimize NIREP fabrication protocols. By ensuring that we can reproducibly conjugate immunoreactive antibodies to the surface of NIREPs, we can apply this methodology to rapidly fabricate a series of antibody-conjugated NIREPs for *in vivo* profiling of molecularly heterogeneous breast cancers. Furthermore, this novel methodology itself will be useful to the wider scientific community. Our expectation is that upon optimizing our coupling procedures, we will quickly progress to a series of antibody-conjugated NIREPs for *in vitro* testing in Year 2. Our preliminary data with positive batches of anti-HER2-NIREPs demonstrate that NIREPs are a powerful
tool for optically distinguishing between BC tumor subtypes in vitro. The ability of antibody-conjugated NIREPs to simultaneously profile multiple cell antigens of interest in vitro would make them a valuable pre-clinical tool for studying the numerous mechanisms of therapeutic resistance. Our studies have further shown that unfunctionalized NIREPs concentrate at tumor sites owing to their nanometer-sized dimensions. This, coupled with their unprecedented brightness, gives NIREPs the potential to profile all sites of metastatic disease in vivo for the first time. This would be transformative in the treatment of advanced stage breast cancer, as it would enable a personalized course of treatment to be determined based on an individual's tumor profile. In addition, the ability to characterize mechanisms of therapy resistance in vivo using NIREPs would drastically improve patient survival and remission rates by enabling prompt intervention, and allowing new treatment strategies to be developed.

References


**Appendices**


(B) Curriculum vitae: Michael J. Therien, Ph.D.

(C) Curriculum vitae: Neil L. Spector, M.D.
Aqueous self-assembly of poly(ethylene oxide)-block-poly(ε-caprolactone) (PEO-b-PCL) copolymers: disparate diblock copolymer compositions give rise to nano- and meso-scale bilayered vesicles†

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Nanoparticles formed from diblock copolymers of FDA approved PEO and PCL have generated considerable interest as in vivo drug delivery vehicles. Herein, we report the synthesis of the most extensive family PEO-b-PCL copolymers that vary over the largest range of number-average molecular weights (Mn: 3.6–57k), PEO weight fractions (fPEO: 0.08–0.33), and PEO chain lengths (0.75–5.8k) reported to date. These polymers were synthesized in order to establish the full range of aqueous phase behaviours of these diblock copolymers and to specifically identify formulations that were able to generate bilayered vesicles (polymersomes). Cryogenic transmission electron microscopy (cryo-TEM) was utilized in order to visualize the morphology of these structures upon aqueous self-assembly of dry polymer films. Nanoscale polymersomes were formed from PEO-b-PCL copolymers over a wide range of PEO weight fractions (fPEO: 0.14–0.27) and PEO molecular weights (0.75–3.8k) after extrusion of aqueous suspensions. Comparative morphology diagrams, which describe the nature of self-assembled structures as a function of diblock copolymer molecular weight and PEO weight fraction, show that in contrast to micron-scale polymersomes, which form only from a limited range of PEO-b-PCL diblock copolymer compositions, a multiplicity of PEO-b-PCL diblock copolymer compositions are able to give rise to nanoscale vesicles. These data underscore that PEO-b-PCL compositions that spontaneously form micron-sized polymersomes, as well as those that have previously been reported to form polymersomes via a cosolvent fabrication system, provide only limited insights into the distribution of PEO-b-PCL diblocks that give rise to nanoscale vesicles. The broad range of polymersome-forming PEO-b-PCL compositions described herein suggest the ability to construct extensive families of nanoscale vesicles of varied bilayer thickness, providing the ability to tune the timescales of vesicle degradation and encapsulant release based on the intended in vivo application.

Introduction

Polymersomes (50 nm to 50 μm diameter polymer vesicles) formed from amphiphilic block copolymers have attracted much attention due to their superior mechanical stabilities and chemical properties relative to those of conventional lipid-based vesicles (liposomes) and micelles.1-5 Polymer vesicles can readily encapsulate water-soluble hydrophilic compounds (drugs, vitamins, fluorophores, etc.) inside of their aqueous cavities, but also have been shown to be capable of encapsulating large hydrophobic molecules6-9 within their thick lamellar membranes. Moreover, the sizes, membrane thicknesses, and stabilities of these synthetic vesicle assemblies can be rationally tuned via various preparative methods6,10 that modulate block copolymer chemical structure, number-average molecular weight, and the ratio of hydrophilic to hydrophobic volume fractions, giving rise to polymersomes with varied characteristics that may be optimized for applications in medical imaging, drug delivery, and for topical cosmetic purposes.5,10

To date, polymersomes have been formed predominantly from amphiphilic diblock copolymers that include poly(ethylene oxide)-b-poly(butadiene) (PEO-b-PBD),6,5 poly(ethylene oxide)-b-poly(ethylethylene) (PEO-b-PEE),2 poly(styrene)-b-poly(ethylene
oxide) (PS-b-PEO), poly(styrene)-b-poly(acrylic acid) (PS-b-PA), poly(ethylene oxide)-b-poly(propylene sulfide) (PEO-PSS), poly(2-(methacryloyloxy)ethylphosphoryl-choline)-b-poly(2-(disopropylamino)ethylmethacrylate) (PMP-PDPA), and poly(styrene)-b-poly(isocyanoalanine) (2-thiophene-3-yl-ethyl) amide (PS-PIAT). None of these well-established polymersome formulations, however, generates fully biodegradable vesicles via aqueous self-assembly. A few biodegradable polymersome compositions have been prepared from amphiphilic diblock copolymers of PEO and aliphatic polyesters/polycarbonates using an organic co-solvent/water injection/extraction method. In contrast to polymersome preparative procedures based on self-assembly (i.e., film hydration, bulk hydration, or electroformation), the co-solvent method requires the organic co-solvent to be completely removed from the aqueous polymersome suspension post-assembly.

We have previously reported the generation of polymersomes via thin-film hydration of the diblock copolymer PEO(2k)-b-PCL(12k), the biomedical utility of PEO-b-PCL diblock copolymers is noteworthy in that they contain two previously FDA-approved building blocks, poly(ethylene oxide) (PEO) and poly(ε-caprolactone) (PCL). Unlike degradable polymersomes formed from blending "bio-inert" and hydrolysable components, PEO-b-PCL-based vesicles are fully bioresorbable, leaving no potentially toxic byproducts upon their degradation. In contrast to other degradable (polypeptide-, polyester-, or polyanhydride-based) polymersomes, PEO(2k)-b-PCL(12k)-based vesicles are formed through spontaneous self-assembly of the pure amphiphilic diblock copolymer, offering manufacturing advantages in terms of cost and safety. Further, these fully bioresorbable polymersomes possess in vivo drug release kinetics appropriate for potential intravascular drug delivery applications.

It is well known that particle sizes greatly influence blood circulation times, reticuloendothelial system (RES) recognition, biodistribution, and the mechanisms of cell uptake. The in vivo uptake of particles and their extent of drug release increase with decreasing particle sizes and increasing particle surface-area-to-volume ratios. As the optimal particle size for prolonged blood stream circulation is ~80–150 nm, and the intracellular uptake of particles having diameters larger than 1 μm is minimal, nanometer-sized bioresorbable vesicles are critical for in vivo drug delivery of encapsulated therapeutics. Congruent with these requirements, Butler and coworkers have further examined the formation of nano-sized vesicles from a handful of commercially available PEO-b-PCL block copolymer compositions featuring mostly small PEO chain lengths (300–2000) and low copolymer molecular weights (Mn: 2.6–7.8k) by the co-solvent injection method.

Expanding upon our initial work, as well as the studies by Butler and colleagues, we describe herein the development of an extensive family of amphiphilic poly(ethylene oxide)-b-poly(caprolactone) (PEO-b-PCL) diblock copolymers with varying hydrophilic PEO block weights (fPEO: 0.08–0.33) and PEO chain lengths (0.75–5.8k); we further screened their ability to assemble into bilayered vesicles through aqueous self-assembly of their respective dry polymer films deposited on Teflon™. These biodegradable PEO-b-PCL diblock copolymers were fabricated by: (i) ring-opening polymerization of ε-caprolactone monomer (ε-CL) followed by coupling to commercially available monomethoxyl PEO (MePEO), and (ii) sequential anionic living polymerization of PEO and ε-CL monomers. The synthesized PEO-b-PCL diblock copolymers possessed number-average molecular weights spanning 3.6–57k, PEO block weight fractions ranging from 0.08–0.33, and polydispersity indices (PDIs) ranging between 1.14 and 1.37 (ESI†). The generated morphologies of PEO-b-PCL copolymers prepared via self-assembled thin-film rehydration techniques were examined. Comparative morphology diagrams that describe the nature of self-assembled structures as a function of diblock copolymer molecular weight and PEO weight fraction exhibit dramatic differences for the nano- and meso-scale size domains. These studies demonstrate that in contrast to the single diblock copolymer formulation of PEO(2k)-b-PCL(12k) (PDI = 1.21) that produces quantitatively micron-sized polymersomes, nanoscale polymersomes can be formed from a multiplicity of PEO-b-PCL diblock copolymer compositions under thin-film rehydration conditions.

Experimental
Polymersome preparation
The thin-film hydration method was employed to assemble the PEO-b-PCL copolymers into their equilibrium aqueous morphologies. Thin-film hydration has been extensively utilized for preparing non-biodegradable polymersomes comprised of PEO-b-PBD diblock copolymers via aqueous self-assembly; an analogous protocol was employed herein for experiments involving PEO-b-PCL copolymers. An organic solution containing the dissolved biodegradable polymer (200 μL of 7 mg mL⁻¹ polymer in CHCl₃) was uniformly coated on the surface of a roughened Teflon™ plate followed by vacuum evaporation for >12 h. Addition of an aqueous solution (e.g., DI water) and heating at 60 °C for 48 h led to spontaneous budding of giant (5–20 μm) biodegradable polymersomes into suspension. In polymersome samples that contained 1 mol% Nile Red, the dye was incorporated into the hydrophobic vesicle bilayer during the self-assembly process noted above, which enabled facile visualization of resultant copolymer aqueous morphology via confocal fluorescence microscopy (ESI†). Nanoscale unilamellar polymersomes were prepared via procedures analogous to those used to formulate small lipid vesicles (sonication, freeze–thaw extraction and extrusion). The sonication procedure involved placing a sample vial containing the aqueous-based solution and a dried thin-film formulation (of polymer uniformly deposited on Teflon™) into a bath sonicator (Fischer Scientific; Model FS20) with constant agitation for 2 h. Freeze–thaw extraction cycles (10) were carried out by alternatively placing the sample vials in liquid N₂ and water baths. A narrow size distribution of nano-sized polymersomes was achieved with 3 extrusions using a Liposofast Basic hand-held extruder equipped with 400 nm polycarbonate membranes (Avestin Inc., Ottawa, Ontario). These nano-sized polymersomes were lyophilized and subject to chromatographic purification (PLgel 5 μm
mixed C, 300 × 7.5 mm, linear MW operating range: 200–2 000 000 g mol⁻¹) in order to verify that no measurable PCL hydrolysis of the PEO-b-PCL polymers occurred under these experimental conditions.

Results and discussion

Synthesis and characterization of biodegradable PEO-b-PCL diblock copolymers

A series of PEO-b-PCL diblock copolymers (Table 1) were synthesized (ESI†) via ring-opening polymerization of ε-CL and commercially available MePEO (Mₙ = 5000, 2000, 1100 and 750). MePEO homopolymers bearing one hydroxyl end group were used as the macroinitiator to activate polymerization (130 °C, 24 h) of ε-CL monomer in the presence of catalyst (tin octoate, SnOct₂). PEO-b-PCL diblock copolymers have been previously synthesized under a variety of catalyzed43,44 and non-catalyzed conditions.45,46 Non-catalyzed ring-opening polymerization of ε-CL must, however, be carried out for several days, which is cumbersome. Of the previously established catalysts, SnOct₂ is the most widely used for the production of biodegradable polyesters, as it is commercially available, easy to handle, soluble in common organic solvents and neat liquids (e.g., cyclic ester monomers), and is a permitted food additive in numerous countries.47-49

Although the synthesis of PEO-b-PCL copolymers from MePEO via ring-opening polymerization of ε-CL is facile, the availability of MePEO homopolymers is limited. As such, we utilized anionic living polymerization of ethylene oxide monomers to produce PEOs over an expansive range of molecular weights (Mₙ); subsequent ε-CL polymerization yields PEO-b-PCL copolymers that vary over the largest range of number-average molecular weights (Mₙ: 3.6–577 k) and PEO weight fractions (fₑₒ): 0.08–0.33) reported to date. An additional advantage of this approach is that the functionality of the PEO terminus of the PEO-b-PCL copolymer can be easily varied (ESI, Scheme S2†). These ethylene oxide polymerization reactions utilized cyanomethyl potassium as the protected initiator, which was prepared by metatation of acetoniitrate with potassium naphthalenide in THF.50-52 While anionic living polymerization had been utilized previously in the syntheses of low molecular weight, high PEO weight fraction PEO-b-PCL polymers [e.g., PEO(2.2k)-b-PCL(1.2k)],53 this strategy, as presented herein, also provides PEO-b-PCL copolymers that possess a larger range of PEO block molecular weights (1.5, 2.6, 3.0, 3.8, and 5.8 kDa), low PEO weight fractions (fₑₒ: 0.10–0.23), and a wider range of diblock Mₙ (7.8–47 k) than has been explored previously.54 ¹H NMR spectroscopy was utilized to characterize the number-average molecular weight of the PEO homopolymers and the corresponding PEO-b-PCL diblock copolymers (ESI†).55-57 GPC was employed to characterize the molecular weight (Mₙ) and molecular weight distribution (Mₙ/Mₙ) (PDI) of each PEO-b-PCL diblock copolymer formulation (see ESI†). GPC data indicate that PEO-b-PCL diblock copolymers synthesized by anionic living polymerization, having PEO molecular weights of 2.6, 3, 3.8 and 5.8 k, exhibited the narrowest molecular weight distributions (PDI: 1.2–1.27). PEO-b-PCL diblock copolymers

<table>
<thead>
<tr>
<th>PEO-b-PCL copolymersa</th>
<th>fₑₒb</th>
<th>Morphologyc (μm)</th>
<th>Morphologyd (nm)</th>
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<tr>
<td>PEO(0.75k)-b-PCL(2.9k)</td>
<td>0.21</td>
<td>MS, IP</td>
<td>V, S</td>
</tr>
<tr>
<td>PEO(0.75k)-b-PCL(5.8k)</td>
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<td>IP</td>
<td>S</td>
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<td>PEO(0.75k)-b-PCL(9k)</td>
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</tr>
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<td>V, S</td>
</tr>
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</tr>
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<td>S</td>
</tr>
<tr>
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<td>IP</td>
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<td>S</td>
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<td>S</td>
</tr>
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<td>PEO(5k)-b-PCL(16k)</td>
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<td>S</td>
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<td>PEO(5k)-b-PCL(22k)</td>
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<td>S</td>
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<td>PEO(5k)-b-PCL(26k)</td>
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<td>S</td>
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<td>PEO(5k)-b-PCL(32k)</td>
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<td>IP</td>
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<td>PEO(5.8k)-b-PCL(24k)</td>
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<td>IP</td>
<td>S, P</td>
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a Number-average molecular weight of PEO-b-PCL diblock copolymers as determined by ¹H NMR spectroscopy. b Weight fraction of the PEO block as determined by ¹H NMR data. c Morphology of particles determined qualitatively from fluorescence confocal microscopic studies of the self-assembled structures formed from thin-film rehydration of 50 : 1 molar ratios of copolymer: Nile Red. Observed polymersome and irregularly shaped particle (IP) diameters ranged from less than 1 μm to greater than 30 μm; microsphere (MS) diameters ranged from ~5–30 μm; vesicle (V) diameters spanned ~5–50 μm. In the cases of mixed morphologies, the major component is reported first. d Morphologies of particles determined qualitatively from cryogenic transmission electron microscopic studies of the self-assembled structures formed from thin-film rehydration of 50 : 1 molar ratios of copolymer: Nile Red followed by extrusion through a 400 nm porous membrane (S = spherical micelles, V = vesicle, P = precipitate; for systems exhibiting mixed morphologies, the majority component is reported first). See Tables S3 and S4.
synthesized from PEO(2k) via ring-opening polymerization showed narrow molecular weight distributions (PDI: 1.1–1.2), while copolymers derived from PEO(5k) displayed distributions that were slightly wider (PDI: 1.32–1.37). These data establish that anionic living polymerization provides an excellent route for the synthesis of PEO-b-PCL diblock copolymers with controlled PEO chain length, modulated PEO/PCL block ratio, and narrow molecular weight distribution (see ESI†).

**Aqueous morphologies of meso-scale PEO-b-PCL diblock copolymers**

The thin-film hydration method was preferentially utilized to assemble meso-scale amphiphilic PEO-b-PCL diblock copolymers into their equilibrium aqueous morphologies [ESI, Table S1†].

Data compiled in Table 1 describe the observed aqueous morphologies for the comprehensive set of PEO-b-PCL diblock copolymers that were fabricated; note that extensive microscopy studies (confocal, TEM) demonstrate that preparations of micron-sized polymersomes gave rise to no measurable quantity of corresponding nanoscale structures.

Large numbers of meso-scale polymersomes were obtained from aqueous hydration and self-assembly of the PEO(2k)-b-PCL(12k) diblock copolymer ($f_{PEO} = 0.14$) (ESI, Fig. S3†). These polymersomes possessed both multimellar and unilamellar bilayered structures. In contrast, micron-sized polymersomes were found to coexist with irregular particles in aqueous preparations of PEO(2–3.8k)-b-PCL(9.5–22.2k) diblock copolymers, where $f_{PEO}$ ranges between 0.12 and 0.19. In aqueous suspensions of PEO-b-PCL diblock copolymers derived from higher (5k or 5.8k) or lower (750–1.5k) molecular weight PEO blocks, no polymersomes were observed regardless of the PEO/PCL ratio (Fig. 1). Unlike conventional vesicle-generating PEO-b-PBD copolymers that have a broad range of compositions compatible with high yields of self-assembled meso-scale vesicles, the range of the PEO weight fraction ($f_{PEO}$: 0.12–0.19), PEO block size (2–3.8k), and total diblock $M_n$ (1.5–26k) compatible with meso-scale polymersome formation for PEO-b-PCL diblock copolymers is relatively narrow (Fig. 1). The reduced PEO weight fractions of these polymersome-forming PEO-b-PCL compositions ($f_{PEO} = 0.14$) contrast sharply to the PEO-b-poly(ethylene) and PEO-b-poly(butadiene) diblock formulations that give rise to the archetypal polymersomal structure ($f_{PEO}: 0.28$–0.39).5,4 The lower PEO weight fractions necessary to generate PCL- and related biodegradable copolymer-based polymersomes may be related to the higher phase transition temperatures for these compositions.27,25

Note, in this regard, Discher and coworkers have also studied the meso-scale morphologies generated from a series of PEO-b-PCL diblock copolymers using organic co-solvent evaporation;33 their work focused upon polymeric compositions that featured PEO/PCL ratios that enabled the formation of worm-like micelles; their results, however, differ from those obtained with the experiments carried out with the broader selection of PEO-b-PCL diblock compositions examined herein, which describe $f_{PEO}$, PEO block size, and total diblock $M_n$ ranges that give rise to meso-scale polymersomal morphologies. Note, for example, that the PEO(2k)-b-PCL(12k) diblock copolymer composition forms largely worm-like structures via the solvent evaporation method,32 but quantitatively self-assembles into vesicles via thin-film hydration (ESI†, Fig. S3). Likewise, (i) PEO(5k)-b-PCL(10k) forms vesicles and spheres via solvent evaporation,33 but self-assembles into microspheres via the thin-film hydration method (Fig. S4†), and (ii) PEO(5.8k)-b-PCL(24k), which has been previously shown to form meso-scale vesicles via solvent injection,38 yields no polymersomes by thin-film hydration. These data indicate that the aqueous morphologies of PEO-b-PCL copolymers are sensitive to preparative method and experimental conditions, which may alter the local arrangement of polymer chains that form corresponding aqueous structures. In this regard, the organic co-solvent water injection/extraction method is well known to produce kinetically trapped morphologies that are sensitive to the order of addition, concentrations and ratios of copolymer and solvent, as well as the injection flow rate.38

In order to elucidate the effects of diblock copolymer molecular weight distribution on vesicle formation, PEO-b-PCL diblock copolymers with varying PEO block size (2.6k, 3k or 3.8k) and narrow molecular distributions (PDI = 1.1) were separated by GPC; no further improvement in the yield of vesicles from these samples was observed relative to PEO-b-PCL diblock copolymers of the same molecular weight having slightly broader molecular weight distributions (PDI: 1.2–1.4). Similarly, the polymersome-forming abilities of PEO-b-PCL diblock copolymer mixtures having much wider molecular weight distributions were examined; scanning laser confocal microscopy of such samples (Fig. 2), shows that polymersomes can be obtained in high yield from a 1: 1: 1 PEO(2k)-b-PCL(9.5k) : PEO(2k)-b-PCL(12k) : PEO(2k)-b-PCL(15k) copolymer blend, suggesting that the molecular weight distribution has little influence on biodegradable polymersome formation from mixtures containing a significant weight fraction of the PEO(2k)-b-PCL(12k) diblock copolymer.

![Fig. 1 Phase diagram of micron-sized particles derived from aqueous hydration of PEO-b-PCL copolymers. Morphologies were determined qualitatively from fluorescence confocal microscopic studies of the self-assembled structures formed from thin-film rehydration of 50 : 1 molar ratios of copolymer : Nile Red. Observed polymersome and irregularly shaped particle (IP) diameters ranged from less than 1 μm to greater than 30 μm; microsphere (MS) diameters ranged from ~5–30 μm; vesicles (V) diameters spanned ~5–50 μm. For systems exhibiting mixed morphologies, the major component is reported first.](image-url)
Nanoscale morphologies of self-assembled PEO-b-PCL diblock copolymers in aqueous suspension

Nanoscale polymersomes are formed from dry thin-films of PEO-b-PCL deposited on Teflon™ upon rehydration in an aqueous solution, along with the addition of sonic energy; several subsequent cycles of freeze–thaw extraction and extrusion (Fig. 3 and 4; see Experimental section, ESI†) yield vesicles of a desired size with narrow distribution (e.g. \( d \sim 100 \pm 15 \) nm). Solution morphologies were characterized utilizing cryo-TEM, which allowed for direct visualization of the aggregate nano-sized structures formed in aqueous suspension (Table 1); note that dynamic light scattering (DLS) measurements confirmed the homogeneous size distributions and were consistent with cryo-TEM experimental data. Nanoscale polymersomes are formed from PEO-b-PCL diblock copolymers of a range of PEO weight fractions (\( f_{PEO} \): 0.14–0.27) and block sizes (PEO molecular weights of 0.75–3.8k). Butler et al. examined a few low molecular weight PEO-b-PCL copolymers that assembled into nm-sized morphologies via co-solvent injection; the results of these investigators mirror the Table 1 data for low molecular weight PEO-b-PCL copolymers that assembled into nm-sized morphologies via self-assembly after thin-film hydration. Note, however, for high molecular weight PEO-b-PCL copolymers with PEO block molecular weight over 3.8k, such as PEO(5k, 5.8k)-PCL diblock copolymers, only spherical micelles and particles are formed under thin-film hydration conditions over a large range of PEO weight fractions (\( f_{PEO} \): 0.09–0.33). It is important to underscore that significant morphological differences are evident for self-assembled meso- and nanoscale structures for a given PEO-b-PCL diblock copolymer. In contrast to morphologies generated on the meso scale, polymersomes dominate observed self-assembled nanoscale morphologies of these PEO-b-PCL diblock copolymers, suggesting disparate formation mechanisms for nano- and meso-sized polymersomes that form under thin film hydration conditions.

As shown in Fig. 5, both hydrophilic volume fraction and PEO chain length affect the observed distribution of self-assembled morphologies on the nanoscale. To further explore this issue, several PEO-b-PCL diblock copolymers were

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**Fig. 2** Scanning fluorescence confocal micrograph (\( \lambda_{ex} = 488 \) nm) of polymersomes derived from a 1 : 1 : 1 mixture of PEO(2k)-b-PCL(9.5k), PEO(2k)-b-PCL(12k), and PEO(2k)-b-PCL(15k), containing membrane-encapsulated Nile Red (peak emission = 603 nm) in DI water at 25 °C.

**Fig. 3** Cryo-TEM images of nanoscale polymersomes derived from PEO-b-PCL (OCL) diblock copolymers. Aqueous suspensions of OCL 1–6 were generated via thin-film hydration and subsequent self-assembly. D (dominant morphology): vesicles define the most prevalently observed morphological structure; C (common morphology): vesicles define one of the commonly observed morphological structures; A (atypical morphology): vesicles are observed, but such structures are less common than other observed morphologies.

**Fig. 4** Cryo-TEM images of nanoscale polymersomes derived from PEO-b-PCL (OCL) diblock copolymers. Aqueous suspensions of OCL A–F were generated via thin-film hydration and subsequent self-assembly. D (dominant morphology): vesicles define the most prevalently observed morphological structure; C (common morphology): vesicles define one of the commonly observed morphological structures; A (atypical morphology): vesicles are observed, but such structures are less common than other observed morphologies.

**OCL Copolymers**

<table>
<thead>
<tr>
<th>OCL</th>
<th>PEO(b)-b-PCL(mk)</th>
<th>( f_{PEO} )</th>
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</thead>
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<td>PEO(2k)-b-PCL(7.4k)</td>
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<td>D</td>
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<tr>
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<td>D</td>
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<tr>
<td>OCL-4</td>
<td>PEO(2k)-b-PCL(15k)</td>
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<td>C</td>
</tr>
<tr>
<td>OCL-5</td>
<td>PEO(2k)-b-PCL(18k)</td>
<td>0.10</td>
<td>A</td>
</tr>
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<td>OCL-6</td>
<td>PEO(2k)-b-PCL(22k)</td>
<td>0.08</td>
<td>None</td>
</tr>
</tbody>
</table>

**Table 1**
generated that featured the same PEO chain length (2k) but which differed with respect to hydrophilic PEO weight fraction (see OCL A–E in Fig. 3); further, other PEO-b-PCL copolymers were also synthesized that exhibited identical PEO weight fractions, but which differed with respect to PEO chain length (see OCL A–E in Fig. 4). The data chronicled in Fig. 3 demonstrate that increasing the molecular weight of the PCL block at a constant PEO chain length \( \left( f_{\text{PEO}} = 0.21 \rightarrow 0.08 \right) \) results in a morphological transition from bilayered vesicles to micellar aggregates. Increasing the length of PCL block augments the hydrophobic content of the amphiphile while decreasing the interfacial curvature, leading to the development of micellar morphologies. Fig. 4 highlights that uniform spherical vesicles are formed from PEO(2k)-b-PCL diblock copolymers when \( f_{\text{PEO}} < 0.12 \), with micelles first appearing when \( f_{\text{PEO}} \) falls below 0.14. For PEO-b-PCL diblock copolymers with \( f_{\text{PEO}} \) fixed at \( \sim 15\% \), PEO chain lengths ranging from 1.1 to 3.8k support polymersome formation at the nanoscale, while micellar aggregates are evident for diblocks having PEO molecular weights <1.1k or >3k.

It is well known that the morphology of diblock copolymer assemblies is determined by the interfacial curvature of the amphiphile;\textsuperscript{14} moreover, previous work has revealed that the surface elasticity of bilayer membranes is scale independent and only depends on the interface.\textsuperscript{37} It is the expectation, therefore, that self-assembled diblock copolymer morphologies elucidated at one size domain provide predictive insights into morphologies that will be evident over other size domains. While this has shown to be true for meso-scale and larger bilayered vesicles, these data emphasize that it is incorrect to extend such predictions to the nanoscale. While the origin of this effect can only be discussed qualitatively, it is likely that the dramatically increased total surface areas and correspondingly decreased surface tensions characteristic of nanoscale bilayered vesicles serve to mitigate destabilizing effects that derive from the augmented interfacial curvature required for vesicle formation, relative to hypothetical micron-sized vesicles derived from an identical diblock copolymer. Likewise, this observed difference in ability to form mesoscopic versus nanoscale vesicles may stem in part from the crystallinity of the hydrophobic PCL chains.\textsuperscript{39} The restricted mobility of the PCL chains may allow the assembly of small sections of bilayers, whereas large length-scale assemblies of lamellar phases may be disallowed. Increased solution energy (via heating and sonication) may also allow for the generation of nano-sized vesicles that would otherwise be energetically unfavourable at the mesoscale for a given diblock copolymer composition; upon solution cooling, increased interchain packing may augment stabilization of the bilayered membrane, yielding the final nanoscale structure. The differences between self-assembled morphologies formed at different length scales should thus be systematically explored for different diblock formulations of a given composition in order to determine if this is a universal effect or one that is dependent on polymer chain flexibility and diblock copolymer transition temperatures. In any event, this work demonstrates clearly that if nanoscale vesicles are the targeted design, there is no substitute for direct screening and characterization of diblock polymer compositions that give rise to self-assembled nanoscale morphologies via cryo-TEM.

**Conclusions**

A series of PEO-b-PCL diblock copolymers varying in PEO block size (\( M_n \): 750, 1100, 2000 and 5000), \( f_{\text{PEO}} (0.08–0.33) \), and \( M_n \) (3.6–57k) were synthesized by ring-opening polymerization of \( \varepsilon \)-CL monomer using commercially available MePPEO as the macro-initiator; anionic living polymerization was also employed to synthesize PEO-b-PCL copolymers with a wider range of controlled PEO block sizes (\( M_n \): 1500, 2600, 3000, 3800, and 5800), \( f_{\text{PEO}} (0.10–0.23) \), and \( M_n \) (ranging from 7.8 to 47k). All copolymers were analysed by GPC and possessed narrow molecular weight distributions (PDI: 1.14–1.37).

The nature of the self-assembled aqueous morphologies derived from these polymers was probed on the micron scale using epifluorescence optical and scanning confocal fluorescence microscopies, and on the nanoscale via cryogenic transmission electron microscopy (cryo-TEM). Key morphological differences were observed to exist between self-assembled meso- and nanoscale structures for a given PEO-b-PCL diblock copolymer. While a single composition [PEO(2k)-b-PCL(12k); PDI = 1.21] provided self-assembled micron-sized structures in which vesicles were the most prevalently observed morphology under thin-film rehydration conditions, corresponding nanoscale polymersomes were formed from a multiplicity of PEO-b-PCL diblock copolymer compositions: these included PEO-b-PCL copolymers having a wide range of PEO weight fractions (\( f_{\text{PEO}}: 0.1–0.27 \)), as well as PCL molecular weights (3.65–26k).

This work shows, for the first time, that morphological differences exist between self-assembled meso- and nanoscale structures for a given PEO-b-PCL diblock copolymer and underscores that PEO-b-PCL diblock copolymer compositions...
that form meso-scale polymersomes provide only limited insight into the distribution of PEO-b-PCL diblocks that give rise to corresponding vesicles on the nanoscale. Because PEO and PCL are FDA-approved polymers, this broad range of polymersome-forming PEO-b-PCL compositions suggests a more extensive utility of these species as nanometric drug delivery vehicles, as polymersomal bilayer thicknesses will undoubtedly modulate timescales of vesicle degradation and corresponding release of polymersomal contents in biologically relevant applications.

**Acknowledgements**

This work was supported by grants from the Department of Defense (W81XWH-13-1-0086), the National Cancer Institute (R01CA115229), and the National Institutes of Health (EB003457-01). M.J.T. and D.A.H. thank the MRSEC Program of the National Science Foundation (DMR-00-79909) for infrastructural support.

**Notes and references**

NAME
Michael J. Therien

POSITION TITLE
William R. Kenan, Jr. Professor

EDUCATION/TRAINING

<table>
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<th>INSTITUTION AND LOCATION</th>
<th>DEGREE</th>
<th>YEAR(s)</th>
<th>FIELD OF STUDY</th>
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<tr>
<td>University of California, Los Angeles, CA</td>
<td>B. S.</td>
<td>1982</td>
<td>Chemistry</td>
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<tr>
<td>University of California, San Diego, CA</td>
<td>Ph.D.</td>
<td>1987</td>
<td>Chemistry</td>
</tr>
<tr>
<td>California Institute of Technology, Pasadena, CA</td>
<td>NIH PostDoc</td>
<td>1987-1990</td>
<td>Chemistry</td>
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RESEARCH AND PROFESSIONAL EXPERIENCE:

Positions Held:

- University of Pennsylvania: Assistant Professor, 1990-1996
- University of Pennsylvania: Associate Professor, 1996-1997
- University of Pennsylvania: Professor of Chemistry, 1997-2002
- Princeton University: Visiting Professor, 2002
- University of Pennsylvania: Alan G. MacDiarmid Professor of Chemistry, 2002-2007
- Universiteit of Lueven: Visiting Professor, 2004; 2005
- Universiteit of Leuven: Francqui International Professor, 2008-2009
- Duke University: Professor of Chemistry, 2008
- Duke University: William R. Kenan, Jr. Professor, 2009-present
- University of Bordeaux: Visiting Professor, 2010

Honors:

National Institutes of Health Postdoctoral Research Fellow (1987-1990); Searle Scholar (1991-1994); Arnold and Mabel Beckman Foundation Young Investigator (1992-1994); National Science Foundation Young Investigator (1993-1998); E. I. DuPont de Nemours Young Faculty Award (1995-1997); Alfred P. Sloan Foundation Fellow (1995-1997); Camille Dreyfus Teacher-Scholar (1997-2002); Journal of Porphyrins and Phthalocyanines Young Investigator Award (2002); Pederson Lecturer, Central Research and Development Division, E. I. DuPont de Nemours (2003); American Chemical Society Philadelphia Section Award (2004); W. Heinlen Hall Lectureship, Bowling Green State University (2005); Elected Fellow, American Association for the Advancement of Science (2005); International Fancqui Chair, Belgium (2008-2009); Fellow, Flemish Academy of Science (2009).

Advisory Positions:

External Reviewer, United States Department of Energy, Nanotechnology Centers (2003-present);
Review Committee Member, Beckman Young Investigator Program (2003-present);
Research Team Leader, NSF-supported Nanoscience and Technology Center (2004-2008);
Scientific Advisory Board, Center for Nanoscale Materials, Argonne National Laboratory (2005-present);
ARPA-E Photovoltaics Review Committee Chair (2009);
External Program Reviewer, Scientific Foundation Ireland (2011);
Molecular Assemblies Research Team Leader, DOE Energy Frontier Research Center for Solar Fuels (2012-present).

Earlier Publications Pertinent to this Application:


**Exemplary Additional Publications over the Three Previous Years (2011-2013):**


BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors in the order listed on Form Page 2.

Follow this format for each person.

<table>
<thead>
<tr>
<th>NAME</th>
<th>POSITION TITLE</th>
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<tr>
<td>Spector, Neil L.</td>
<td>Associate Professor of Medicine (Oncology)</td>
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<th>eRA COMMONS USER NAME (credential, e.g., agency login)</th>
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<td>Spect002</td>
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EDUCATION/TRAINING  (Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)

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<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
<th>MM/YY</th>
<th>FIELD OF STUDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>University of North Carolina, Chapel Hill</td>
<td>B.S.</td>
<td>5/1978</td>
<td>Pre-Med</td>
</tr>
<tr>
<td>New Jersey Medical School UMDNJ</td>
<td>MD.</td>
<td>6/1982</td>
<td>Medicine</td>
</tr>
<tr>
<td>Parkland Memorial Hospital, UT-Southwestern</td>
<td></td>
<td>6/1982- 6/1986</td>
<td>Internship &amp; Residency</td>
</tr>
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</table>

A. Personal Statement. I have a long standing interest in elucidating mechanisms involved in tumorigenesis and the development of molecule to target these pathways. My group has performed extensive work in targeting HER2 and other EGFR family members and has a successful track record in translating research from the bench to the clinic. My group led the translational research program that was instrumental in the development of lapatinib, the first small molecule inhibitor of the EGFR and HER2 tyrosine kinases approved for the treatment of women with HER2+ breast cancer. Our work led to insight into the molecular signatures of tumors that were more likely to respond to lapatinib, and conversely those that were likely to be resistant. We successfully developed the first reported models of acquired resistance to HER2 TKIs that mimic the clinical setting. Using these models, we showed that successful inhibition of PI3K-Akt signaling in lapatinib-treated HER2+ breast cancer cells led to de-repression of the FOXO3a transcription factor with consequential activation of estrogen receptor (ER) signaling. Co-treatment with anti-estrogens and lapatinib could abrogate lapatinib resistance in pre-clinical models, which provided the scientific basis for a clinical development plan combining an aromatase inhibitor with lapatinib, a combination approved by the FDA as a first-line therapy for the treatment of advanced stage ER+/HER2+ breast cancers. In addition, we have identified a number of other molecular mechanisms of therapeutic resistance to HER2 targeted therapies. Since coming to Duke in 2006, I have been eager to continue my interests in drug development as the co-leader of the Developmental Therapeutics Program (DTP) in the Duke Cancer Institute. I am on a number of multi-investigator grants specifically designed to take novel therapeutics discovered at Duke and bring them into the clinic. In my leadership role in the DTP, I have sought to create an environment that will facilitate the critical partnerships necessary to translate discoveries made by Duke investigators, into the clinic.

B. Positions and Honors

1986-1988 Clinical Hematology and Medical Oncology, Massachusetts General Hospital, Harvard Medical School, Boston, MA (Fellowship)
C. Selected Peer-reviewed Publications


